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The Characterisation of Genetic Diversity of a Collection of Perennial Ryegrass (*Lolium perenne* L.)

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Submission for Ph.D.

University of Dublin, Trinity College

Declaration

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Abstract

Perennial ryegrass (*Lolium perenne* L.) is a member of the Poaceae family, is native to Europe, the Near East and North Africa and is grown in all the temperate climate areas of the world as a forage and turf grass. Due to its persistence, palatability and nutritive value for ruminants, it is a principal component of pastures, and the most important forage species in Ireland. The primary aim of this thesis was to characterise the level of diversity in a large genetic resource collection of *L. perenne* germplasm held at Teagasc, Oak Park. Molecular markers, both chloroplast and nuclear SSRs, biochemical characters (water soluble carbohydrate, crude protein, and dry matter), and morphological characters (vegetative and flowering) were used to characterise this diversity, as well as population differentiation, and geographic patterns. Levels of diversity in all systems were found to be high in this collection.

Primers to amplify microsatellite markers from the chloroplast genome of *Lolium perenne* were designed and optimized using *de novo* sequencing and *in silico* sequences. With one exception, each locus was polymorphic with a range from two to nine alleles in *L. perenne*. The newly developed primer pairs cross-amplified in different species of *Lolium* and in 50 other grass species representing nine grass subfamilies. These markers were then used to characterise chloroplast genetic diversity at allelic and haplotypic level in 104 accessions of *Lolium perenne,* other *Lolium* species, *Festuca* species and ×*Festulolium* cultivars. Furthermore, genetic relationships between the accessions and biogeographic distribution of haplotypes were investigated using a range of population genetic diversity measures and an Analysis of Molecular Variance (AMOVA). An extremely high number of 511 haplotypes was detected in 1,575 individuals possibly attributable to natural and anthropogenic migration. Much of the *L. perenne* European ecotype diversity (61%) could be attributed to within population variance. Plastid gene pools and maternal lineages for *L. perenne* could be clearly identified. Evidence was found showing a most likely migration route of *L. perenne* into Ireland from southern regions of Europe northwards.

Morphological variation of 13 vegetative and reproductive traits was characterised for 2,481 individuals from 50 *L. perenne* accessions, a mixture of Irish and European ecotypes and cultivars. Considerable levels of among and within population variation was found across traits. Principal component analysis and UPGMA dendrograms were able to separate ecotypes from cultivars. Cultivars generally had later dates of ear emergence, better spring and summer growth, longer rachis length and more spikelets per spike than ecotypes. Correlation and regression analysis were used to assess relationships between traits and strong positive relationships were seen between reproductive characters, i.e. rachis length with spikelets per spike, florets per spikelet and glume length. The strong relationship between rachis length and the other reproductive characters suggested that rachis length could be used as a predictor for reproductive performance. Later flowering was correlated with improved spring and summer growth.

Water soluble carbohydrate (WSC; glucose and fructose determined by HPLC), crude protein (determined via LECO analysis), and dry matter contents were recorded for 1,320 individuals, pooled into 132 samples from 33 *L. perenne* ecotypes and cultivars at five different harvest time points across the 2004 growing season. While, in general, the cultivars had higher WSC contents than the ecotypes, individual ecotypes did show potential to be used in breeding programmes, as they showed higher values than all other accessions at particular cutting points. In correlation analyses, positive relationships were shown between dry matter and glucose both early and late in the growing season, and this was in agreement with the amount of leaves compared to stem at these times in the growing season. PCA analysis allowed the separation either between cultivars and ecotypes, or between tetraploid cultivars and the rest of the accessions at four out of five cutting points. In the ANOVA analysis, cutting point was the most significant factor influencing the variation in the traits.

Eight nuclear SSR markers were used to characterize genetic diversity in 928 individuals from 40 diploid ecotypes and cultivars of *L. perenne*. High levels of genetic diversity (0.82, Nei's gene diversity, over all accessions) and high numbers of alleles (22.25 average number of alleles per locus) was found. An average polymorphic information content (PIC) value of 0.81 across all loci was found. When deviations from Hardy-Weinberg equilibrium were tested, the majority of populations

had an excess of homozygotes. Very low levels of linkage disequilibrium were found between pairs of loci tested. AMOVA analysis and *F* statistics were used to test partitioning of variation, and most variation was found within populations (e.g. 31% for glume length in ecotypes). UPGMA, PCA and STRUCTURE analysis all gave similar patterns of relationships between populations, where relationships with high bootstrap support on the UPGMA dendrogram were also seen in the other analyses.

The overall results of the thesis are discussed in the context of plant breeding programmes and natural population genetic variation. Strategies for incorporation of the results of the thesis (and the novel markers developed within) into plant breeding programmes are suggested.

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Chapter 1

General introduction to the characterisation of genetic diversity of a collection of perennial ryegrass (*Lolium perenne* L.)

1.1 Introduction

1.1.1 *Lolium perenne* and close relatives

Lolium perenne L. (perennial ryegrass; family Poaceae) is native to Europe, the Near East and Africa, and is cultivated in all the temperate areas of the world as a forage and turf species. It is believed to have originated in the Mediterranean area (Cresswell *et al.* 2001), from where it spread to Europe, North Africa and East Asia. *Lolium perenne* has been cultivated as a forage grass in the British Isles for the last 300 years, but has only been commercially bred in the last 80 years, with natural populations still being grown on farms until the 1980's. In Ireland, 90% of farmed land is grassland (Connolly, 2001) and *L. perenne* is the most important forage grass for agriculture because of its high palatability and digestibility (Delagarde *et al.* 2000) as well as its persistence and vigour. In the UK, 75% of all agricultural land use (50% of all land use) is accounted for by *L. perenne*, and its economic value in terms of end products (milk, meat) was estimated to be £6 billion sterling in 2002 (DEFRA, 2002). Genetically, *L. perenne* is usually diploid (2n = 14), is an obligate outbreeder and is perennial. Taxonomically it belongs to the grass tribe Poeae and subfamily Pooideae. This subfamily also includes several cereal genera such as wheat (*Triticum* L.), barley (*Hordeum* L.), rye (*Secale* L.) and oats (*Avena* L.) and the main genera of high value for forage such as *Festuca* L., and *Poa* L.

The international plant names index (IPNI; www.ipni.org) lists over 80 different species names within *Lolium* but most of these are synonyms because less than ten species are currently recognized (excluding inter-generic hybrids). According to Clayton *et al.* (2006), the genus *Lolium* contains seven species, *L. canariense* Steud., *L. multiflorum* Lam., L. *perenne*, *L. persicum* Boiss., *L. remotum* Schrank, *L. rigidum* Gaud., and *L. temulentum* L. Several interspecific and intergeneric hybrids have also been recorded, for example, *L.* ×*hybridum* Hausskn (a hybrid between *L. multiflorum*

and *L. perenne*), *F. gigantean* × *L. perenne*, *F. arundinacea* × *L. perenne*. There are also various ×*Festuloliums* described, for example, ×*Festulolium braunii* (*L. multiflorum* × *F. pratensis*), and ×*Festulolium L. multiflorum* × *F. arundinacea*. The morphology of the genus *Lolium* has been described since the 18th century (Loos & Jarvis, 1992). In the 20th century, taxonomic relationships within the genus were investigated using morphological characteristics by several authors (Essad, 1954; Naylor, 1960; Terrell, 1968; Bulinska-Radomska & Lester, 1985; Bulinska-Radomska & Lester, 1988; Loos, 1993). Separation of autogamous from allogamous species within the genus was found in several of the studies (Essad, 1954; Bulinska-Radomska & Lester, 1985; Loos, 1993). Within both of these groups, morphological intergradation was found and the species were often difficult to distinguish from each other using these characters (Naylor, 1960; Terrell, 1968; Bulinska-Radomska & Lester, 1985; Loos, 1993). Naylor's analysis of species differentiation between *L. perenne* and *L. multiflorum* (= *L. italicum* A. Braun) indicated that interbreeding occurs easily but results in loss of fitness in the progeny. This indicates that the two species are closely allied and probably separated phylogenetically relatively recently. Morphological intergradation was also recorded between *L. perenne* and *L. multiflorum* by Bulinska-Radomska & Lester, (1985) and also between these species and *L. rigidum.* In contrast, in an analysis by Loos, (1993), the allogamous species (*L. perenne, L. multiflorum,* and *L. rigidum*) were clearly distinct from each other but the autogamous species (*L. loliaceum* Hand.-Mazz., *L. persicum, L. remotum* and *L. temulentum*) were difficult to distinguish from each other.

In the largest morphological analysis of the genus to date, where approximately 5,000 samples were examined (Terrell, 1968), taxonomic groupings within the genus were not found to be distinct from each other. However, several groupings were suggested. The first grouping included *L. persicum, L. remotum* and *L. temulentum*, which were proposed to have been derived from the same stock in southwest Asia or Central Europe (Terrell, 1968). They are only known as weeds of cultivated grasses and probably evolved alongside primitive agriculture. The second group included *L. perenne* and *L. multiflorum*. *Lolium rigidum,* divided into several elements, made up the third grouping, along with *Lolium subulatum*. One of the elements (*strictum-rottbolloides*) was a weedy group and evolved after introgression from *L. perenne* and *L. multiflorum*. *Lolium subulatum* was considered an off-shoot of this element. The

fourth group was comprised of *Lolium canariense* and was most similar to the second group. *Lolium canariense* was believed to have originated as a result of isolation after dispersal of group two or three to the North Atlantic islands. While local populations of taxa within *Lolium* had distinctive characteristics, when many populations were analysed together, the taxa were bridged by almost continuous variation. Morphological characters have also been used to study the closely related species of the *Lolium/Festuca* sect. *Bovinae* complex (Terrell, 1968; Bulinska-Radomska & Lester, 1988). In DNA-based studies, *L. perenne* and other *Lolium* species tend to group together with the broad-leaved *Festuca* species, and separate from the fine-leaved *Festuca* species (e.g. Gaut *et al.* 2000)

1.1.2 Breeding varieties of *Lolium perenne*

Commercial breeding of *L. perenne* began in the 1930's (Humphreys *et al.* 2006), with the "S" series of varieties being released from the Welsh Plant Breeding Station to support grass re-seeding. Characteristics which were important in initial breeding programmes were yield and persistency. In the late 1960's, tetraploid varieties of *L. perenne* were developed in Holland using colchicine treatment. Generally, they give better establishment than diploid varieties, higher tiller density, higher water soluble carbohydrate (WSC) content, preferential grazing, higher yield but lower persistency (Connolly, 2001). In the 1970's, while yield, persistency and disease resistance were still important, breeding focus began to switch to improved nutritional value, nitrogen use efficiency and extended seasonal growth. Also, improved yield and performance in ruminants was also a focus.

Techniques for measurement of characters of interest, such as near-infrared spectroscopy (NIRS) have been incorporated in breeding work since the 1980's and have facilitated the breeding of traits such as high *in vitro* dry matter and WSC into the new *L. perenne* varieties. Modern techniques such as detection of quantitative trait loci (QTL) and linkage mapping are being applied to detect gene regions for characters of interest and allow development of markers for marker assisted selection. Also, advances in techniques such as high-throughput genotyping and marker assisted selection (MAS) make breeding more efficient (Humphreys *et al.* 2006), particularly

for characters which are expensive to evaluate, or which occur late in the growing season.

Generally two methods of selection are used in the Teagasc grass breeding programme in Oak Park when breeding varieties in *L. perenne* – the full-sib and half-sib progeny tests (Connolly, 2001). Half-sib selection involves preliminary spaced plant selection for parents with similar heading date. Parental genotypes are then clonally propagated to provide adequate seeds for field testing. Parental genotypes are then polycrossed to generate half-sib seed, which were tested in field plots. The best are selected from the parents to develop new varieties, meanwhile the parents are maintained in clonal swards. This method is labour intensive and time consuming and the preferred method now is to use full-sib testing. In full-sib testing, source populations are pair crossed, which generates full-sib families, which are then multiplied to give enough seed for field trials. Superior pair cross families are retained and original seed is used to develop synthetic varieties. These are then sent for evaluation at international test centres and are only multiplied and marketed when they are added to national recommended variety lists. MAS and genotyping techniques could make these methods of breeding varieties much quicker and more efficient in the future because it can improve selection efficiency at each stage of the breeding cycle. The main objectives of the Teagasc grass breeding programme are: increased total annual yield, improved seasonal yield in spring and autumn, increased persistency, improved sward density, reduced stem in aftermath re-growth, and improved disease resistance (Connolly, 2001). Currently, seven cultivars of *L. perenne* are on the recommended list of grass varieties for Ireland (DAF, 2007). Since 1992, the Teagasc grass breeding programme has been in a commercial alliance with DLF-Trifolium, Denmark, which gives Teagasc a link to a wide evaluation network and research of other breeding centres.

1.1.3 Genetic resources

Germplasm collections are considered important because they conserve genetic variation within and between species and provide a source of material for exploitation. They also allow for the characterisation of plant material via, for example, taxonomy, phylogenetics, population genetics and parentage assessment. Seed collections were

initially started in the 1930's (Tanksley & McCouch, 1997) as their potential was realised by scientists. The Russian Nikolai Vavilov (1887-1943) was a botanist who collected seeds from all over the world. He set up one of the first seed banks, in Leningrad (now St Petersburg). It is now known as the Vavilov Institute of Plant Industry. Currently there are at least 700 documented seed collections in the world and they are overseen by Biodiversity International (formerly the International Plant Genetic Resources institute; www.biodiversityinternational.org). Current trends in agriculture mean that the contents of these collections are becoming more important. In modern agriculture, the high increases in yields in many important crops have been achieved through high inputs of fertiliser, chemicals and water. However, as water becomes scarcer and environmental concerns increase, such high inputs are not feasible for a sustainable agriculture (Humphreys *et al.* 2006). Exploitation of genetic resources is an alternative method to conventional breeding for the improvement of crops (Tanksley & McCouch, 1997). Domestication of plants and modern breeding has narrowed the genetic base of many crop species. Alleles are lost from varieties during this process. Such narrowing of the genetic base makes plants vulnerable to disease, pests and abiotic stresses (Harlan, 1984). These alleles can only be recovered by the reintroduction of these alleles from wild relatives of the varieties. For the use of collections in such breeding programmes to be useful, they must be characterised in an efficient manner. Traditionally, such collections have been screened for a clearly defined desirable physical character recognisable in a phenotype (Humphreys *et al.* 2006). When an accession is found with the character in question, it is crossed with elite breeding material to create a new variety. Such an approach is useful when the trait of interest is controlled by a single or small number of genes (eg. for resistance traits). However, most traits of interest to breeders (eg. yield) are controlled by many genes and so such crosses do not capture all the genetic variation connected with the phenotypic character. A more modern method of screening genetic resources is by using genetic markers to find QTL for traits of interest, and then use these QTL to develop marker assisted selection strategies (Humphreys *et al.* 2006). Genetic markers can also be utilised to select the widest genetic range of populations for addition to these collections.

The Teagasc Oak Park collection of germplasm holds 419 Irish *L. perenne* accessions collected from old Irish pasture ecosystems (Connolly, 2000). This collection was

made between the years 1980 and 1982 as part of the *Lolium* Core Collection Project which was coordinated by the European Co-operative Programme for Genetic Resources (ECPGR). The populations originated from collection sites where according to the farmer, no reseeding had been done for 50 years or more.

1.1.4 Diversity

Traditionally, characterisation of crop genetic diversity has been based on morphological traits. Morphological traits have advantages, such as being easy to detect and measure, and their relevance to germplasm users and breeders. Disadvantages, however, include complex genetic control of many morphological traits, making the morphological traits less useful for genetic diversity characterisation. Furthermore, they can be influenced strongly by environmental conditions (Lombard *et al.* 2001). However, these traits are useful especially when used in conjunction with other markers from other sources, especially DNA (Gilliland *et al.* 2000; Lisa *et al.* 2004). Biochemical characters are also used to determine genetic diversity in crops (Ougham *et al.* 1996; Gilliland *et al.* 2000) because many of these characters, such as WSC and protein, are of interest to breeders and holders of germplasm. However, biochemical characters can also be influenced strongly by environmental conditions. In contrast to morphological and biochemical traits, molecular markers based on DNA polymorphism are generally not affected by the environment. DNA characters are almost limitless for the characterization of genetic resources (a small genome like *Arabidopsis* contains c. 140 million base pairs (mbp) of DNA and rice contains 389 mbp). The genome of *Lolium* has not yet been sequenced but is estimated to contain 389mbp (International Rice Genome Sequencing Project, 2005). DNA markers also are reliable to study and efficient to obtain. The different types of DNA (nuclear, chloroplast, mitochondrial, ribosomal etc.) as well as the different type of marker system can be utilised for genetic diversity studies which have different objectives. These systems are described in detail in latter chapters of this thesis (especially Chapters 2 and 3).

1.1.5 General aims of the thesis

The primary aim of this work was to characterise the diversity of perennial ryegrass populations from Ireland and Europe as well as cultivated varieties held in the germplasm collection of Teagasc (Oak Park). Little is known about the Irish ecotype material housed at Oak Park. Chloroplast and nuclear microsatellite markers, and morphological and biochemical characters were applied and were used to determine genetic diversity, to assess the relationships between populations, to determine phylogeographic pattern, and to develop markers suitable for future plant breeding initiatives such as QTL mapping and MAS. More specifically, the objectives of this thesis were to:

(1) design and optimize a novel set of chloroplast simple sequence repeat (SSR) markers for *L. perenne* (Chapter 2) for this genetic diversity study.
(2) describe cpDNA allelic and haplotypic diversity in natural and breeding populations of *Lolium* including Irish and other European *L. perenne* ecotypes and bred *L. perenne* and ×*Festulolium* cultivars (Chapter 2).
(3) assess the potential of the set of cpSSR markers for plastid genome identification and to determine their value for plant breeding and for the definition of cytoplasmic pools (Chapter 2).
(4) determine the level of biogeographic population genetic structure in Irish and European *L. perenne* populations using cpDNA and to gain insights into their phylogeography (Chapter 2).
(5) assess morphological variation in *L. perenne*, using measurements of morphological characters from a large collection of plants using summary statistics, t-tests, ANOVA (analysis of variance) and multivariate ordination (Chapter 3).
(6) compare the morphological diversity results to geography (their provenance) and patterns of diversity determined using plastid DNA microsatellites (Chapter 3).
(7) investigate diversity of Irish *L. perenne* accessions in comparison to cultivars with respect to a number of biochemical traits, over the growing season,

including fructose, glucose, total WSC, crude protein and dry matter production (Chapter 4).

(8) test nuclear SSR markers for the characterization of variation in the collection (Chapter 5) and to obtain new markers suitable for QTL/association mapping of morphological and biochemical variation and for MAS initiatives.

(9) investigate nuclear DNA variation in a collection of *L. perenne* accessions to record the diversity of accessions, to determine the scale of differentiation among these accessions and to seek explanations for the patterns of diversity that were recorded (Chapter 5).

Two peer-reviewed publications have already been published in international journals from Chapter 2 of this thesis (McGrath et al. 2006; McGrath et al. 2007; see appendix 8.10) and others are in preparation from each of the other chapters.

Chapter 2

Characterisation of accessions of *Lolium perenne* L. and related species accessions using chloroplast SSR markers

2.1 Introduction

2.1.1 Chloroplast DNA

Plastids, which are found in all plants, contain DNA in circular molecules (Dean & Schmidt, 1995) and contain genes which are mainly used in the photosynthesis pathways (Watson & Murphy, 1993). There are many types of plastid including proplastids, etioplasts, amyloplasts, chromoplasts and chloroplasts (Neuhaus & Emes, 2000). However, the predominant plastid type in the leaf is the chloroplast and because of this the word chloroplast DNA is used widely in the literature to mean plastid, the term chloroplast is therefore generally used hereafter.

Chloroplast genomes of plants are generally uniparentally inherited, haploid, non-recombinant and have conserved gene order (Provan *et al.* 2001), making chloroplast DNA (cpDNA) a useful tool for studying inter-relationships of plants at many taxonomic levels (*e.g.* Catalan *et al.* 1997; Hodkinson *et al.* 2002; Hashimoto *et al.* 2004).

2.1.2 Chloroplast simple sequence repeat (SSR) markers

Although cpDNA generally has lower variability than nuclear DNA (Wolfe *et al.* 1987), chloroplast simple sequence repeat (cpSSR) loci have been shown to be polymorphic particularly at mononucleotide repeat loci (Powell *et al.* 1995) and can be used to investigate plant population structure, diversity and differentiation (reviewed by Provan *et al.* 2001). Chloroplastic markers have been used in *L. perenne* to study phylogenetic relationships using restriction site (Darbyshire & Warwick, 1992; Charmet *et al.* 1997; Balfourier *et al.* 2000) and DNA sequence variation (Catalan *et al.* 2004; Torrecilla *et al.* 2004). Chloroplast microsatellite (cpSSR) markers have been previously used successfully to assess variation and chloroplast

DNA (cpDNA) diversity in a range of other plant species (Provan *et al.* 2001; Harbourne *et al.* 2005; Flannery *et al.* 2006). However, there is a need for the development of highly polymorphic cpDNA markers for *Lolium* and other members of the *Festuca-Lolium* complex.

2.1.3 Use of chloroplast DNA markers to assess relationships in the *Festuca/Lolium* complex

cpDNA Restriction Fragment Length Polymorphism (RFLP) markers and DNA sequencing have been used to assess the phylogenetic relationships of *L. perenne* to other *Lolium* and *Festuca* species (Darbyshire & Warwick, 1992; Catalan *et al.* 1997; Charmet *et al.* 1997; Catalan *et al.* 2004; Torrecilla *et al.* 2004). These studies showed the separation of narrow-leaved fescues (e.g. *F. alpina*, *F. ovina*) from the broad-leaved fescues (e.g. *F. arundinacea, F. pratensis*), with *Lolium* species grouping either close to the broad-leaved fescue group (Catalan *et al.* 1997) or within this group (Darbyshire & Warwick, 1992). Within *Lolium,* self-pollinating species tend to separate phylogenetically from the open-pollinating species. In the study undertaken by Catalan *et al.* (2004), using chloroplast *trn*L-F and nuclear ribosomal ITS sequences, two autogamous species, *L. canariense* and *L. rigidum*, grouped together while the allogamous species *L. perenne* grouped with a second allogamous species, *L. multiflorum.*

2.1.4 Use of chloroplast DNA markers to characterise genetic variation in *Lolium perenne*

Genetic characterization of natural and breeding populations of *L. perenne* has so far largely utilized nuclear molecular DNA markers (e.g. Cresswell *et al.* 2001; Kubik *et al.* 2001; Bolaric *et al.* 2005a). Few studies have assessed chloroplast or mitochondrial organelle diversity partly because easily applicable organelle markers have, until recently, not been easily produced (Huang *et al.* 2002; McGrath *et al.* 2006). cpSSRs have the potential to be valuable tools for plant breeding and genetic resource characterization activities (Flannery *et al.* 2006). Chloroplast DNA variation can be used to monitor the transmission of chloroplast genomes during hybridisation and introgression (Hodkinson *et al.* 2002). cpSSRs can also be used in breeding

programmes as cultivar identifiers as an addition to nuclear DNA markers (Joshi *et al.* 1999). Furthermore, detailed characterization of plastid type is essential in studies investigating nucleo-cytoplasmic effects (Hallden *et al.* 1993) since plastid signals controlling nuclear gene expression can have both positive and negative effects on gene expression (Gray, 2005). Several chloroplast genes may have importance for genetic engineering such as those involved in synthesis of fatty acids, amino acids and vitamins (Saski *et al.* 2005) or for the directed manipulation of plant lines in breeding programmes (Daniell *et al.* 2005). cpSSR markers have not been used before to study plastid types for plant breeding purposes in *Lolium* species.

2.1.5 Use of chloroplast DNA markers to determine biogeographic patterns in *Lolium perenne*

Lolium perenne is thought to have originated in the Near East, with Europe as a secondary centre of origin (Balfourier *et al.* 2000). It has subsequently been introduced to almost all of the rest of the temperate world (Charmet *et al.* 1996). cpDNA RFLP polymorphisms have been used to assess phylogeographic structure in wild *Lolium* populations and to infer methods and pathways of geographic migration of *Lolium* populations (Balfourier *et al.* 2000). Balfourier *et al.* (2000) recognized three major clusters of haplotypes in their European sample of *Lolium*. Their results suggest a single origin for *Lolium* as well as a geographical structure following an east/west cline, matching known historical processes such as the emergence of agriculture and cereal crops from the Fertile Crescent 10,000 years ago and the spread of these crops towards Europe. As yet, cpDNA SSRs have not been used to study plastid diversity in populations of *L. perenne* or other *Lolium* species; neither have they been used to study the phylogeography of these species or *Festuca* species. Given the agronomic importance of *L. perenne* for European agriculture, migration routes from its centres of origin require investigation.

2.2 Aims

The aims of this chapter are to:

(1) design and optimize a novel set of chloroplast SSR markers for *Lolium perenne,*

(2) describe cpDNA allelic and haplotypic diversity in natural and breeding populations of *Lolium* including Irish and other European *L. perenne* ecotypes and bred *L. perenne* and ×*Festulolium* cultivars,

(3) assess the potential of the set of cpSSR markers for plastid genome identification and to assess their value for plant breeding and for the definition of cytoplasmic pools, and

(4) determine the level of biogeographic population genetic structure in Irish and European *L. perenne* populations and to gain insights into their phylogeography.

2.3 Methods

2.3.1 Selection of samples and target DNA regions for sequencing

Total genomic DNA was extracted from three *Lolium perenne* individuals (the cultivar 'Magician', and the ecotypes 2419 Roscommon and 2483 Wexford), two *Festuca arundinacea* individuals (the cultivars 'Dovey' and 'Festorina'), two *Lolium multiflorum* individuals (the cultivars 'Nival' and 'Multimo'), two *Festuca pratensis* individuals (the cultivars Barprest and Wendelmol) and a *Saccharum* sp. (accession number 108 TCD) using a modified CTAB extraction method (Doyle & Doyle, 1987). Four target chloroplast DNA regions were chosen for sequencing (*trn*L intron, *trn*L-F intergenic spacer, *rps*16 region, and *atp*B-*rbc*L intergenic spacer), and the primers of Taberlet *et al.* (1991; *trn*L and *trn*L-F region), Oxelman *et al.* (1997; *rps*16 region) and Samuel *et al.* (1997; *atp*B-*rbc*L region) were used to amplify these plastid genome regions in the individual plants.

2.3.2 Amplification of chloroplast genes

For amplification of each gene region, a master mix was prepared according to the conditions in Table 2.3.1. A volume of 1µl DNA (±100ng) was added to each tube, and an aliquot of master mix was added to the tubes to bring the volume to 50µl. The contents of the tubes were mixed using a vortex and spun down using a microcentrifuge. The samples were loaded on to a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, USA) under the conditions outlined in Table 2.3.2.

Table 2.3.1 Master mix components with volumes and concentrations for PCR amplification of chloroplast DNA regions.

Component	Volume per sample	Concentration/amount
10X PCR buffer (Promega)	5 µl	1X
DNTPs (each at 10mM)	1 µl	0.2 mM
$MgCl_2$ (25mM)	4 µl	2 mM
Forward primer (100ngul-1)	0.5 µl	50 ng (or 1ng μl^{-1})
Reverse primer (100ngul-1)	0.5 µl	100 ng/µl 50 ng (or 1ng μl^{-1})
Taq DNA polymerase (Promega) 5 $unitsul^{-1}$	0.25 µl	0.25 units
Sterile ultra-pure H_2O	37.75 µl	-
Total	49 µl	-

Table 2.3.2 Thermal cycling conditions of amplification for the chloroplast gene regions.

Process	Temperature	Time	Cycles
Denaturation	97°C	1 minute	
Annealing	52°C	1 minute	30
Extension	72°C	3 minutes	
Final extension	72°C	7 minutes	

2.3.3 Verification of amplification success using agarose gel electrophoresis

An assessment of the success of amplification of chloroplast DNA regions was performed using electrophoresis on an agarose gel (1.2% GibcoBRL) containing ethidium bromide stain. Stained DNA fluoresces in the presence of UV light (260 nm).

Protocol for verifying PCR amplification success

A 1.2% w/v agarose gel in 1x TBE[1] was prepared in a Duran bottle. 80 ml agarose gel was then aliquotted and 1µl ethidium bromide ($10mgl^{-1}$) was added and mixed with gentle swirling. The agarose mixture was allowed to cool slightly and a gel casting boat was prepared. The cooled gel was poured into the casting boat and allowed to set for 20-25 minutes. The combs and tape were removed and the gel was placed in an electrophoresis tank and covered in 1x TBE[1].

[1] TBE – contains 1M Tris pH 8, boric acid and 0.5M EDTA

4µl of amplified DNA from each sample was mixed with 1 µl loading dye[1]. A 100bp ladder (Gibco-BRL) and each PCR reaction were loaded onto the gel. The rig was connected to the power supply (EC-Apparatus Corporation EC 105 power pack) and run for approximately 20 minutes at 100V.

The gel was then removed from the rig and placed on a UV light box (Dual Intensity Transilluminator UVP) and a digital image of the gel was taken using the Scientific Imaging System from Digital Science (Kodak ID 2.0.2) gel photography software.

2.3.4 Purification of amplified gene regions

Amplified products were purified prior to cycle sequencing using the Jet Quick purification kit (Genomed). The manufacturer provided the following information regarding the provided constituents of the kit: Binding buffer (H1) contained guanidine hydrochloride and isopropanol. Wash buffer (H2) contained NaCl, Tris-HCl and EDTA.

Protocol for purification of amplified gene regions

A mixture of 400µl binding solution and 50µl sample was added to the centre of labelled spin cartridges (in 2ml wash tubes). The tubes were then centrifuged at 12,000g for 1 minute. The flow-through was discarded and 500µl wash buffer (containing ethanol) was added to the centre of the spin cartridges. The samples were centrifuged at 12,000g for 1 minute and the flow-through was again discarded. The samples were centrifuged again at 12,000g for 1 minute to remove the remaining wash buffer from the tubes. The spin cartridges were placed into labelled recovery tubes and 50µl of warm sterile ultra-pure water (65°C) was added to each to dissolve the amplified DNA. The tubes were allowed to incubate at room temperature for 1 minute and centrifuged again at 12,000g for 2 minutes. The spin cartridges were discarded and the samples labelled and kept in the freezer until required.

[1] Loading dye – 0.25% bromophenol blue,40% sucrose

2.3.5 Cycle sequencing reactions

Samples were prepared for the cycle sequencing reactions by aliquotting 3µl of the purified PCR products into labelled flat-topped tubes. A master mix containing Applied Biosystems *Taq* Dye-deoxy/terminator cycle sequencing mix V.1.1 (PINK mix[1]) and sequencing buffer along with sterile water was prepared according to Table 2.3.3. The forward and reverse primers were diluted according to Table 2.3.3 and added to separate master mixes.

Table 2.3.3 Master mix components, volumes and concentrations per sample for amplification of target DNA regions in cycle sequencing reaction.

Component	Volume
PINK mix	1µl
Sterile ultra-pure H_2O	1.8µl
Sequencing buffer[2] (10X)	3.5µl
Primer (Forward/Reverse; 5ng/mL)	0.7µl
Total volume	7µl

7µl of the master mix was added to each sample. Samples were mixed and spun down and loaded onto the Applied Biosystems 9700 thermocycler under the thermal cycling conditions outlined in Table 2.3.4.

Table 2.3.4 Thermal cycling conditions for amplification of forward and reverse sequences of target DNA regions prior to sequencing.

Temperature	Time	Cycles
96°C	10 seconds	
50°C	5 seconds	25 cycles
60°C	4 minutes	

2.3.6 Purification of products prior to sequencing

Each amplified sample was purified by mixing 50µl ethanol (EtOH; 100%) with 2µl sodium acetate (NaOAc; 3M) for each sample. 52µl of the mixture was added to each amplified sample and incubated at room temperature for 5 minutes. The samples were

[1] PINK: Big Dye® Terminator V1.1. Cycle Sequencing RR-100, Applied Biosystems, Warrington, UK
[2] 200mM Tris-HCl, 5mM $MgCl_2$ (pH 9.0)

then placed on ice for 5-10 minutes and then centrifuged at 13,000g for 25 minutes. The samples were drained onto clean tissue to remove the alcohol. 300µl EtOH (70%) was added to the pellet at the bottom of each tube. The samples were centrifuged at 13,000g for 15 minutes and drained onto clean tissue paper. The process was repeated once more. The drained tubes were placed between sheets of clean tissue paper and left overnight to ensure the remainder of the alcohol had evaporated off.

2.3.7 Preparation of samples for sequencing

Purified samples were prepared for sequencing by adding 25µl of Template Suppression Reagent (TSR; Applied Biosystems) into each tube. The contents of the tubes were mixed on a vortex and then incubated at 95°C for four minutes. The samples were cooled on ice and centrifuged down. The lids of the tubes were then removed and septa were inserted onto each tube. Samples were loaded onto an ABI prism 310 Genetic Analyser (Applied Biosystems) set to Big Dye® Terminator short-read, Run Module: Seq. Pop6 rapid (1.0mL)E using Pop6 polymer for 55 minutes per sample. The raw sequence data was automatically saved and compiled using Sequence Analysis Version 3.4.1 (Applied Biosystems).

2.3.8 Assembling of DNA sequences

Forward and reverse sequences from each sample were assembled to form a contig and so that any ambiguities in the sequence could be rectified. This was done by importing sequences from both directions (forward and reverse) into AutoAssembler Version 2.0 (Applied Biosystems). The initial and final few base pairs (10-20bp) of each sequence were deleted due to their unreliability and a contig sequence was produced by combining the sequences from both directions and checking ambiguities against each other.

2.3.9 Alignment of DNA sequences

The contig sequence from each sample at each of the gene regions was then aligned with other samples from the same region to form a matrix. The alignment of samples from the same gene region was carried out using a combination of visual alignment

using PAUP 4 (Swofford, 2003) and the alignment software Seq-Al V2.0a1 (Rambout, 1999), which was used to convert sequences into Nexus format. Sequences from GenBank (NCBI database) from other chloroplast DNA regions were included in the data set. ClustalX V1.8 (Thompson *et al.* 1997) was used to compile the sequences and align them to each other automatically. The aligned sequences were imported into PAUP 4 (Swofford, 2003) and checked by eye and alterations were made where required. Table 2.3.5 lists the samples and source of each gene sequence used for alignment.

Table 2.3.5 Gene regions, species names, and accession numbers for aligned sequences.

Gene Region	Species	NCBI accession number	Reference
*trn*L intron and *trn*L- F intergenic spacer region	*Festuca arundinacea*	-	This study
	Festuca pratensis	-	"
	Lolium multiflorum	-	"
	Lolium perenne	DQ123585	"
*rps*16	*Festuca arundinacea*	-	This study
	Festuca pratensis	-	"
	Lolium multiflorum	-	"
	Lolium perenne	DQ131606	"
*atp*B-*rbc*L	*Festuca arundinacea*	-	This study
	Festuca pratensis	-	"
	Lolium multiflorum	-	"
	Lolium perenne	DQ123586	"
16S	*Aegilops speltoides*	AJ555401	Rudnoy *et al.* (2004)
	Aegilops tauschii	AJ555402	,,
	Triticum turgidum	AJ555400	"
	Triticum aestivum	AJ239003	Kovacs *et al.* (2000)
*ndh*F	*Poa pratensis*	U21980	Clark *et al.* (1995)
	,,	AF267706	Redinbaugh *et al.* (2000)
	Poa angustifolia	U71010	Catalan *et al.* (1997)
	Sesleria argentea	U71011	,,
	Deschampsia cespitosa	U71012	,,
	Festuca arundinacea	U71013	,,
	Lolium perenne	U71014	,,
	Festuca rubra	U71015	,,
	Dactylis glomerata	U71016	,,

	Poa fendleiana	AF236868	Larsen *et al.* unpublished
	Poa secunda	AF236867	„
*rpl*2-*trn*H	*Zea mays*	X53066	Kavousi *et al.* (1990)
	„	X12851	Bowman *et al.* (1988)
	„	X62070	Hoch *et al.* (1991)
	„	AY044158	Adams *et al.* (2001)
	Oryza sativa	L40578	Moon & Wu (1988)
	„	M22826	„
	„	X12844	Moon *et al.* (1988)
	Hordeum vulgare	X78185	Hess *et al.* (1994)
	Triticum aestivum	AJ295995	Subramanian *et al.* unpublished
23S-5S	*Poa pratensis*	L41587	Goremykin *et al.* (1996)
	"	L29442	Bobrova *et al.* unpublished
*trn*H-*psb*A	*Secale cereale*	X13327	Kolosov *et al.* (1989)
	Triticum aestivum	M12352	Hanleybowdoin & Chua (1988)
	Hordeum vulgare	M38374	Efimov *et al.* (1988a)
	"	X07942	Boyer & Mullet (1988)
	"	X07521	Efimov *et al.* (1988b)
	Pharlaris minor	AY294643	Tripathi *et al.* unpublished
	"	AY211527	"
	Oryza sativa	NM_197617	Yu *et al.* (2003)
	"	M36191	Wu *et al.* (1987)
	Zea mays	AF543684	Netto *et al.* unpublished
	"	M27567	Sederoff *et al.* (1986)
	Poa annua	AF131886	Mengistu *et al.* (2000)
	"	AF131887	"
	Lolium perenne	AF363674	Larsen, unpublished
	Phragmites australis	AY016310	Saltonstall,

			(2001)
*psb*B	*Secale cereale*	X07672	Bukharov *et al.* (1988)
*pet*B	*Hordeum vulgare*	X14107	Reverdatto *et al.* (1989)
*trn*V	*Pennisetum glaucum*	AY694130	Nallar *et al.* unpublished
*rpo*A-*pet*D spacer	*Psathyrostachys stoloniformis*	Z77754	Petersen & Seberg (1997)
*rpo*C2	*Nardus stricta*	L25382	Cummings *et al.* (1994)

2.3.10 Identification of chloroplast microsatellite regions

Chloroplast DNA regions were searched for microsatellite motifs using a modified version of the MISA perl script (www2.unil.ch/software/). Microsatellite motifs in gene regions where there was more than one sequence were checked for variability (by comparing the aligned sequences). Possible polymorphic regions suitable for marker development were noted.

2.3.11 Primer design

Conserved regions flanking actual or possible polymorphic microsatellite regions were identified and searched for suitable primers using the web-based PRIMER 3 software (Rozen & Skaletsky, 2003). Suitable primers were chosen based on the following guidelines (Loffert *et al.* 1997; Sambrook & Russell, 2001):

- *Primer length:* Each primer should be between 18 – 30 base pairs long. This should be long enough to allow the amplification of an unique template sequence. Members of a primer pair should not have more than 3 base pairs difference in length.
- *Base composition:* G/C composition should be between 40 – 60% with an even distribution of bases along the length of the primer.
- *3' terminal sequence:* A run of more than 3 G/C bases should be avoided as this can cause non-specific annealing. A thymidine base at the 3' end should be avoided as this can cause mis-priming. NNGC or NNCG terminal sequences should be avoided as this promotes the formation of hairpin structures and primer dimers.

- *Melting temperatures (T_m):* T_m is calculated using the following equation:

$$T_m = 2(A + T) + 4(G+C)$$

Equation 2.3.1 Calculation of the melting temperature of primer sequences (Sambrook & Russell, 2001).

T_m values of pairs of primers should not differ by >5°C. The annealing temperature of the PCR product is generally 5°C lower than the calculated T_m value.

- *Complementarity/Self-complementarity:* Inverted repeat sequences or self-complementary sequences should be avoided as these can cause formation of hairpin structures. 3' terminal sequences on one member of a primer pair should not be complementary to any part of the sequence of the other member of the primer pair as this can cause primer dimers.

The primers that were designed are outlined in Table 2.3.6. Forward primers were taken directly from the DNA sequence, while reverse primers were the reverse and complement of the 3' end of the forward strand.

Table 2.3.6 Sequences, accession numbers and gene regions of primers designed for the amplification of chloroplast microsatellites.

SSR ID	Accession No.	Gene region	Primer Sequence
TeaCpSSR1F	DQ123586	*atp*B-*rbc*L	ATTGATTTGGGTTGCGCTAT
TeaCpSSR1R			TCATTAAAGAAAATTGAGGGCA TA
TeaCpSSR2F	DQ123585	*trn*L and *trn*L-F intergenic spacer region	TCCATTCCAATTGAATATTTTGT
TeaCpSSR2R			AGTCCCTTTATCCCCAAACC
TeaCpSSR3F	DG123585	*trn*L and *trn*L-F intergenic spacer region	GCAAACGATTAATCATGGAACC
TeaCpSSR3R			TTGTGAGGGTTCAAGTCCCT
TeaCpSSR4F	L41587	23S-5S ITS region	ACGAACGAACGATTTGAACC
TeaCpSSR4R			TGAAGCCCCAATTCTTGACT
TeaCpSSR5F	AF363674	*psb*A	GCTATGCATGGTTCCTTGGT
TeaCpSSR5R			TTCCTACTACAGGCCAAGCAG
TeaCpSSR6F	AY694130	*trn*V	CGGATTCTAACCGTAGACCTTC
TeaCpSSR6R			TCAAAGCCAGGAAGCAATCT
TeaCpSSR7F	X53066	*trn*H	GGAATTTGCAATAATGCGATG

TeaCpSSR7R			TCGATCGAGGTATGGAGGTC
TeaCpSSR8F	Z77754	*rpo*A-*pet*D intergenic spacer	TTGACAGTTTTCGTATGGAAGA
TeaCpSSR8R			GATTGTGCCAAAGATGCAAA
TeaCpSSR9F	DQ123585	*trn*L and *trn*L-F intergenic spacer region	AACCCGGTTTTTCGGTTTAT
TeaCpSSR9R			TCCTGACCTTTTCTTGTGCAT
TeaCpSSR10F	DQ131606	*rps*16	TTCGCTCGAAATGAGACAAA
TeaCpSSR10R			CCTCATACGGCTCGAGAAAA
TeaCpSSR11F	L25382	*rpo*C2	GCTTATTTTGACGATCCACGA
TeaCpSSR11R			TGGGCTGCCATACTCTTCTT
TeaCpSSR12F	U71014	*ndh*F	TGGAGCAATGGATAATGGAA
TeaCpSSR12R			CCGCGATTATATGACCAACTG
TeaCpSSR13F	DQ123586	*atp*B-*rbc*L	TGGTAAATCAAATCCACGGTTTA
TeaCpSSR13R			GGCGTAAATCCAACTTTAGCA
TeaCpSSR14F	DQ123585	*trn*L and *trn*L-F intergenic spacer region	TACCAAAGGATCCGGACAAA
TeaCpSSR14R			TCCTGACCTTTTCTTGTGCAT
TeaCpSSR15F	DQ123585	*trn*L and *trn*L-F intergenic spacer region	TCCATTCCAATTGAATATTTTGT
TeaCpSSR15R			TCAAGTCCCTTTATCCCCAA
TeaCpSSR16F	DQ131606	*rps16*	TGAAGTCCTCTCCACCTCAAA
TeaCpSSR16R			GGGACCGAGGTAGATAAATAAC G
TeaCpSSR17F	DQ131606	*rps16*	GCTCTTGGCTCGCAATAGTC
TeaCpSSR17R			CCTATCTTCAAAAAGAGGGCTTT C
TeaCpSSR18F	DQ131606	*rps16*	GCTCTTGGCTCGCAATAGTC
TeaCpSSR18R			TTGGCCATTTTTATTAGTTTTCA
TeaCpSSR19F	DQ131606	*rps16*	TCTATTCCTCCCGAACCAAA
TeaCpSSR19R			CCTATCTTCAAAAAGAGGGCTTT C
TeaCpSSR20F	U71014	*ndh*F	CAACAACAAAGAATGGAGTTGC
TeaCpSSR20R			TTCCATTATCCATTGCTCCA
TeaCpSSR21F	X13327	*psb*A	GGAAAGGCAATTCTTGCATC
TeaCpSSR21R			TTCTCCAGCATTTGATTCCTT
TeaCpSSR22F	X07672	*psb*B	TCATCATATTGCTGCGGGTA
TeaCpSSR22R			TAATTCGATTGGGGTCGTTG
TeaCpSSR23F	X14107	*pet*B	GTAGTTCGACCGCGGAATTT
TeaCpSSR23R			CAGTCTGGTTGCGAGGTCTT
TeaCpSSR24F	AY694130	*trn*V	GCAATCGATCGTCGAGTTTA
TeaCpSSR24R			TGTTGGGTTTTTGAAACAGG
TeaCpSSR25F	AY016310	*trn*H-*psb*A intergenic spacer	TGGACATAGGATGCCACTCTT
TeaCpSSR25R			ATTGTATGGCCAACCATTGC
TeaCpSSR26F	M22826	*trn*H-*rpl*22 intergenic spacer region	CGGCATTTCACGAGTTATGA
TeaCpSSR26R			AAGGTTATTCCCCGCTTACC

2.3.12 Primer testing – unlabelled primers

Primer testing was carried out on each primer pair. A master mix was prepared according to the conditions in Table 2.3.7. A volume of 1µl DNA (±100ng) of three samples (from population 2250 Tipperary) was added to each tube. For each primer, 1µl of sterile ultrapure water was used instead of DNA as a negative control sample. An aliquot of master mix was added to the tubes to bring the volume to 10µl. The contents of the tubes were mixed using a vortex and spun down using a microcentrifuge. The samples were loaded on to a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, USA) under the cycling conditions outlined in Table 2.3.8.

Table 2.3.7 Master mix components with volumes and concentrations for initial PCR testing of unlabelled primer pairs.

Component	**Volume per sample**	**Concentration/amount**
10X PCR buffer (Promega)	1 µl	1X
dNTPs	1.25 µl	5mM
Forward primer	0.25 µl	100 ng/ µl
Reverse primer	0.25 µl	100 ng/ µl
Taq DNA polymerase (Promega)	0.2 µl	0.25 units
Sterile ultra-pure H_2O	6.05 µl	-
Total volume	9 µl	

Table 2.3.8 PCR amplification conditions for initial testing of unlabelled microsatellite primer pairs.

Process	**Temperature**	**Time**	**Cycles**
Initial denaturation	95°C	5 minutes	
Denaturation	95°C	1 minute	
Annealing	60°C	1 minute	35
Extension	72°C	1 minute	
Final extension	72°C	10 minutes	

Amplification success was determined by agarose gel electrophoresis as outlined in section 2.3.3.

Primer pairs which were not shown to successfully amplify DNA in the first test were then tested under varying $MgCl_2$ concentrations. A master mix was prepared according to the conditions in Table 2.3.9. A volume of 1µl DNA (±100ng) of two

samples A and B (from population 2250 Tipperary) was added to each tube. For each primer and each $MgCl_2$ concentration, 1µl of sterile ultrapure water was used instead of DNA as a negative control sample. An aliquot of master mix was added to the tubes to bring the volume to 10µl. The contents of the tubes were mixed using a vortex and spun down using a microcentrifuge. The samples were loaded on to a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, USA) under the conditions outlined in Table 2.3.10.

Table 2.3.9 Master mix components with volumes and concentrations for PCR testing of unlabelled primer pairs over a magnesium gradient.

Component	Volume per sample	Concentration
10X PCR buffer (Promega)	1 µl	
dNTPs	1.25 µl	5mM
$MgCl_2$	0.2 µl or 0.4 µl	2.5mM or 3mM
Forward primer	0.25 µl	100ng/ µl
Reverse primer	0.25 µl	100ng/ µl
Taq DNA polymerase (Promega)	0.2 µl	0.25 units
Sterile ultra-pure H_2O	5.85 µl	-
Total volume	9 µl	

Table 2.3.10 Thermal cycling conditions of amplification for testing of unlabelled primer pairs over a magnesium gradient.

Process	Temperature	Time	Cycles
Initial denaturation	95°C	5 minutes	
Denaturation	95°C	1 minute	
Annealing	60°C	1 minute	35
Extension	72°C	1 minute	
Final extension	72°C	10 minutes	

Amplification success was determined by agarose gel electrophoresis as outlined in section 2.3.3.

Each primer pair, with no successful amplification tested with varied $MgCl_2$ concentrations, was tested a second time on a temperature gradient from 45°C to 65°C. A master mix was prepared according to the conditions in Table 2.3.11. A volume of 1µl DNA (±100ng) of one sample A (from population 2250 Tipperary) was added to each tube. For each primer and each temperature, 1µl of sterile ultrapure water was used instead of DNA as a negative sample. An aliquot of master mix was

added to the tubes to bring the volume to 10µl. The contents of the tubes were mixed using a vortex and spun down using a microcentrifuge. The samples were loaded on to a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, USA) under the conditions outlined in Table 2.3.12.

Table 2.3.11 Master mix components with volumes and concentrations for PCR testing of unlabelled primer pairs over different temperatures.

Component	Volume per sample	Concentration
10X PCR buffer (Promega)	1 µl	
dNTPs	1.25 µl	5mM
Forward primer	0.25 µl	100ng/ µl
Reverse primer	0.25 µl	100ng/ µl
Taq DNA polymerase (Promega)	0.2 µl	0.25 units
Sterile ultra-pure H_2O	6.05 µl	-
Total volume	9 µl	

Table 2.3.12 Conditions of amplification for testing of unlabelled primer pairs over different temperatures.

Process	Temperature	Time	Cycles
Initial denaturation	94°C	3 minutes	
Denaturation	94°C	1 minute	
Annealing	45°C - 65°C	1 minute	35
Extension	72°C	1 minute	
Final extension	72°C	5 minutes	

Amplification success was determined by agarose gel electrophoresis as outlined in section 2.3.3.

2.3.13 Plastid microsatellite primer testing – labelled primers

Based on the tests of the unlabelled primer pairs, twelve primer pairs were ordered as fluorescently labelled primer pairs. The final master mix conditions are given in Table 2.3.13. Final $MgCl_2$ concentrations and annealing temperatures for each of the twelve fluorescently labelled primer pairs are given in Table 2.3.14.

Table 2.3.13 Final master mix components with volumes and concentrations for PCR testing of labelled plastid microsatellite primer pairs.

Component	Volume per sample	Concentration
10X PCR buffer (Promega)	1 µl	
dNTPs	1.25 µl	5mM
$MgCl_2$	0 µl /0.2 µl/0.4 µl	2mM/2.5mM/3mM
Forward primer	0.25 µl	100ng/ µl
Reverse primer	0.25 µl	100ng/ µl
Taq DNA polymerase (Promega)	0.2 µl	0.25 units
Sterile ultra-pure H_2O	6.05 µl/5.85 µl/5.65 µl	-
Total volume	9 µl	

Table 2.3.14 Final $MgCl_2$ concentrations, annealing temperatures and fluorescent labels, determined to be optimal for amplification, for twelve labelled plastid microsatellite primer pairs.

Primer	Fluorescent Label	$MgCl_2$ concentration	Annealing Temperature
TeaCpSSR1	VIC™	2.5mM	60
TeaCpSSR2	NED™	2.5mM	45
TeaCpSSR3	FAM™	3mM	60
TeaCpSSR4	NED™	2mM	60
TeaCpSSR5	VIC™	2.5mM	60
TeaCpSSR6	PET™	2mM	45
TeaCpSSR7	NED™	2.5mM	60
TeaCpSSR8	PET™	2.5mM	45
TeaCpSSR9	FAM™	2.5mM	45
TeaCpSSR10	PET™	2.5mM	60
TeaCpSSR11	FAM™	3mM	60
TeaCpSSR12	VIC™	2.5mM	60

The final thermalcycling conditions are given in Table 2.3.15.

Table 2.3.15 Final thermal cycling conditions for amplification of labelled primer pairs.

Process	Temperature	Time	Cycles
Initial denaturation	95°C	5 minutes	
Denaturation	95°C	1 minute	
Annealing	45°C/60°C	1 minute	35
Extension	72°C	1 minute	
Final extension	72°C	10 minutes	

2.3.14 Analysis of plastid microsatellite amplification products

Amplified PCR products heated at 65°C for 30 minutes on a Px2 Thermal Cycler (Thermo Electron Corporation) to avoid non-uniform PolyA tails. PCR products were diluted in sterile ultrapure water to achieve optimum fluorescence intensities for runs on the ABI3100 (Table 2.3.16). Four primer pairs with different fluorescent labels could be pooled together for a single microsatellite analysis run on the ABI3100. Final volumes pooling of PCR products were given in Table 2.3.16.

Table 2.3.16 Final dilution factors and pooling amounts for twelve fluorescently labelled primer pairs.

Primer	Group	Dilution factor	Pooling volume
TeaCpSSR1	1	1 in 20	2 µl
TeaCpSSR2	3	1 in 40	2 µl
TeaCpSSR3	3	1 in 30	1 µl
TeaCpSSR4	1	1 in 20	2 µl
TeaCpSSR5	3	1 in 20	0.5 µl
TeaCpSSR6	1	1 in 10	0.5 µl
TeaCpSSR7	2	1 in 40	2 µl
TeaCpSSR8	3	1 to 1	2 µl
TeaCpSSR9	1	1 in 10	1 µl
TeaCpSSR10	2	1 in 10	0.5 µl
TeaCpSSR11	2	1 in 30	1 µl
TeaCpSSR12	2	1 in 20	2 µl

0.5 µl of the pooled amplification products were added to 9.5 µl of a LIZ 500 internal sizing standard:Formamide (CH_3NO) mix (1:36). The samples were denatured on a heating block for 10 minutes at 95°C, transferred to an ice tray for two minutes and centrifuged. PCR products were sized using LIZ 500 sizing standard on an ABI 3100 automated DNA sequencer with Data Collection Software version 1.0 (Applied Biosystems). PCR products were sized using GENEMAPPER™ version 3.7 software (Applied Biosystems).

2.3.15 Testing of primer pairs for cross-amplification across the Poaceae

The twelve labelled primer pairs were used to test for cross-amplification in the Poaceae. 51 species were chosen to represent nine of the ten subfamilies of the Poaceae (Appendices 8.1 and 8.2).

A master mix was prepared according to the conditions in Table 2.3.13 with the $MgCl_2$ concentrations and annealing temperatures for each primer pair given in Table 2.3.14. A volume of 1µl DNA (±100ng) of each sample was added to each tube. An aliquot of master mix was added to the tubes to bring the volume to 10µl. The contents of the tubes were mixed using a vortex and spun down using a microcentrifuge. The samples were loaded on to a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, USA) under the conditions outlined in Table 2.3.15. Amplified products were analysed as described in Section 2.3.14. Size ranges and allele numbers were determined for amplification products of each primer pair.

2.3.16 Selection of samples for analysis

In total, 104 grass accessions were studied in the main analysis (Appendices 8.1 and 8.2). 78 *L. perenne* accessions were included. These 78 accessions consisted of 30 Irish *L. perenne* ecotypes, 32 European *L. perenne* ecotypes and 16 commercial *L. perenne* varieties (*cv.*). In addition, eleven other *Lolium* species, six *Festuca* species and nine ×*Festulolium* varieties were used. The term ecotype is used broadly in this thesis to define locally adapted populations. The Irish ecotypes were selected from the Teagasc Oak Park collection holding 419 Irish *L. perenne* accessions collected from old Irish pasture ecosystems (Connolly, 2000). This collection was made between the years 1980 and 1982 as part of the *Lolium* Core Collection Project which was coordinated by the European Co-operative Programme for Genetic Resources (ECPGR). The populations originated from collection sites where, according to the farmer, no reseeding had been done for 50 years or more. For this study, accessions were selected from the Teagasc Oak Park collection to cover a wide spread of diverse geographic regions from the Republic of Ireland. The other European accessions were selected to provide a wide European geographic coverage to allow genetic diversity

comparisons to be made with the rest of Europe and to help address possible migration routes of *L. perenne*.

2.3.17 Growth of plant material

Seeds were germinated on seed testing paper, seedlings transferred to pots and the plants were raised in the greenhouse. The ecotype accessions and cultivated varieties were subsequently planted as spaced plants in the field at Oak Park.

2.3.18 Harvesting and extraction of DNA from plant material

Leaf material from each plant was harvested into 96-well deep well plates. The samples were dried using a freeze drier (Ilshin Lab. Co. Ltd.). A single 5mm glass bead was placed in each well and the samples were then ground with a bead mill (Retsch MM300). DNA was extracted from the samples using a magnetic bead based method (Qiagen MagAttract Plant DNA Core Kit) which had been modified for use on the HamiltonStar automated liquid handling system.

Protocol for extraction of DNA

600 µl Buffer RLT was added to ground leaf material in each well of a 96-well deep well plate in order to lyse the cells. The plate was sealed and shaken in an upright position 20 times back and forth. The plate was then centrifuged at 2,000xg for five minutes.

65 µl of Buffer RB was added to each well of a 96-well microplate. 20 µl resuspended MagAttract Suspension A was added to each well of the 96-well microplate containing the Buffer RB. 200 µl of each supernatant was transferred to the 96-well microplate containing MagAttract Suspension A and Buffer RB. The samples were mixed thoroughly by pipetting up and down several times. The samples were incubated at room temperature for two minutes and then shaken on the robotic plate shaker for a further two minutes. The 96-well microplate was then transferred onto a magnet and allowed to incubate for twenty seconds in order to separate the MagAttract particles. The supernatant was then removed. 200 µl isopropanol and 3.52 µl of RNase A was added to Buffer RPW. 200 ul of this was added to each well of the

96-well microplate and the pelleted MagAttract particles were thoroughly resuspended by pipetting up and down. The microplate was transferred to the magnet, the MagAttract particles allowed to separate for 20 seconds and the supernatant removed. 200 µl ethanol (100%) was added to each well of the microplate and the MagAttract pellets were resuspended thoroughly by pipetting up and down. The microplate was placed on the magnet, the MagAttract particles were allowed to separate for 20 seconds and the supernatant was removed. A second ethanol wash was performed following the same steps. The plate was incubated at 60°C for 10 minutes to ensure the removal of any remaining alcohol. 100 µl AE buffer was added to each well of the microplate, mixed by shaking on the robotic shaker plate and incubated at room temperature for five minutes. The microplate was placed on the magnet, the MagAttract particles allowed to separate for 20 seconds and the supernatant removed to a clean 96-well microplate.

Samples were amplified according to the methods described in Section 2.3.15 and amplified products were analysed according to the methods described in Section 2.3.14.

2.3.19 Data analysis

Allelic and haplotypic variation

Allele numbers and size ranges were calculated for each accession. Haplotype numbers (including numbers of unique and shared haplotypes) were calculated for each accession.

Genetic distance between populations

A genetic distance matrix (Appendix 8.3) was calculated based on Nei's standard genetic distance measure (Nei, 1972), using allele data (characters) without size information. This measure calculates the genetic distance between populations in terms of the number of gene substitutions between loci according to the Equation 2.3.2 (Nei, 1972).

$$D_S = -\log(1 - d_{XY}) + [\log(1 - d_X) + \log(1 - d_Y)]/2$$

Equation 2.3.2 Standard genetic distance (D_S), where the quantities d_X, d_Y, and d_{XY} are the probabilities that two alleles are different when randomly drawn from two different populations (d_{XY}) or from the same population (d_X, and d_Y).

$$d_X = \sum_{i \neq j} \sum x_i x_j$$

$$d_Y = \sum_{i \neq j} \sum y_i y_j$$

$$d_{XY} = \sum_{i \neq j} \sum x_i y_j$$

Where x_i and y_j are the frequencies of the ith and jth alleles at a locus in populations X and Y respectively. To extend this measure of multiple loci, averages of d_X, d_Y, and d_{XY} are taken over all loci.

Distances based on a stepwise mutation model such as the delta mu-squared distance (D_{DM}; Goldstein *et al.* 1995; Flannery *et al.* 2006) were not used because the size variation of alleles could be attributed to both SSR length variation and other types of substitutions (non-SSR indels).

From this matrix, a dendrogram showing the similarities between populations was constructed using the unweighted pair group method using arithmetic means (UPGMA) method (Sneath & Sokal, 1973) as implemented in POPGENE (Yeh & Boyle, 1997). Bootstrapping analysis was performed on the UPGMA data with 1,000 replicates as implemented in NTSYSpc V2.2 software (Rohlf, 2005).

Mantel test

A geographic distance matrix was constructed (Appendix 8.4), using the 56 accessions where an exact geographical origin was known. A Mantel test was used to correlate the pairwise comparisons in the geographic distance matrix and the genetic distance matrix using NTSYSpc V2.2 (Rohlf, 2005). A total of 10,000 permutations were employed to test for significance.

Mantel's test is based on a simple cross product term:

$$z = \sum_{i=1}^{n} \sum_{j=1}^{n} x_{ij} y_{ij}$$

and is normalized (Equation 2.3.3) to rescale the statistic to the range of a conventional correlation coefficient ($-1 \leq r \leq 1$).

$$r = \frac{1}{(n-1)} \sum_{i=1}^{n} \sum_{j=1}^{n} \frac{(x_{ij} - \bar{x})}{s_x} \cdot \frac{(y_{ij} - \bar{y})}{s_y}$$

Equation 2.3.3 Mantel's statistic where x and y are variables measured at locations i and j and n is the number of elements in the distance matrices ($=m(m-1)/2$ for m sample locations), and s_x and s_y are standard deviations for variables x and y.

Genetic diversity measures

Nei's (1973) gene diversity measure was calculated for each population according to Equation 2.3.4.

$$h = 1 - \sum x_j^2$$

Equation 2.3.4 Nei's (1973) gene diversity measure, h, where x_j is the frequency of the jth allele at each locus. Over multiple loci, an average value was taken.

Nei's gene diversity is a measure of the probability that two copies of the same gene (in this case microsatelite alleles from a particular locus) chosen at random in a population will have different alleles.

The total diversity (H_T), the mean within population diversity (H_S), and the coefficient of variation (G_{ST}) were calculated following the procedures of Nei (1987) for subdivided populations according to Equations 2.3.5 to Equation 2.3.10 (Nei, 1987) using the programme POPGENE (Yeh & Boyle, 1997).

$$J_T = \left(\sum J_k + \sum_{k \neq l} J_{kl} \right) / s^2$$

Equation 2.3.5 Gene identity in the total population, J_T, where $J_{kl} = \sum_l x_{ki} x_{li}$ is the gene identity between the kth and lth subpopulations.

$$J_S = \sum J_k / s = \sum \overline{x_i^2}$$

Equation 2.3.6 Average gene identity within subpopulations, J_S.

$$D_{ST} = \sum_k \sum_l D_{kl} / s^2$$

Equation 2.3.7 Average gene diversity between subpopulations, D_{ST}, including the comparisons of the subpopulations themselves.

$$H_S = 1 - J_S$$

Equation 2.3.8 Average gene diversity within subpopulations, H_S.

$$H_T = H_S + D_{ST}$$

Equation 2.3.9 Gene diversity in the total population, H_T.

$$G_{ST} = D_{ST}/H_T$$

Equation 2.3.10 Nei's coefficient of differentiation, G_{ST}, the relative magnitude of gene differentiation among subpopulations.

AMOVA analyses

An analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) was carried out with ARLEQUIN 2.0 software (Schneider *et al.* 2000) based on the number of different haplotypes, which is the equivalent of a weighted F_{ST} over all loci when estimating genetic structure (Weir & Cockerham, 1984; Michalakis & Excoffier, 1996). The level of significance for variance component estimates was calculated by non-parametric permutation procedures using 1,000 permutations. The data were partitioned in several combinations to display among and within population variation of Irish and European *L. perenne* accessions, to assess biogeographic differentiation and to address possible migration routes of *L. perenne*.

AMOVAs were calculated to test for evidence of geographic patterns of genetic structuring. In addition we used AMOVAs to test if there was evidence of migration of *Lolium* (a) following a Mediterranean route; this involved comparisons of Near

Eastern vs. Southern European and Southern European vs. Western European *L. perenne* accession groups, (b) following a Danubian migration route; this involved comparisons of Near Eastern vs. Southern European, Southern European vs. Eastern European, and Eastern European vs. Northern European *L. perenne* accession groups, (c) following a Northern African route, this involved comparisons of Near Eastern vs. Northern African and Northern Africa vs. Southern European *L. perenne* accession groups, (d) following a northerly retreat route of the glaciers after the last ice age; this involved comparison of all *L. perenne* ecotype groups south of the Alps (Near East, North Africa and Southern Europe) against all *L. perenne* ecotype groups North of the Alps (Northern Europe, Eastern Europe, Western Europe, Ireland), and (e) following routes into Ireland consistent with migration from neighbouring geographical regions; this involved comparisons of Irish *L. perenne* ecotypes with three European accession groups (Southern Europe, Western Europe and Northern Europe).

Shared haplotypes

An Edward's Venn diagram was constructed to display shared haplotypes between six accession groups: Irish *L. perenne* ecotypes, European/Near Eastern *L. perenne* ecotypes, *L. perenne* cultivars, other *Lolium* species, ×*Festulolium* cultivars and *Festuca* species.

Geographical groupings

From the genetic distance matrix, a dendrogram showing the similarities between six major groups of accessions (Irish *L. perenne* ecotypes, European/Near Eastern *L. perenne* ecotypes, divided into six geographical groups, *L. perenne* cultivars, other *Lolium* species, ×*Festulolium* cultivars and *Festuca* species) was constructed using the unweighted pair group method using arithmetic means (UPGMA) method (Sneath & Sokal, 1973) as implemented in POPGENE (Yeh & Boyle, 1997). Bootstrapping analysis was performed on the UPGMA data with 1,000 replicates as implemented in NTSYSpc V2.2 software (Rohlf, 2005).

2.4 Results

2.4.1 Sequencing of chloroplast gene regions

Amplification of the three gene regions was successful for all but three of the samples used (*Lolium perenne* 'Magician', *trn*L-F; *Festuca arundinacea* 'Festorina', *rps*16; *Festuca pratensis* 'Barpresto', all gene regions)

Lengths of sequences for each samples are given in Table 2.4.1. Sequences are given in Appendix 8.5.

Table 2.4.1 Length of sequences obtained in each gene region.

Species	**Sample**	***trn*L-F intergenic spacer region**	***rps*16**	***atp*B-*rbc*L intergenic spacer region**
Lolium perenne	2419 Roscommon	955	775	803
Lolium perenne	2483 Wexford	952	540	1018
Lolium perenne	Magician	-	766	801
Lolium multiflorum	Nival	950	753	792
Lolium multiflorum	Multimo	929	537	809
Festuca arundinacea	Festorina	943	749	-
Festuca arundinacea	Dovey	950	770	806
Festuca pratensis	Wendelmold	963	541	808
Festuca pratensis	Barpresto	-	-	-
Saccharum sp.	108	862	566	787

- : No amplification

2.4.2 Testing of primers

The unlabelled primer pairs were first tested in a general PCR programme and checked for successful amplification using agarose gel electrophoresis (Figures 2.4.1 and 2.4.2). Successful amplification was seen in three out of twenty six primer pairs (TeaCpSSR1, TeaCpSSR5 and TeaCpSSR13).

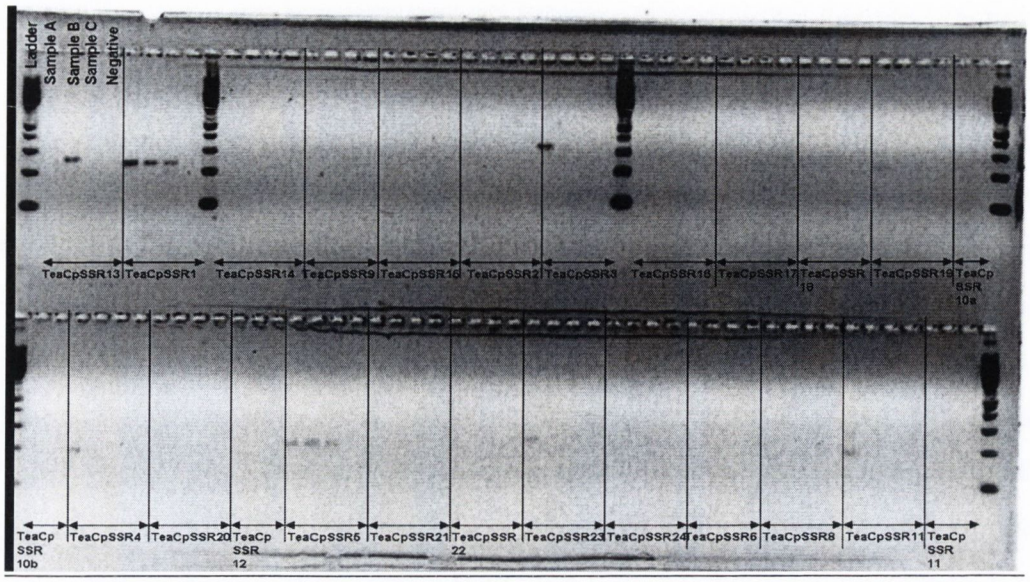

Figure 2.4.1 3% Agarose gel electrophoresis image of plastid SSR PCR products with 24 of 26 unlabelled primer pairs TeaCpSSR1 – TeaCpSSR6, TeaCpSSR8 - TeaCpSSR25. Ladder: 100bp ladder. 1.5% TBE. Sample A, B, C: individuals from population 2250 Tipperary.

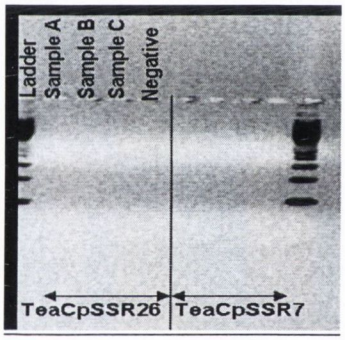

Figure 2.4.2 3% Agarose gel electrophoresis image of plastid SSR PCR products with the remaining two unlabelled primer pairs TeaCpSSR7 and TeaCpSSR26. Ladder: 100bp ladder. 1.5% TBE. Sample A, B, C: individuals from population 2250 Tipperary.

The remaining 23 primer pairs were then tested over two different $MgCl_2$ concentrations and checked for successful amplification using agarose gel electrophoresis (Figures 2.4.3 and 2.4.4). Successful amplification was seen in four primer pairs for both concentrations (TeaCpSSR3, TeaCpSSR4, TeaCpSSR7 and TeaCpSSR23; Figures 2.4.3 and 2.4.4) and in five primer pairs for one concentration (TeaCpSSR10, TeaCpSSR12, TeaCpSSR19, TeaCpSSR20, and TeaCpSSR 22; Figures 2.4.3 and 2.4.4).

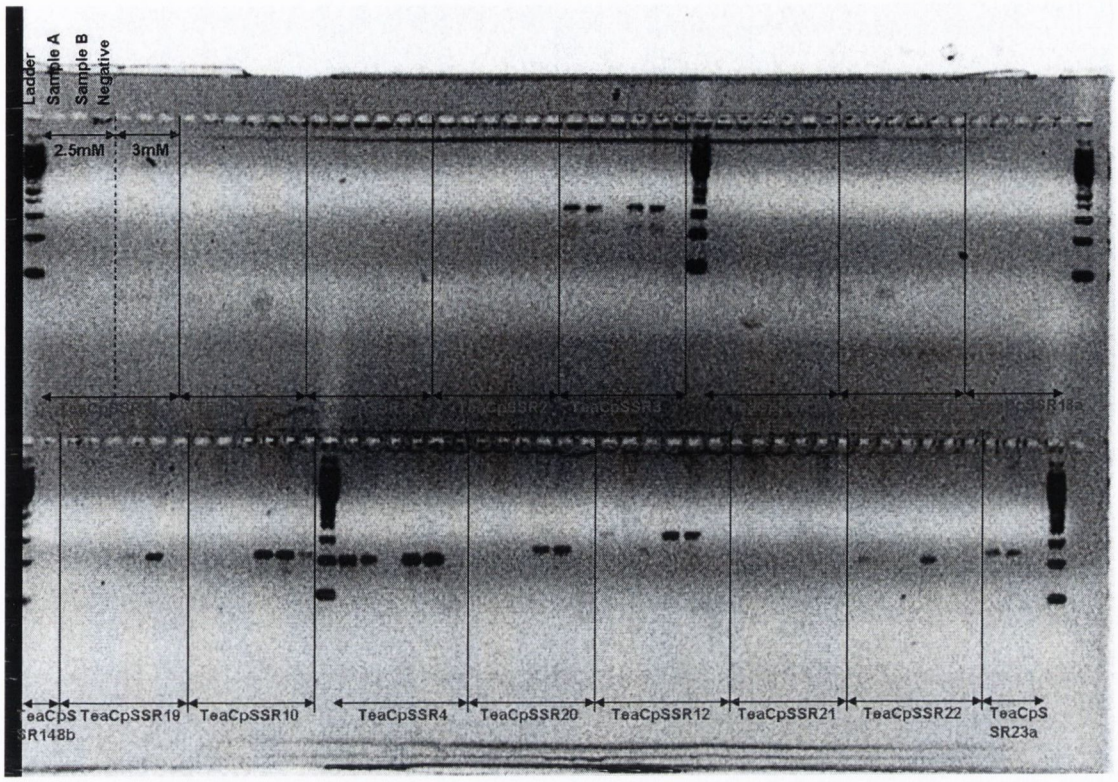

Figure 2.4.3 3% Agarose gel electrophoresis image of plastid SSR PCR products with 16 of 23 unlabelled primer pairs over two $MgCl_2$ concentrations (2.5mM and 3.0mM). Ladder: 100bp ladder. 1.5% TBE. Sample A and B: individuals from population 2250 Tipperary.

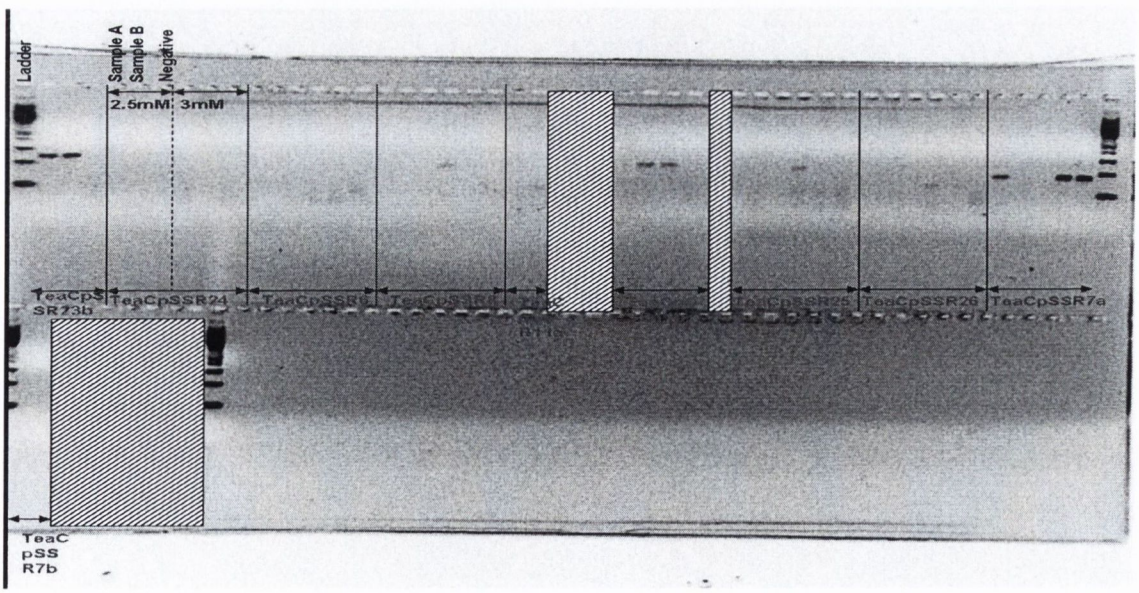

Figure 2.4.4 3% Agarose gel electrophoresis image of plastid SSR PCR products with 7 of 23 unlabelled primer pairs over two $MgCl_2$ concentrations (2.5mM and 3.0mM).

Ladder: 100bp ladder. 1.5% TBE. Sample A and B: individuals from population 2250 Tipperary.

The remaining 14 primer pairs were then tested over a temperature gradient and checked for successful amplification using agarose gel electrophoresis (Figures 2.4.5, 2.4.6, 2.4.7 and 2.4.8). Successful amplification was seen in eight of the 14 primer pairs (TeaCpSSR2, 46.5°C; TeaCpSSR6, 45°C; TeaCpSSR8, 45°C; TeaCpSSR9, 45°C; TeaCpSSR15, 45°; TeaCpSSR21, 46.5°C; TeaCpSSR25, 45°C; and TeaCpSSR26, 45°C).

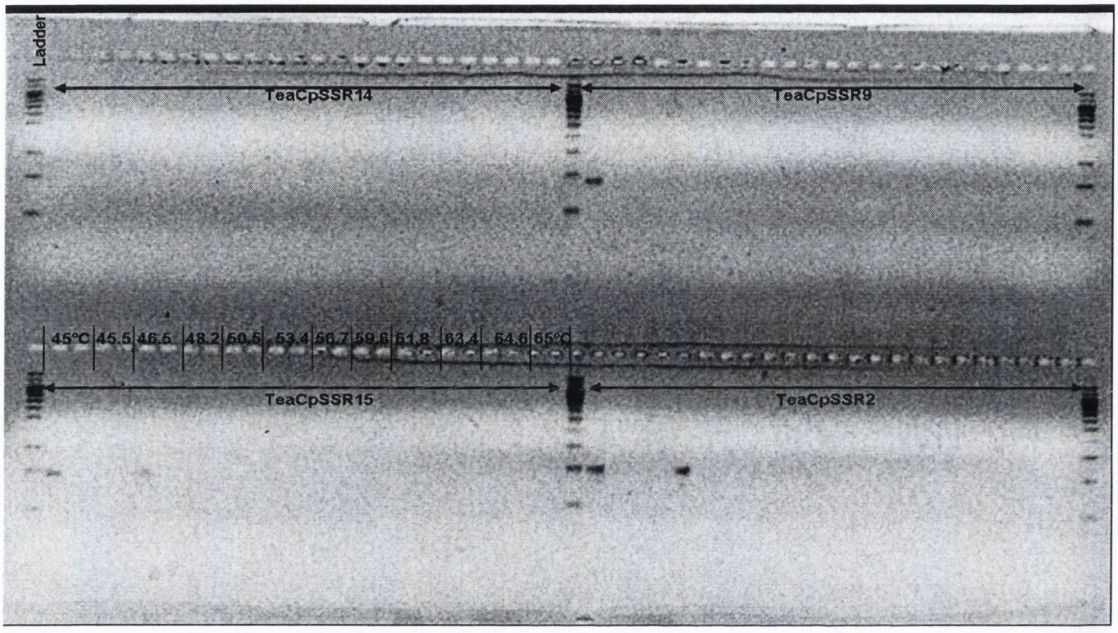

Figure 2.4.5 3% Agarose gel electrophoresis image of plastid SSR PCR products with 4 of 14 unlabelled primer pairs (TeaCpSSR2, TeaCpSSR9, TeaCpSSR14 and TeaCpSSR15) over a temperature gradient from 45°C to 65°. Ladder: 100bp ladder. 1.5% TBE. Sample A: individual from population 2250 Tipperary.

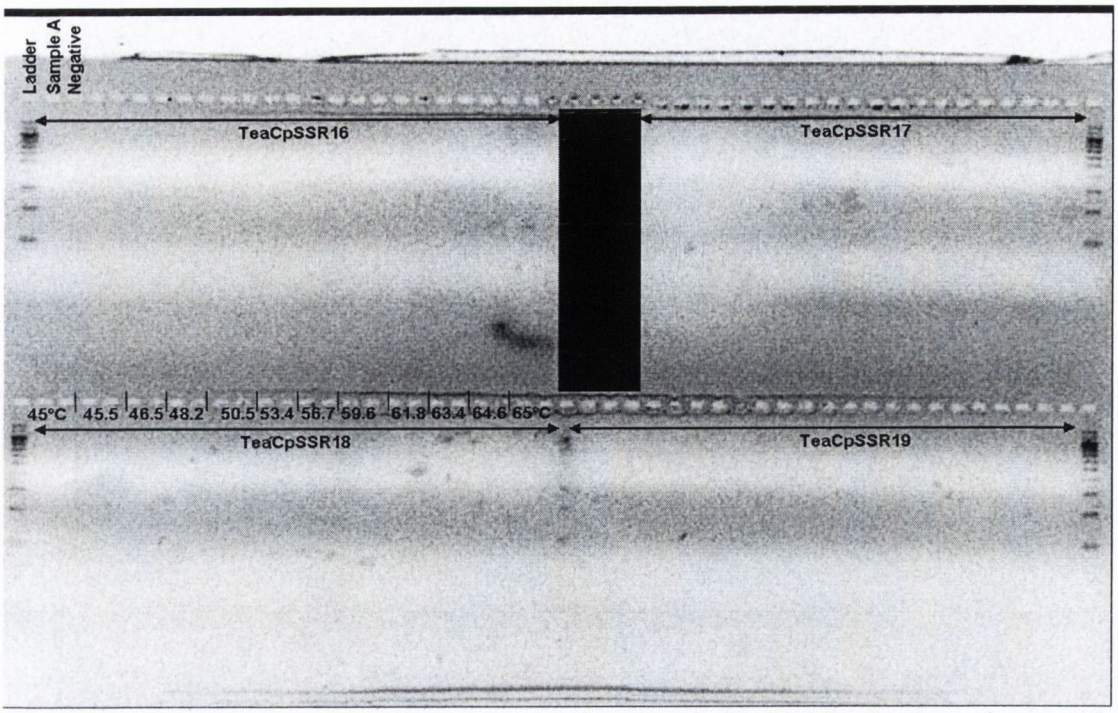

Figure 2.4.6 3%Agarose gel electrophoresis image of plastid SSR PCR products with 4 of 14 unlabelled primer pairs (TeaCpSSR16 to TeaCpSSR19) over a temperature gradient from 45°C to 65°. Ladder: 100bp ladder. 1.5% TBE. Sample A: individual from population 2250 Tipperary.

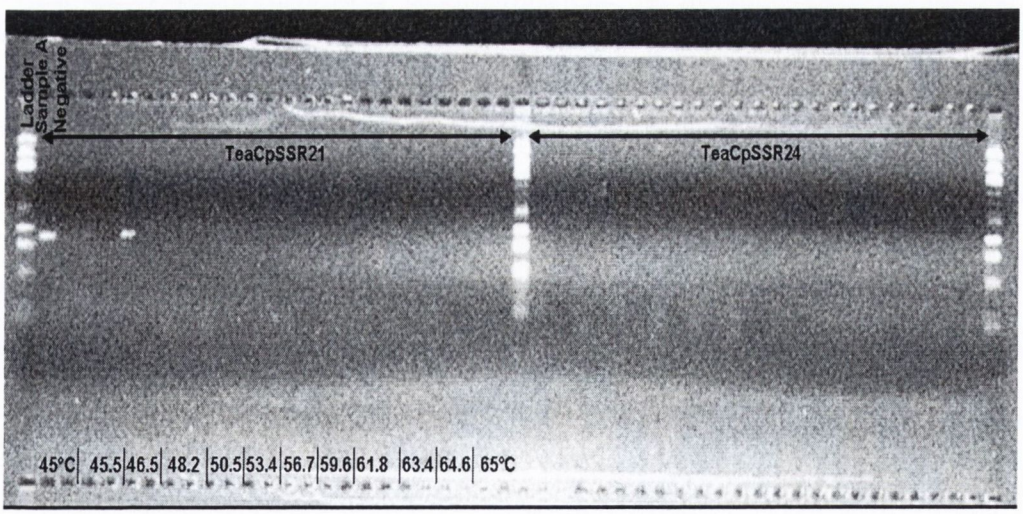

Figure 2.4.7 3%Agarose gel electrophoresis image of plastid SSR PCR products with 2 of 14 unlabelled primer pairs (TeaCpSSR21 and TeaCpSSR24) over a temperature gradient from 45°C to 65°. Ladder: 100bp ladder. 1.5% TBE. Sample A: individual from population 2250 Tipperary.

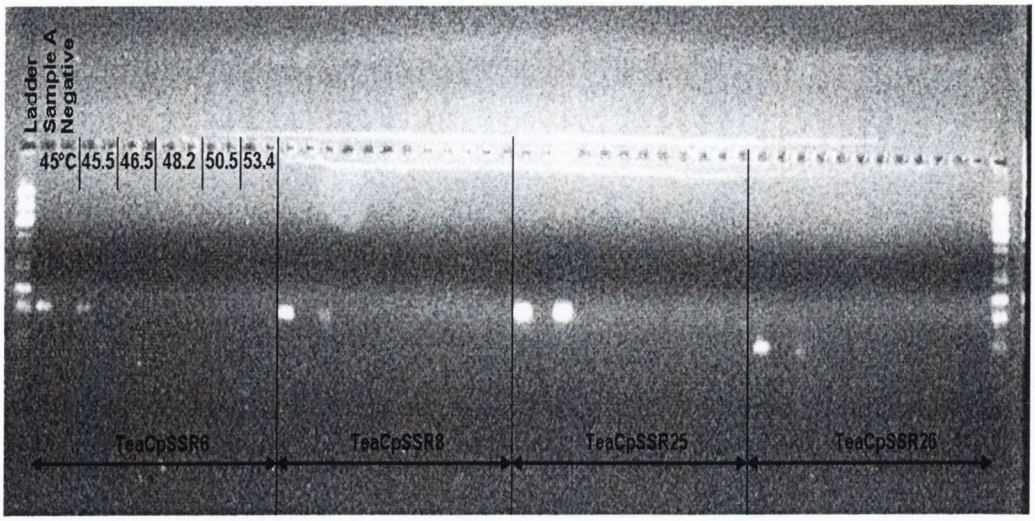

Figure 2.4.8 3%Agarose gel electrophoresis image of plastid SSR PCR products with 4 of 14 unlabelled primer pairs over a temperature gradient from 45°C to 65°. Ladder: 100bp ladder. 1.5% TBE. Sample A: individual from population 2250 Tipperary.

2.4.3 Testing of primer pairs for cross-amplification across the Poaceae

Size ranges and allele numbers were determined for each of the loci tested (Table 2.4.2). A single locus (TeaSSR10) was monomorphic in *L. perenne* but polymorphic across all grasses (Table 2.4.2). The eleven remaining loci were polymorphic within both *L. perenne* and all the other grasses. In *L. perenne*, the allele numbers ranged from locus TeaSSR10 with one allele to locus TeaSSR3 with nine alleles. All twelve loci also amplified in *L. temulentum* and *L. hybridum*. In the other grasses, allele numbers ranged from loci with two alleles (TeaSSR5, TeaSSR11 and TeaSSR12) to locus TeaSSR3 with ten alleles. Particularly, cpSSRs from the regions *trn*L-F, 23S-5S and *trn*V were highly polymorphic. Successful cross species amplification of the markers was found at all twelve loci, with less successful cross species amplification in more distantly related grasses.

Table 2.4.2 Allele sizes (in b.p.), size ranges and allele numbers of the twelve *de novo* developed cpSSR markers in 51 grass species representing nine subfamilies of Poaceae.

Subfamily	Species	Chloroplast Marker Amplification											
		TeaSSR1	TeaSSR2	TeaSSR3	TeaSSR4	TeaSSR5	TeaSSR6	TeaSSR7	TeaSSR8	TeaSSR9	TeaSSR10	TeaSSR11	TeaSSR12
Anomochlooideae	*Streptochaeta spicata*	229	-	-	194	209	196	-	-	-	-	-	-
Arundinoideae	*Arundo donax*	229	-	-	195	209	195	-	-	194	-	-	-
Bambusoideae	*Phyllostachys flexuosa*	229	197	312	200	-	195	-	-	189	-	-	-
"	*Phyllostachys nuda*	229	-	312	194	-	-	172	181	189	-	-	-
Centothecoideae	*Chasmanthium latifolium*	-	-	-	-	-	195	-	-	-	-	-	-
"	*Orthoclada laxa*	229	-	-	195	212	196	-	-	190	-	-	-
Chloridoideae	*Chloris* sp.	-	-	-	195	-	194	-	-	-	-	-	-
"	*Eleusine coricana*	-	196	312	200	209	195	-	-	-	-	-	-
"	*Eragrostis chloromatus*	-	196	302	194	209	195	171	-	-	-	-	-
Danthonioideae	*Danthonia decumbens*	229	196	-	194	212	196	-	181	194	-	-	-
Ehrhartoideae	*Oryza sativa*	-	-	312	194	209	195	172	-	-	-	-	-
Panicoideae	*Miscanthus sinensis*	229	196	311	199	209	199	-	-	-	-	-	-
"	*Saccharum arundinaceum*	-	196	-	200	209	-	-	-	-	-	-	-
"	*Saccharum spontaneum*	229	196	311	199	212	201	-	-	-	-	-	-
"	*Zea diploperennis*	-	196	-	200	209	-	171	-	-	-	-	-
"	*Zea mays*	229	196	312	199	209	199	171	181	194	-	-	-
"	*Pharus latifolius*	229	196	-	195	209	195	-	-	189	-	-	-
Pooideae	*Aegilops speltoides*	229	-	312	194	-	195	-	-	194	-	-	-
"	*Agrostis canina*	229	196	312, 313, 316	195, 196	209, 212	188	172	179	-	-	-	-
"	*Agrostis capillaris*	229	196	313	194, 195	212	195, 196	172	184	-	-	195	-
"	*Agrostis stolonifera*	-	196, 197	311, 312	195, 196	209, 212	188	172	179	-	-	-	-
"	*Alopecurus pratensis*	228, 229	202	311, 312	194, 195	209, 212	195	171, 172	179	-	-	-	-
"	*Avena sativa*	-	196	312	200	-	-	172	181	-	-	-	-
"	*Briza media*	229	-	-	195, 196	209, 212	188	172, 173	-	-	-	-	-
"	*Bromus erectus*	229	196	311, 312	185, 186, 188	209	188	-	178	-	-	-	-
"	*Cynosurus cristatus*	229	197	305	193, 194, 195	209, 212	197	172	179	194	-	-	-
"	*Dactylis glomerata*	227, 229, 230	196	312, 314, 315	194, 195	209, 212	195	172	179, 180 181	194	219	-	-
	Festuca arundinacea	229	196	313	194	209	195	172	181	-	-	-	-

"	*Festuca gigantea*	-	196	313	194	209, 212	195	-	181	-	-	-	-
"	*Festuca ovina*	229	196	312, 314, 317	194, 195	209, 212	195	172, 173	179, 180 181	-	-	-	-
	Festuca pratense	229	196	313	194	209	195	172	181	194	-	-	-
"	*Festuca vivipara*	229, 230	196	314, 317	194, 195	209, 212	195	172, 173	180	-	-	-	-
"	*Festuca rubra*	229	196	311, 312	194, 195	209, 212	195	172	179, 180	189, 194	-	-	-
"	*Holcus lanatus*	-	196, 197	312	194, 195	212	195	174	180	194	-	-	-
"	*Hordeum vulgare*	-	196	-	-	209	-	172	181	194	219	194	-
"	*Koeleria macrantha*	-	196, 197	312, 313	195, 196	212	188	-	179	194	-	-	-
"	*Lolium canariense*	227, 229	196	308, 312, 313, 314	194, 195	209, 212	195	171, 172, 173	181	-	-	-	-
"	*Lolium hybridum*	227, 229	196	312	194, 195	209, 212	195	172	181	189, 194	219	194, 195	305
"	*Lolium multiflorum*	227, 229	196, 197	312, 314, 315	194, 195	209, 212	195	172	181	178	218, 219	-	-
"	*Lolium perenne*	217-230	196-200	305-318	193-200	209-212	194-203	172-173	179-182	175-194	219	194-196	301-307
"	*Lolium persicum*	228, 229	196	313, 317	194, 195	209, 212	195	-	181	-	-	194, 195	-
"	*Lolium remotum*	229	196	316	194, 195	212	195	172	181	176	219	-	304, 305
"	*Lolium rigidum*	229	196	312, 318	194, 195	209	195	172	181	176	215, 219	-	304
"	*Lolium subulatum*	228, 229	196	314, 317	194, 195	212	195	174	181	-	219	-	-
"	*Lolium temulentum*	227	196, 197	311, 312, 317	195	209	195	172	181	176	219	194, 195	305
"	*Phleum pratense*	229	196	311, 312, 317	194	-	201	172	-	189	-	-	-
"	*Poa pratensis*	229	197	-	194, 195	-	195	172, 177	179	194	219	-	-
"	*Poa palustris*	229	196	-	194, 195	209, 212	195	172, 177	-	-	-	-	-
"	*Secale cereale*	-	196	-	200	209	-	172	-	-	-	194	-
"	*Triticum aestivum*	-	-	312	-	209	-	-	181	-	-	194	-
"	×*Triticosecale*	-	196	-	-	209	-	172	181	194	218, 219	194	-
Size ranges *L. perenne* (other species)		217-230 (227-230)	196-200 (196-202)	305-318 (305-318)	193-200 (185-200)	209-212 (209-212)	194-203 (188-201)	172-173 (171-177)	179-182 (178-184)	175-194 (176-194)	219 (215-219)	194-196 (194-195)	301-307 (304-305)
Allele numbers *L. perenne* (other species)		8 (4)	8 (3)	9 (10)	7 (9)	8 (2)	3 (7)	3 (5)	8 (5)	3 (5)	1 (3)	7 (2)	8 (2)

- = no amplification

2.4.4 Data analysis on full dataset

Ten primer pairs were chosen from the twelve primer pairs tested initially because of ease of scoring. A total of 1,575 individuals across 104 accessions were genotyped using the twelve markers.

Allele information

All ten cpSSR marker loci were found to be polymorphic, ranging from marker TeaCpSSR7 with four alleles, to marker TeaCpSSR3 with 22 alleles (Table 2.4.3). The distribution of alleles in the populations varied between *L. perenne* ecotypes, *L. perenne* cultivars and the groups of other species. At locus TeaCpSSR8, there was only one allele present in the ×*Festulolium* cultivars, but ten alleles present in Irish *L. perenne* ecotypes. Loci TeaCpSSR3 and TeaCpSSR8 had the largest number of alleles for *L. perenne* ecotypes. Loci TeaCpSSR2, TeaCpSSR3 and TeaCpSSR4 had the largest number of alleles for *L. perenne* cultivars. Locus TeaCpSSR3 was extremely rich in alleles, including eleven alleles for the other tested *Lolium* species. Generally there were more alleles unique to *L. perenne* ecotypes than in all the other species groups. Marker locus TeaCpSSR8 was the richest locus for unique alleles in *L. perenne* in general. For locus TeaCpSSR8, there were five alleles unique to the Irish *L. perenne* ecotypes, five alleles unique to the European/Near Eastern ecotypes, and one allele unique to the other *Lolium* species. None of the alleles at this locus were unique to the ×*Festulolium* cultivars or to *Festuca* species. Locus TeaCpSSR7 was an exception with no allele being unique to *L. perenne* accessions. None of the alleles, across all loci, were diagnostic by themselves for a single population, but some were for a defined group of populations. However, unique alleles were present only in groups containing more than one allele. Three alleles were unique for non-*L. perenne Lolium* species (at loci TeaCpSSR3 and TeaCpSSR8). Generally across all ten marker loci no unique alleles for *Festuca* and ×*Festulolium* accessions were found (Table 2.4.3).

Haplotype information

The 104 tested populations had a large amount of haplotypic variation (Table 2.4.4, Figure 2.4.9). Of the 511 haplotypes present, 363 of these were unique to individual populations. Generally with a few exceptions each of the 104 populations had a range of unique haplotypes (Table 2.4.4). Eleven populations had no unique haplotypes, while four populations (*L. temulentum* L10, *F. arundinacea, F. gigantea* and *F. pratensis*) were composed of completely unique haplotypes (Table 2.4.4). No single haplotype was present in all groups of populations. 112 of the haplotypes were only present in Irish *L. perenne* ecotypes (Figure 2.4.9) of which 97 were unique to single populations. Thus 15 haplotypes were shared only among Irish *L. perenne* ecotypes and were diagnostic for these accessions (Table 2.4.5). 124 haplotypes were unique to European *L. perenne* ecotypes of which 106 were unique to single populations. 18 haplotypes could be considered as diagnostic for European *L. perenne* ecotype accessions (Table 2.4.5), especially for ecotypes of the Northern European and Western European geographical regions. 45 haplotypes were found only in *L. perenne* cultivars of which 42 haplotypes were unique to single populations. Three haplotypes were shared among *L. perenne* cultivars (Table 2.4.5), and were found in cultivars 'Aurora', 'Cancan', 'Magician' and 'Shandon'. 52 unique haplotypes were detected in ×*Festulolium* cultivars of which 32 were unique to single populations. 74 haplotypes were specific to other *Lolium* species of which 69 were unique to single populations. 21 haplotypes were found to be specific for *Festuca* species of which 20 were unique to single populations. In total, 29 haplotypes were shared between ×*Festulolium* accessions and *Lolium* accessions, of which 26 haplotypes were shared with *L. perenne* ecotypes (Figure 2.4.9).

Table 2.4.3 Number of alleles (and unique alleles) per locus in each group of accessions.

	N	TeaCpSSR1	TeaCpSSR2	TeaCpSSR3	TeaCpSSR4	TeaCpSSR5	TeaCpSSR7	TeaCpSSR8	TeaCpSSR10	TeaCpSSR11	TeaCpSSR12	Total number of alleles/ group
Irish *Lolium perenne* ecotypes	480	6 (2)	6 (1)	11 (1)	6 (1)	7 (3)	3	10 (5)	4 (1)	3 (1)	4 (2)	60
European and other geographic regional *L. perenne* ecotypes (total)	496	3 (1)	8 (3)	16 (3)	5	3 (1)	2	9 (5)	4 (2)	4 (2)	4 (1)	58
Northern Europe △	100	2	2	11	3	2	2	3	1	2	3	31
North Africa □	48	2	3	5	3	2	2	3	2	1	2	25
Near East ▲	60	2	4	4	2	2	2	1	1	2	4	24
Southern Europe ■	128	3	4	8	4	2	2	3	3	2	2	33
Western Europe ○	80	2	5	6	2	2	2	4	2	3	3	31
Eastern Europe ●	80	2	3	5	3	3	2	1	1	2	2	24
L. perenne varieties	259	5 (1)	5	8	4 (1)	5 (2)	2	2	3	3 (1)	4 (2)	41
×*Festulolium*	140	2	1	8	2	2	2	1	1	1	2	22
Other *Lolium* species	136	2	3	11 (2)	2	2	4	2 (1)	1	3	3	33
Festuca species	64	1	1	5	2	2	2	4	2	1	1	21
Total number of alleles/ locus		8	10	22	7	10	4	17	6	7	8	99

N: Number of individuals

The numbers of unique alleles are shown in parentheses.

Table 2.4.4 Group, haplotype numbers and diversity information on accessions used in this study.

Species	Accession Number	Group [a]	N[b]	H[c]	N Haplotypes/ population	N Unique haplotypes
L. perenne	IRL-OP-02337	I 1	15	0.180	9	2
L. perenne	IRL-OP-02059	I 2	16	0.202	12	1
L. perenne	IRL-OP-02007	I 3	16	0.177	11	2
L. perenne	IRL-OP-02011	I 4	14	0.207	11	0
L. perenne	IRL-OP-02015	I 5	16	0.268	15	5
L. perenne	IRL-OP-02048	I 6	16	0.202	10	2
L. perenne	IRL-OP-02192	I 7	16	0.197	11	1
L. perenne	IRL-OP-02312	I 8	16	0.219	12	3
L. perenne	IRL-OP-02320	I 9	16	0.202	11	2
L. perenne	IRL-OP-02064	I 10	16	0.190	10	1
L. perenne	IRL-OP-02078	I 11	18	0.324	15	5
L. perenne	IRL-OP-02230	I 12	16	0.333	15	8
L. perenne	IRL-OP-02128	I 13	16	0.122	5	2
L. perenne	IRL-OP-02538	I 14	16	0.198	8	2
L. perenne	IRL-OP-02274	I 15	16	0.174	9	1
L. perenne	IRL-OP-02480	I 16	16	0.276	16	5
L. perenne	IRL-OP-02442	I 17	16	0.270	15	2
L. perenne	IRL-OP-02444	I 18	16	0.191	10	1
L. perenne	IRL-OP-02068	I 19	15	0.287	14	7
L. perenne	IRL-OP-02241	I 20	16	0.253	13	5
L. perenne	IRL-OP-02419	I 21	16	0.253	14	3
L. perenne	IRL-OP-02258	I 22	16	0.191	11	3
L. perenne	IRL-OP-02272	I 23	16	0.301	16	6
L. perenne	IRL-OP-02250	I 24	17	0.260	13	2
L. perenne	IRL-OP-02267	I 25	16	0.292	15	4
L. perenne	IRL-OP-02269	I 26	17	0.248	15	1
L. perenne	IRL-OP-02173	I 27	16	0.273	12	2
L. perenne	IRL-OP-02483	I 28	16	0.262	16	1
L. perenne	IRL-OP-02491	I 29	16	0.248	14	1
L. perenne	IRL-OP-02018	I 30	16	0.286	14	4
L. perenne	GR 5092	△1	16	0.199	12	2
L. perenne	PI 598445	△2	12	0.146	8	0
L. perenne	ABY-Ba 12896	△3	16	0.128	8	5
L. perenne	NGB14250	△4	12	0.285	11	2
L. perenne	16-7-62-2 Nordic	△5	16	0.268	13	5
L. perenne	PI 619024	△6	12	0.106	6	2
L. perenne	W6 9339	△7	16	0.247	14	2
L. perenne	PI 610958	□8	16	0.260	14	7
L. perenne	ABY-Ba 11315	□9	16	0.206	12	5
L. perenne	E1	□10	16	0.066	4	3
L. perenne	W6 11325	▲11	16	0.114	6	1
L. perenne	PI 598512	▲12	16	0.188	9	1
L. perenne	PI 547390	▲13	12	0.118	5	1
L. perenne	PI 317452	▲14	16	0.165	9	1
L. perenne	No 10 Spain	■15	16	0.250	13	6
L. perenne	3408 Italy	■16	16	0.264	15	4
L. perenne	W6 16127	■17	15	0.191	9	8
L. perenne	3013 Romania	■18	17	0.235	15	3
L. perenne	3199 Romania Podoloni	■19	16	0.235	14	0

L. perenne	920 Bulgaria	■20	16	0.206	11	0
L. perenne	PI 418701	■21	16	0.120	6	1
L. perenne	ABY-Ba 11478	■22	16	0.132	14	3
L. perenne	W6 9286	○23	16	0.155	9	2
L. perenne	ABY-Ba 11514	○24	16	0.182	10	3
L. perenne	CPI 44924	○25	16	0.205	11	0
L. perenne	GR 5095	○26	16	0.205	12	2
L. perenne	GR 5105	○27	16	0.238	12	5
L. perenne	PI 274637	●28	16	0.158	10	1
L. perenne	PI 267058	●29	16	0.099	5	0
L. perenne	PI 182857	●30	16	0.232	6	6
L. perenne	PI 321397	●31	16	0.232	12	5
L. perenne	IV-51-161 Hungary	●32	16	0.245	14	3
L. perenne	cv. Aurora	V 1	17	0.249	12	1
L. perenne	cv. Barlenna	V 2	16	0.229	10	3
L. perenne	cv. Cancan	V 3	16	0.302	15	5
L. perenne	cv. Cashel	V 4	16	0.172	5	1
L. perenne	cv. Fennema	V 5	16	0.207	11	3
L. perenne	cv. Greengold	V 6	17	0.161	9	0
L. perenne	cv. Magician	V 7	16	0.254	14	3
L. perenne	cv. Millenium	V 8	16	0.157	9	0
L. perenne	cv. Navan	V 9	16	0.272	11	3
L. perenne	cv. Odenwaelder	V 10	16	0.259	14	3
L. perenne	cv. Portstewart	V 11	16	0.193	9	2
L. perenne	cv. Premo	V 12	16	0.167	9	0
L. perenne	cv. S24	V 13	17	0.199	12	4
L. perenne	cv. Sarsfield	V 14	16	0.238	12	1
L. perenne	cv. Shandon	V 15	16	0.213	11	2
L. perenne	cv. Talbot	V 16	16	0.235	13	4
L. canariense	PI 320544	L 1	16	0.239	7	6
L. hybridum	ABY-Ba 13122	L 2	16	0.157	9	9
L. hybridum	GR11849/94	L 3	8	0.109	5	3
L. multiflorum	GR11855/98	L 4	8	0.231	6	4
L. persicum	PI 229764	L 5	16	0.065	3	3
L. remotum	GR11839/99a	L 6	8	0.052	3	1
L. rigidum	GR11848/91	L 7	8	0.066	4	4
L. subulatum	PI 197310	L 8	16	0.156	6	6
L. temulentum	ABY-Ba 13643	L 9	16	0.227	13	10
L. temulentum	ABY-Ba 8917	L 10	16	0.174	9	9
L. temulentum	GR11880/82	L 11	8	0.147	5	5
×*Festulolium braunii*	cv. Perun	F 1	16	0.240	13	9
×*Festulolium braunii*	cv. HD 14 DK	F 2	16	0.247	13	6
×*Festulolium braunii*	cv. Paulita	F 3	16	0.207	11	5
xFestulolium braunii	cv. Achilles	F 4	16	0.187	10	1
×*Festulolium Lolium multiflorum* × *Festuca arundinacea*	cv. Lesana	F 5	16	0.213	13	0
×*Festulolium Lolium multiflorum* ×	cv. Becva	F 6	16	0.251	13	1

Festuca arundinacea						
×*Festulolium Lolium multiflorum* × *Festuca arundinacea*	cv. Lofa	F 7	16	0.273	15	10
×*Festulolium Lolium multiflorum* × *Festuca arundinacea*	cv. Korina	F 8	16	0.215	11	0
×*Festulolium Lolium multiflorum* × *Festuca arundinacea*	cv. Felina	F 9	12	0.214	8	2
Festuca arundinacea	cv. Dovey	NL 1	8	0	1	1
Festuca gigantea	PI 440362	NL 2	8	0.122	5	5
Festuca ovina	PI 634304	NL 3	16	0.148	9	6
Festuca pratensis	cv. Northland	NL 4	8	0	1	1
Festuca rubra	IRL-OP-02174	NL 5	8	0.115	4	3
Festuca vivipara	PI 251118	NL 6	16	0.109	5	3

[a]Group: I = Irish ecotype, △= Northern Europe group 1, □ = North Africa group 2, ▲= Near East group 3, ■ = Southern Europe group 4, ❍ = Western Europe group 5, ● = Eastern Europe group 6, V = *Lolium perenne* variety, NL = non-*Lolium* species, L = *Lolium* species; [b]N = number of individuals, [c]H = Nei's (1973) gene diversity

Table 2.4.5 Diagnostic haplotypes for population groups of *Lolium perenne, Festuca* species and ×*Festulolium* varieties.

Diagnostic haplotypes for groups	TeaCp SSR1	TeaCp SSR2	TeaCp SSR3	TeaCp SSR4	TeaCp SSR5	TeaCp SSR7	TeaCp SSR8	TeaCp SSR10	TeaCp SSR11	TeaCp SSR12
Irish *L. perenne* group										
Irish 1	228	197	312	194	211	172	181	219	195	305
Irish 2	228	200	312	195	211	172	181	219	195	305
Irish 3	229	197	311	194	210	172	181	219	195	305
Irish 4	229	181	311	194	211	172	181	219	195	305
Irish 5	229	197	311	194	211	172	181	219	195	305
Irish 6	229	197	311	194	211	172	181	219	195	306
Irish 7	229	196	311	194	211	172	199	219	195	305
Irish 8	229	196	311	194	211	172	199	219	195	306
Irish 9	229	197	311	195	210	172	181	219	195	305
Irish 10	229	197	311	195	210	172	181	219	195	306
Irish 11	229	197	312	195	210	172	181	219	195	305
Irish 12	229	197	312	195	210	172	181	219	195	306
Irish 13	229	196	312	200	211	172	181	219	195	305
Irish 14	229	196	312	200	211	172	181	219	195	306
Irish 15	228	197	312	194	211	172	181	219	195	305
European *L. perenne* group										
Europe 1	228	196	312	194	210	173	178	219	195	306
Europe 2	229	196	312	194	211	173	181	219	194	306
Europe 3	229	200	310	194	211	173	181	219	195	306
Europe 4	229	196	310	195	211	172	181	219	195	306
Europe 5	229	196	310	194	211	172	181	219	195	305
Europe 6	229	196	310	194	211	172	181	219	195	306
Europe 7	229	200	312	194	210	173	181	219	195	305
Europe 8	229	200	312	194	210	173	181	219	195	306
Europe 9	229	200	311	194	211	173	181	219	195	305
Europe 10	229	200	311	194	211	173	181	219	195	306
Europe 11	229	200	312	194	211	173	181	219	195	305
Europe 12	229	200	312	194	211	173	181	219	195	306
Europe 15	229	196	310	195	210	172	181	219	194	304
Europe 16	229	196	310	195	210	172	181	219	194	305
Europe 17	229	196	311	195	210	172	189	219	194	305

Europe 18	229	196	312	195	182	172	181	219	195	305
Europe 19	228	200	312	194	210	173	181	219	195	305
Europe 20	228	200	312	194	210	173	181	219	195	306
Commercial *L. perenne* varieties										
Cultivar 1	228	200	311	195	211	173	181	219	195	305
Cultivar 2	228	212	311	194	211	172	181	219	195	305
Cultivar 3	228	212	311	194	211	172	181	219	195	306
Lolium species groups										
Lolium 1	229	196	315	194	211	172	181	219	-	-
Lolium 2	229	196	317	195	211	172	181	219	-	-
Lolium 3	229	196	315	195	210	172	181	219	194	305
Lolium 4	229	196	315	195	210	172	181	219	194	306
Lolium 5	229	196	315	195	210	172	181	219	-	-
×*Festulolium* varieties										
×*Festulolium* 1	228	196	313	194	210	172	181	219	195	306
×*Festulolium* 2	228	196	313	195	210	172	181	219	195	306
×*Festulolium* 3	228	196	314	195	210	172	181	219	195	306
×*Festulolium* 4	228	196	313	195	211	172	181	219	195	305
×*Festulolium* 5	228	196	313	195	211	172	181	219	195	306
×*Festulolium* 6	228	196	313	195	210	173	181	219	195	305
×*Festulolium* 7	228	196	313	195	210	173	181	219	195	306
×*Festulolium* 8	228	196	313	195	211	173	181	219	195	305
×*Festulolium* 9	228	196	313	195	211	173	181	219	195	306
×*Festulolium* 10	229	196	313	194	211	172	181	219	195	305
×*Festulolium* 11	229	196	313	194	210	173	181	219	195	305
×*Festulolium* 12	229	196	313	194	211	173	181	219	195	305
×*Festulolium* 13	229	196	313	194	211	173	181	219	195	306
×*Festulolium* 14	228	196	313	194	211	172	181	219	195	306
×*Festulolium* 15	229	196	313	195	210	173	181	219	195	305
×*Festulolium* 16	229	196	313	195	210	173	181	219	195	306
×*Festulolium* 17	228	196	313	194	210	173	181	219	195	305
×*Festulolium* 18	228	196	313	194	210	173	181	219	195	306
×*Festulolium* 19	228	196	313	194	211	173	181	219	195	305
×*Festulolium* 20	228	196	313	194	211	173	181	219	195	306

Festuca group										
Festuca 1	229	194	195	172	-	212	180	211	196	312
Festuca 2	229	195	195	172	-	212	180	211	196	314
Festuca 3	229	195	195	172	-	212	180	211	196	317

- : No amplification

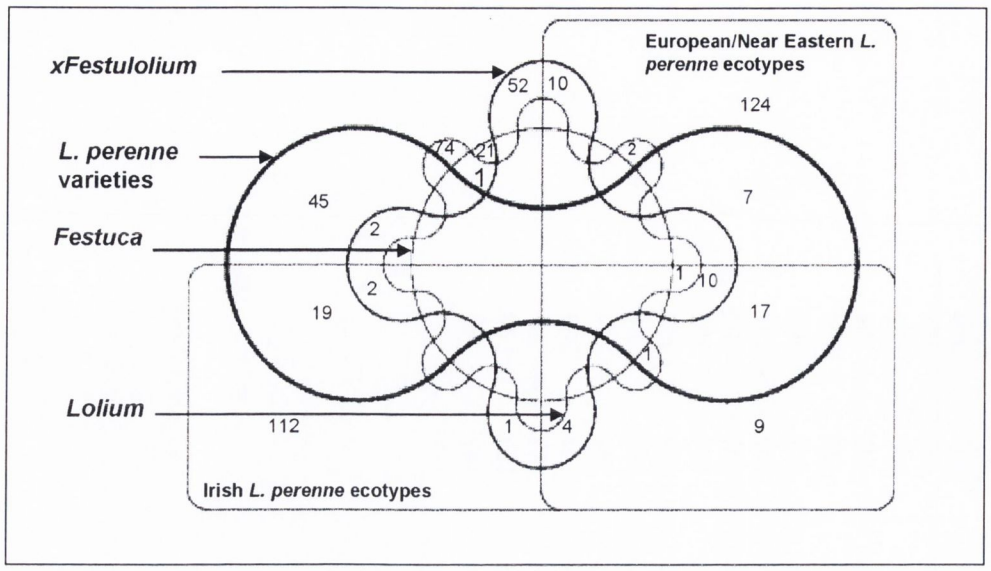

Figure 2.4.9 Edwards' Venn diagram demonstrating shared and unique haplotypes for the Irish *L. perenne* ecotypes, European/Near Eastern *L. perenne* ecotypes, *L. perenne* commercial varieties, other *Lolium* species, ×*Festulolium* varieties, and *Festuca* species. Shared haplotypes among groups are in intersects.

Genetic distance between populations

The genetic distance matrix between the populations is shown in Appendix 8.3. The UPGMA dendrogram constructed from this matrix is shown in Figure 2.4.10. The source of geographical groupings is shown in Figure 2.4.11. One group of *Festuca* species was resolved outlying all other accessions. This group consisted of *F. ovina*, *F. rubra,* and *F. vivipara* (Figure 2.4.10: group I) and was supported by a bootstrap value of 99%. A single accession of *L. perenne*, ■17, was also an outlying accession and was isolated from the rest of the *L. perenne* ecotypes as was another accession, *L. multiflorum* L4, (Figure 2.4.11: groups V, VI). The majority of the tree could be split in several major groups (II, III, IVa, and IVb). Group II contained two further *Festuca* species and a *L. temulentum* accession (L11). The grouping of *F. pratensis* and *L. temulentum* (L11) was supported by a bootstrap value of 100%. Group III contained a number of *Lolium* species. Accessions within this group were strongly supported by bootstrap values. *Lolium hybridum* and *L. rigidum* could be clearly separated from each other. The other major group was divided into two subgroups (groups IVa and IVb). Group IVa contained the majority of the Irish *L. perenne* ecotypes and commercial *L. perenne* cultivars, with the exception of accessions I9 and I13. Further

exceptions in group IVa were three European *L. perenne* accessions ■15, ■18 and ■19. Group IVb contained most of the European *L. perenne* ecotype accessions and two cultivars 'Barlenna' and 'Talbot'. Also present in this group was an assemblage of ×*Festulolium* cultivars and *F. gigantea.* These ones were however clearly separated from the European *L. perenne* accessions in group IVb. Exceptions were the ×*Festulolium* cultivars 'Lesana', 'Becva' and 'Korina', which grouped with accessions in group IVa. These three ×*Festulolium* cultivars had *L. multiflorum* as the female parent. There was no bootstrap support for many major groups on the dendrogram.

A second UPGMA dendrogram showing the similarities between eleven groups of accessions was constructed to support the AMOVA analysis and to investigate the broad-scale geographical structuring (Figure 2.4.12). The group of *Festuca* species were outlying all other groups (Figure 2.4.12, group I). The rest of the dendrogram was split into two major groups (Figure 2.4.12, groups II and III). The first group (II) contained the Irish *L. perenne* ecotypes, the *L. perenne* cultivars, the *Lolium* species and the *Festulolium* cultivars. The second group (III) contained all European/Near Eastern *L. perenne* ecotypes and could be split into two subgroups (IIIa and IIIb). Subgroup IIIa consisted of the Southern European, Western European and Northern European ecotypes, while subgroup IIIb consisted of the North African, Near Eastern and Eastern European ecotypes. There was moderate to good bootstrap support for many branches of the tree.

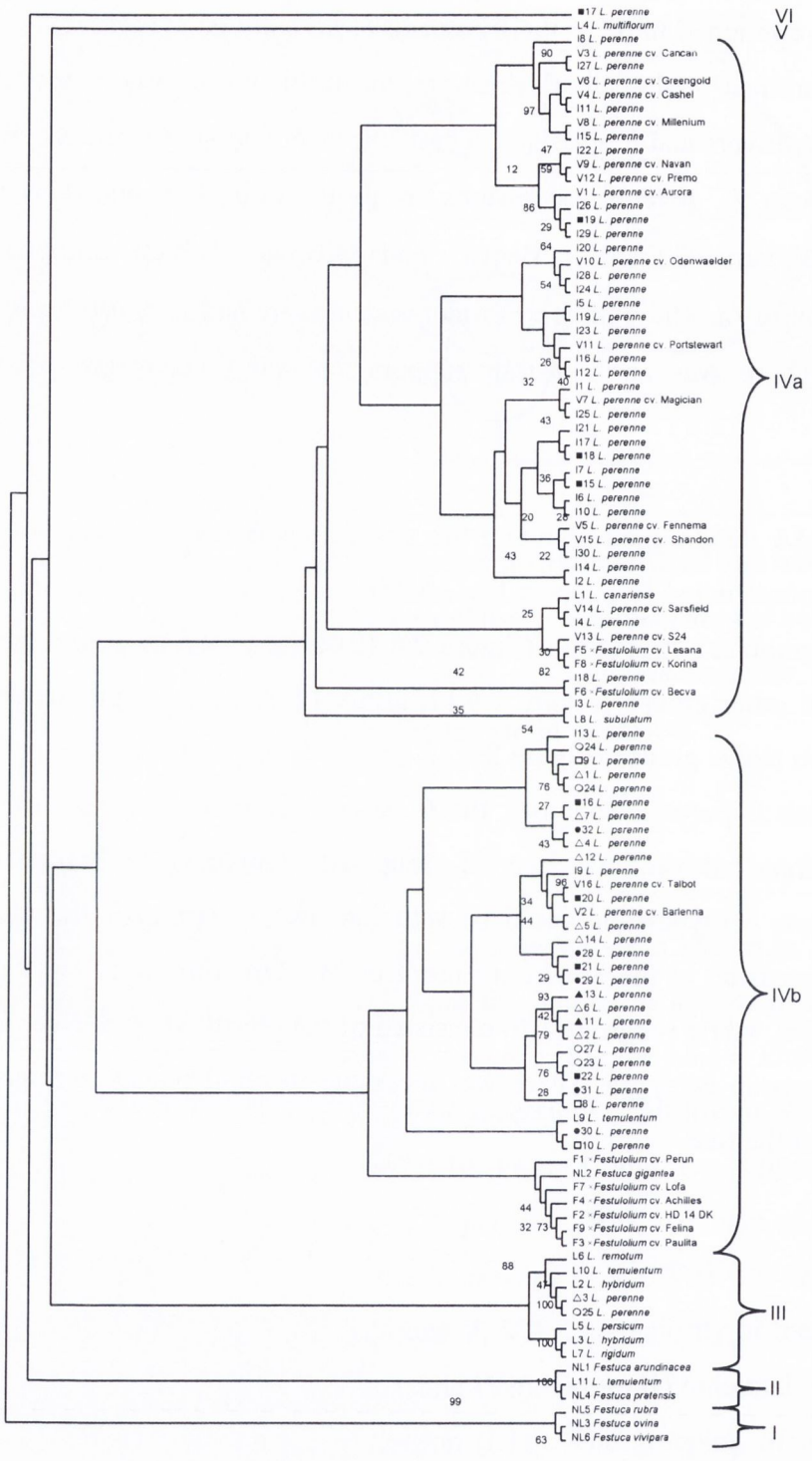

Figure 2.4.10 Unrooted dendrogram showing similarities between populations, constructed using the unweighted pair group method with arithmetic means (UPGMA) method (Sneath & Sokal, 1973) as implemented in POPGENE (Yeh & Boyle, 1997), based on Nei's genetic distance measures (Nei, 1972). Numbers on the branches are percentage bootstrap values generated in NTSYSpc V2.2 (Rohlf,

2005).[a]V: V represents a grade of two outlying accessions. Different symbols represent a geographical group: △ = Northern Europe □ = North Africa ▲ = Near East ■ = Southern Europe ❍ =Western Europe ● = Eastern Europe, I = Ireland.

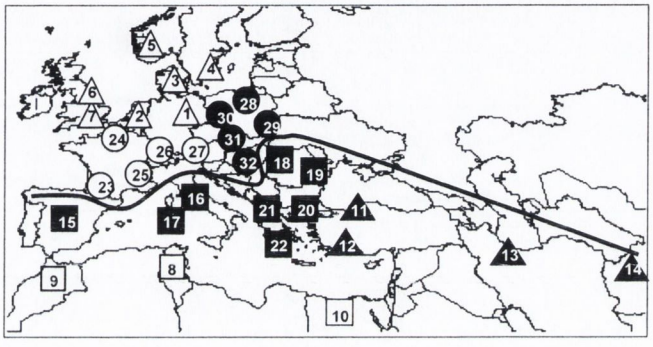

Figure 2.4.11 Map of the distribution of six geographical *Lolium perenne* accession groups included in this study. Different symbols represent a geographical group: △ = Northern Europe □ = North Africa ▲ = Near East ■ = Southern Europe ❍ =Western Europe ● = Eastern Europe, I = Ireland. Accessions north of the bold line were in the category "north of the Alps", and those south of the line were in the category "south of the Alps".

Source of map: http://geography.about.com/library/blank/blxeurasia.htm

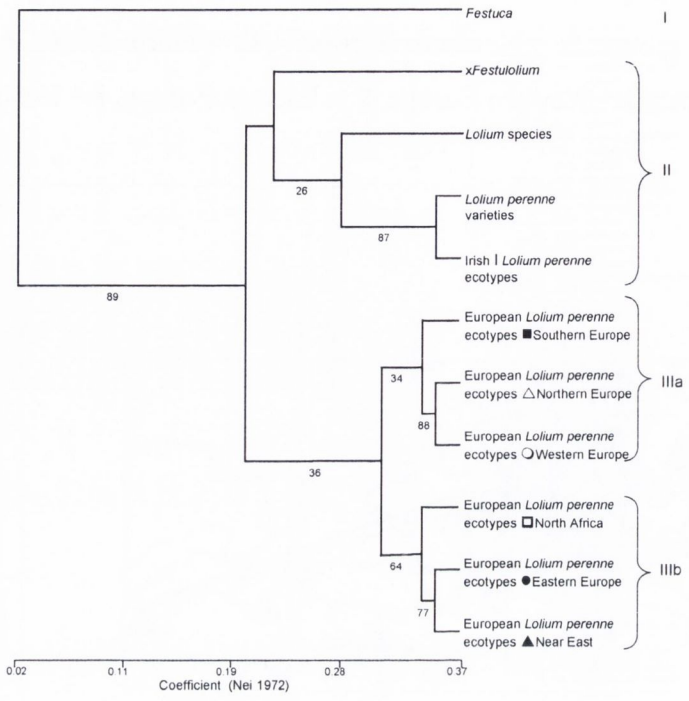

Figure 2.4.12 Unrooted dendrogram showing similarities between groups of accessions, constructed using the unweighted pair group method with arithmetic means (UPGMA) method (Sneath & Sokal, 1973) as implemented in NTSYSpc V2.2 (Rohlf, 2005), based on Nei's genetic distance measures (Nei, 1972). Numbers on the branches are percentage bootstrap values generated in NTSYSpc V2.2 (Rohlf, 2005).

Genetic diversity

Nei's gene diversity value (H; Nei, 1972) within accessions ranged between zero (NL1 and NL4) and 0.333 with the Irish ecotype I12 (Table 2.4.4). Only one haplotype was present in the *F. arundinacea* and *F. pratensis* populations, thus their h values were zero. The lowest h value of 0.052 was found in a *L. remotum* population (L6, Table 2.4.4). H values were higher in the Irish and European/Near Eastern *L. perenne* ecotypes ranging between 0.190 and 0.236. In the other groups of accessions, values for cultivars were h = 0.219, other *Lolium* species h = 0.148, ×*Festulolium* cultivars h = 0.201, and *Festuca* species h = 0.083.

The total gene diversity (H_t; Table 2.4.6) based on Nei's gene diversity in subdivided populations (Nei, 1987) ranged from the lowest values in Near Eastern *L. perenne*

accessions (H_t = 0.233) to the highest values in Southern European *L. perenne* accessions (H_t = 0.359). H_t values for other accession groups were within this range. The gene diversity within subdivided populations (H_s) ranged between 0.077 for *Festuca* species and 0.238 for Irish *L. perenne* ecotypes. The highest H_s value in the Irish *L. perenne* ecotypes was closely followed by the value for *×Festulolium* accessions (H_s = 0.227). The H_s value for Near Eastern *L. perenne* ecotypes was the lowest (H_s = 0.146) among *L. perenne* accessions. G_{st} values ranged from 0.238 in the Irish *L. perenne* ecotypes to 0.716 in the *Festuca* species. As G_{st} becomes closer to one, the populations within the groups are more markedly different from each other. The *×Festulolium* cultivars had exactly the same G_{st} value (0.285) as commercial *L. perenne* cultivars.

Table 2.4.6 Diversity statistics based on Nei's analysis of gene diversity in subdivided populations (Nei, 1987) for geographical groups of *Lolium perenne* ecotypes, *L. perenne* breeding varieties, *Lolium* species, *×Festulolium* varieties and *Festuca* species.

	N	H_t	H_s	G_{st}
Irish *Lolium perenne* ecotypes	480	0.312	0.238	0.238
European and other geographic regional *L. perenne* ecotypes (total)	496	0.330	0.188	0.431
Northern Europe △	100	0.327	0.197	0.397
North Africa □	48	0.254	0.177	0.301
Near East ▲	60	0.233	0.146	0.373
Southern Europe ■	128	0.359	0.217	0.395
Western Europe ○	80	0.347	0.191	0.449
Eastern Europe ●	80	0.247	0.166	0.328
L. perenne varieties	259	0.302	0.216	0.285
×Festulolium	140	0.318	0.227	0.285
Other *Lolium* species	136	0.341	0.142	0.585
Festuca species	64	0.269	0.077	0.716

N = Number of individuals; H_t, Total gene diversity; H_s, Diversity within subdivided populations; G_{st}, Coefficient of genetic differentiation

AMOVA results

AMOVA analysis was carried out on fifteen different subgroups of accessions, to test for differences in genetic structure within and between Irish and European ecotypes, to test possible geographic migration routes, and to test for differences between and within *L. perenne* accessions and *×Festulolium* cultivars (Table 2.4.7). In general, the

variation among populations accounted for less of the total variation than that found within populations. For example, for the Irish and European *L. perenne* ecotype comparison, the within population variation accounted for 63% of total variation. The among population variation accounted for 26% of total variation and among group variation accounted for 11% of total variation. These results were comparable to the variation found within and among partitions in the *L. perenne* and ×*Festulolium* comparison (64% within population variation, 17% among population variation and 19% among group variation). However there was more variation among groups and less variation at the among population level (Table 2.4.7).

Within population variation of Irish *L. perenne* ecotypes accounted for 82% of the variation and among population variation accounted for 18%. For the European *L. perenne* ecotypes the situation was different. The within population variation accounted for only 61% of the total variation, and the among population for 39% (Table 2.4.7). For all AMOVA calculations all results were highly significant ($p \leq 0.001$). For analyses comparing the phylogeographic structure in the European/Near Eastern ecotypes, the percentages of variation accounted for by among and within population variation were similar for all the partitions tested (data not shown).

For all calculations comparing migration routes the among group and among population variation was highly significant ($p \leq 0.001$). All within population variations were not significant. In both tests for evidence of a Mediterranean migration route, among group variation was less than or equal to zero (Table 2.4.7). For the two tests investigating phylogeographic structure on a possible Danubian migration route (Southern vs. Western Europe and Southern vs. Eastern Europe) among group variations were low but significant, three and four %, respectively (Table 2.4.7). Similarly, for both tests investigating phylogeographic structure on a possible North African migration route, among group variations were low but significant, two and four %, respectively (Table 2.4.7). A post-glacial migration route appeared to be possible since among group variation for this possible post glacial migration route was zero ($p \leq 0.001$). For migration into Ireland from three neighbouring geographical groups, the lowest among group variation was found in the partition between Southern European and Irish *L. perenne* ecotypes with only 5%.

The values for Western European and Northern European groups were higher, each 10%, respectively.

Table 2.4.7 Analysis of molecular variance (AMOVA) for Irish and European *Lolium perenne* accessions, ×*Festulolium* varieties, and subgroups within European/Near Eastern *L. perenne* ecotype accessions.

Source of Variation	Migration route	d.f.[a]	SSD[b]	Variance component	Variance (%)	P[c]
Irish ecotypes	N/A					
Among populations		29	140.17	0.23	18	***
Within populations		449	493.29	1.10	82	***
European ecotypes	N/A					
Among populations		31	312.84	0.59	39	***
Within populations		462	427.57	0.93	61	***
Irish ecotypes vs. European ecotypes	N/A					
Among groups (Irish ecotypes vs. European ecotypes)		1	95.24	0.18	11	***
Among populations/within groups		61	453.01	0.42	26	***
Within populations		911	920.86	1.01	63	***
Irish L. perenne ecotypes vs. ×Festulolium varieties	N/A					
Among groups (Irish *L. perenne* ecotypes v ×*Festulolium* varieties)		1	74.98	0.32	19	***
Among populations/within groups		38	201.84	0.28	17	***
Within populations		580	622.31	1.07	64	***
European L. perenne ecotypes vs. ×Festulolium varieties	N/A					
Among groups (European *L. perenne* ecotypes v ×*Festulolium* varieties)		1	47.00	0.17	10	***
Among populations/within groups		40	374.51	0.56	34	***
Within populations		593	556.59	0.94	56	***
Near East ▲ v Southern Europe ■	Mediterranean, Danubian					
Among groups (Near Eastern ecotypes v Southern European ecotypes)		1	8.95	-0.01	-1	***
Southern Europe ■ v Western Europe ○	Mediterranean					
Among groups (Southern European		1	11.76	0.00	0	***

ecotypes v Western European ecotypes)						
Southern Europe ■ v Eastern Europe ●	Danubian					
Among groups (Southern European ecotypes v Eastern European ecotypes)		1	18.48	0.06	4	***
Eastern Europe ● v Northern Europe △	Danubian					
Among groups (Eastern European ecotypes v Northern European ecotypes)		1	12.92	0.04	3	***
Near East ▲ v North Africa □	North African					
Among groups (Near Eastern ecotypes v North African ecotypes)		1	10.01	0.03	2	***
North Africa □ v Southern Europe ■	North African					
Among groups (North African ecotypes v Southern European ecotypes)		1	15.65	0.08	4	***
All north of the alps ecotypes v all south of the alps ecotypes	Post-glacial					
Among groups (All northern ecotypes v all southern ecotypes)		1	10.63	0.00	0	***
Among populations/within groups		31	312.45	0.59	39	***
Within populations		477	444.82	0.93	61	N/S
Southern Europe ■ v Irish ecotypes	Into Ireland					
Among groups (Southern European ecotypes v Irish ecotypes)		1	21.91	0.08	5	***
Among populations/within groups		36	216.10	0.31	21	***
Within populations		568	630.13	1.09	74	**
Western Europe ○ v Irish ecotypes	Into Ireland					
Among groups (Western European ecotypes v Irish ecotypes)		1	26.49	0.15	10	***
Among populations/within groups		33	192.76	0.30	19	***
Within populations		524	564.79	1.08	71	***
Northern Europe △ v Irish ecotypes	Into Ireland					
Among groups (Northern European ecotypes v Irish ecotypes)		1	31.30	0.16	10	***

Among populations/within groups	35	198.72	0.29	19	***
Within populations	542	583.69	1.08	71	***

[a]d.f.: degrees of freedom, [b]SSD: Sum of squared differences, [c]p: ** indicates significance value P = <0.01, *** indicates significance value P = <0.001, N/S = not significant, N/A = not applicable

2.5 Discussion

2.5.1 Characterisation of cpDNA diversity at allelic and haplotypic level

All accessions studied displayed a high level of cpDNA SSR allelic variation and considerable numbers of haplotypes were found within ecotypes, within cultivars and within closely related groups of accessions. The high allelic variation also allowed a high total number of 511 haplotypes to be detected with an average of 10.375 haplotypes per accession. Partially this extremely high allelic variation could be explained by the importance of *L. perenne* as a widely cultivated agricultural species. Seed dispersal is the main factor affecting maternal plastid diversity over geographic space. Seeds could have been moved deliberately and accidentally by grazers, birds and wind or by human involvement including seed trade. We have detected numerous chloroplast haplotypes within *Lolium* populations which would suggest that seed dispersal is high between populations over large geographical areas. The high haplotype diversity contrasts with that found in other species groups studied in Ireland. For example, Kelleher *et al.* (2004) used plastid DNA markers (PCR-RFLPs) to characterise plastid types in Irish oak. They found low diversity in comparison with the rest of Europe and low diversity within populations (many showing no haplotype variation). However, other studies such as Echt *et al.* (1998) at nine SSR loci detected 25 alleles and 23 different haplotypes in 159 individuals of red pine (*Pinus resinosa*). Relatively high levels of haplotype variation have been found within and between Irish populations of the outbreeding *Fraxinus excelsior* (Harbourne *et al.* 2005). The results presented here for *Lolium* are the first to characterise an allogamous perennial grass in Ireland. Clearly the breeding system and cultivation history of the species are contributing factors to the high diversity of plastid DNA recorded within and among populations. Rapid molecular evolution of the SSR markers may also be a contributing factor.

Some of the marker loci tested in our study were more variable than others. There are several possible reasons for this. For example, the locus with the least amount of variation, TeaCpSSR7, is located within a gene (*trn*H), whereas the locus with the highest amount of variation, TeaCpSSR3, is located in an intergenic spacer region (*trn*L and *trn*L-F intergenic spacer). This would be in accordance with the theoretical

expectations for the evolution rate of these particular genomic regions (e.g. Wolfe *et al.* 1987). Moreover, the length of the cpSSR PCR product could play a role. At locus TeaCpSSR7 the length of the PCR product is shorter than the product at locus TeaCpSSR3 (McGrath *et al.* 2006). The length of the PCR product could reduce the theoretical possible likelihood of variation for a given length of sequence. Furthermore, longer cpSSR loci are known to display higher levels of molecular divergence than shorter cpSSR loci (Provan *et al.* 2001) partially because slipped strand mis-pairing during DNA replication is greater within these regions.

Individual accessions showed a range of variation in gene diversity values, particularly among the ecotypes. For example, gene diversity values in Irish *L. perenne* ecotypes ranged from 0.122 to 0.333 (Table 2.4.4). Different factors acting on the individual populations may have affected the cytoplasmic diversity of the ecotypes. Isolation and fragmentation of individual populations could reduce diversity values, whereas increased movement of seed between certain ecotypes could have caused proportionally increased diversity.

The diversity of the Irish *L. perenne* ecotype populations was slightly less than (but more or less equal to) European *L. perenne* populations (H_t: 0.312 and 0.330, respectively). Lower diversity might be expected because of the geographic position of Ireland as an island that has isolated Irish *L. perenne* ecotypes from the populations on the continent. If continental Europe was the centre of origin for *L. perenne,* the Irish diversity levels may be expected to be lower. However, plastid diversity was not markedly lower in Irish than in European *L. perenne* ecotypes and this could possibly be due to the thorough collection strategy of the Irish team in the ECPGR collection that aimed to maximize the ecogeographical spread of Irish *L. perenne* populations. The G_{st} value of the Irish *L. perenne* ecotypes is almost half that of the European/Near Eastern *L. perenne* ecotypes (0.238 and 0.431, respectively). This indicates that the European/Near Eastern *L. perenne* ecotypes are more markedly different from each other than the Irish *L. perenne* ecotypes are from each other. Because of this it may be argued that Irish ecotype accessions could be considered as a big meta-population. However significant AMOVA variance components among Irish populations would contradict such a meta-population theory.

Haplotypes were also shown to be highly heterogenous within populations, with only eleven out of 104 populations containing no unique haplotypes (Table 2.4.4). This level of heterogeneity in breeding populations indicated that seed for these populations was derived from many maternal lines, which is in accordance with breeding principles for allogamous forage species (Acquaah, 2006). Fifteen haplotypes were found in a study of 447 *L. perenne* and *L. rigidum* individuals (3%) by Balfourier *et al.* (2000), 41 haplotypes in a study of 168 *Fraxinus excelsior* individuals (24%) by Harbourne *et al.* (2005) compared to 511 haplotypes in 1,575 individuals (32%) in this study. While 27% of haplotypes detected in the study by Balfourier *et al.* (2000) were unique to single populations, 71% of haplotypes detected in this study were unique to single populations.

2.5.2 Plastid genome identification for breeding purposes and identification of cytoplasmic gene pools

While none of the alleles for each of the ten cpSSRs were diagnostic for individual populations, several of the alleles found were unique to specific population groups (Table 2.4.3). It is possible that germplasm from these collections could be identified by genotyping these cpSSR markers if sufficient numbers of individuals are tested. Particularly useful for this purpose could be marker TeaCpSSR8 where half of the alleles were unique to Irish or European *L. perenne* ecotype accessions, respectively (Table 2.4.3). At the haplotype level, the majority of haplotypes detected were unique to specific groups of populations (Figure 2.4.9). While the high level of heterogeneity of haplotypes within populations made it difficult to assign individuals to specific populations, it was possible to use these haplotypes to assign individuals to specific groups. For example, 22% of haplotypes were specific to the Irish *L. perenne* ecotypes, and 24% of haplotypes were specific to the other European *L. perenne* ecotypes. These haplotypes have potential to distinguish geographic *L. perenne* ecotypes and accessions (Table 2.4.5).

The high level of variation, both allelic and haplotypic, in the European and Irish *L. perenne* ecotype collection in comparison with *L. perenne* cultivars suggests that the full cytoplasmic diversity is still underexploited in breeding material (Table 2.4.3 and Figure 2.4.9). Ecotypes with unique plastid variation, not present in breeding material,

could be useful to expand the cytoplasmic gene pool for breeding of the species. A wide variation in plastid type can be useful to enhance the possibility of yield gains and yield stability as demonstrated on a data set for potato (Provan *et al.* 1999a). For both UPGMA dendrograms (Figures 2.4.10 and 2.4.12), the ten cpSSR markers were able to distinguish among Irish and European *L. perenne* ecotypes. This outcome indicated the usefulness of these ten cpSSR markers to identify cytoplasmic gene pools in ecotypes and breeding *L. perenne* germplasm.

Identification of plastid type is also useful for the study of introgression and hybridisation (Johannessen *et al.* 2005; Hodkinson *et al.* 2002), as plastid marker information can identify the source of introgression and can be used in parentage analysis. For example, *L. temulentum* (L11) grouped with two *Festuca* species NL1 and NL4 (Figure 2, group II). This could be an indication of introgression of the plastid genome from *Festuca* species into *Lolium.* AMOVA analysis of Irish and European and Near Eastern *L. perenne* ecotypes versus the ×*Festulolium* cultivars showed there was almost twice as much of the among group variation between Irish *L. perenne* ecotypes and ×*Festulolium* cultivars than between the European/Near Eastern *L. perenne* ecotypes and ×*Festulolium* cultivars (Table 2.4.7). This could suggest more movement of cytoplasmic material between European ecotypes and ×*Festulolium* cultivars than with Irish *L. perenne* ecotypes. Six out of nine ×*Festulolium* cultivars grouped with the European *L. perenne* ecotypes (Figure 2.4.10), which also could indicate introgression from European and Near Eastern *L. perenne* ecotypes into ×*Festulolium.*

Plastid identification could also be used to verify that seed or seedlings derived from crosses in breeding programmes were assigned to the correct maternal parent (*e.g.* Gauthier *et al,* 1997). This could be particularly helpful for *Lolium* breeding in which multiple maternal lines are used in plant breeding (top cross breeding).

2.5.3 Phylogenetic and phylogeographic genetic structure of *Lolium*

Studying plastid DNA variation can contribute to phylogenetic analysis. UPGMA data demonstrated that two broad-leaved *Festuca* species, *F. arundinacea* and *F. pratensis,*

and three narrow-leaved *Festuca species, F. ovina, F. rubra* and *F. vivipara,* grouped together, respectively (Figure 2.4.10). The broad-leaved *Festuca* species grouped closer to *Lolium*. Both of these findings were in agreement with previous studies (Darbyshire & Warwick, 1992; Catalan *et al.* 1997; Charmet *et al.* 1997; Catalan *et al.* 2004; Torrecilla *et al.* 2004). However, some unusual groupings have occurred in the UPGMA dendrogram (Figure 2.4.10). For example, one of the European *L. perenne* ecotypes (■17) was separate from all other *Lolium* accessions. This particular accession was from Sardinia where previously a lot of diversity was found for other species as well *(e.g.* Papa *et al.* 1998). Moreover, unlike other studies (Catalan *et al.* 1997; Charmet *et al.* 1997; Catalan *et al.* 2004; Torrecilla *et al.* 2004), no separation of allogamous and autogamous *Lolium* species was found. These unusual groupings could be explained by high homoplasy in the dataset caused by rapid molecular evolution at the loci studied. Parallel evolution at these loci would therefore be expected to be high and this would obscure phylogenetic signal of the markers (Flannery *et al.* 2006).

A loose correlation of genetic and geographic distances was detected with a Mantel test for the ecotypes where an exact geographic position was available ($r = 0.33$). While studies have tested the correlation between nuclear and geographic distances such as Cresswell *et al.* (2001), this is the first study to test the correlation between plastid genetic and geographic distances in *L. perenne*. We believe the lack of correlation is due to both the high within population plastid diversity and the high degree of seed-mediated gene flow, natural and human related.

The AMOVA analyses indicated that most of the variation in populations used in this study was within groups and individual populations, but that there was also significant population genetic structuring among groups (Table 2.4.7). Generally higher among population variance component values are comparable to AMOVA analysis results of other studies of *L. perenne* populations using nuclear markers (*e.g.* Bolaric *et al.* 2005).

The results for the AMOVA analysis showing the proportion of variance within or among groups were also useful for assessing broad-scale biogeographical patterns. For comparisons among groups examining the Mediterranean migration route in

relation to other possible migration routes, the variance components between groups for the Danubian and North African routes, were zero or close to zero. When a variance component is close to zero, it can mean that there is no population genetic structure (Schneider *et al.* 2000). Close to zero or zero values can also be an indication that samples among groups are more closely related to each other than samples within groups. This would indicate that these population groups are closer to each other than to groups showing a higher among group variance component. For this data set it could be an indication of a Mediterranean movement of *L. perenne* from the Near East across Southern Europe into North Western Europe, Ireland. This is in accordance with one movement theory of *L. perenne* across Europe as proposed by Balfourier *et al.* (2000). This finding was furthermore substantiated in our study by the result of an AMOVA for post glacial partitioning of south of the Alps ecotypes against North of the Alps ecotypes. Their among group variation was zero as well (Table 2.4.7). The post glacial movement hypothesis can be further supported by our UPGMA data (Figure 2.4.12). Southern European, Northern European and Western European *L. perenne* ecotypes grouped together and were distinct from the other European/Near Eastern *L. perenne* ecotypes. This indicated that these population groups were more closely related to each other than to other geographic groups and that movement of seed between these groups has occurred. Finally, the hypothesis that *L. perenne* most likely moved from the South into Ireland can be supported by the lowest among group variation for movement in the AMOVA analysis with the Southern European group (Table 2.4.7: 5%).

2.6 Conclusion

Novel primers were designed which amplified across nine of the ten grass subfamilies. They have potential to be used in many other grass species. They have already been applied to many other genera (e.g. Sungkaew *et al.* unpublished for bamboos; Terrawatanonon *et al.* unpublished for panicoid grasses).

Allelic and haplotypic variation was extremely high within and between Irish and European *L. perenne* ecotypes. Migration of seed material by natural or anthropogenic means, including breeding, could contribute to this high level of variability. High plastid diversity was clearly persisting in populations. The cpSSR markers were shown to be extremely useful for characterizing variation in our accessions and have enabled the identification of cytoplasmic genepools and maternal lineages. The plastid type of individual populations could not be unambiguously identified, but groups of populations could be successfully identified. This suggests that an increase in the number of cpSSR markers would increase the likelihood of identifying individuals within population groups (characterisation of the other SSR markers initiated in this chapter is ongoing; Diekmann *et al.* in preparation). Our findings describe broad scale biogeographical patterns of population genetic structure in this highly heterogenous crop species. Furthermore some evidence was provided to support possible broadscale prehistorical geographical migrations. The results are consistent with a likely pathway of postglacial migration from Southern Europe to Northwest Europe including Ireland.

Chapter 3

Morphological diversity of a collection of *Lolium perenne* L. ecotypes and varieties

3.1 Introduction

3.1.1 Morphology of a grass plant

Grasses show a huge diversity in gross morphology. Some are herbaceous, some woody, some aquatic. They, apart from some outlying groups within the family (Anomochlooideae, Pharoideae), are all characterised by the standard grass spikelet containing one or more florets with specialised structures known as glumes, lemmas, paleas and lodicules (Hubbard, 1984). *Lolium* is in this sense a fairly 'typical' grass genus. It has eight species (Clayton & Renvoize, 1986) and belongs to the Pooideae subfamily and Poeae tribe of grasses. The generic description of *Lolium* from Clayton and Renvoize (1986) is as follows:

"Annual or perennial. Raceme with spikelets in 2 opposite rows edgeways on and partially sunk in rachis. Spikelets several-many-flowered; lower glume absent (except in terminal spikelet); upper glume abaxial, shorter than lemma to as long as spikelet, coriaceous; lemma membranous to coriaceous, with or without a subterminal awn. Hilum linear. All species are more or less interfertile; consequently they intergrade morphologically and are very difficult to separate. Most of them will hybridize with *Festuca arundinacea* and its allies."

Lolium perenne is a tufted perennial grass (Figure 3.1.1) which can range in height from ten to 90 centimetres. Its culms are slender, tend to have two to four nodes and are smooth. Its leaves are green, smooth, and possess membranous ligules with auricles. The blades are folded in the shoots. The spikes can be straight or slightly curved. The spikelets, which are stalkless, alternate on both sides of the axis.

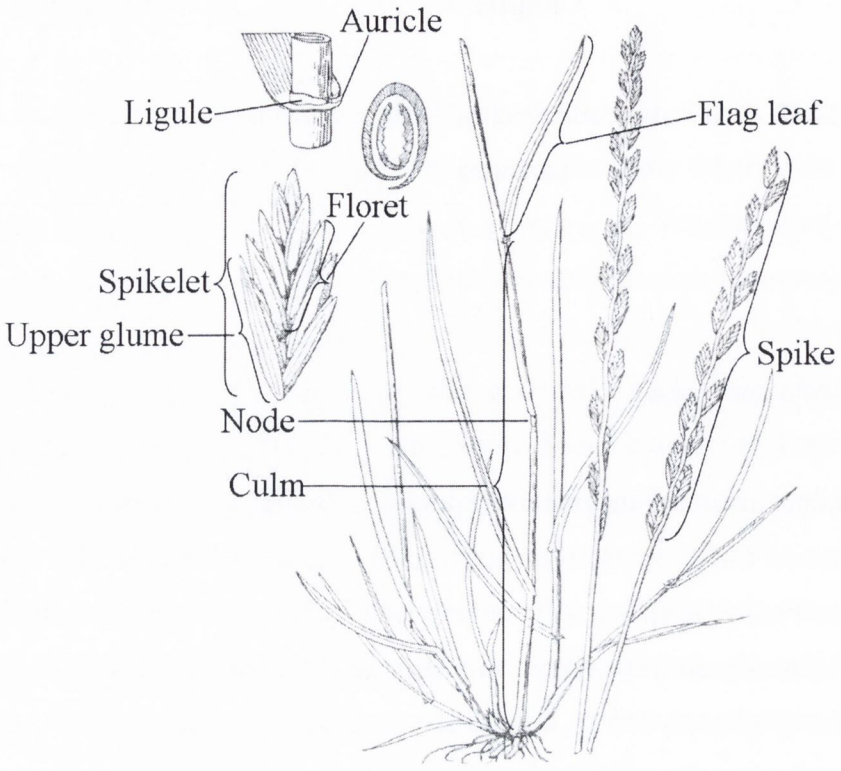

Figure 3.1.1 Morphology of *Lolium perenne*. Source: Hubbard (1984).

3.1.2 Morphological characters and their importance in breeding and systematics

Morphological characters were the earliest markers used in the management of germplasm. They provide an indirect method of analysing genetic diversity at the same time as assessing genotypic performance under normal growing environments. They have been successfully used in many plant species for genetic diversity analysis (e.g .: Cavagnaro *et al.* 2006; Alvarez *et al.* 2007; Routray *et al.* 2007; Zhang *et al.* 2007) and cultivar development (Lafitte *et al.* 2002; Miko *et al.* 2003; Rumball *et al.* 2003a; Rumball *et al.* 2003b). Within *L. perenne*, morphological characters have been used to assess genetic diversity in several studies (Loos, 1993; Kolliker *et al.* 1999; Gilliland *et al.* 2000; Roldan-Ruiz *et al.* 2001; Van Treuren *et al.* 2005; Hazard *et al.* 2006). The most commonly used morphological characters in genetic diversity studies are a mixture of vegetative characters such as plant height at ear emergence and 30 days afterwards, growth habit, length and width of the flag leaf at ear emergence; and reproductive characters such as date of ear emergence, ear length, spikelets per spike, length of spikelet, and glume length.

Morphological characters are also used by the International Union for Protection of Varieties (UPOV) in DUS (distinctness, uniformity and stability) testing of new varieties (UPOV, 2002). The characteristics which are used in this testing must fulfil several basic requirements, namely that the expression of the particular characteristic results from a single genotype or group of genotypes, that it is consistent and repeatable in a particular environment, is sufficiently variable between varieties, that it allows uniformity and stability requirements to be fulfilled, and that it is capable of precise definition. In the case of *L. perenne* varieties, the characters examined in DUS testing (UPOV, 2006) include a single qualitative character (ploidy level) and several quantitative characters (growth habit with and without vernalisation, leaf length and width at the vegetative state, intensity of leaf colour, plant width after vernalisation and at ear emergence, plant height after vernalisation and at ear emergence, tendency to form inflorescences without vernalisation, date of ear emergence after vernalisation, flag leaf length and width, flag leaf length/width ratio, length of the longest stem excluding the inflorescence, length of the upper internode, length of the inflorescence, number of spikelets, density of inflorescence, outer glume length and length of spikelets).

Morphological characters, particularly vegetative characters, and characters such as date of ear emergence are important forage characteristics that are of importance in animal performance. Growth characteristics, such as length and width of leaves, spring growth and summer growth, are important indicators of quality and performance in *L. perenne* (Orr *et al.* 2004; Orr *et al.* 2005; Smit *et al.* 2005).

Manipulating inflorescence/reproductive characters (such as spikelets per spike, and florets per spikelet) during cultivar development in *L. perenne* is important. Breeding varieties for increased quality can cause a loss in characteristics associated with high seed numbers and number of fertile seeds (Marshall & Wilkins, 2003). New cultivars of *L. perenne,* need improved quality characters and increased yield, but also require increased numbers of seeds in order to make multiplication of the new cultivar a viable option.

Morphological characteristics often have advantages over neutral molecular markers for genetic resource characterisation because they record phenotypic variation and

directly relate to important agronomic characters. They can, however, have a number of limitations such as low polymorphism, low heritability, late expression and vulnerability (phenotypic plasticity) to environmental influences.

3.1.3 Studies of *Lolium perenne* morphology

Morphological characters have been used to investigate genetic variability within the genus *Lolium* (Loos, 1993; Loos, 1994; Fernando *et al.* 1997; Kolliker *et al.* 1999; Gilliland *et al.* 2000; Roldan-Ruiz *et al.* 2001; Van Treuren *et al.* 2005). Loos (1993) and Kolliker *et al.* (1999) focused on using morphological characters to investigate genetic variation at the interspecies level. Using 51 ecotypic populations from seven *Lolium* species, principal component analysis (PCA) and canonical variate analysis (CVA) of the morphological data, Loos (1993) showed that populations from the same species grouped together, and that allogamous and autogamous species were separated in PCA mainly by date of ear emergence, number of florets per spikelet and plant height and width. Less variation was seen between the allogamous species than the autogamous species. Using three cultivars each of *L. perenne, Festuca pratensis* and *Dactylis glomerata*, Kolliker *et al.* (1999) used both morphological characters and random amplified polymorphic DNA (RAPD) markers to analyse species and cultivar relationships within and between the three species. While cultivars within species could not be separated with this analysis, higher levels of variation were seen between genotypes within cultivars and between species. Large differences in morphological characters were seen between *L. perenne* and *Festuca pratensis* which was suggested to be due to different environmental adaptations. Gilliland *et al.* (2000) and Roldan-Ruiz *et al.* (2001) used UPOV-listed morphological characters to assess genetic differentiation between cultivars of *L. perenne*. Using twelve diploid populations divided into five groups, Gilliland *et al.* (2000) assessed the ability of morphological characters to separate the groups, and to distinguish 'Initial varieties' from their related 'Essential derived variety'. A variety is deemed to be essentially derived from another (initial) variety if it is predominantly derived from the initial variety, and it retains the essential characteristics resulting from the genotype, or combination of genotypes, of the initial variety. An ability to separate these types of varieties is important from a breeders' rights perspective. PCA separated the five groups of varieties. The initial varieties and essential derived varieties were distinct from each

other, and the magnitude of the difference reflected their known breeding histories. Roldan-Ruiz *et al.* (2001) compared amplified fragment length polymorphism (AFLP) and morphological analysis on 16 varieties of *L. perenne*. Using morphological data, all varieties were found to be distinct following UPOV guidelines. The 'turf' and 'forage' varieties separated from each other, reflecting the different breeding strategies employed for each type. Loos (1994), Fernando *et al.* (1997) and Van Treuren *et al.* (2005) used morphological characters to investigate genetic variation among ecotypes of *L. perenne*. Loos (1994) used morphological characters to analyse differentiation between 21 Dutch ecotypes, 15 European ecotypes and six cultivars which had been mostly derived from Dutch material. PCA showed that Dutch populations were clearly separated from both European ecotypes and the cultivars, based on date of ear emergence, leaf size and plant length. No geographical pattern of morphological differentiation was clear from their data. Fernando *et al.* (1997) analysed 20 populations from a range of habitats with isoenzymes and morphological characters. Populations from conventional grasslands were separated by PCA from the other populations mainly by date of ear emergence, yield and winter damage characters. Again, no relationship between geography and genetic distance was found with these data. Van Treuren *et al.* (2005) analysed 16 Dutch ecotypes and eight cultivars using morphological characters and AFLP markers. Less variation was found in cultivars than in ecotypes, and ecotypes were separated from the cultivars in a PCA, mainly with date of ear emergence and plant vigour characters.

3.1.4 Comparisons of morphological analysis and molecular genetic analysis

The combination of morphological and molecular methods in genetic resource characterisation requires further discussion. Several studies have used morphological and molecular methods together to analyse relationships in *L. perenne* (Fernando *et al.* 1997; Kolliker *et al.* 1999; Gilliland *et al.* 2000; Roldan-Ruiz *et al.* 2000; Roldan-Ruiz *et al.* 2001) and three of these studies directly compare morphological and genetic methods in *L.* perenne (Gilliland *et al.* 2000; Roldan-Ruiz *et al.* 2000; Roldan-Ruiz *et al.* 2001). In the analysis of Fernando *et al.* (1997), isozymes and morphological characters were used. In individual populations, different levels of variation were seen using the different methods. Kolliker *et al.* (1999) used RAPD markers and morphological characters, and both methods showed a lower level of

variation in *Festuca pratensis* compared to *L. perenne*. Gilliland *et al.* (2000) and Roldan-Ruiz *et al.* (2000) analysed the same collection of cultivars with morphological characters, AFLP markers and allozymes. The AFLP markers and morphological characters gave the same clustering of varieties into groups and the same relationships between members of a group. Allozymes put the varieties into the same groupings as AFLP and morphological characters, but only seed protein allozymes showed congruent relationships with AFLP and morphology between varieties within groups. Roldan-Ruiz *et al.* (2001) used morphological characters, sequence tagged site (STS) markers and AFLP markers to analyse relationships between 16 varieties of *Lolium perenne*. Using correlation analysis, no correspondence was seen between morphological characters and either STS or AFLP markers. The only correspondence seen between methods was seen between varieties which were very similar or very distant from each other.

3.2 Aims

This chapter generally aimed to assess morphological variation in *Lolium perenne*, using measurements of morphological characters from a large collection of plants using summary statistics, t-tests, ANOVA (analysis of variance) and multivariate ordination (PCA). It also aimed to compare the morphological results to geography and patterns of diversity determined using plastid DNA microsatellites (cpSSRs). Specific objectives were to:

(1) describe morphological diversity in a collection of Irish *Lolium perenne* ecotypes, along with European *L. perenne* ecotypes and cultivars,

(2) determine if populations or geographic groups of populations can be differentiated using morphological measures and characters,

(3) determine if morphological data have a geographic pattern,

(4) determine if morphological information describes a similar pattern of diversity as chloroplast DNA data,

(5) determine if morphological characters are dependent on each other by means of correlation and regression analyses, and

(6) evaluate characters in use for variety registration such as those used by UPOV in DUS testing to determine distinctness, uniformity and stability.

3.3 Materials and methods

3.3.1 Selection of samples for analysis

A total of 2,481 individuals from a selection of 50 *Lolium perenne* populations (populations) were used to investigate morphological diversity (Appendix 8.1). These populations have been previously used in the work reported in Chapter 2 to investigate chloroplast DNA diversity and population genetic structure and pattern. Between 46 and 50 individuals per population were analysed (Appendix 8.1).

3.3.2 Growth of plant material

Seeds were germinated, and plants transferred to the field in Oak Park, Carlow as described in Chapter 2. Plants were laid out in the field as spaced plants in 2m × 4.5m blocks with 5 plants in each row, 0.5m apart. Blocks were spaced 1m apart from each other in rows of 17 blocks. In 2004 plants were managed under a conservation cut regime with five cuts. After each cut 80kg/ha nitrogen was applied. In 2005 80kg/ha nitrogen fertilizer was applied before the flowering season began.

3.3.3 Scoring of characters

Each plant was scored for the following morphological characters in 2005 (Table 3.3.1): spring growth (on a scale of 1 excellent to 9 very poor), late summer growth (*ditto*), date of ear emergence (recorded in days from April 1st 2005) and presence of awns were recorded. Measurements were taken with a tape measure or Vernier callipers for the following characters: height at ear emergence (cm), length and width of flag leaf at ear emergence (cm), height 30 days after ear emergence (cm), rachis length (cm), awn length (mm) and glume length (mm). Counting was done for the number of spikelets per spike and number of florets per spikelet. For all quantitative characters, four measurements per single plant were taken, and the mean of the measurements taken. For qualitative characters, a single record per plant was taken. Where a plant was lost during the growing season, all results for that plant were removed from the analysis.

Table 3.3.1 Description of characters examined.

Character of interest	Description of character
Spring growth	Visual assessment of the growth of the plant since the first cutting.
Summer growth	Visual assessment of the growth of the plant since the second cutting.
Presence of awns	Presence or absence
Height at ear emergence	Height of the plant in cm from the base of the first tiller to the tip of the spike.
Length of flag leaf at ear emergence	Length of the flag leaf in cm from the ligule to the tip of the blade.
Width of flag leaf at ear emergence	Maximum width of the flag leaf in mm.
Height 30 days at ear emergence	Height of the plant in cm from the base of the first tiller to the tip of the spike.
Rachis length	Length of the rachis from the base of the first spikelet to the base of the terminal spikelet.
Awn length	Length of the awn from the tip of the lemma to the tip of the awn.
Glume length	Length of the lower glume of the lowest spikelet from base to tip.
Spikelets per spike	Number of spikelets per spike
Florets per spikelet	Number of florets per spikelet
Date of ear emergence	Number of days after April 1st

3.3.4 Data analysis

Data analysis for basic statistics, data transformations, correlations and regression analyses were performed using Minitab® Version 15 Statistical Software (Minitab Incorporated, 2000). All equations are given in boxes 3.3.1 to 3.3.4.

For quantitative data, means and standard deviations were calculated for each plant, for each population and for four population groups (Irish ecotypes, European ecotypes, and diploid cultivated varieties and tetraploid cultivated varieties). For all normally distributed characters, two-tailed two sample t-tests (Equation 3.3.1; Box 3.3.1) were used to determine if the means of each group were significantly different from each other. For non-normally distributed characters, Mann-Whitney U tests (Equation 3.3.2; Box 3.3.1) were used to determine if the means of the groups were significantly different from each other. Individual populations were distinguished from each other using the Ryan-Einot-Gabriel-Welsch multiple range test (Ramsey, 1978) according to Equation 3.3.3 (Box 3.3.1) and performed using the Statistical

Analysis System (SAS®) software, Version 9.1 of the SAS System for Windows 2002-2003, SAS Institute Inc., Cary, NC, USA).

Box 3.3.1

$$t = \frac{\bar{x}_1 - \bar{x}_2}{se(\bar{x}_1 - \bar{x}_2)}$$

Equation 3.3.1 T-statistic (Altman, 1991, p211), where $\bar{x}_1$ is the mean of sample 1, $\bar{x}_2$ is the mean of sample 2, and $se(\bar{x}_1 - \bar{x}_2)$ is the standard error of the mean difference. This t-statistic is then compared with the t-distribution with $n_1 + n_2 - 2$ degrees of freedom.

$$U = n_1 n_2 + \frac{1}{2} n_1 (n_1 + 1) - T$$

Equation 3.3.2 Mann-Whitney U statistic (Altman, 1991, p195), where n_1 = sample size of group 1, n_2 = sample size of group 2, and T = the sum of the ranks in the smaller group.

$$\bar{y}_i - \bar{y}_j \geq q(\gamma_p; p, \nu) \frac{s}{\sqrt{n}}$$

Equation 3.3.3 Ryan-Einot-Gabriel-Welsch test, where homogeneity of means $\bar{y}_i, \ldots, \bar{y}_j < j$ is rejected, where $p = j\text{-}i+1$.

$$D = \max\{D^+, D^-\}$$

Equation 3.3.4 Kolmogorov-Smirnov test statistic (START, 2003),
where $D^+ = \max_i \{i/n - Z_{(i)}\}$, $D^- = \max_i \{Z_{(i)} - (i-1)/n)\}$, $Z_{(i)} = F(X_{(i)})$, F(x) is the probability distribution function of the normal distribution, $X_{(i)}$ is the ith order statistics of a random sample, $1 \leq i \leq n$ and n is the sample size.

Normality tests

A histogram for each character was constructed to determine, visually, if the data followed a normal distribution. Normality tests were performed using the Kolmogorov-Smirnov test according to Equation 3.3.4 (Box 3.3.1) and probability

plots were constructed for the test statistics. A character was regarded as being normally distributed, if the p-value was greater than the value of the Kolmogorov-Smirnov test statistic. For the character 'spikelets per spike', values which were considered as outliers in the probability plots were removed, and the tests for normality were repeated.

Data transformation for non-normal distributed characters

Where data were determined not to be normally distributed, data transformation was performed and the tests for normality as described above were repeated. The following transformations were attempted: log transformation, square root transformation, reciprocal transformation and natural log transformation.

For characters which were not normally distributed after data transformation, Johnson's transformations were attempted. Johnson's transformation optimally selects one of three families of distribution: S_B, S_L and S_U where B, L and U refer to the variable being bounded, log-normal, and unbounded respectively (Chou *et al.* 1998; Equations 3.3.5, 3.3.6, 3.3.7; Box 3.3.2). For a transformation function to be fitted to the data, the selected transformation function must have the largest p-value and is greater than the selected p-value (0.05)

Box 3.3.2

$$S_B = \gamma + \eta \ln\left[\frac{(x-\varepsilon)}{(\lambda+\varepsilon-x))}\right]$$

Equation 3.3.5 S_B transformation function (Chou *et al.* 1998), with a range of $\eta, \lambda > 0, -\infty < \gamma < \infty, -\infty < \varepsilon < \infty, \varepsilon < x < \varepsilon + \lambda$.

$$S_L = \gamma + \eta \ln(x-\varepsilon)$$

Equation 3.3.6 S_L transformation function (Chou *et al.* 1998), with a range of $\eta > 0, -\infty < \gamma < \infty, -\infty < \varepsilon < \infty, \varepsilon < x$.

$$S_U = \gamma + \eta \mathrm{Sinh}^{-1}\left[\frac{(x-\varepsilon)}{\lambda}\right]$$

Equation 3.3.7 S_U transformation function (Chou *et al.* 1998), where $\sinh^{-1}(x) = \ln\left[x + \sqrt{(1+x^2)}\right]$, and has a range of $\eta, \lambda > 0$, $-\infty < \gamma < \infty$, $-\infty < \varepsilon < \infty$, $-\infty < x < \infty$.

Correlations between characters

Pearson correlation coefficients were calculated for each pair of normally distributed characters according to Equation 3.3.8 (Box 3.3.3). The Pearson's correlation coefficient measures the degree of linear relationship between two variables (Altman, 1991, p. 278). Where a character could not be transformed to normality (date of ear emergence, spring growth and summer growth), Spearman's rank order correlations were carried out between the non-normally distributed characters and the other characters according to Equation 3.3.9 (Box 3.3.3). Spearman's rank correlation coefficient is obtained by ranking the observations for each character (Altman, 1991, p. 295). Where the correlations between a pair of characters were significant ($p<0.05$) and the Pearson's or Spearman's correlation coefficient > 0.4, scatterplots of the pair of characters were constructed.

Linear regression analysis

For pairs of characters which showed relatively strong significant correlations (rachis length *versus* spikelets per spike, rachis length *versus* florets per spike, and rachis length *versus* glume length), linear regression analysis was carried out.

Before performing linear regressions a check was performed to determine if the data met the following assumptions: (1) data were normally distributed, (2) the variability of variable Y was the same as for variable X, (3) the relationships of variables X and Y were linear (Altman, 1991, p. 303). Residual values for each observation were calculated. The residual is defined as the difference between the observed values and predicted or fitted values that is not explained by the fitted model (Altman, 1991, p. 301). The residual of an observation is calculated according to Equation 3.3.10 (Box 3.3.3). Histograms and normality plots for the residual values were plotted. Residual values were also plotted against the X values.

In simple linear regression, the data are fitted to the model shown in Equation 3.3.11 (Box 3.3.3). The regression coefficient β_o is given by Equation 3.3.12 (Box 3.3.3). The standard error of the coefficient, SE Coeff., is given in Equation 3.3.13 (Box 3.3.3). The coefficient of determination, R^2, indicates how much variation in the response is explained by the model and is calculated using the equation given in Equation 3.3.14 (Box 3.3.3). The R^2 value is adjusted to the number of predictors in the model (R^2 adj.) and is given in Equation 3.3.15 (Box 3.3.3).

The sums of squared distances were calculated to determine the total variation in the data ($\sum (y_i - \bar{y})^2$, where y_i = ith observed response value and $\bar{y}$ = mean response), the proportion of the variation explained by the model ($\sum (\hat{y}_i - \bar{y})^2$, where $\bar{y}$ = mean response, and $\hat{y}_i$ = ith fitted response), and the proportion not explained by the model and attributed to error ($\sum (y_i - \hat{y})^2$, where y_i = ith observed response value and $\hat{y}_i$ = ith fitted response) (Altman, 1991, Chap. 11).

Mean square regression ($\frac{\sum (\hat{y}_i - \bar{y})^2}{p}$, where $\bar{y}$ = mean response, $\hat{y}_i$ = ith fitted response and p = number of terms in the model) and mean square error ($\frac{\sum (y_i - \hat{y}_i)^2}{n - p - 1}$, where y_i = ith observed response value, $\hat{y}_i$ = ith fitted response, n = number of observations, and p = number of terms in the model) were calculated (Altman, 1991, Chap. 11).

For each regression analysis, a fitted line plot was constructed with the 95% confidence interval and 95% prediction interval fitted to the plot. The 95% confidence interval was determined according to Equation 3.3.16 (Box 3.3.3). The 95% prediction interval was determined according to Equation 3.3.17 (Box 3.3.3) (Altman, 1991, Chapter 11).

Stepwise regression

In order to determine if more than one character has an influence on the reproductive characters, stepwise regression was performed on the following sets of characters:

1. Spikelets per spike *versus* rachis length, florets per spikelet (log transformed data), and glume length (log transformed data).
2. Florets per spikelet (log transformed data) *versus* rachis length, spikelets per spike and glume length (log transformed data).
3. Glume length (log transformed data) *versus* rachis length, spikelets per spike and florets per spikelets (log transformed data).

The first step in stepwise regression is to calculate the F statistic and p value for each variable in the model. F is calculated according to Equation 3.3.18 (Box 3.3.3). When the p-value for any variable is greater than the specified value of alpha, the variable with the largest p-value is removed from the model. The regression equation is re-calculated. If a variable cannot be removed from the model, an attempt is made to add a variable. For every variable not in the model, F statistics and p-values are calculated for each variable. F is calculated according to Equation 3.3.19 (Box 3.3.3). When the p-value for any variable is smaller than the specified value of alpha, the variable with the smallest p-value is added to the model. The regression equation is re-calculated. When no more variables can be added or removed from the model, the stepwise procedure ends (Altman, 1991, Chapter 11).

Box 3.3.3

$$\rho = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{(n-1)s_x s_y}$$

Equation 3.3.8 Pearson's correlation coefficient, where $\bar{x}$ is the sample mean for the first variable, S is the standard deviation for the first variable, $\bar{y}$ is the sample mean for the second variable, S is the standard deviation for the second variable and n is the number of samples (Altman, 1991, p 293)

$$\rho_s = 1 - \frac{6\sum_{i=1}^{n} d_i^2}{N^3 - N}$$

Equation 3.3.9 Spearman's correlation coefficient, where d_i is the difference in the ranks for each of the N subjects being studies (Altman, 1991, p 295).

$$e_i = y_i - \hat{y}_i$$

Equation 3.3.10 Residual of an observation, e_i, where y_i = ith observed response value, $\hat{y}_i$ = ith fitted response (Altman, 1991, p 313)

$$Y = \beta_o + \beta_k X_k + e$$

Equation 3.3.11 Simple linear regression model, where Y = response, X = predictor, β_k = kth population regression coefficient and e = error term ~ N(0, 1) (Altman, 1991, p 302).

$$\beta_o = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sum (x_i - \bar{x})^2}$$

Equation 3.3.12 Regression coefficient β_o, where x_i = ith predictor value, $\bar{x}$ = mean predictor, y_i = ith observed response value, $\bar{y}$ = mean response (Altman, 1991, p 311).

$$\text{SE Coeff.} = \frac{s}{\sqrt{\sum (x_i - \bar{x})^2}}$$

Equation 3.3.13 Standard error of the coefficient, SE Coeff., where x_i = ith predictor value, $\bar{x}$ = mean predictor and s = standard deviation (Altman, 1991, p 314).

$$R^2 = 1 - \frac{\sum (y_i - \hat{y}_i)^2}{\sum (y_i - \bar{y})^2}$$

Equation 3.3.14 Coefficient of determination, R^2, where y_i = ith observed response value, $\bar{y}$ = mean response, and $\hat{y}_i$ = ith fitted response (Altman, 1991, p 308).

$$R^2 adj = 1 - \left(\frac{\sum (y_i - \hat{y}_i)^2}{\sum (y_i - \bar{y}_i)^2} \right) \left(\frac{n-1}{n-p-1} \right)$$

Equation 3.3.15 Adjusted R^2 value (R^2 adj.), where y_i = i^{th} observed response value, $\bar{y}$ = mean response, and $\hat{y}_i$ = i^{th} fitted response, n = number of observations, p = number of terms in the model (Altman, 1991, p 345).

$$95\%\ \text{Confidence interval} = \hat{y}_h \pm t_{\left(1-\frac{\alpha}{2},n-p\right)} * s\left(\hat{y}_h\right)$$

Equation 3.3.16 95% Confidence interval, where α = chosen alpha value, n = number of observations, p = number of parameters and

$s\left(\hat{y}_h\right) = \sqrt{MSE\left(X_h'\left(X'X\right)^{-1}X_h\right)} = \sqrt{X_h' s^2\{b\} X_h}$, MSE = mean square error, and $s^2\{b\}$ = variance of the coefficients (Altman, 1991, p 313).

$$95\%\ \text{prediction interval} = \hat{Y}_o \pm t_{\left(1-\frac{\alpha}{2},n-p\right)} * s(pred), s\left(pred\right) = \sqrt{s^2\left(1 + X_o'\left(X'X\right)^{-1}X_o\right)}$$

Equation 3.3.17 95% prediction interval, where $\hat{Y}_o$ = fitted response value for a given set of predictor values, α = level of significance, n = number of observations, p = number of terms in the model, s^2 = mean square error, X = response matrix, X_o = matrix of given predictor values (Altman, 1991, p 315).

$$F_{(1,n-j-1)} = \frac{SSE_{(j-x_r)} - SSE_j}{MSE_j}$$

Equation 3.3.18 F statistic, where n = number of observations, j = number of variables, $SSE_{(j\text{-}xr)}$ = SS error for the model that does not contain x_r, SSE_j = SS error and MSE_j = MS error for the model that contains x_r.

$$F_{(1,n-j-1)} = \frac{SSE_j - SSE_{(j+x_a)}}{MSE_{(j+x_a)}}$$

Equation 3.3.19 F statistic, where n = number of observations, j = number of variables, SSE_j = SS error for the model before x_a is added, $SSE_{(j+xa)}$ = SS error and $MSE_{(j+xa)}$ = MS error for the model after x_a is added.

Principal components analysis

PCA was performed on the population means data using NTSYSpc V2.2 software (Rohlf, 2005).

Original data were standardised using the STAND module according to Equation 3.3.20 (Box 3.3.4). A Euclidean distance matrix was calculated from the standardised data using the SIMINT module according to Equation 3.3.21 (Box 3.3.4). The resulting distance matrix was transformed to scalar product form in order that eigenvalues and eigenvectors could be determined, using the DCENTER module. This 'double centers' the distance matrix by first replacing the off-diagonal element, d_{ij}, with $-\frac{1}{2}d_{ij}^2$. The row and column means are then subtracted from each element and the grand mean is added on. Eigenvalues and eigenvectors were then computed using the EIGEN module. The scalars and the matrix F are found according to the Equation 3.3.22 (Box 3.3.4). In order to determine which characters influenced the separation of populations in each dimension, a canonical variates analysis (CVA) was performed on the data using the POOLVC and CVA modules.

PCA was also performed on the chloroplast microsatellite genetic distance matrix (Appendix 8.3) which was based on Nei's genetic distance (Nei, 1972) for the subset of populations which were also analysed for morphology (Appendix 8.1). The distance matrix was transformed to scalar product form in order that eigenvalues and eigenvectors could be determined, using the DCENTER module and eigenvalues and eigenvectors were then computed using the EIGEN module as above.

Graphs of the eigenvectors and eigenvalues for two dimensions were constructed.

Box 3.3.4

$$y' = \frac{y - \bar{y}_i}{Std(y_i)} - c$$

Equation 3.3.20 Standardisation equation (Milligan & Cooper, 1987) where y = variable of interest, $\bar{y}_i$ = mean of y, $Std(y_i)$ = Standard deviation of y and c = constant.

$$E_i = \sqrt{\sum_{i=1}^{n} (x_i - y_i)^2}$$

Equation 3.3.21 Euclidean distance (Sneath & Sokal, 1973), E_i, between two individuals x and y in dimension i, where n = number of individuals.

$$Af_i = \lambda_i f_i$$

Equation 3.3.22 Matrix F, where A is the n x n symmetric matrix to be operated on, λ_i is the ith eigenvalue, and f_i is the ith eigenvector.

Dendrogram

From the Euclidean distance matrix, a dendrogram showing the similarities between populations was constructed using the unweighted pair group method using arithmetic means (UPGMA) method (Sneath & Sokal, 1973) as implemented in the SAHN module of NTSYSpc V2.2 software (Rohlf, 2005). Bootstrapping analysis was performed on the UPGMA data with 1,000 replicates as implemented in NTSYSpc V2.2 software (Rohlf, 2005).

Mantel test

A Mantel test was used to correlate the pairwise comparisons in the geographic distance matrix (Appendix 8.4) and the Euclidean distance matrix (Appendix 8.6) using the 28 ecotypes where an exact geographical origin was known and which were also used in the morphological analysis, using NTSYSpc V2.2 (Rohlf, 2005). A total of 10,000 permutations were employed to test for significance. A second Mantel test was used to correlate the pairwise comparisons in the Euclidean morphology distance

matrix (Appendix 8.6) and the chloroplast genetic distance matrix (Appendix 8.3) using the 50 populations which were studied in both analyses. A total of 10,000 permutations were employed to test for significance.

ANOVA analysis

One-way analysis of variance (ANOVA) tests were performed to determine the variation between different groups of populations (between populations, between cultivars and ecotypes, between Irish and European ecotypes, and between diploid and tetraploid cultivars). The percentage of variation between groups and its significance was determined. The difference between pairs of populations were determined using the Ryan-Einot-Gabriel-Welsch test statistic (Equation 3.3.4) and between pairs of groups was determined using the t-test and Mann Whitney U tests according to Equations 3.3.1 and 3.3.2.

3.4 Results

3.4.1 Data description

Mean rachis length

For the character mean rachis length ecotype 3408 Italy had the shortest mean rachis length (15.48cm) while cultivar Magician had the longest (24.89cm, Table 3.4.1). The lowest and highest values in the ranges of mean rachis length were higher in cultivars (17.21cm in Greengold to 24.89cm in Magician) than in ecotypes (15.48cm in 3408 Italy to 23.37cm in IV-51-161 Hungary). The standard deviation of rachis length ranged from 1.75cm in the ecotype IRL-OP-02258 to 3.95cm in the cultivar Fennema. The lowest and highest values in the ranges of standard deviations were higher in the cultivars (2.95cm in Sarsfield to 3.95cm in Fennema) than in the ecotypes (1.76cm in IRL-OP-02258 to 3.79cm in IRL-OP-02444). The minimum individual value was a rachis length of 10.38cm in ecotype IRL-OP-02258, while the maximum individual value was 33.95cm in cultivar Magician. Significant differences, as measured by Ryan-Einot-Gabriel-Welsch tests, between populations were primarily seen between the cultivar Magician and most of the Irish (and some of the European) ecotypes, with Magician having longer rachis lengths than the populations from which it is significantly different. Significant differences were also seen between a European population (3408 Italy) and most of the cultivars, some Irish and some European ecotypes, with 3408 Italy having shorter rachis lengths than the other populations. Some of the Irish ecotypes (IRL-OP-02078, IRL-OP-02128, and IRL-OP-02258) are significantly different from some European populations, and cultivars, and have shorter rachis lengths.

Mean spikelets per spike

The ecotype 3408 Italy had the least mean value for spikelets per spike (16.87) while the cultivar Cancan had the most (25.22, Table 3.4.1). The lowest and highest values in the ranges of mean number of spikelets per spike were higher in cultivars (18.61 in Greengold to 25.22 in Cancan) than in ecotypes (16.87 in 3408 Italy to 24.13 in IV-51-161 Hungary). The standard deviation of spikelets per spike ranged from 1.50 in

ecotype IRL-OP-02048 to 4.16 in the cultivar Fennema. The lowest and highest values in the ranges of standard deviations were higher in the cultivars (1.88 in Sarsfield to 4.16 in Fennema) than in the ecotypes (1.5 in IRL-OP-02444 to 3.84 in 3199 Romania Podoloni). The minimum individual value was 5.25 spikelets per spike in ecotype IRL-OP-02419 while the maximum individual value was 33.5 spikelets per spike in the cultivar Cancan. Significant differences between populations are seen between Irish populations (IRL-OP-02078 and IRL-OP-02258) and the European ecotypes and cultivars, with the Irish ecotypes having less spikelets per spike. Again, 3408 Italy is significantly different from some Irish ecotypes, most European ecotypes and most cultivars with lower numbers of spikelets per spike. Cultivar Cancan is significantly different from several Irish ecotypes, having a higher number of spikelets per spike.

Mean florets per spikelet

The ecotype IRL-OP-02078 had the least mean number of florets per spikelet (4.56) while the cultivar Cashel had most (9.10, Table 3.4.1). The lowest and highest values in the ranges of mean number of florets per spikelet were higher in cultivars (4.7 in Sarsfield to 9.1 in Cashel) than in ecotypes (4.56 in IRL-OP-02078 to 8.33 in 16-7-62-2 Nordic). The standard deviations of florets per spikelet ranged from 0.82 in ecotype IRL-OP-02059 to 4.65 in ecotype IRL-OP-02272. However, the next lowest standard deviation was 2.42 in ecotype IRL-OP-02312. The higher standard deviation appears to be caused by an exceptionally high number of florets per spikelet (34.35) in one individual of IRL-OP-02272. Ignoring this population, the ranges of standard deviation of florets per spikelet are similar for cultivars (0.9 in Sarsfield to 2.26 in S24) and ecotypes (0.82 in IRL-OP-02059 to 2.42 in IRL-OP-02312). The minimum individual value of florets per spikelet was 0.5 in ecotype IRL-OP-02173 while the maximum individual value of florets per spikelet was 34.25 in IRL-OP-02272. Ignoring this population, the next highest individual value of florets per spikelet was 18 in IRL-OP-02419. Most significant differences between populations for this character were seen between the cultivars Cashel and Magician and most Irish ecotypes, most European ecotypes and several of the other cultivars, with more florets per spikelet. European ecotype 16-7-62-2 Nordic was significantly different from most Irish ecotypes, two European ecotypes, and most cultivars.

Mean glume length

Ecotype 3408 Italy had the shortest mean glume length (7.03mm), while the cultivar Magician had the longest glumes (10.98mm, Table 3.4.1). The ranges of mean glume length were similar for both cultivars as were their highest and lowest values (7.97mm in Premo to 10.98mm in Magician) and ecotypes (7.03mm in 3408 Italy to 10.83mm in IRL-OP-02068). The standard deviations of glume length ranged from 1.05mm in cultivar Premo to 2.02mm in cultivar Aurora. The lowest and highest values in the ranges of standard deviations were higher in cultivars (1.05mm in Premo to 2.02mm in Aurora) than in ecotypes (1.17mm in 16-7-62-2 Nordic to 1.73mm in No. 10 Spain). The minimum individual value for glume length was 3.63mm in cultivar Millennium to 18.34mm in cultivar Aurora. European ecotypes 3408 Italy and 3199 Romania Podoloni are significantly different from most Irish ecotypes and most cultivars, having shorter glume lengths.

Mean height at ear emergence

The ecotype IRL-OP-02064 had the lowest height at ear emergence (31.65cm) while the cultivar Odenwaelder had the highest (33.22cm, Table 3.4.2). The ranges of mean height at ear emergence were similar for both cultivars (32.12cm in Cancan to 33.22cm in Odenwaelder) and ecotypes (31.65cm in IRL-OP-02064 to 33.21cm in IRL-OP-02241). The standard deviations of height at ear emergence ranged from 1.76cm in cultivar Navan to 2.94cm in ecotype IRL-OP-02250. Standard deviations were similar in cultivars (1.76cm in Navan to 2.71cm in Portstewart) and ecotypes (1.83cm in IRL-OP-02011 to 2.94cm in IRL-OP-02250). The minimum individual value for height at ear emergence was 25.5cm in cultivar Aurora, while the maximum individual value was 39.25cm in the ecotype IRL-OP-02192. There were no significant differences between any of the pairs of populations.

Mean height 30 days after ear emergence

The ecotype IRL-OP-2059 had the lowest mean height 30 days after ear emergence (38.1cm), while the cultivar Navan had the highest (43.24cm, Table 3.4.2),. The

highest and lowest values in ranges for mean height 30 days after ear emergence were higher in cultivars (42.12cm in Aurora to 43.24cm in Navan) than in ecotypes (38.1cm in IRL-OP-02059 to 43.16cm in 16-7-62-2 Nordic. The standard deviations of height 30 days after ear emergence ranged from 1.84cm in the ecotype IRL-OP-02018 to 2.67cm in the cultivar Fennema. The ranges of standard deviations were similar in both cultivars (1.98cm in Cashel to 2.67cm in Fennema) and ecotypes (1.84cm in IRL-OP-02018 to 2.66cm in IRL-OP-02267). The minimum individual value for height 30 days after ear emergence was 33.09cm in ecotype IRL-OP-02059 while the maximum individual value was 49.5cm in ecotype 3408 Italy. Irish ecotype IRL-OP-02059 was significantly different from most of the other ecotypes and cultivars, being lower in height 30 days after ear emergence. Irish ecotype IRL-OP-02337 was significantly different from all but one of the European ecotypes (most cultivars had lower height values than ecotypes).

Mean length of flag leaf

Ecotype IRL-OP-02048 had the shortest flag leaf (14.51cm) while the ecotype IRL-OP-02128 had the longest (15.55cm, Table 3.4.3). The ranges for mean length of flag leaf and their highest and lowest values were similar in cultivars (14.55cm in Sarsfield to 15.36cm in Premo) and in ecotypes (14.51cm in IRL-OP-02048 to 15.55cm in IRL-OP-02128). The standard deviations of length of flag leaf ranged from 1.18cm in the ecotype IRL-OP-02015 to 2.13cm in the cultivar Shandon. The lowest and highest values in the ranges of standard deviations were higher in cultivars (1.37cm in Navan to 2.13cm in Shandon) than in the ecotypes (1.18cm in IRL-OP-02015 to 1.98cm in IRL-OP-02267). The minimum individual value for length of flag leaf was 10.25cm in IRL-OP-02538, while the maximum individual value for length of flag leaf was 19.75cm in IRL-OP-02018). There were no significant differences between any of the pairs of populations.

Mean width of flag leaf

Ecotype No. 10 Spain had the narrowest flag leaf (3.81mm), while the ecotype IRL-OP-02241 had the widest (4.24mm, Table 3.4.2). The ranges for mean width of flag leaf and their minimum and maximum values were similar for both cultivars (3.84mm

in Premo to 4.21mm in Millennium) and for ecotypes (3.81mm in No. 10 Spain to 4.24mm in IRL-OP-02241). The standard deviations of width of flag leaf ranged from 0.56mm in cultivar Shandon to 0.9mm in cultivar Cashel. Ranges of standard deviations were slightly higher in cultivars (0.56mm in Shandon to 0.9mm in Cashel) than in ecotypes (0.58mm in IRL-OP-02173 to 0.79mm in IRL-OP-02059). The minimum individual value of width of flag leaf was 2mm in the ecotype IRL-OP-02538, while the maximum individual value was 6mm in cultivars Cashel and Talbot, and the ecotype IRL-OP-02538. There were no significant differences between any of the populations.

Mean spring growth

All the cultivars, with the exception of Premo, had the highest mean scores for spring growth (Table 3.4.3). The mean scores for spring growth ranged from a high score of 1 in the cultivars Aurora, Cancan, Magician and Millennium, to a low score of 7 in ecotypes IRL-OP-02444, IRL-OP-02250 and 920 Bulgaria. The highest and lowest values in the ranges of mean scores were higher in the cultivars (from 5 in Premo to 1 in Aurora, Cancan, Magician and Millennium) than in the ecotypes (from 7 in 920 Bulgaria, IRL-OP-02259, and IRL-OP-02444 to 3 in 3408 Italy and IRL-OP-02483). Standard deviations ranged from a low of 0.14 in the cultivar Cancan to a high of 2.51 in the ecotype IRL-OP-02007. The highest and lowest values in the ranges of standard deviations were lower in the cultivars (0.14 in Cancan to 1.88 Cashel) than in the ecotypes (1.03 in 920 Bulgaria to 2.51 in IRL-OP-02007). The minimum individual score was 9 which was seen in all ecotypes with the exception of IRL-OP-02015, IRL-OP-02192, 3408 Italy and 3199 Romania Podoloni. The maximum individual score was 1 which was seen in all the varieties (with the exception of Navan and Barlenna) and the ecotypes 3408 Italy, IRL-OP-02048, IRL-OP-02269 and IRL-OP-02419. Significant differences between populations were mainly seen between cultivars and the Irish and European ecotypes, with the cultivars having better spring growth than the ecotypes.

Mean summer growth

The lowest value for summer growth was 7 in the ecotype 920 Bulgaria while the highest value was 1 in cultivar Cancan (Table 3.4.3). Ranges of mean scores for summer growth differed in cultivars (from 5 in Premo to 1 in Cancan) compared to the ecotypes (from 7 in 920 Bulgaria to 2 in 3408 Italy). Standard deviations ranged from 0.47 in cultivar Cancan to 2.18 in ecotype IRL-OP-02272. The lowest and highest values in the ranges of standard deviations were lower in cultivars (0.47 in Cancan to1.53 in Shandon) than in the ecotypes (1.03 in IRL-OP-02048 to 2.18 in IRL-OP-02272). The minimum individual score was 9 in the ecotypes IRL-OP-02272, IRL-OP-02274, IRL-OP-02007, IRL-OP-02312, IRL-OP-02267, IRL-OP-02059, IRL-OP-02337, IRL-OP-02250, IRL-OP-02192, IRL-OP-02173, IRL-OP-02258, IRL-OP-02444, IRL-OP-02419, IRL-OP-02442 and 920 Bulgaria. The maximum individual score was 1 in cultivars Barlenna, Navan and Premo, and the cultivars 3408 Italy and IRL-OP-02269. Similar significant differences between populations to spring growth were seen for summer growth, with even more populations being significantly different from each other. Again, the cultivars showed better summer growth than the ecotypes.

Mean date of ear emergence

For the character date of ear emergence, the mean values (measured in days after April 1st) ranged from 23 days (April 23rd) in cultivar Aurora to 67 days (June 6th) in cultivar Sarsfield (Table 3.4.3). Aurora showed an extremely early date of ear emergence. With the exception of Aurora, the lowest and highest values in the range of date of ear emergence were later in cultivars (42 days in S24, May 12th, to 67 days, June 6th, in Sarsfield) than in the ecotypes (35 days, May 5th, in 3408 Italy to 62 days, June 1st, in IRL-OP-02018). Standard deviations ranged from 1.07 in cultivar Premo to 4.74 in ecotype IRL-OP-02059. The lowest and highest values in the ranges of standard deviations were higher in ecotypes (from 1.36 in IRL-OP-02442 to 4.74 in IRL-OP-02059) than in cultivars (from 1.07 in Premo to 3.63 in Odenwaelder). The minimum individual date of ear emergence was 19 days, April 19th, in cultivar Aurora, while the maximum individual date of ear emergence was 70 days, June 9th, in cultivar Sarsfield. Almost all populations were significantly different from each

other, with the Irish ecotypes generally having an earlier heading date than the cultivars.

Presence of awns

Awns were only present in six individuals: two individuals from the ecotype IRL-OP-02015 and single individuals from the ecotypes IRL-OP-02250, No. 10 Spain and 3408 Italy and from the cultivar Millennium. This character was not further analysed due to lack of variation.

Table 3.4.1 Summary statistics (mean, standard deviation, minimum and maximum) for each population for the reproductive quantitative characters rachis length, spikelets per spike, florets per spike and glume length.

Population Number	Group[1]	Rachis length				Spikelets per spike				Florets per spikelet				Glume length			
		X^2	SD^3	min^4	max^4	X	SD	min	max	X	SD	min	max	X	SD	min	max
IRL-OP-02337	I 1	$20.75^{cdefghij}$	3.47	12.60	29.65	$21.53^{cdefghijklmn}$	2.42	17.50	28.75	6.38^{defghi}	1.31	3.00	9.25	10.71^{abcd}	1.59	5.86	13.97
IRL-OP-02059	I 2	19.12^{ijkl}	3.19	14.05	24.05	20.041^{mnopq}	2.74	14.50	25.50	$5.49^{ghijklm}$	0.82	3.25	7.00	9.53^{defgh}	1.64	6.10	12.99
IRL-OP-02007	I 3	19.32^{ijklm}	2.90	13.00	24.85	19.54^{nopqr}	2.19	15.75	24.00	5.36^{hijklm}	1.22	3.25	9.25	8.99^{fghijk}	1.36	6.08	12.78
IRL-OP-02011	I 4	$19.73^{efghijk}$	3.03	13.45	26.15	$20.68^{fghijklmnop}$	2.05	16.75	25.50	$5.55^{ghijklm}$	1.18	3.50	9.00	9.25^{efghi}	1.20	7.07	11.74
IRL-OP-02015	I 5	$21.07^{bcdefghi}$	2.98	15.20	27.50	19.68^{mnopqr}	2.19	14.50	25.00	7.58^{cde}	1.67	3.00	12.25	9.42^{efghi}	1.23	6.93	11.35
IRL-OP-02048	I 6	19.83^{hijkl}	2.40	15.08	26.58	$21.83^{cdefghijklm}$	1.50	18.75	24.50	5.28^{hijklm}	1.08	2.75	8.00	8.64^{hijkl}	1.20	6.51	10.92
IRL-OP-02192	I 7	19.53^{ijkl}	2.69	13.75	25.33	$21.31^{efghijklmn}$	2.40	16.50	26.25	$5.36^{ghijklm}$	1.03	3.50	7.75	8.12^{ijkl}	1.38	5.72	10.90
IRL-OP-02312	I 8	20.081^{ghijk}	2.82	13.68	25.98	22.64^{bcdefg}	3.33	12.50	29.75	6.69^{defgh}	2.42	3.00	13.50	9.51^{efghi}	1.51	7.09	13.41
IRL-OP-02064	I 10	19.40^{ijkl}	3.45	13.45	28.63	22.53^{bcdefg}	2.45	16.75	26.25	$5.82^{fghijkl}$	1.07	3.50	8.25	7.87^{klm}	1.19	5.38	11.14
IRL-OP-02078	I 11	16.36^{mn}	2.42	11.43	21.48	17.60^{qrs}	2.36	13.75	21.50	4.56^{m}	1.08	3.00	7.50	9.01^{fghijk}	1.27	6.50	12.56
IRL-OP-02230	I 12	18.80^{ijklm}	3.63	13.13	26.98	$19.88^{klmnopq}$	3.14	14.00	24.75	4.74^{klm}	1.05	3.00	6.75	8.81^{fghijk}	1.58	6.37	13.30
IRL-OP-02128	I 13	17.141^{mn}	3.36	10.50	26.65	$19.73^{ijklmnop}$	2.98	10.50	25.75	$5.61^{ghijklm}$	1.30	2.75	9.25	8.73^{ghijk}	1.60	5.38	12.53
IRL-OP-02538	I 14	19.57^{ghijkl}	2.97	14.73	25.85	$20.59^{fghijklmnop}$	2.74	15.00	27.00	$5.55^{ghijklm}$	1.04	3.50	7.50	9.98^{abcdef}	1.56	6.96	13.80
IRL-OP-02274	I 15	$20.20^{efghijk}$	2.38	16.40	26.68	$20.07^{jklmnop}$	2.18	16.75	25.50	$6.01^{efghijk}$	1.08	4.00	7.75	$9.88^{abcdefg}$	1.49	7.72	12.72
IRL-OP-02442	I 17	17.69^{klm}	3.05	11.45	26.43	18.58^{opqrs}	2.67	11.25	24.00	4.98^{klm}	1.20	3.25	8.00	$9.79^{bcdefgh}$	1.40	7.11	13.78
IRL-OP-02444	I 18	18.93^{ijklm}	3.79	10.95	28.90	$20.24^{hijklmnop}$	3.32	12.25	28.50	$5.55^{ghijklm}$	1.22	3.75	9.50	8.90^{fghijk}	1.56	5.70	12.59
IRL-OP-02068	I 19	22.41^{bcde}	2.79	16.53	27.40	$21.20^{defghijklmn}$	2.51	15.00	26.25	7.73^{abc}	1.68	4.75	11.50	10.83^{ab}	1.59	7.95	15.14
IRL-OP-02241	I 20	18.95^{ijklm}	2.49	13.63	23.75	19.83^{mnopqr}	2.64	13.00	24.50	$5.49^{ghijklm}$	1.12	3.50	8.50	$9.93^{abcdefg}$	1.36	7.18	13.29
IRL-OP-02419	I 21	19.66^{ghijkl}	2.82	11.95	25.70	19.65^{mnopqr}	3.05	5.25	25.50	6.16^{efghij}	2.32	3.00	18.00	9.22^{efghij}	1.52	6.55	13.07
IRL-OP-02258	I 22	16.50^{mn}	1.76	12.65	20.33	17.53^{rs}	2.10	13.00	21.50	5.21^{ijklm}	1.19	3.00	8.00	$9.71^{bcdefgh}$	1.32	5.88	12.29
IRL-OP-02272	I 23	22.40^{bcdef}	3.02	16.33	27.98	22.38^{bcdef}	2.56	18.50	29.75	7.79^{abc}	4.65	4.75	34.25	9.53^{efgh}	1.43	7.23	13.94
IRL-OP-02250	I 24	18.52^{jklm}	3.14	10.38	26.08	$21.12^{fghijklmnop}$	1.97	16.25	24.75	$5.82^{fghijkl}$	1.61	2.50	10.50	9.45^{cdefgh}	1.40	5.99	12.34
IRL-OP-02267	I 25	19.90^{hijkl}	3.12	14.83	26.80	$21.91^{cdefghijklmn}$	3.46	16.50	28.25	$5.38^{ghijklm}$	1.23	3.75	10.00	8.64^{hijkl}	1.40	6.02	11.47
IRL-OP-02269	I 26	18.74^{jklm}	3.58	12.63	26.80	19.67^{nopqr}	3.56	11.50	26.25	$5.82^{fghijkl}$	1.68	1.25	8.75	$9.67^{bcdefgh}$	1.27	7.42	12.47
IRL-OP-02173	I 27	$21.27^{bcdefghi}$	3.13	15.23	29.63	$21.87^{cdefghijklm}$	2.58	16.25	29.25	$5.72^{ghijklm}$	1.59	0.50	8.75	9.18^{efghij}	1.56	6.00	12.58
IRL-OP-02483	I 28	$21.06^{bcdefghi}$	2.89	15.46	26.50	$20.99^{fghijklmno}$	2.40	15.25	26.25	6.93^{cdef}	1.06	4.50	9.50	10.38^{abcde}	1.38	7.52	13.41
IRL-OP-02018	I 30	19.27^{ijkl}	2.84	12.80	23.98	$20.34^{ghijklmnop}$	2.74	14.75	25.50	$5.84^{fghijkl}$	1.05	4.00	8.50	8.84^{fghijk}	1.39	5.73	11.58
16-7-62-2 Nordic	△5	22.77^{abc}	3.19	14.43	27.13	$22.22^{bcdefghi}$	2.90	16.00	28.75	8.33^{ab}	1.22	4.75	10.67	9.14^{efghij}	1.17	7.23	12.18

No 10 Spain	■15	18.88^{ijklm}	3.17	11.00	25.68	20.53^{fghijklmnop}	2.52	12.00	25.25	7.13^{cde}	1.88	3.75	13.75	8.87^{fghijk}	1.73	4.05	13.93
3408 Italy	■16	15.48^{n}	2.90	11.00	23.20	16.87^{s}	2.74	11.50	25.75	5.99^{fghijkl}	1.42	3.50	10.50	7.03^{m}	1.34	4.15	10.15
3013 Romania	■18	21.40^{bcdefghi}	2.69	17.85	28.75	23.48^{abcd}	2.75	18.13	30.75	6.34^{efghij}	1.34	3.50	8.50	8.14^{jklm}	1.40	5.74	11.63
3199 Romania	■19	23.10^{bcd}	3.25	12.48	29.85	23.48^{abcde}	3.84	6.00	31.50	5.86^{fghijklm}	1.48	2.50	8.50	7.53^{lm}	1.47	4.03	13.12
Podoloni																	
920 Bulgaria	■20	20.49^{defghij}	3.70	12.73	31.18	20.55^{ghijklmnop}	3.08	13.75	27.50	5.27^{jklm}	1.17	3.33	8.25	8.73^{fghijk}	1.34	5.48	11.45
IV-51-161	●32	23.37^{ab}	2.96	16.80	30.03	24.13^{ab}	3.13	17.50	32.25	6.46^{defg}	1.24	3.50	8.75	8.41^{hijkl}	1.35	4.89	11.91
Hungary																	
cv. Aurora	V 1	22.69^{bcd}	3.12	17.68	29.48	23.13^{abcde}	2.00	16.75	27.00	6.13^{efghijk}	1.07	4.25	9.50	9.67^{bcdefgh}	2.02	6.53	18.34
cv. Barlenna	V 2	20.44^{cdefghij}	3.38	12.33	26.45	21.72^{bcdefghij}	3.14	13.00	26.75	5.44^{ghijklm}	0.91	3.25	7.25	8.79^{fghijk}	1.31	5.56	12.00
cv. Cancan	V 3	21.02^{bcdefghi}	3.03	15.80	28.70	25.22^{a}	3.63	17.25	33.50	5.18^{ijklm}	0.97	3.50	7.50	9.26^{efghij}	1.40	6.67	14.28
cv. Cashel	V 4	23.68^{ab}	2.90	16.60	30.33	22.68^{bcdef}	2.61	18.50	29.00	9.10^{a}	1.25	7.00	12.00	9.61^{efgh}	1.41	7.22	13.44
cv. Fennema	V 5	21.04^{bcdefghi}	3.95	12.68	27.90	21.91^{bcdefghi}	4.16	7.75	28.75	5.49^{ghijklm}	1.41	3.25	9.50	8.90^{fghijk}	1.76	6.18	13.63
cv. Greengold	V 6	17.21^{lmn}	2.50	12.73	23.23	18.61^{pqrs}	2.22	14.00	22.25	4.83^{klm}	1.23	2.00	8.00	8.64^{efghij}	1.34	7.00	11.57
cv. Magician	V 7	24.89^{a}	3.27	17.08	33.95	23.15^{abcde}	2.59	17.00	28.50	8.49^{ab}	1.35	5.50	11.75	10.98^{a}	1.41	8.27	13.70
cv.	V 8	23.19^{abc}	2.92	16.28	28.20	22.74^{bcdefgh}	2.39	16.75	29.25	5.62^{ghijklm}	1.03	3.50	8.00	10.69^{abc}	1.88	3.63	14.24
Millennium																	
cv. Navan	V 9	22.87^{bcd}	3.45	14.05	33.80	20.38^{fghijklmnop}	2.42	14.50	26.00	5.84^{fghijkl}	1.18	3.50	8.75	9.89^{abcdefg}	1.51	5.88	13.61
cv.	V 10	21.98^{bcdefg}	2.90	15.63	28.90	23.55^{abc}	3.29	17.25	32.25	5.84^{fghijkl}	1.32	3.50	8.75	9.21^{efghij}	1.18	6.20	12.46
Odenwaelder																	
cv. Portstewart	V 11	20.86^{cdefghij}	3.01	16.05	28.15	22.17^{bcdefghijk}	2.42	18.00	27.50	5.30^{ghijklm}	1.09	3.50	8.50	8.93^{fghijk}	1.48	6.62	12.32
cv. Premo	V 12	20.34^{defghij}	2.79	14.08	25.65	21.68^{bcdefghijkl}	2.66	17.00	27.25	5.94^{efghijk}	1.30	3.25	8.50	7.97^{jklm}	1.05	6.43	10.54
cv. S24	V 13	21.95^{bcdefgh}	2.89	15.80	28.70	22.37^{bcdefgh}	3.23	14.75	28.75	8.04^{abc}	2.26	4.50	19.25	8.92^{fghijk}	1.17	6.96	12.61
cv. Sarsfield	V 14	21.09^{bcdefghi}	2.35	15.83	25.73	20.68^{fghijklmnop}	1.88	17.50	25.00	4.70l^{m}	0.90	2.75	6.50	8.79^{fghijk}	1.21	5.05	11.74
cv. Shandon	V 15	20.41^{defghij}	2.81	14.05	26.88	21.98^{bcdefghijkl}	2.82	16.50	26.75	7.41^{bcd}	1.34	5.00	10.50	9.75^{bcdefgh}	1.37	7.08	13.47
cv. Talbot	V 16	21.27^{bcdefghib}	2.60	15.68	25.20	22.10^{bcdefghijkl}	2.08	16.00	26.25	5.99^{fghijk}	1.17	3.00	8.00	9.73^{bcdefgh}	1.46	6.47	13.74

[1]Group: I = Irish ecotype, △= Northern Europe group, ■ = Southern Europe group, ● = Eastern Europe group, V = *Lolium perenne* variety; [2]*X*: arithmetic mean; [3]SD: standard deviation; [4]*min*: minimum value, [5]*max*: maximum value, Means (*X*) followed by a common letter are not significantly different at $p \leq 0.05$ with the Ryan-Einot-Gabriel-Welsch test.

Table 3.4.2 Summary statistics (mean, standard deviation, minimum and maximum) for each population for the vegetative quantitative characters height at ear emergence, height 30 days after ear emergence, length of flag leaf and width of flag leaf.

Population Number	Group [1]	Height at ear emergence				Height 30 days after ear emergence				Length of flag leaf				Width of flag leaf			
		X[2]	*SD*[3]	*min*[4]	*max*	*X*	*SD*	*min*	*max*	*X*	*SD*	*min*	*max*	*X*	*SD*	*min*	*max*
IRL-OP-02337	I 1	32.29[a]	2.41	27.25	37.75	42.29[a]	2.46	36.50	47.25	15.04[a]	1.50	12.00	18.75	3.99[a]	0.65	2.75	5.75
IRL-OP-02059	I 2	32.60[a]	2.66	27.00	39.25	38.10[b]	2.10	33.09	43.03	14.74[a]	1.51	11.75	18.00	3.95[a]	0.79	2.75	5.75
IRL-OP-02007	I 3	32.57[a]	2.76	26.75	38.75	42.56[a]	2.55	38.00	49.00	15.08[a]	1.57	11.25	18.00	4.00[a]	0.61	2.75	5.75
IRL-OP-02011	I 4	33.19[a]	1.83	29.75	38.25	42.71[a]	2.33	38.50	47.25	15.08[a]	1.67	11.00	19.25	4.08[a]	0.59	2.75	5.50
IRL-OP-02015	I 5	32.39[a]	2.70	26.50	37.25	42.32[a]	2.44	38.25	48.25	15.16[a]	1.18	12.00	17.00	3.89[a]	0.78	2.25	5.50
IRL-OP-02048	I 6	32.36[a]	1.93	28.75	35.75	42.48[a]	2.34	38.75	47.75	14.51[a]	1.70	11.25	18.25	3.95[a]	0.77	2.25	5.25
IRL-OP-02192	I 7	32.77[a]	2.37	28.50	39.25	42.30[a]	1.95	38.25	47.25	14.74[a]	1.42	11.00	17.00	4.00[a]	0.65	2.75	5.25
IRL-OP-02312	I 8	32.98[a]	2.31	28.50	39.00	42.42[a]	2.15	38.00	48.75	14.96[a]	1.23	13.00	18.25	3.88[a]	0.77	2.25	5.25
IRL-OP-02064	I 10	31.65[a]	2.66	25.75	37.75	41.76[a]	2.03	38.25	46.25	14.90[a]	1.69	12.00	18.25	4.04[a]	0.77	2.50	5.75
IRL-OP-02078	I 11	32.42[a]	1.87	28.00	35.25	41.97[a]	2.65	35.75	46.75	14.89[a]	1.32	12.00	17.75	4.19[a]	0.58	3.00	5.25
IRL-OP-02230	I 12	32.41[a]	2.28	28.00	37.50	42.00[a]	2.21	38.00	47.00	15.43[a]	1.51	12.00	18.75	3.83[a]	0.78	2.50	5.75
IRL-OP-02128	I 13	32.68[a]	2.44	28.00	39.25	43.13[a]	2.37	38.50	47.00	15.55[a]	1.58	12.75	19.00	3.92[a]	0.77	2.25	5.50
IRL-OP-02538	I 14	33.12[a]	2.74	27.25	38.50	42.40[a]	2.21	38.25	48.00	15.31[a]	1.49	10.25	18.75	4.18[a]	0.77	2.00	6.00
IRL-OP-02274	I 15	32.80[a]	2.81	27.25	37.00	42.51[a]	2.46	38.00	46.00	15.19[a]	1.29	11.00	17.25	4.09[a]	0.70	2.75	5.50
IRL-OP-02442	I 17	32.51[a]	2.60	26.00	37.25	42.48[a]	2.28	36.75	47.50	14.80[a]	1.56	11.50	17.50	3.85[a]	0.73	2.75	5.50
IRL-OP-02444	I 18	32.47[a]	2.26	28.25	37.25	43.10	2.43	37.75	47.75	15.15[a]	1.57	11.75	18.50	3.91[a]	0.69	3.00	5.50
IRL-OP-02068	I 19	32.54[a]	2.29	28.00	37.00	42.54[a]	2.17	38.25	48.00	15.13[a]	1.59	11.75	18.25	4.16[a]	0.59	3.00	5.75
IRL-OP-02241	I 20	33.21[a]	2.26	28.25	38.50	42.46[a]	2.26	37.00	48.00	15.09[a]	1.63	12.50	18.00	4.24[a]	0.69	3.00	5.75
IRL-OP-02419	I 21	32.57[a]	2.37	27.00	36.00	41.77[a]	2.50	36.50	46.25	14.72[a]	1.48	11.00	17.75	4.05[a]	0.67	2.50	5.50
IRL-OP-02258	I 22	32.35[a]	1.91	28.75	37.50	42.33[a]	2.20	38.00	46.25	14.92[a]	1.50	11.50	19.50	3.94[a]	0.68	2.75	5.50
IRL-OP-02272	I 23	32.24[a]	2.30	27.75	36.75	41.91[a]	2.03	38.00	45.75	15.49[a]	1.37	12.00	17.75	4.07[a]	0.62	2.75	5.00
IRL-OP-02250	I 24	32.91[a]	2.94	27.00	39.00	42.13[a]	2.55	37.50	46.50	14.93[a]	1.48	10.50	17.75	3.89[a]	0.75	2.25	5.25
IRL-OP-02267	I 25	32.77[a]	2.20	27.25	37.00	42.75[a]	2.66	37.75	47.75	14.89[a]	1.98	11.25	19.50	3.98[a]	0.65	2.75	5.50
IRL-OP-02269	I 26	32.57[a]	2.32	27.25	37.00	42.66[a]	2.13	38.00	47.00	14.80[a]	1.54	11.50	18.50	3.95[a]	0.64	2.50	5.25
IRL-OP-02173	I 27	31.86[a]	2.09	27.50	35.25	42.81[a]	2.06	38.75	46.25	14.61[a]	1.70	10.75	18.50	4.04[a]	0.58	3.00	5.25
IRL-OP-02483	I 28	32.36[a]	1.89	28.25	36.75	41.99[a]	2.46	36.75	47.25	15.09[a]	1.46	11.25	18.50	4.14[a]	0.75	3.00	5.75
IRL-OP-02018	I 30	32.88[a]	2.56	28.00	38.75	42.79[a]	1.84	38.25	47.25	15.33[a]	1.68	12.50	19.75	3.88[a]	0.76	2.25	5.25
16-7-62-2 Nordic	△5	32.47[a]	2.24	28.00	36.75	43.16[a]	2.03	37.25	47.75	15.17[a]	1.53	12.25	18.25	4.20[a]	0.70	2.25	5.75
No 10 Spain	■15	32.96[a]	2.52	28.25	38.25	42.44[a]	2.43	37.00	47.00	15.23[a]	1.18	13.00	17.50	3.81[a]	0.71	2.25	5.25

3408 Italy	■16	32.10[a]	2.33	27.50	37.00	42.44	2.36	37.00	49.50	15.35[a]	1.66	11.75	18.25	4.02[a]	0.69	2.50	5.25
3013 Romania	■18	32.33[a]	2.14	27.75	36.25	42.87	2.22	37.25	47.25	14.89[a]	1.49	11.75	18.50	3.98[a]	0.68	2.25	5.50
3199 Romania Podoloni	■19	32.54[a]	1.84	28.50	37.00	42.34	2.56	37.25	47.25	14.85[a]	1.74	11.75	18.75	3.83[a]	0.66	2.50	5.00
920 Bulgaria	■20	32.37[a]	2.55	26.50	37.75	42.67	2.40	37.50	46.75	14.88[a]	1.62	12.50	18.50	4.14[a]	0.73	2.75	5.50
IV-51-161 Hungary	●32	32.09[a]	2.43	27.75	38.25	41.87[a]	2.12	36.50	46.50	14.93[a]	1.67	12.00	19.50	4.17[a]	0.75	2.50	5.75
cv. Aurora	V 1	32.24[a]	2.49	25.50	38.00	42.12[a]	2.42	37.00	47.25	14.99[a]	1.46	12.50	18.00	3.89[a]	0.68	2.50	5.50
cv. Barlenna	V 2	32.54[a]	2.22	27.50	38.25	42.81	2.55	36.00	47.75	15.11[a]	1.49	12.00	18.00	4.15[a]	0.66	2.75	5.75
cv. Cancan	V 3	32.12[a]	1.94	28.50	37.50	42.67	2.09	38.25	47.25	15.13[a]	1.46	10.75	18.25	3.97[a]	0.66	2.50	5.50
cv. Cashel	V 4	32.48[a]	2.51	26.75	38.00	42.19[a]	1.98	38.00	46.75	15.05[a]	1.70	11.50	18.75	4.02[a]	0.90	2.50	6.00
cv. Fennema	V 5	32.76[a]	2.07	27.25	37.75	42.46	2.67	37.25	47.75	14.84[a]	1.46	11.75	17.75	4.16[a]	0.81	2.25	5.75
cv. Greengold	V 6	32.73[a]	2.47	26.75	37.50	42.56[a]	2.26	37.50	48.00	14.72[a]	1.44	11.50	16.75	4.12[a]	0.67	2.75	5.25
cv. Magician	V 7	32.60[a]	2.65	27.50	38.25	42.99	2.55	37.50	48.00	14.67[a]	1.54	11.25	17.50	4.19[a]	0.74	3.00	5.75
cv. Millenium	V 8	32.33[a]	2.24	28.00	37.00	42.34	2.32	38.00	46.25	15.11[a]	1.53	12.25	18.50	4.21[a]	0.74	2.50	5.75
cv. Navan	V 9	32.54[a]	1.76	28.25	36.75	43.24	2.15	39.25	47.50	14.82[a]	1.37	12.50	18.25	4.09[a]	0.69	2.75	5.50
cv. Odenwaelder	V 10	33.22[a]	2.58	27.25	38.25	42.70	2.07	39.00	47.25	14.91[a]	1.52	11.25	18.00	4.10[a]	0.73	2.75	5.75
cv. Portstewart	V 11	32.74[a]	2.71	27.00	38.00	42.42[a]	2.50	36.00	47.50	15.21[a]	1.69	11.00	18.25	3.95[a]	0.72	2.50	5.50
cv. Premo	V 12	32.50[a]	1.78	28.75	36.25	42.44[a]	2.60	35.50	47.00	15.36[a]	1.54	12.50	18.25	3.84[a]	0.71	3.00	5.75
cv. S24	V 13	33.11[a]	2.15	28.00	37.25	42.82	2.00	37.50	46.50	15.16[a]	1.70	12.00	18.75	3.94[a]	0.75	2.25	5.50
cv. Sarsfield	V 14	32.68[a]	2.68	26.50	37.00	42.21[a]	2.55	36.25	46.50	14.55[a]	1.53	11.00	18.00	3.98[a]	0.62	3.00	5.25
cv. Shandon	V 15	32.34[a]	2.42	28.00	36.25	42.74	2.41	37.75	47.25	14.86[a]	2.13	11.25	19.50	3.91[a]	0.56	3.00	5.25
cv. Talbot	V 16	32.21[a]	2.45	27.00	37.50	42.64	2.43	37.75	48.00	14.97[a]	1.42	11.50	18.25	4.14[a]	0.70	2.75	6.00

[1]Group: I = Irish ecotype, △= Northern Europe group, ■ = Southern Europe group, ● = Eastern Europe group 6, V = *Lolium perenne* variety; [2]*X*: arithmetic mean; [3]SD: standard deviation; [4]*min*: minimum value, [5]*max*: maximum value, Means (*X*) followed by a common letter are not significantly different at $p \leq 0.05$ with the Ryan-Einot-Gabriel-Welsch test.

Table 3.4.3 Summary statistics (mean, standard deviation, minimum and maximum) for each population for the qualitative characters spring growth, summer growth and date of ear emergence.

	Group[1]	Spring Growth				Summer Growth				Ear Emergence			
		X[2]	SD[3]	min[4]	max[5]	X	SD	min	max	X	SD	min	max
IRL-OP-02337	I 1	6^{abcdef}	2.37	0	9	7^{a}	1.61	4	9	55^{e}	2.81	50	60
IRL-OP-02059	I 2	6^{abcde}	1.82	2	9	7^{abc}	1.58	4	9	52^{fg}	4.74	44	59
IRL-OP-02007	I 3	6^{abcd}	2.51	2	9	6^{cdef}	1.85	3	9	59^{d}	2.29	55	63
IRL-OP-02011	I 4	$5^{cdefghij}$	2.08	2	9	4^{ghi}	1.5	2	7	41^{qr}	1.39	39	43
IRL-OP-02015	I 5	4^{mnopq}	1.63	2	7	4^{ghi}	1.36	2	6	38st	1.54	36	40
IRL-OP-02048	I 6	$4^{ijklmnop}$	1.79	1	9	3^{ijk}	1.03	2	5	48^{kl}	3.56	41	53
IRL-OP-02192	I 7	$5^{defghij}$	1.34	2	8	6^{abcd}	1.67	4	9	43^{opq}	2.66	39	46
IRL-OP-02312	I 8	$4^{hijklmnop}$	1.51	2	9	6^{abcde}	2.13	3	9	52^{gh}	3.07	47	56
IRL-OP-02064	I 10	$4^{ijklmnop}$	1.44	2	9	4^{gh}	1.41	2	6	51^{ghi}	2.37	47	54
IRL-OP-02078	I 11	$5^{ghijklm}$	1.90	0	9	6^{abcd}	1.47	4	8	41^{qr}	1.57	39	44
IRL-OP-02230	I 12	$5^{efghijk}$	1.88	2	9	5^{defg}	1.84	3	8	49^{jk}	3.73	43	54
IRL-OP-02128	I 13	4^{mnopq}	1.57	2	9	4^{gh}	1.83	2	7	40^{rs}	1.96	36	43
IRL-OP-02538	I 14	4^{lmnopq}	1.86	0	9	4^{ghi}	1.79	2	7	41^{qr}	1.68	39	44
IRL-OP-02274	I 15	$5^{bcdefgh}$	2.18	3	9	6^{bcdef}	1.82	3	9	41^{qr}	1.68	39	44
IRL-OP-02442	I 17	$4^{hijklmno}$	1.81	2	9	7^{a}	1.39	5	9	38st	1.36	36	40
IRL-OP-02444	I 18	7^{ab}	1.79	2	9	7^{ab}	1.76	4	9	58^{d}	3.23	53	64
IRL-OP-02068	I 19	$4^{hijklmno}$	2.10	2	9	$5f^{gh}$	2.01	2	8	44no	1.72	41	47
IRL-OP-02241	I 20	$4^{hijklmno}$	1.49	3	9	5^{cdef}	1.72	3	8	47^{lm}	2.81	43	51
IRL-OP-02419	I 21	$6^{abcdefg}$	2.02	1	9	6^{abcd}	2.00	4	9	45^{mn}	3.77	39	51
IRL-OP-02258	I 22	$5^{cdefghi}$	2.19	2	9	7^{abc}	1.73	4	9	42^{pqr}	1.39	39	44
IRL-OP-02272	I 23	6^{abc}	2.31	2	9	5^{efg}	2.18	2	9	53^{fg}	3.77	47	59
IRL-OP-02250	I 24	7^{ab}	1.57	3	9	7^{abc}	1.66	4	9	43^{opq}	2.37	39	47
IRL-OP-02267	I 25	6^{abcdef}	2.01	3	9	6^{abcd}	1.57	4	9	50^{hij}	1.73	47	53
IRL-OP-02269	I 26	$4^{jklmnop}$	1.89	1	9	2^{klm}	1.09	1	4	41^{pqr}	4.11	35	48
IRL-OP-02173	I 27	$5^{cdefghi}$	1.55	3	9	6^{abcd}	1.68	4	9	58^{d}	2.01	55	61
IRL-OP-02483	I 28	3^{nopq}	1.93	0	9	4^{ghi}	1.85	2	7	38^{t}	1.43	36	40
IRL-OP-02018	I 30	$5^{fghijkl}$	1.53	2	9	5^{defg}	1.60	3	8	62^{c}	3.99	54	67
16-7-62-2 Nordic	△5	$5^{hijklmn}$	1.40	3	9	5^{fgh}	1.47	3	7	43^{opq}	1.68	40	45
No 10 Spain	■15	$4^{klmnopq}$	2.31	0	9	5^{fgh}	1.31	3	7	45^{mn}	3.9	39	51
3408 Italy	■16	3^{nopq}	1.40	1	8	2^{klmn}	1.05	1	4	35^{u}	4.31	30	40

3013 Romania	■18	$5^{cdefghi}$	1.39	2	9	5^{efgh}	1.36	3	7	55^{e}	1.89	53	58
3199 Romania Podoloni	■19	$4^{hijklmnop}$	1.03	3	8	5^{efgh}	1.39	3	7	55^{e}	1.55	53	57
920 Bulgaria	■20	7^{a}	1.84	3	9	7^{a}	1.36	5	9	49^{ijk}	2.17	46	53
IV-51-161 Hungary	●32	$5^{ghijklm}$	1.27	2	9	5^{fgh}	1.51	3	7	45^{mn}	1.86	43	48
cv. Aurora	V 1	1^{t}	0.53	1	4	3^{klm}	1.01	1	4	23^{v}	2.41	19	27
cv. Barlenna	V 2	3^{nopq}	0.57	3	5	3^{ijk}	1.09	2	5	48^{kl}	2.26	45	51
cv. Cancan	V 3	1^{t}	0.14	1	2	1^{n}	0.47	1	2	64^{b}	3.16	59	69
cv. Cashel	V 4	3^{qrs}	1.88	1	9	3^{jk}	1.49	1	5	45^{mn}	3.36	39	50
cv. Fennema	V 5	3^{pqr}	1.28	1	7	2^{klm}	1.07	1	4	46^{mn}	1.67	43	48
cv. Greengold	V 6	2st	0.96	1	5	2^{klm}	1.11	1	4	58^{d}	2.21	55	61
cv. Magician	V 7	1^{t}	0.74	1	5	$2l^{mn}$	0.74	1	3	43^{op}	2.84	39	47
cv. Millenium	V 8	1^{t}	0.62	1	4	2^{mn}	0.50	1	2	61^{c}	1.63	59	64
cv. Navan	V 9	3^{opqr}	1.35	2	8	4^{ijh}	1.32	2	6	62^{c}	1.36	59	63
cv. Odenwaelder	V 10	3^{qrs}	1.07	1	5	3^{kl}	1.02	1	4	58^{d}	3.63	51	63
cv. Portstewart	V 11	3^{qrs}	1.58	1	9	2^{klm}	1.21	1	4	64^{b}	1.97	61	67
cv. Premo	V 12	$5^{hijklmn}$	1.71	0	9	5^{efgh}	1.45	3	7	52^{fg}	1.07	51	54
cv. S24	V 13	3^{qrs}	1.23	1	6	3^{kl}	1.07	1	4	42^{pq}	1.31	40	44
cv. Sarsfield	V 14	2st	0.83	0	4	2^{klm}	1.11	1	4	67^{a}	1.74	65	70
cv. Shandon	V 15	2^{rst}	1.13	1	5	3^{kl}	1.53	1	5	58^{d}	3.14	53	63
cv. Talbot	V 16	3^{qrs}	1.21	1	8	2^{klmn}	1.12	1	4	54^{ef}	2.09	51	57

[1]Group: I = Irish ecotype, △= Northern Europe group, ■ = Southern Europe group, ● = Eastern Europe group 6, V = *Lolium perenne* variety; [2]*X*: arithmetic mean; [3]SD: standard deviation; [4]*min*: minimum value, [5]*max*: maximum value, Means (*X*) followed by a common letter are not significantly different at $p \leq 0.05$ with the Ryan-Einot-Gabriel-Welsch test.

Character summaries per group (overall, cultivars, diploid cultivars, tetraploid cultivars, ecotypes, Irish ecotypes, European ecotypes)

The character rachis length had an overall mean of 20.36cm, while the mean of ecotypes was lower at 19.73cm, and the mean of cultivars was 21.62cm (Table 3.4.4). This difference was highly significant with $p < 0.001$ (Table 3.4.5). Within ecotypes, the difference between Irish (mean: 19.45cm) and European (mean: 20.77cm) was highly significant at $p < 0.0001$. Within cultivars, the difference between diploid cultivars (mean: 21.46cm) and tetraploid cultivars (mean: 22cm) was significant at $p < 0.05$.

The character spikelets per spike had an overall mean of 21.19, while the mean of ecotypes was lower at 20.65, and the mean of cultivars was 22.26 (Table 3.4.4). This difference was highly significant with $p < 0.001$ (Table 3.4.5). Within ecotypes, the difference between Irish (mean: 20.38) and European (mean: 21.66) was highly significant with $p < 0.0001$. Within cultivars, the difference between diploid cultivars (mean: 22.73) and tetraploid cultivars (mean: 21.2) was highly significant with $p < 0.0001$.

The character florets per spikelet had an overall mean of 6.05, while the mean of ecotypes was lower at 5.96, and the mean of cultivars was 6.23 (Table 3.4.4). This difference was significant at $p < 0.05$ (Table 3.4.5). Within ecotypes, the difference between Irish (mean: 5.83) and European (mean: 6.48) was highly significant with $p < 0.0001$. Within cultivars, the difference between diploid cultivars (mean: 6.37) and tetraploid cultivars (mean: 5.94) was highly significant with $p < 0.001$.

The character glume length had an overall mean of 9.22mm, while the mean of ecotypes was lower at 9.12mm, and the mean of cultivars was 9.43mm (Table 3.4.4). This difference was highly significant with $p < 0.001$ (Table 3.4.5). Within ecotypes, the difference between Irish (mean: 9.35mm) and European (mean: 8.23mm) was highly significant with $p < 0.0001$. Within cultivars, the difference between diploid cultivars (mean: 9.2mm) and tetraploid cultivars (mean: 9.47) was highly significant ($p < 0.0001$).

The character date of ear emergence had an overall mean of 48.96 days after April 1st, while the mean of ecotypes was earlier at 47.07 days after April 1st, and the mean of cultivars was 52.89 days after April 1st (Table 3.4.4). This difference was not significant (Table 3.4.5). Within ecotypes, the difference between Irish (mean: 47.09 days after April 1st) and European (mean: 46.97 days after April 1st) was highly significant ($p < 0.0001$). Within cultivars, the difference between diploid cultivars (mean: 50.41 days after April 1st) and tetraploid cultivars (mean: 58.37 days after April 1st) was highly significant ($p < 0.0001$).

The character spring growth had an overall mean of 4.10, while the mean of ecotypes was lower at 4.93, and cultivars showed better spring growth with a mean 2.39 (Table 3.4.4). This difference was highly significant ($p < 0.0001$) (Table 3.4.5). Within ecotypes, the difference between Irish (mean: 4.99) and European (mean: 4.67) was less pronounced but significant ($p < 0.05$). Within cultivars, the difference between diploid cultivars (mean: 2.62) and tetraploid cultivars (mean: 1.89) was highly significant ($p < 0.0001$).

The character summer growth had an overall mean of 4.51, while the mean of ecotypes was lower at 5.08, and the mean of cultivars was 2.70 (Table 3.4.4). This difference was highly significant ($p < 0.0001$) (Table 3.4.5). Within ecotypes, the difference between Irish (mean: 5.51) and European (mean: 4.89) was highly significant ($p < 0.0001$). Within cultivars, the difference between diploid cultivars (mean: 2.79) and tetraploid cultivars (mean: 2.50) was significant ($p < 0.05$).

The remaining characters, height at ear emergence (overall mean 32.52cm, Table 3.4.4), height 30 days after ear emergence (overall mean 42.43cm), length of flag leaf (overall mean 15cm) and width of flag leaf (4.02mm), showed no significant differences between groups of populations (Table 3.4.5) with the exception of height 30 days after ear emergence with showed a significant ($p < 0.05$) difference between ecotypes (mean: 42.35) and cultivars (mean: 42.58), and length of flag leaf which showed a significant difference ($p < 0.05$) between diploid (mean: 15.02cm) and tetraploid (mean: 14.77cm) cultivars.

Variation in the characters (standard deviations, Table 3.4.4) was highest in the production characters, spring growth (54.39%) and summer growth (49.67%), and in the reproductive character florets per spikelet (29.59%). It was lowest in the generative characters, height at ear emergence (7.26%), height 30 days after ear emergence (5.63%) and length of flag leaf (4.33%). The other characters had intermediate variation, that is, rachis length (17.63%), spikelets per spike (14.74%), width of flag leaf (17.41%), and date of ear emergence (18.83%).

Table 3.4.4 Overall and group means and standard deviations (in parentheses) for the characters: rachis length, spikelets per spike, florets per spikelet, glume length, height at ear emergence, height 30 days after ear emergence, length of flag leaf, width of flag leaf, date of ear emergence, spring growth, and summer growth.

	Rachis length	Spikelets per spike	Florets per spikelet	Glume length	Height at ear emergence	Height 30 days after ear emergence	Length of flag leaf	Width of flag leaf	Date of ear emergence	Spring growth	Summer growth
Overall	20.36	21.19	6.05	9.22	32.52	42.43	15.00	4.02	48.96	4.10	4.51
	(3.59)	(3.23)	(1.79)	(1.72)	(2.36)	(2.39)	(1.54)	(0.70)	(9.22)	(2.23)	(2.24)
Cultivars	21.62	22.26	6.23	9.43	32.55	42.58	14.94	4.05	52.89	2.39	2.70
	(3.40)	(3.16)	(1.78)	(1.87)	(2.33)	(2.33)	(1.56)	(0.72)	(11.17)	(1.47)	(1.42)
Diploid	21.46	22.73	6.36	9.20	32.53	42.55	15.02	4.02	50.41	2.62	2.79
	(3.16)	(3.16)	(1.77)	(1.50)	(2.34)	(2.32)	(1.60)	(0.72)	(11.40)	(1.54)	(1.46)
Tetraploid	22.00	21.20	5.94	9.95	32.57	42.63	14.77	4.10	58.38	1.89	2.50
	(3.87)	(2.93)	(1.75)	(2.44)	(2.32)	(2.36)	(1.47)	(0.69)	(8.35)	(1.18)	(1.31)
Ecotypes	19.73	20.65	5.96	9.12	32.51	42.35	15.03	4.00	47.07	4.93	5.38
	(3.52)	(3.12)	(1.79)	(1.63)	(2.37)	(2.41)	(1.53)	(0.70)	(7.42)	(2.06)	(2.03)
Irish	19.45	20.38	5.83	9.35	32.55	42.31	15.03	3.99	47.09	4.99	5.51
	(3.29)	(2.86)	(1.79)	(1.57)	(2.39)	(2.44)	(1.52)	(0.69)	(7.50)	(2.09)	(2.05)
European	20.77	21.66	6.48	8.23	32.39	42.50	15.06	4.02	46.97	4.67	4.89
	(4.11)	(3.79)	(1.71)	(1.56)	(2.29)	(2.31)	(1.55)	(0.72)	(7.10)	(1.90)	(1.90)

Table 3.4.5 Results of t-tests and Mann-Whitney U tests between groups of observations for the characters: rachis length, spikelets per spike, florets per spikelet, glume length, height at ear emergence, height 30 days after ear emergence, length of flag leaf, width of flag leaf, date of ear emergence, spring growth, and summer growth.

	Rachis length†	Spikelets per spike†	Florets per spikelet†	Glume length†	Height at ear emergence†	Height 30 days after ear emergence†	Length of flag leaf‡	Width of flag leaf‡	Date of ear emergence‡	Spring growth‡	Summer growth‡
Cultivated varieties *versus* ecotypes	<0.0001	<0.0001	<0.005	<0.0001	N/S	<0.05	NS	NS	<0.0001	<0.0001	<0.0001
Irish ecotypes *versus* European ecotypes	<0.0001	<0.0001	<0.0001	<0.0001	NS	NS	NS	NS	N/S	<0.05	<0.0001
Diploid cultivars *versus* tetraploid cultivars	<0.05	<0.0001	<0.001	<0.0001	NS	NS	<0.05	NS	<0.0001	<0.0001	<0.05

*NS: Non significant; †Differences between groups tested with t-tests; ‡Differences between groups tested with Mann-Whitney U tests.

3.4.2 Data analysis

Normality tests for characters

Histograms were constructed for each character to display the distribution of their variability (Figures 3.4.1 and Figure 3.4.2). The histograms for rachis length, length of flag leaf, width of flag leaf, height at ear emergence and height at 30 days after ear emergence had a normal distribution appearance (in a bell-shaped curve). The histograms for the other characters appeared to be skewed, either to the left (spikelets per spike, and date of ear emergence) or to the right of the bell curve (florets per spikelet, glume length, spring growth and summer growth).

Probability plots were constructed for each character using the Kolmogorov-Smirnov test statistic (Figures 3.4.3 and 3.4.4). Rachis length, length of flag leaf, width of flag leaf, height at ear emergence and height at 30 days after ear emergence followed a straight line in these plots indicating normal distribution. The other characters deviated from a straight line in the tails of the distributions, indicating that these characters may not be normally distributed.

The indications from the histograms and probability plots were confirmed using the Kolmogorov-Smirnov test statistic (Table 3.4.6). A character is deemed normally distributed if the value of the Kolmogorov test statistic is smaller than the corresponding p-value.

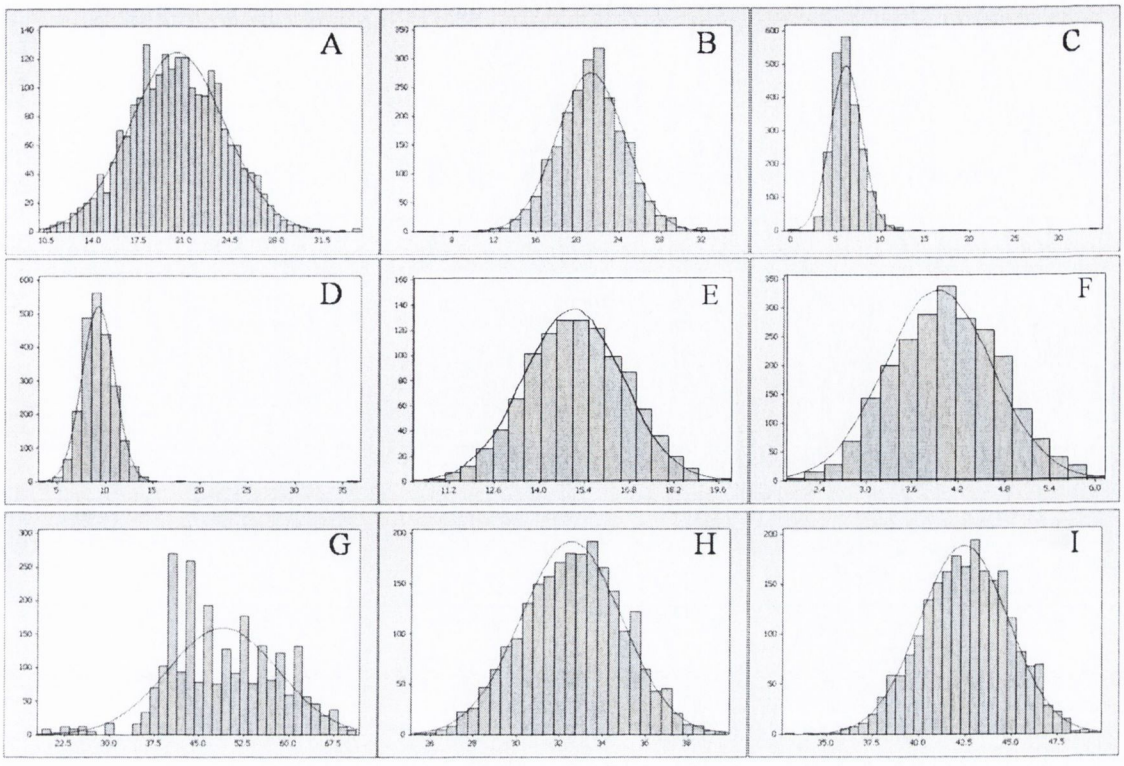

Figure 3.4.1 Histograms with fitted normal distribution curves for the following characters: A: Rachis length, B: Spikelets per spike, C: Florets per spikelet, D: Glume length, E: Length of flag leaf, F: Width of flag leaf, G: Date of ear emergence, H: Height at ear emergence, I: Height 30 days after ear emergence. Y-axis: Frequency. X-axis: value of character of interest.

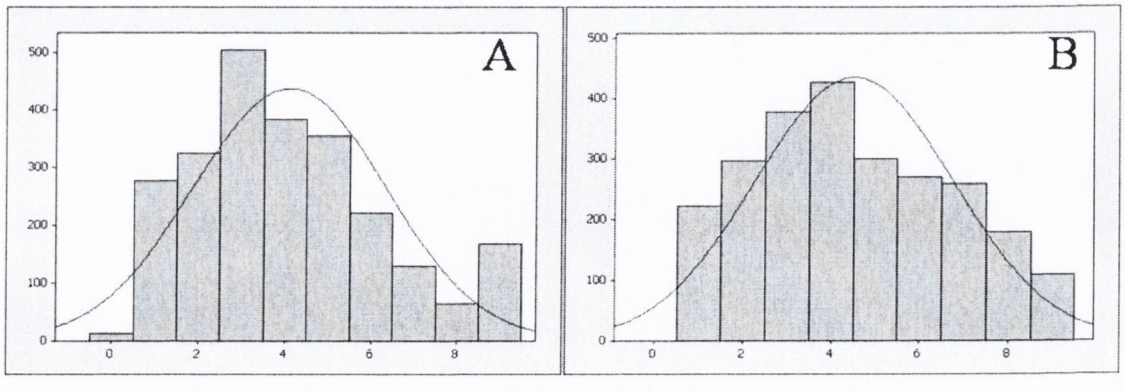

Figure 3.4.2 Histograms with fitted normal distribution curves for the following characters: A: Spring growth, B: Summer growth. Y-axis: Frequency. X-axis: value of character.

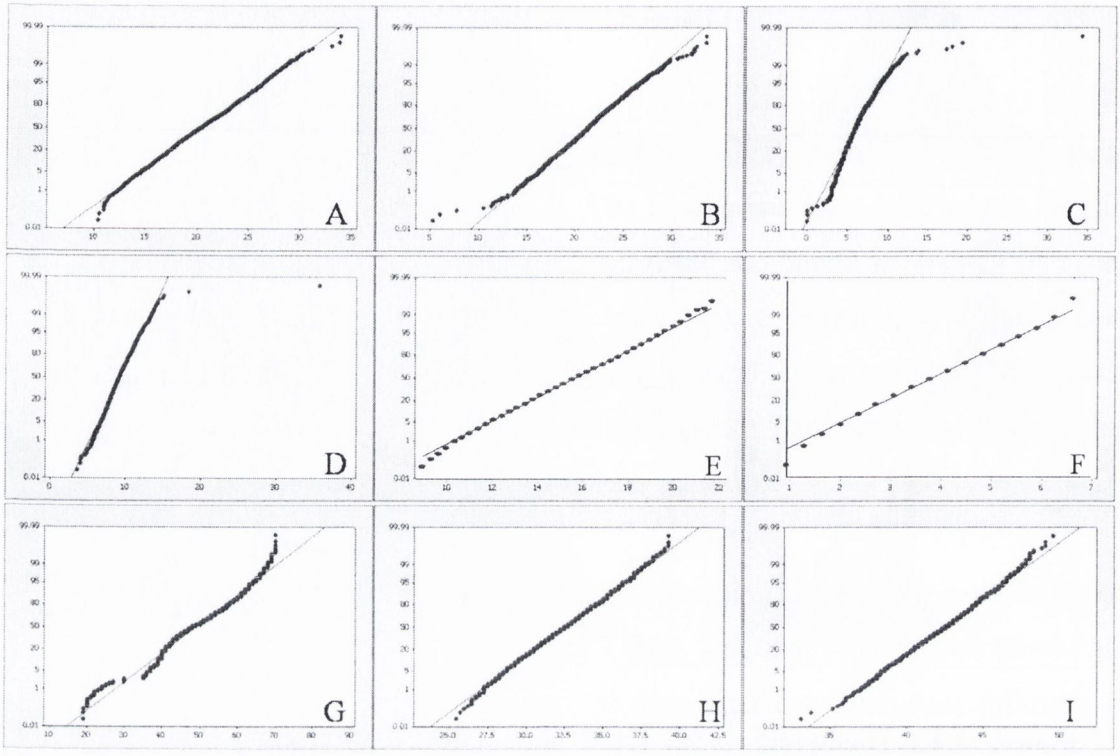

Figure 3.4.3 Probability plots using the Kolmogorov-Smirnov test for the following characters: A: Rachis length (cm), B: Spikelets per spike, C: Florets per spikelet, D: Glume length (mm), E: Length of flag leaf (cm), F: Width of flag leaf (mm), G: Date of ear emergence, H: Height at ear emergence, I: Height 30 days after ear emergence. Y-axis: Percentage. X-axis: character value of interest.

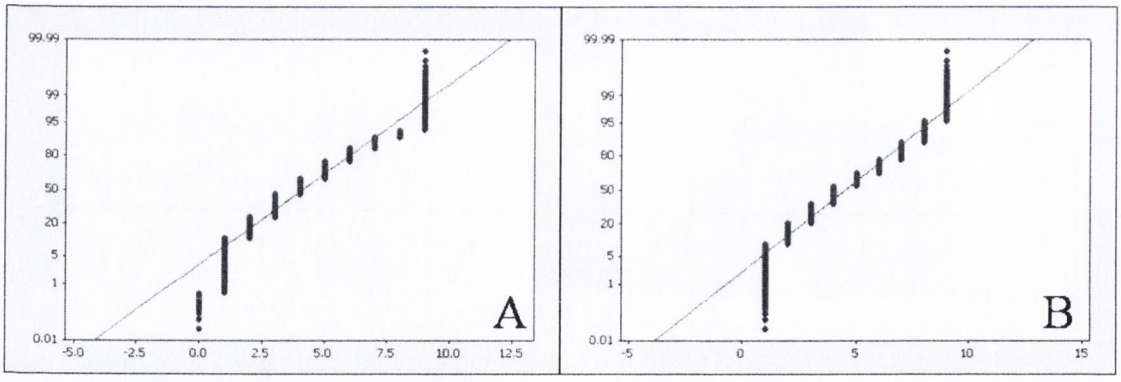

Figure 3.4.4 Probability plots using the Kolmogorov-Smirnov test for the following characters: A: Spring growth, B: Summer growth. Y-axis: Percentage. X-axis: character value of interest.

Table 3.4.6 Kolmogorov-Smirnov statistics and p-values for each character.

Charater	Kolmogorov-Smirnov statistic	p-value
Rachis length	0.020	0.032
Spikelets per spike*	0.022	0.015
Florets per spikelet*	0.074	<0.010
Glume length*	0.036	<0.010
Length of flag leaf	0.013	>0.150
Width of flag leaf	0.015	>0.150
Date of ear emergence*	0.055	<0.010
Height at ear emergence	0.013	>0.150
Height 30 days after ear emergence	0.018	0.063
Spring growth*	0.056	<0.010
Summer growth*	0.045	<0.010

*Non-normally distributed characters

For the character spikelets per spike, outliers were removed from the data, a histogram and normality plot were constructed and normality tests repeated. The character now appeared normally distributed in both the histogram (Figure 3.4.5) and the normality plot (Figure 3.4.6). The indications from the histogram and probability plot were confirmed using the Kolmogorov-Smirnov test statistic (Kolmogorov-Smirnov statistic: 0.018, p-value: 0.074).

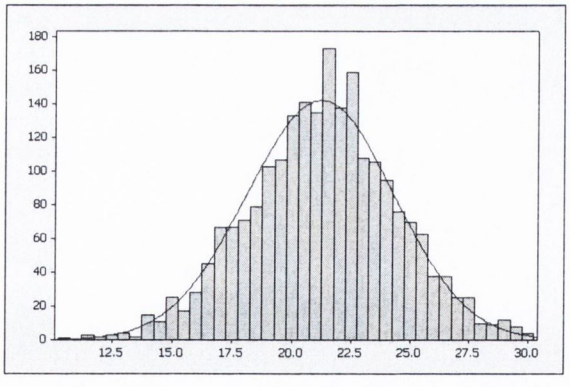

Figure 3.4.5 Histogram with fitted normal distribution curves for the character spikelets per spike. Y-axis: Frequency. X-axis: value of character.

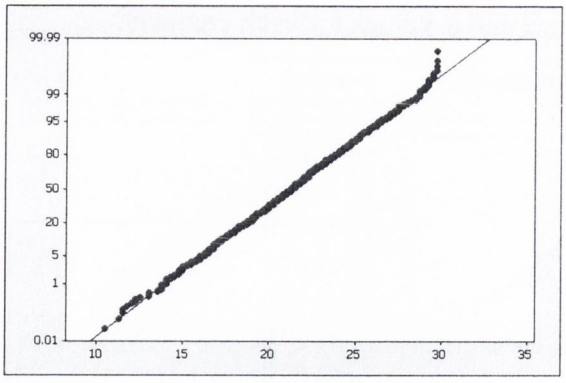

Figure 3.4.6 Probability plots using the Kolmogorov-Smirnov test for the character spikelets per spike. Y-axis: Percentage. X-axis: character value of interest.

Data transformation for non-normal distributed characters

(1) Log transformation

Data from the non-normally distributed characters, florets per spikelet, glume length, date of ear emergence, spring growth and summer growth, were transformed using a log transformation. Histograms with fitted normal distributions were constructed (Figure 3.4.7). The histograms for data of the log transformed characters, florets per spikelet and glume length, appeared to be normally distributed. The data of other characters were still either skewed to the left (date of ear emergence) or did not have enough values in the tails of the distributions (spring growth and summer growth).

Probability plots were constructed for each character using the Kolmogorov-Smirnov test statistic (Figure 3.4.8). Log transformed data for the characters florets per spikelet and glume length followed a straight line in the probability plots. The characters date of ear emergence, spring growth and summer growth were still deviating from a straight line in the tails of the distributions. Indications of normality in the plots were confirmed with the Kolmogorov-Smirnov test statistic (Table 3.4.7).

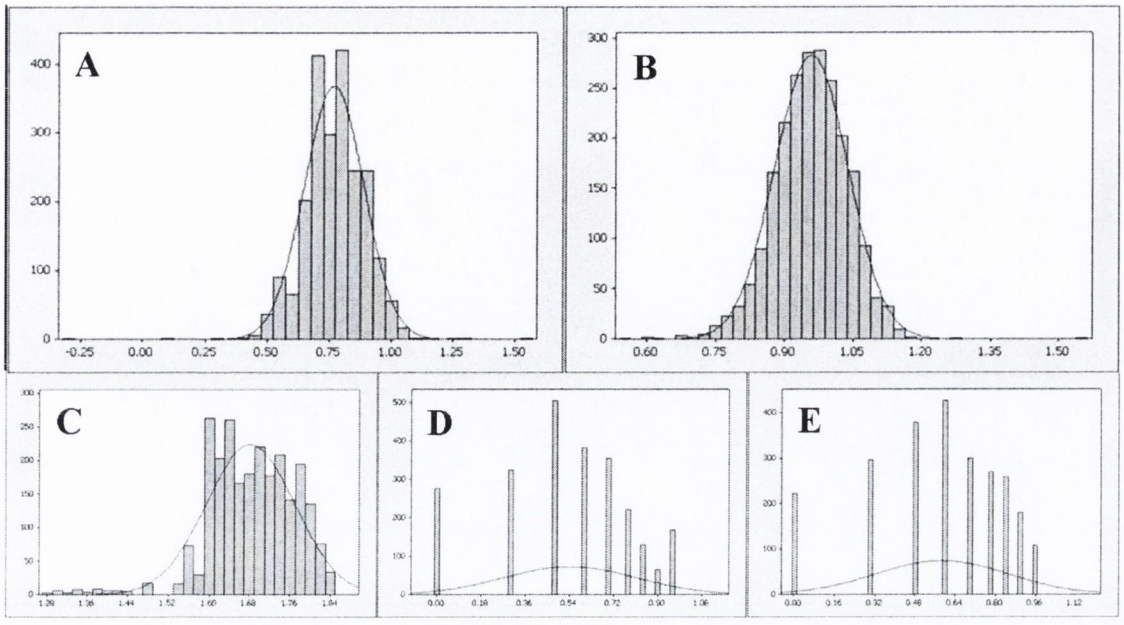

Figure 3.4.7 Histograms with fitted normal distribution curves for the following log transformed data of characters: A: florets per spikelet, B: glume length (mm), C: date of ear emergence, D: spring growth, E: summer growth. Y-axis: Frequency. X-axis: Data for log transformed character of interest.

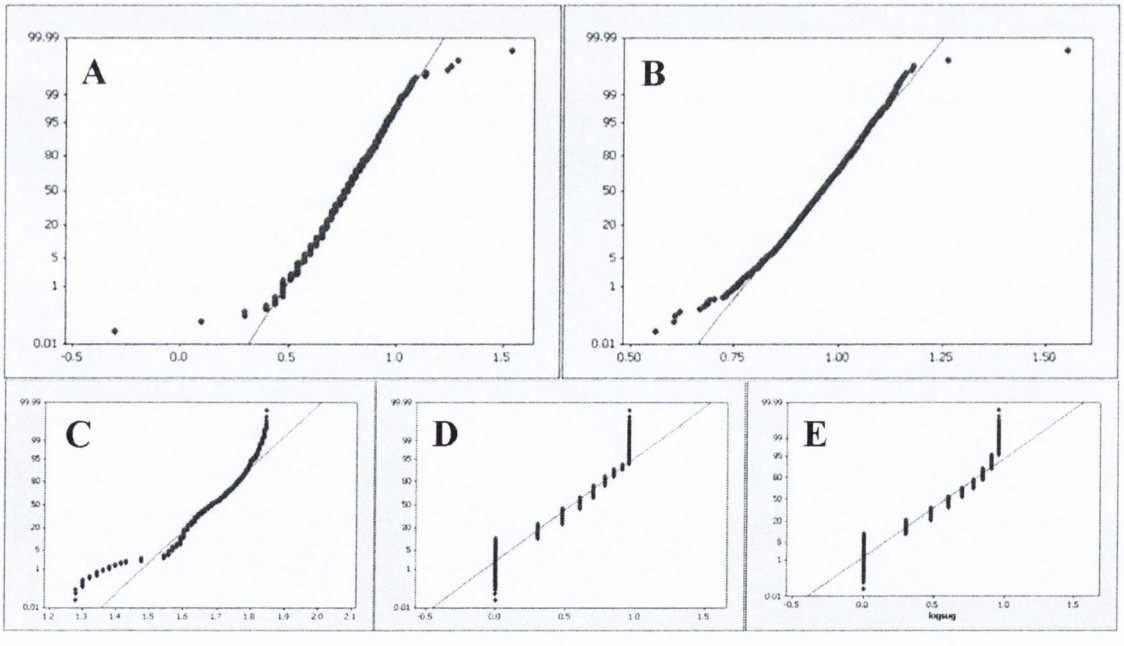

Figure 3.4.8 Probability plots using the Kolmogorov-Smirnov test for the log transformed data of characters: A: florets per spikelet, B: glume length (mm), C: date of ear emergence, D: spring growth, E: summer growth. Y-axis: percentage. X-axis: character value of interest.

Table 3.4.7 Kolmogorov-Smirnov statistics and p-values for each log transformed character.

Character	Kolmogorov-Smirnov statistic	p-value
Florets per spikelet	0.019	0.047
Glume length	0.021	0.047
Date of ear emergence*	0.053	<0.010
Spring growth*	0.053	<0.010
Summer growth*	0.069	<0.010

*Non-normal characters

Square root, reciprocal or natural log transformations did not transform any of the four remaining non-normally distributed characters to normality (Appendix 8.7).

(2) Johnson transformation

Johnson transformations were unable to transform the remaining characters to normality. For the character date of ear emergence, this was possibly because the data followed a binomial distribution. For spring and summer growth, this was probably because there were no data at the upper and lower ends of the distribution.

Correlations between pairs of characters

Pearson's correlation coefficients were calculated for each pair of normally distributed characters, and Spearman rank correlation coefficients were calculated for each pair of non-normally distributed character (Table 3.4.8). Three correlations had significant positive correlation coefficients with more than 0.4 (rachis length *versus* spikelets per spike, rachis length *versus* florets per spikelet, and rachis length *versus* glume length). There were also weaker significant positive correlations (spikelets per spike *versus* florets per spikelet, spikelets per spike *versus* date of ear emergence, florets per spikelet *versus* glume length and spring growth *versus* summer growth) and weaker significant negative correlations (rachis length *versus* spring growth, rachis length *versus* summer growth, and florets per spikelet *versus* date of ear emergence). For the pairs of characters with the strongest significant correlations, scatterplots were constructed (Figure 3.4.9). The other characters which had

significant correlation coefficients had either weak positive correlations or weak negative correlations.

Table 3.4.8 Pearson and Spearman correlation coefficients and p-values (in brackets) for each pair of characters.

	Rachis length	Spikelets per spike	Florets per spikelet[b]	Glume length[b]	Length of flag leaf	Width of flag leaf	Ear emergence[a]	Height at ear emergence	Height 30 days after ear emergence	Spring growth[a]
Spikelets per spike	0.556									
	(<0.0001)									
Florets per spikelet[b]	0.402	0.166								
	(<0.0001)	**(<0.0001)**								
Glume length[b]	0.444	0.009	0.308							
	(<0.0001)	(0.675)	**(<0.0001)**							
Length of flag leaf	0.007	-0.005	0.015	0.004						
	(0.743)	(0.811)	(0.492)	(0.864)						
Width of flag leaf	0.053	-0.003	0.038	0.068	-0.035					
	(0.013)	(0.991)	(0.073)	**(0.001)**	(0.101)					
Ear emergence[a]	0.1104	0.133	-0.142	-0.0635	-0.023	-0.015				
	(<0.0001)	**(<0.0001)**	**(<0.0001)**	**(0.003)**	(0.281)	(0.476)				
Height at ear emergence	0.018	0.002	0.025	0.048	0.033	0.013	-0.023			
	(0.403)	(0.931)	(0.237)	**(0.022)**	(0.118)	(0.552)	(0.290)			
Height 30 days after ear emergence	0.019	0.003	0.016	0.008	-0.012	0.002	0.021	0.009		
	(0.361)	(0.901)	(0.450)	(0.719)	(0.561)	(0.907)	(0.324)	(0.679)		
Spring growth[a]	-0.133	-0.167	-0.040	-0.061	0.002	0.004	-0.066	-0.006	-0.009	
	(<0.0001)	**(<0.0001)**	(0.060)	**(0.004)**	(0.912)	(0.848)	**(0.001)**	(0.786)	(0.689)	
Summer growth[a]	-0.155	-0.143	-0.076	-0.020	0.002	-0.044	-0.092	0.009	-0.033	0.492
	(<0.0001)	**(<0.0001)**	**(0.004)**	(0.355)	(0.911)	(0.040)	**(<0.0001)**	(0.669)	(0.117)	**(<0.0001)**

[a]Data were correlated via Spearman correlation, [b]Data were log transformed to normality. P-values less than 0.05 are highlighted in bold text

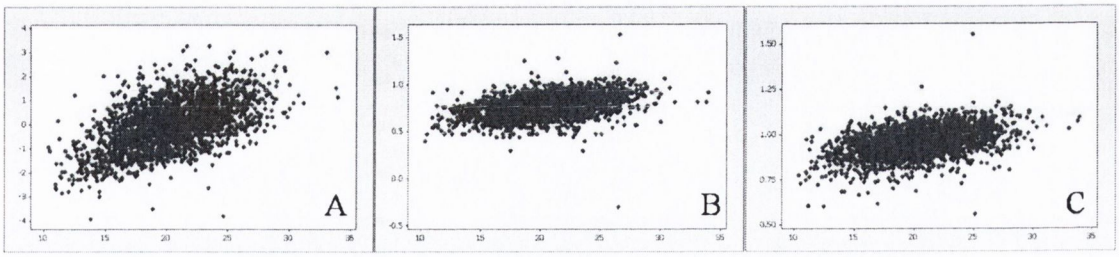

Figure 3.4.9 Scatterplots showing correlations between the following pairs of characters: A: spikelets per spike *versus* rachis length, B: florets per spikelet (log transformed data) *versus* rachis length and C: glume length (log transformed data) *versus* rachis length.

Linear regression analysis

Linear regression analysis was performed for three pairs of characters which showed the strongest significant correlations:

1. Rachis length (X) *versus* spikelets per spike (Y,).
2. Rachis length (X) *versus* florets per spikelet (Y, log transformed).
3. Rachis length (X) *versus* glume length (Y, log transformed).

The histograms of the residuals (Figure 3.4.10) for each pair of characters appeared to be approximately normally distributed, and the normality plots (Figure 3.4.11) for each character followed a straight line. The scatterplots of the residuals *versus* the X values (Figure 3.4.12) showed the values were evenly scattered. These findings indicated that the assumptions for the residuals were met for these three pairs of characters.

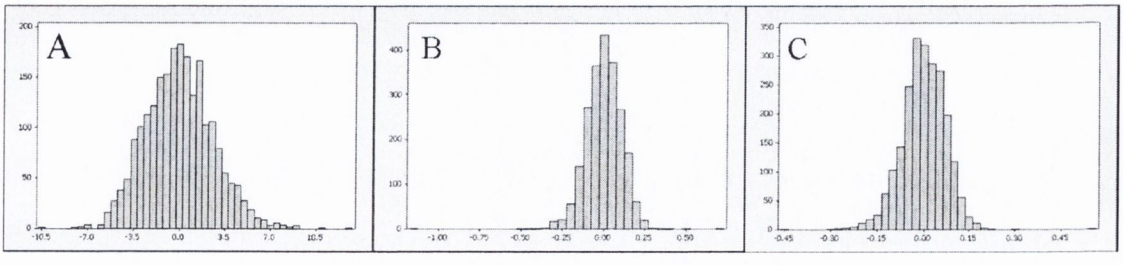

Figure 3.4.10 Histograms of the residuals for the following pairs of characters: A: Rachis length *versus* spikelets per spike, B: Rachis length *versus* florets per spikelet (log transformed data), C: Rachis length *versus* glume length (log transformed).

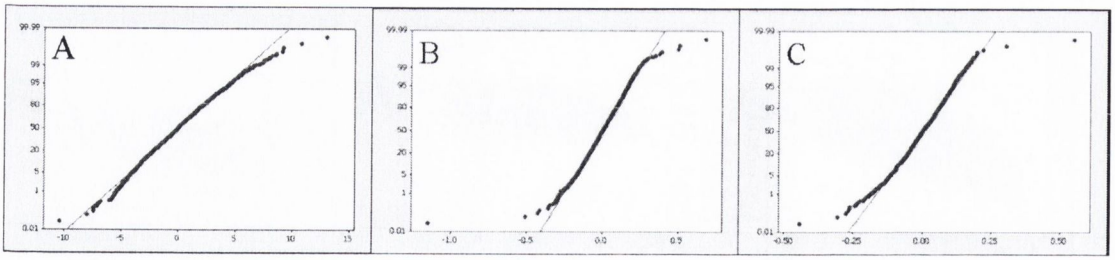

Figure 3.4.11 Probability plots of the residuals for the following pairs of characters: A: Rachis length *versus* spikelets per spike, B: Rachis length *versus* florets per spikelet (log transformed data), C: Rachis length *versus* glume length (log transformed data).

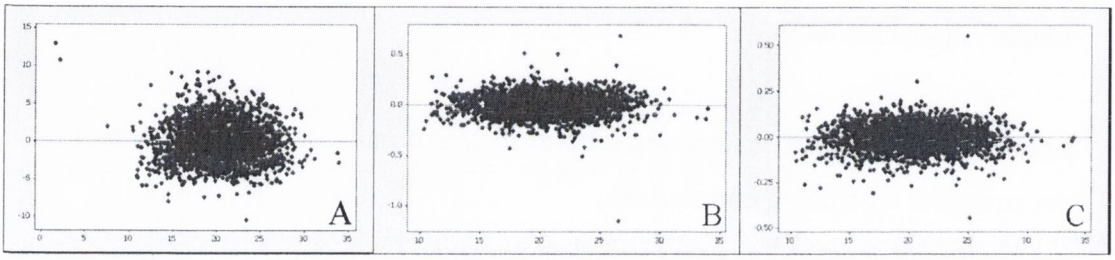

Figure 3.4.12 Scatterplots of residuals *versus* X values for the following pairs of characters: A: Rachis length *versus* spikelets per spike, B: Rachis length *versus* florets per spikelet (log transformed data), C: Rachis length *versus* glume length (log transformed data).

1. Rachis length *versus* spikelets per spike.

The regression equation (Equation 3.4.1) showed that as rachis length increased, the number of spikelets per spike increased and the p values in Table 3.4.9 indicate that the coefficient values in this equation were significant at $p<0.001$. The R^2 value indicated that 29.6% of the variation in spikelets per spike was accounted for by the relationship with rachis length. The R^2 adjusted value (29.5%) was very close to the R^2 value so the sample size did not have an effect on the percentage of variation explained. Table 3.4.10 indicates that the R^2 value was significant to $p < 0.0001$. The scatterplot of rachis length *versus* spikelets per spike (Figure 3.4.13) with the fitted regression line and prediction interval indicates that the fit of the regression line is very good. The majority of the points on the scatterplot fall within the 95% prediction interval ($-0.1 \leq x \leq 16.5$) for the regression equation.

$$Y = -11.72 + 0.4662X$$

Equation 3.4.1 Regression equation for rachis length *versus* spikelets per spike.

Table 3.4.9 Coefficients, standard errors, t values and p values for the regression equation of rachis length *versus* spikelets per spike.

Predictor	Coefficient	Standard error of the coefficient	t	p
Constant	-3.222	0.103	-31.24	0.0001
Rachis length	0.158	0.005	31.68	0.0001

Table 3.4.10 ANOVA table for regression equation of rachis length *versus* spikelets per spike.

Source	Degrees of freedom	Sums of squares	Mean squares	F statistic	p
Regression	1	6339.3	6339.28	929.97	0.0001
Residual error	2214	15092.0	6.82		
Total	2215	21431.3			

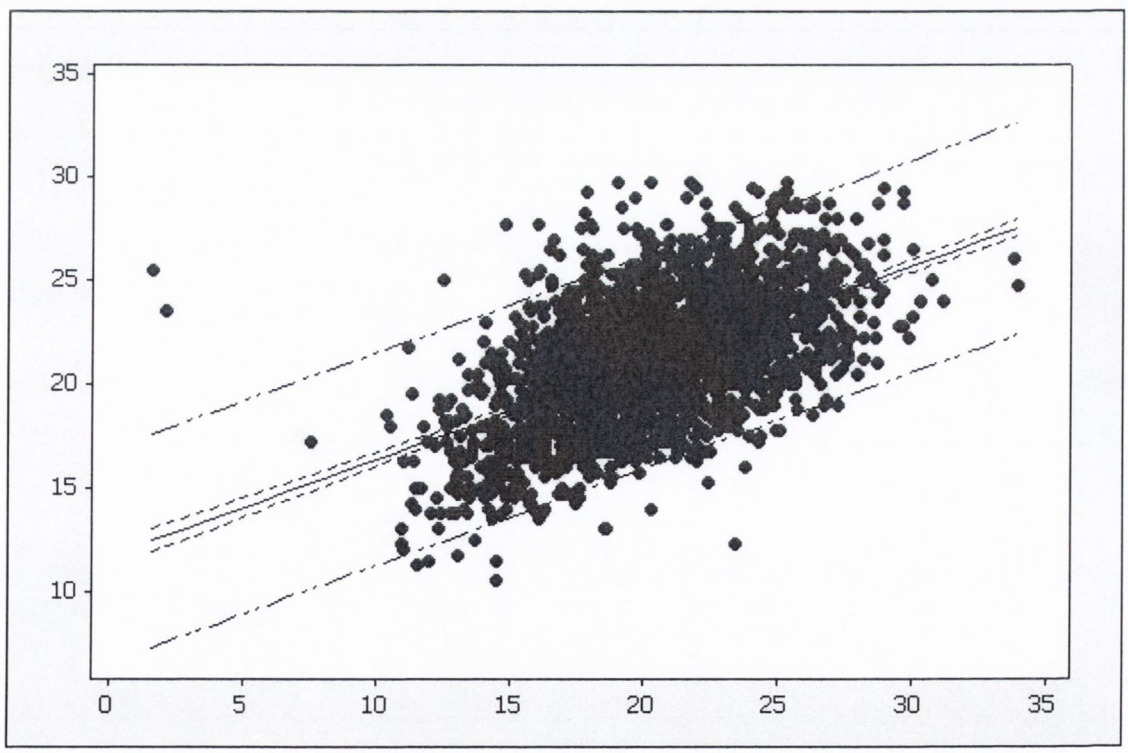

Figure 3.4.13 Scatterplot of rachis length *versus* spikelets per spike with regression line (-), 95% confidence interval (-----), and fitted 95% prediction interval (———).

2. Rachis length *versus* florets per spikelet (log transformed data).

The regression equation (Equation 3.4.2) showed that as rachis length increased, the log transformed number of florets per spikelet increased and the p values in Table 3.4.11 indicate that the coefficient values in this equation were significant at $p<0.001$. The pattern was similar with untransformed data (data not shown). The R^2 value indicated that 16.2% of the variation in log transformed florets per spikelet was accounted for by the relationship with rachis length. The R^2 adjusted value was the same as the R^2 value so the sample size did not have an effect on the percentage of variation explained. Table 3.4.12 indicates that the R^2 value was significant to $p < 0.0001$. The scatterplot of rachis length *versus* log transformed florets per spikelet (Figure 3.4.14) with the fitted regression line and prediction interval indicates that the fit of the regression line is very good. The majority of the points on the scatterplot fall within the 95% prediction interval ($0.407 \leq x \leq 0.833$) for the regression equation.

$$Y = 0.494 + 0.0134X$$

Equation 3.4.2 Regression equation for rachis length *versus* florets per spikelet (log transformed data).

Table 3.4.11 Coefficients, standard errors, t values and p values for the regression equation of rachis length *versus* florets per spikelet (log transformed data).

Predictor	Coefficient	Standard error of the coefficient	t	p
Constant	0.494	0.013	36.76	0.0001
Rachis length	0.013	0.001	20.67	0.0001

Table 3.4.12 ANOVA table for the regression equation of rachis length *versus* florets per spikelet (log transformed data).

Source	Degrees of freedom	Sums of squares	Mean squares	F statistic	p
Regression	1	5.145	5.145	427.29	0.0001
Residual error	2212	26.633	0.012		
Total	2213	31.777			

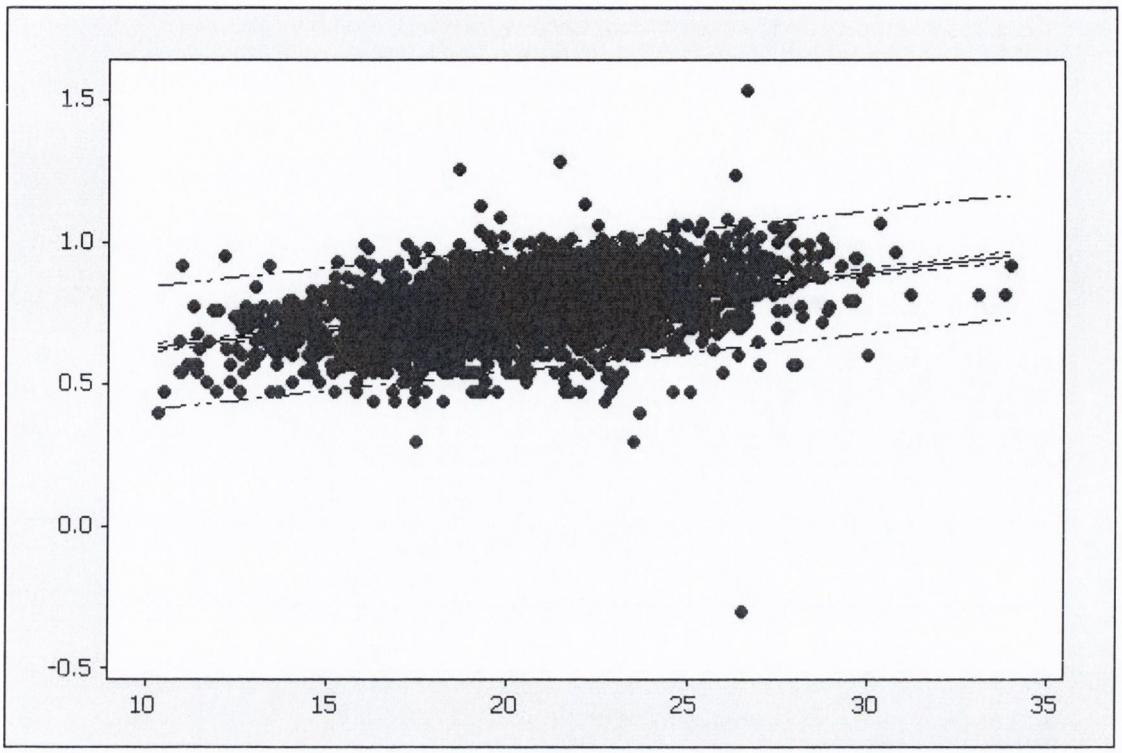

Figure 3.4.14 Scatterplot of rachis length *versus* florets per spikelet (log transformed data) with regression line (-), 95% confidence interval (-----), and fitted 95% prediction interval (-------).

3. Rachis length *versus* glume length (log transformed data).

The regression equation (Equation 3.4.3) showed that as rachis length increased, the log transformed glume length increased and the p values in Table 3.4.13 indicate that the coefficient values in this equation were significant at $p<0.001$. The R^2 value indicated that 19.7% of the variation in log transformed glume length was accounted for by the relationship with rachis length. The R^2 adjusted value (19.6%) was almost the same as the R^2 value so the sample size did not have an effect on the percentage of variation explained. Table 3.4.14 indicates that the R^2 value was significant to $p < 0.0001$. The scatterplot of rachis length *versus* glume length (Figure 3.4.15) with the fitted regression line and prediction interval indicates that the fit of the regression line is very good. The majority of the points on the scatterplot fall within the 95% prediction interval ($0.707 \leq x \leq 0.99$) for the regression equation.

$$Y = 0.758 + 0.00979X$$

Equation 3.4.3 Regression equation for rachis length *versus* glume length (log transformed data).

Table 3.4.13 Coefficients, standard errors, t values and p values for the regression equation of rachis length *versus* glume length (log transformed data).

Predictor	Coefficient	Standard error of the coefficient	t	p
Constant	0.758	0.009	87.65	0.0001
Glume length (log transformed)	0.010	0.000	23.43	0.0001

Table 3.4.14 ANOVA table for the regression equation of rachis length *versus* glume length (log transformed data).

Source	Degrees of freedom	Sums of squares	Mean squares	F statistic	p
Regression	1	2.779	2.779	549.1	0.0001
Residual error	2241	11.341	0.005		
Total	2242	14.119			

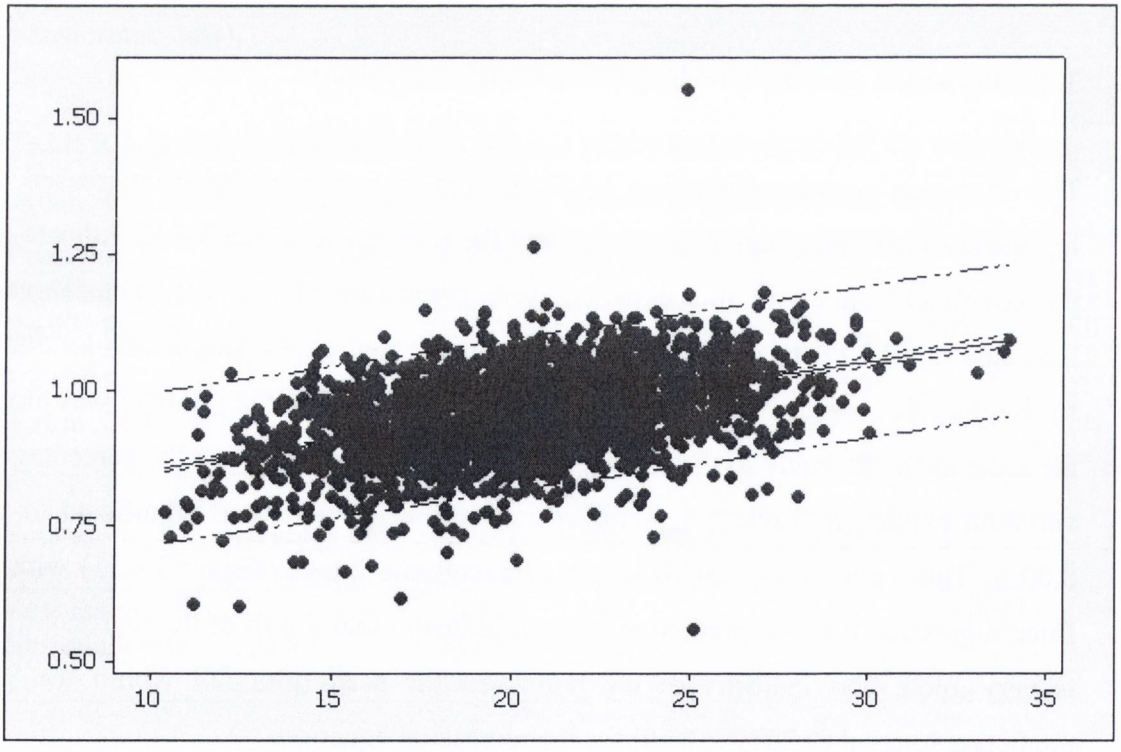

Figure 3.4.15 Scatterplot of rachis length *versus* glume length (log transformed data) with regression line (-), 95% confidence interval (-----), and fitted 95% prediction interval (---------).

Stepwise regression analysis

Stepwise regression analysis was performed for three sets of characters.

1. Spikelets per spike *versus* rachis length, florets per spikelet (log transformed data) and glume length (log transformed data).

The regression equation (Equation 3.4.4) indicated that as both rachis length and log transformed glume length increased, the number of spikelets per spike increased and the values in Table 3.4.15 indicated that these values were significant ($p<0.0001$). The pattern was similar with untransformed data (data not shown). The R^2 value indicated that 35.97% (R^2 adjusted: 35.91%) of the variation in spikelets per spike is accounted for by the relationship between spikelets per spike and rachis length and log transformed glume length.

$$\text{Spikelets per spike} = 20.35 + 0.569(\text{Rachis length}) - 11.20(\text{Glume length})$$

Equation 3.4.4 Regression equation for spikelets per spike *versus* rachis length and glume length (log transformed).

Table 3.4.15 Table of values for stepwise regression analysis of spikelets per spike *versus* rachis length, florets per spikelet (log transformed) and glume length (log transformed).

Variable	Coefficient	t	p
Constant	20.35		
Rachis length	0.57	35.03	0.0001
Glume length (log transformed)	-11.20	-15.12	0.0001

2. Florets per spikelet (log transformed) *versus* rachis length, and glume length (log transformed).

The regression equation (Equation 3.4.5) indicated that as both rachis length and log transformed glume length increased, the number of log transformed florets per spikelet increased and the values in Table 3.4.16 indicated that these values were significant ($p<0.0001$). The pattern was similar with untransformed data (data not

shown). The R^2 value indicated that 19.02% (R^2 adjusted: 18.94%) of the variation in log transformed florets per spikelet is accounted for by the relationship between log transformed florets per spikelet and rachis length and log transformed glume length.

$$\text{Florets per spikelet} = 0.3066 + 0.011(\text{Rachis length}) + 0.251(\text{Glume length})$$

Equation 3.4.5 Regression equation for florets per spikelet (log transformed) *versus* rachis length and glume length (log transformed).

Table 3.4.16 Table of values for stepwise regression analysis of florets per spikelet (log transformed) *versus* rachis length and glume length (log transformed).

Variable	Coefficient	t	p
Constant	0.307		
Rachis length	0.011	15.66	0.0001
Glume length (log transformed)	0.251	7.89	0.0001

3. Glume length (log transformed) *versus* rachis length, spikelets per spike and florets per spikelets (log transformed).

The regression equation (Equation 3.4.6) indicated that as rachis length, spikelets per spike and log transformed florets per spikelet increased, log transformed glume length increased and the values in Table 3.4.17 indicated that these values were significant ($p<0.0001$). The pattern was similar with untransformed data (data not shown). The R^2 value indicated that 28.40% (R^2 adjusted: 28.30%) of the variation in log transformed glume length is accounted for by the relationship between rachis length, spikelets per spike and log transformed florets per spikelet.

$$\text{Glume length} = 0.812 + 0.012(\text{Rachis length}) - 0.008(\text{Spikelets per spike}) + 0.096(\text{Florets per}$$

Equation 3.4.6 Regression equation for glume length (log transformed) *versus* rachis length, spikelets per spike and florets per spikelet (log transformed).

Table 3.4.17 Table of values for stepwise regression analysis of the pair of characters glume length (log transformed data) *versus* rachis length, spikelets per spike and florets per spikelets (log transformed data).

Variable	Coefficient	t	p
Constant	0.812		
Rachis length	0.012	23.57	0.0001
Spikelets per spike	-0.008	-14.74	0.0001
Florets per spikelet (log transformed)	0.096	7.19	0.0001

Principal components analysis

The first three eigenvalues (Table 3.4.18) of the morphological PCA explained more than 50% of the variation in the dataset, with the first eigenvalue explaining 27.29%, the second eigenvalue explaining 14.96% and the third eigenvalue explaining a further 11.87%. The remainder of the variation was explained by the next eight eigenvalues. When the eigenvectors were plotted for the first two dimensions (Figure 3.4.16) a good separation was found between the cultivars, which were mostly in the two right hand quadrants of the diagram, and the ecotypic material (in the left two quadrants). When the first dimension was plotted against the third, and the fourth dimension, a similar split was seen, but when the second dimension was plotted against the third and fourth dimensions, a similar split was not seen. This would indicate that it was the first dimension which was mostly splitting the varieties from the ecotypes. After canonical variates analysis, the scores for each character (Table 3.4.19) showed the relative importance of each character to separation in the PCA at the first axis and indicated that the characters rachis length, spikelets per spike, spring growth, summer growth and date of ear emergence were the main characters which caused the split between ecotypes and cultivars.

Table 3.4.18 Eigenvalues and percentage of the variation in the morphological data explained by each dimension.

Axis	Eigenvalue	Percentage variance explained	Cumulative percentage variance explained
1	1.47	27.29	27.29
2	0.80	14.96	42.24
3	0.63	11.87	54.11
4	0.61	11.47	65.58
5	0.49	9.15	74.73
6	0.45	8.35	83.08
7	0.33	6.14	89.22
8	0.30	5.66	94.88
9	0.16	3.04	97.92
10	0.06	1.19	99.10
11	0.05	0.90	100.00



Figure 3.4.16 Principal components analysis diagram in two dimensions for morphological data showing separation between ecotypes and cultivars. X axis: Dimension 1, Y axis: Dimension 2. □: Irish ecotype, ○: European ecotype, Δ: Cultivar, ▲: Tetraploid cultivar. Numbers of the populations are given in Appendix 8.1. Dimension 1 explained 27.29% of the variation and dimension 2 explained 14.96% of the variation.

Table 3.4.19 Scores for each character for the first dimension of the principal components analysis for the morphological data.

Character	Score
Rachis length	0.599
Spikelets per spike	0.500
Florets per spikelet	0.072
Glume length	0.086
Height at ear emergence	0.011
Height 30 days after ear emergence	-0.201
Length of flag leaf	-0.019
Width of flag leaf	0.011
Spring growth	-0.812
Summer growth	-0.867
Date of ear emergence	1.940

Eigenvectors and eigenvalues were calculated for the chloroplast genetic distance matrix. The percentage accounted for by each eigenvalue was determined (Table 3.4.20). The first four dimensions explained more than 80% of the variation, with the first dimension explaining 34.05% of the variation, the second dimension explaining 25.51%, the third dimension explaining 13.93% and the fourth dimension explaining 11.91% of the variation. In comparison with the principal components analysis for morphological data (Figure 3.4.16), the diagram showing the first dimension plotted against the second dimension (Figure 3.4.17) did not appear to show such a clear distinction between ecotypes and cultivars or present any other meaningful groupings. When the grouping from the UPGMA dendrogram in Chapter 2 (Figure 2.4.10) were overlaid on the morphological principal components diagram, no clear distinction was seen between ecotypes and varieties. However, the cultivars were more in the right hand quadrants. Similar patterns were seen when the first dimension was plotted against the other dimensions.

Table 3.4.20 Eigenvalues and percentage of the variation in the chloroplast DNA dataset explained by each axis.

Axis	Eigenvalue	Percentage variance explained	Cumulative percentage variance explained
1	1.04	34.05	34.05
2	0.72	25.51	57.56
3	0.43	13.93	71.49
4	0.36	11.91	83.40
5	0.24	7.69	91.09
6	0.17	5.45	96.54
7	0.10	3.26	99.80
8	0.03	0.20	100.00

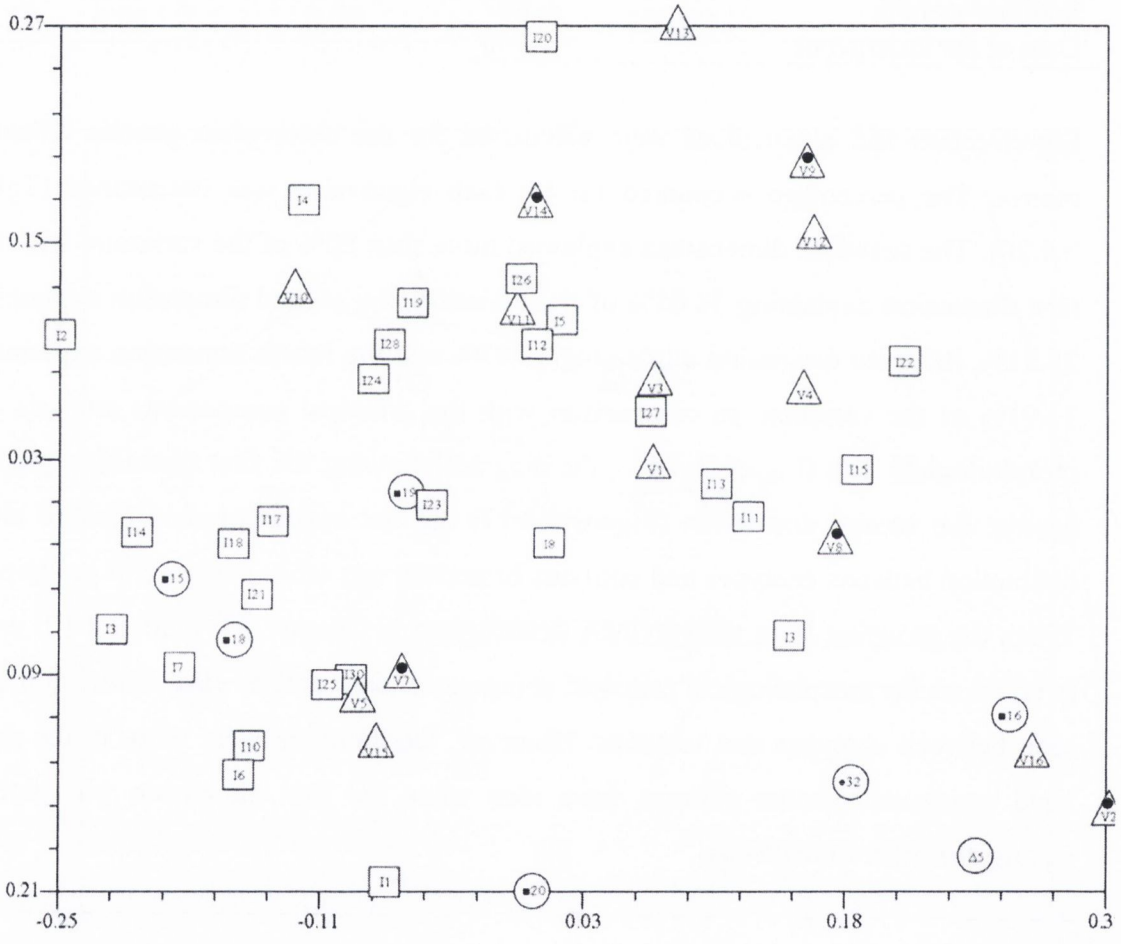

Figure 3.4.17 Principal components analysis diagram in two dimensions for chloroplast data. X axis: Dimension 1, Y axis: Dimension 2. □: Irish ecotype, ○: European ecotype, Δ: Cultivar, ▲: Tetraploid cultivar. Numbers of the populations are given in Appendix 8.1. Dimension 1 explained 34.05% of the variation and dimension 2 explained 25.51% of the variation.

Dendrogram

The UPGMA dendrogram based on Euclidean distances (Figure 3.4.18) consisted of two major groups (I and II, Figure 3.4.18). The first major group was split into two subgroups (Ia and Ib, Figure 3.4.18). The first subgroup (Ia) consisted of eight cultivars. The second subgroup (Ib) consisted of six ecotypes and five cultivars. The second major group (II) consisted of all the other ecotypes and the three remaining cultivars (Premo, Barlenna, and Greengold). There was moderate bootstrap support for some of the branches of the tree.

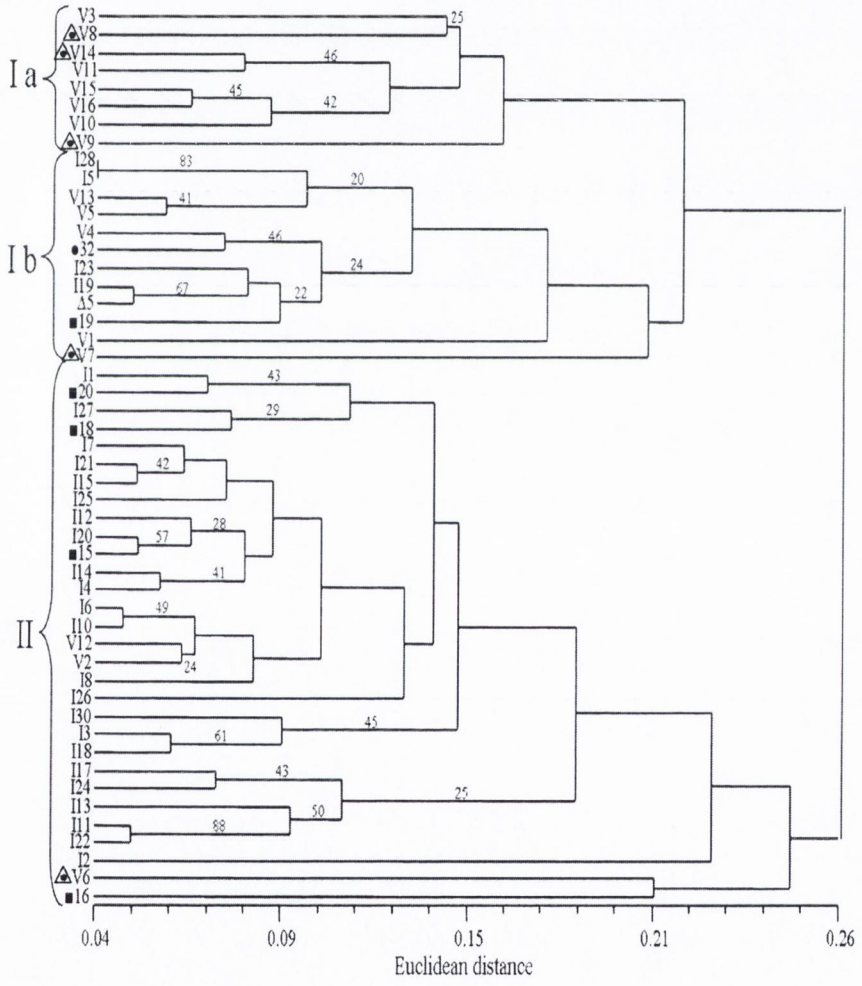

Figure 3.4.18 Unrooted dendrogram for morphological data showing similarities between populations, constructed using the unweighted pair group method with arithmetic means (UPGMA) method (Sneath & Sokal, 1973) as implemented in NTSYSpc V2.2 (Rohlf, 2005), based on the Euclidean distance measure. Numbers on the branches are percentage bootstrap values generated in NTSYSpc V2.2 (Rohlf,

2005). Different symbols represent a geographical group: △ = Northern Europe ■ = Southern Europe ● = Eastern Europe, I = Ireland, V = Cultivar, ◬ = Tetraploid cultivar.

The UPGMA dendrogram based on the genetic distance matrix of the cpDNA (Figure 3.4.19) can be divided into two major groups (I and II). The first major group consisted of all the Irish ecotypes, all the cultivars with the exception of Talbot and Barlenna, and a single European ecotype (3199 Romania Podoloni). The second major group (II) consisted of all the remaining European ecotypes and the cultivars Talbot and Barlenna. There was little consistency between the dendrogram based on morphological distance and the dendrogram based on chloroplast genetic distance.

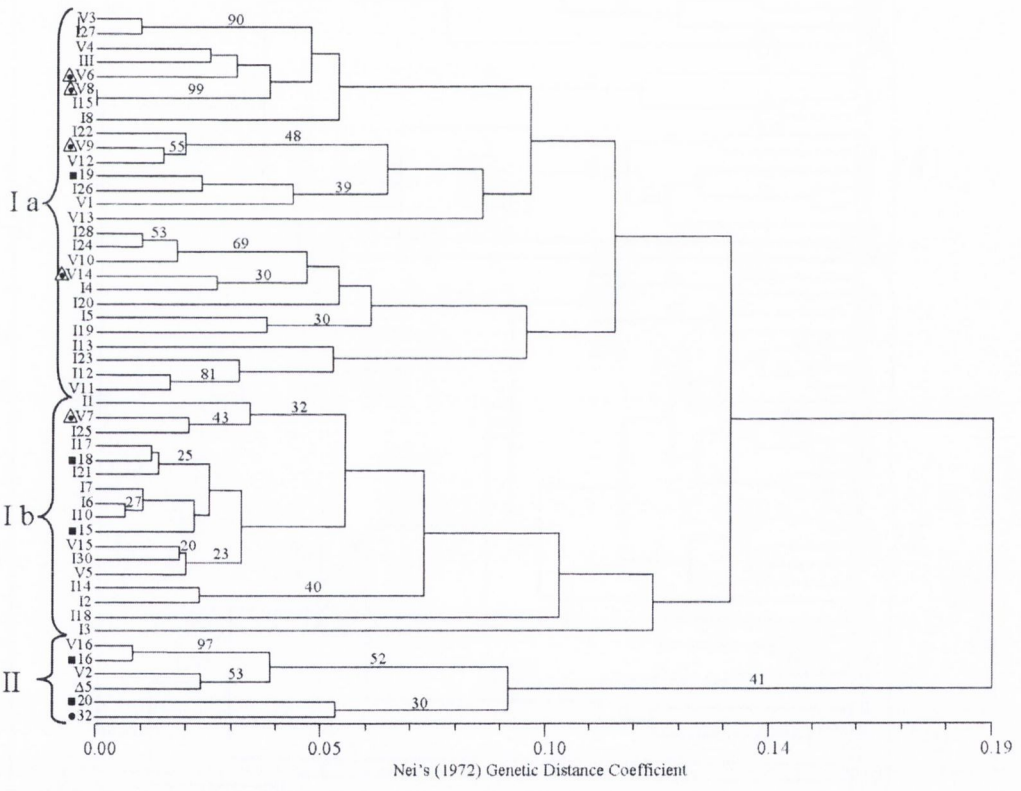

Figure 3.4.19 Unrooted dendrogram for chloroplast data showing similarities between populations, constructed using the unweighted pair group method with arithmetic means (UPGMA) method (Sneath & Sokal, 1973) as implemented in NTSYSpc V2.2 (Rohlf, 2005), based on Nei's 1972 genetic distance measure of the cpDNA. Numbers on the branches are percentage bootstrap values generated in NTSYSpc V2.2 (Rohlf, 2005). Different symbols represent a geographical group: △ = Northern Europe ■

= Southern Europe ● = Eastern Europe, I = Ireland, V = Cultivar, ◬ = Tetraploid cultivar.

Mantel test

A loose correlation between geographic distance and Euclidean distance was found for 28 populations (r = 0.324). This value was not significant at $p < 0.05$ ($p = 0.07$). This would indicate that there was little or no association between geographic distance and Euclidean morphological distance. A loose correlation was also found between Euclidean distance and genetic distance of the cpDNA (r = 0.244) and this value was also not significant at $p < 0.05$. This is consistent with PCA and UPGMA dendrogram results that also failed to show any obvious geographical structuring.

ANOVA analysis

For all characters, with the exception of height at ear emergence, height 30 days after ear emergence, length of flag leaf and width of flag leaf; most variation was found among populations (Table 3.4.21), with most variation among populations found for the characters date of ear emergence (41.36%), spring growth (40.86%) and summer growth (49.42%). The characters rachis length, spikelets per spike and florets per spikelet showed similar levels of among population variation (30.19%, 29.12% and 32.27%, respectively). Less variation was seen between populations for glume length (24.55%) and height 30 days after ear emergence (6.90%). No significant variation between populations was seen for height at ear emergence, length of flag leaf and width of flag leaf. Most within population variation was seen in height at ear emergence (50.59%), height 30 days after ear emergence (30.23%), length of flag leaf (55.97%), and width of flag leaf (50.92%), with cultivars generally having higher within population variation than ecotypes, except for height at ear emergence. Less within-population variation was seen for rachis length (6.75%), spikelets per spike (7.41%), florets per spikelet (6.26%) and glume length (8.34%). With the exception of florets per spikelet, cultivars had higher within population variation than ecotypes. The least within population variation was seen in date of ear emergence (0.25%), spring growth (3.21%) and summer growth (2.40%). Within population variation in all characters for both cultivars and ecotypes was very similar.

Ryan-Einot-Gabriel-Welsch tests results (Table 3.4.1) showed that the significant differences between population groups for rachis length were generally between ecotypes and cultivars. The class groupings showed that generally the cultivars grouped together with higher means than the ecotypes. Similar results were seen for spikelets per spike. For florets per spikelet, European ecotypes and cultivars grouped together with higher means than the Irish ecotypes. Significant differences between populations for the character glume length were mainly between 3408 Italy and 3013 Romania Podoloni and the rest of the populations, with less obvious groupings between cultivars and ecotypes. There were no significant differences between any of the pairs of populations for height at ear emergence, length of flag leaf and width of flag leaf (Table 3.4.2). The variation between populations for height 30 days after ear emergence was between the ecotype IRL-OP-02059 Clare and most of the other populations. For date of ear emergence, there were significant differences between almost all of the pairs of populations (Table 3.4.3). There were no clear groupings of populations as shown by the grouping of mean classes. For spring growth and summer growth, most of the variation was caused by differences between ecotypes and cultivars. For spring and summer, most of the ecotypes grouped together with worse mean growth scores than the cultivars.

For comparisons between cultivars and ecotypes, most variation was seen in spring growth (23.08%), summer growth (27.34%), date of ear emergence (10.17%), rachis length (6.13%) and spikelets per spike (5.33%). Lower variation was seen between cultivars and ecotypes for the characters florets per spikelet (0.48%), glume length (0.74%), and height 30 days after ear emergence (0.19%). The remaining characters (height at ear emergence, length of flag leaf and width of flag leaf did not show significant variation between cultivars and ecotypes. Differences were positive between cultivars and ecotypes for the characters rachis length, spikelets per spike, florets per spikelet, glume length, height 30 days after ear emergence, and date of ear emergence, while there were negative differences between cultivars and ecotypes for spring growth and summer growth.

Most variation in comparisons between diploid and tetraploid cultivars was seen for date of ear emergence (12.14%), spring growth (5.33%), glume length (3.12) and

florets per spikelet (2.79%). Lower variation was seen between diploid and tetraploid cultivars for the characters rachis length (0.55%) and length of flag leaf (0.53%). The remaining characters (spikelets per spike, height at ear emergence, height 30 days after ear emergence, width of flag leaf and summer growth) had no significant variation in comparisons between diploid and tetraploid cultivars. Positive differences between diploid and tetraploid cultivars were seen for the characters florets per spikelet, length of flag leaf, and spring growth, while negative differences were seen for the characters rachis length, glume length, and date of ear emergence.

Between Irish and European ecotypes, most variation is seen for the character glume length (8.65%). Less variation was seen between Irish and European ecotypes for rachis length (2.31%), spikelets per spike (3.00%), florets per spikelet (2.67%) and spring growth (0.28%). No significant differences were seen between Irish and European ecotypes for the characters height at ear emergence, height 30 days after ear emergence, length of flag leaf, width of flag leaf, and date of ear emergence. Positive differences were seen between Irish and European ecotypes for the characters rachis length, spikelets per spike, and florets per spikelet, while negative differences were seen for the characters glume length, spring growth and summer growth.

Table 3.4.21 One-way ANOVA analysis results with percentage between group variation, p-value, and difference between groups for each of the characters: rachis length, spikelets per spike, florets per spike, glume length, height at ear emergence, height 30 days after ear emergence, length of flag leaf, width of flag leaf, date of ear emergence, spring growth and summer growth.

	Comparison	Rachis length	Spikelets per spike	Florets per spikelet	Glume length	Height at ear emergence	Height 30 days after ear emergence	Length of flag leaf	Width of flag leaf	Date of ear emergence	Spring growth	Summer growth
Overall	**Cultivar**	6.13%	5.33%	0.48%	0.74%	0%	0.19%	0.08%	0.11%	10.17%	23.08%	27.34%
	versus	<0.0001	<0.0001	<0.05	<0.001	NS[a]	<0.05	NS	NS	<0.0001	<0.0001	<0.0001
	Ecotype	1.89*[b]	0.55*	0.02*	0.01*	0.03	0.22*	-0.09	0.05	0.76*	-1.23*	-1.69*
Cultivars	**Diploid**	0.55%	0.44%	2.79%	3.12%	0%	0.02%	0.53%	0.22%	12.14%	5.33%	0.25%
	versus	<0.05	<0.0001	<0.001	<0.0001	NS	NS	<0.05	NS	<0.0001	<0.0001	<0.05
	tetraploid	-0.55*	0.10	0.16*	-0.03*	-0.04	-0.08	0.25*	-0.07	-1.07*	0.35*	0.15
Ecotypes	**Irish *versus***	2.31%	3.00%	2.67%	8.65%	0.09%	0%	0%	0.03%	0.04%	0.28%	1.85%
	European	<0.0001	<0.0001	<0.0001	<0.0001	NS	NS	NS	NS	NS	<0.05	<0.0001
		1.31*	0.46*	0.05*	-0.06*	-0.17	0.19	0.03	0.03	-0.04	-0.16*	-0.41*
Among populations	**Overall**	30.19%	29.12%	32.27%	24.55%	1.97%	6.90%	2.12%	2.51%	41.36%	40.86%	49.42%
		<0.0001	<0.0001	<0.0001	<0.0001	NS	<0.0001	NS	NS	<0.0001	<0.0001	<0.0001
Within populations	**Overall**	6.75%	7.41%	6.26%	8.34%	50.59%	30.23%	55.97%	50.92%	0.25%	3.21%	2.40%
		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	Cultivars	7.22%	7.91%	2.19%	11.86%	49.75%	54.38%	54.87%	49.23%	0.10%	2.98%	3.49%
		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	N/S	<0.0001	<0.0001
	Diploid	18.26%	16.32%	2.45%	17.02%	40.53%	61.40%	69.74%	49.11%	0.10%	3.23%	3.84%
		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	N/S	<0.0001	<0.0001
	Tetraploid	2.74%	3.94%	1.34%	12.49%	84.85%	40.36%	50.23%	54.79%	0.10%	2.78%	2.37%
		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	N/S	<0.0001	<0.0001
	Ecotypes	5.65%	5.86%	6.76%	5.73%	52.48%	18.20%	47.94%	45.64%	0.34%	7.05%	3.90%
		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	N/S	<0.0001	<0.0001
	Irish	9.32%	9.25%	8.51%	8.43%	54.25%	46.41%	49.74%	15.57%	7.68%	4.79%	0.34%
		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	N/S
	European	2.39%	2.90%	3.76%	6.318%	48.77%	51.25%	32.62%	54.54%	4.52%	1.96%	0.28%
		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	N/S

[a]NS: non-significant, [b]* Differences between groups are significant

3.5 Discussion

3.5.1 Morphological diversity

Different levels of variation were seen across the different characters both within and among populations. Among populations, vegetative characters (height at ear emergence, height 30 days after ear emergence, length of flag leaf and width of flag leaf) showed the least variation. Within individual populations, the vegetative characters height at ear emergence, height 30 days after ear emergence, and length of flag leaf, showed the lowest ranges of variation. However, overall within population variation is the highest in the characters height at ear emergence, height 30 days after ear emergence, length of flag leaf and width of flag leaf. While low levels of variation would be expected for the cultivated material, where consistency of these characters have been selected for during breeding programmes, higher levels of variation should be expected in populations of the ecotypic material. The higher level of overall within population variation could explain this. Reproductive characters showed higher levels of variation among populations. Moderate levels (e.g. 8.34% for glume length) of within population variation were also seen for reproductive characters. Similar results were seen for both ecotypes and cultivars in the study of Dutch populations (Van Treuren *et al.* 2005, ranging from 12.7% to 31% in ecotypes, and ranging from 10.4% to 21.6% in cultivars), as well as in studies of morphological variations in cultivars alone (Gilliland *et al.* 2000; Roldan-Ruiz *et al.* 2001). High levels of among population variation were seen for date of ear emergence, that is, among population variation accounted for 41.36% of the variation observed (Table 3.4.21). However, very low levels of within population variation were seen for date of ear emergence (measured from standard deviations in Table 3.4.3) ranging from 2.06% (in cultivar Premo) to 12.3% (in ecotype 3408 Italy). Also very low overall within population variation was found for date of ear emergence (Table 3.4.21) (0.25%). High levels of among population variation in date of ear emergence were also seen in other studies (Gilliland *et al.* 2000; Roldan-Ruiz *et al.* 2001, Van Treuren *et al.* 2005). The low level of within population variation combined with the high among population variation in date of ear emergence for both ecotypes and cultivars could be as a result of adaptation to environmental factors such as day length, temperature and precipitation that may influence fitness via amount of seed set (in the case of

ecotypes) and as a result of selection for optimal forage potential during breeding (in the case of cultivars). A lot of the variation in spring and summer growth was seen within population (Table 3.4.21) and in the ecotypes, and these ecotypes would be adapted to local environmental conditions and so have varied growth in spring and summer.

Similar results have been seen in other studies (Naylor, 1960; Loos, 1994; Kolliker *et al.* 1999; Gilliland *et al.* 2000; Roldan-Ruiz *et al.* 2001; Van Treuren *et al.* 2005). For instance, Kolliker *et al.* (1999) found that 80% of the overall variation in *L. perenne* was accounted for by variation among populations. Some of the ecotypes which had better values (significantly and positively different) in the reproductive characters or which were similar to the better cultivars have the potential to be used to breed new varieties.

There was also a wide range of diversity between different groups of populations (ecotypes, both Irish and European; and cultivars, both diploid and tetraploid) (Table 3.4.21). The largest range of diversity between groups was seen in the characters spring growth, summer growth, date of ear emergence. T-tests (Table 3.4.5) confirmed the differences in groups in the most variable characters (rachis length, spikelets per spike, florets per spikelet, glume length, date of ear emergence, spring growth and summer growth) and the lack of differences between groups in the least variable characters (length of flag leaf, width of flag leaf, height at ear emergence). For differences between cultivars and ecotypes, in production characters (spring growth, summer growth) higher amounts of variation were recorded for between group variation (Table 3.4.21), while more moderate amounts of between group variation were seen for date of ear emergence, rachis length and spikelets per spike. In the production characters, the cultivars had higher values for these characters than the ecotypes, indicating that the potential of the ecotype group as a whole for breeding of these production characters is limited and less than the cultivars.

3.5.2 Separation of populations

More variation was seen between ecotypes and cultivars than either between diploid and tetraploid cultivars, or between Irish and European ecotypes (Table 3.4.21).

Ecotypes and cultivars were separated from each other both by PCA and UPGMA analyses. Similar separations of ecotypes and cultivars were seen in studies of Dutch ecotypes (Loos, 1994; Van Treuren *et al.* 2005). CVA scores for the PCA analysis (Table 3.4.19) showed that date of ear emergence, spring growth and summer growth, and to a lesser extent, height 30 days after ear emergence, spikelets per spike and rachis length are the characters that contribute most to the separation. Populations with later date of ear emergence, good spring and summer growth, longer rachis length, more spikelets and lower height after ear emergence grouped together, which was in agreement with Loos (1994). Van Treuren *et al.* (2005) also found that date of ear emergence, rachis length, and spikelets per spike separated ecotypes from cultivars. This is a reflection of the breeding history of cultivars, which would normally be selected for later heading date, and good growth in the growing season. Based on these results, cultivars which would eventually be selected for commercial breeding would be expected to have more spikelets per spike and longer rachis length.

The fact that these characters separated ecotypes from cultivars is in agreement with what might be expected from the processes that shaped their characteristics. In the case of ecotypes these characters would have been moulded by adaptive evolution to produce locally adapted ecotypes; in the case of cultivars the characters would have been influenced by evolutionary history of their progenitors but also by recent breeding efforts where characters have been selected via artificial selection. While ecotypes and cultivars were separated from each other, ploidy level differences between populations did not account for the groupings seen (Figure 3.4.16, Figure 3.4.18). This could be considered surprising because tetraploids in *L. perenne* have been shown to have increased leaf and plant size (Sugiyama, 2005) relative to diploids. In this study, leaf length and such characters were the least variable characters and did not contribute to any split between any populations. While height at ear emergence showed more variation in tetraploid varieties than in diploid varieties (Table 3.4.21), the fact that only five populations of the total of 33 analysed were tetraploid may have contributed to the fact that this variation did not contribute to a visual splitting of populations.

The lack of geographical structure in the morphological data was shown by the Mantel test, which gave a very poor correlation between Euclidean distance of the

morphological data and geographical distance between the populations (r = 0.324), and also by the PCA and UPGMA analysis which showed no clustering of geographically close populations. The lack of geographical pattern was seen in other studies, both at the country level (Loos, 1994; Van Treuren *et al.* 2005) and at the European level (Fernando *et al.* 1997). While morphological characters were able to separate European and Dutch populations (Loos, 1994), Dutch populations from different geographical regions were not distinguishable. Similar results for Dutch ecotypes were seen by Van Treuren *et al.* (2005). In a wider European context, Fernando *et al.* (1997) did not show any geographical pattern among European *L. perenne* ecotypes using morphological data. This lack of geographical structuring of populations was also seen in the cpDNA data (see Chapter 2) and could be a result of the rapid spread of *L. perenne* across Europe with agriculture and also because of seed and pollen mediated geneflow. *Lolium perenne* is also an allogamous species and its obligate outbreeding would enhance geneflow over geographical distance and reduce population substructuring.

There was little consistency between the cpDNA and morphological data results for either the PCA or UPGMA analysis. While the PCA and UPGMA data for morphology separated the European and Irish ecotypes from the varieties (Figures 3.4.16 and 3.4.18), the PCA and UPGMA analysis of the cpDNA data showed a separation of Irish ecotypes and cultivars from the European ecotypes (Figures 3.4.17 and 3.4.19). Mantel testing also showed a very poor correlation between Euclidean distance and genetic distance determined with the cpDNA markers (r=0.244). Such differences between the different genetic diversity measures could be expected from their different modes of evolution. Morphological characters such as date of ear emergence, and reproductive characters could be expected to separate ecotypes from cultivars because breeding objectives for cultivars would give different results (e.g. later flowering) from ecotypes allowed to adapt to local environments and competitive stresses. Artificial selection has caused convergence in morphological form that masks patterns of morphological variation determined by natural evolutionary processes of adaptation and geneflow. CpDNA data would have resulted from a different process of evolution where cultivars are derived from the same maternal lines. Most morphological characters in contrast would be determined biparentally.

3.5.3 Relationships between characters.

Positive relationships were seen between rachis length and reproductive characters (spikelets per spike, florets per spikelet, and glume length) in both correlation and regression analyses. While it would seem intuitive that with increased rachis length, the number of spikelets increase (because there is simply more space available for spikelets), this would not explain the positive relationship between rachis length and florets per spikelet, and between rachis length and glume length. Numbers of spikelets and numbers of florets are directly related to inflorescence branching processes. The more branching within a rachis the more spikelets will be produced; the more branching within a spikelet the more florets will be produced, unless reproductive structures fail to develop from these branches. While quantitative trait loci (QTL) studies in sorghum (Brown *et al.* 2006), have found a low correlation between the number of primary and secondary inflorescence branches, QTL studies in rice (Li *et al.* 2006) found moderate correlation between primary and secondary branch number and also between number of branches at both orders of branching and numbers of spikelets. This suggests that in rice, regulation of branching is related at all orders of branching. Similar correlations were seen in this study, i.e. the moderately significant correlations between rachis length, spikelets per spike, and florets per spikelet. This could indicate that regulation of branching in *L. perenne* could be controlled in a similar way to rice. QTL studies undertaken by Brown *et al.* (2006) in sorghum and by Upadyayula *et al.* (2006) in maize suggest that allelic variation in genes controlling branch length (*ramosa* gene in maize) causes morphological variation in inflorescence branch length within a species. Such allelic variation in *L. perenne* could account for the high levels of variation seen between the different populations and groups of populations of *L. perenne* in this study. Interestingly the *ramosa* gene is not expressed in the branch meristem but in the position marking the start of the bract subtending the branch (Bortiri *et al.* 2006) and its DNA sequence is conserved among the grasses that have been studied to date. The fact that the *ramosa* gene is expressed in the tissue where the glume begins may explain the relationship between rachis length and glume length seen in these analyses. This is because the *ramosa* gene product controls branch length and primary and secondary branches and so levels of the *ramosa* gene product would be expected to produce a relationship in branch length between

primary (rachis) and secondary branches (spikelet) and thus between spikelet and the length of its parts. Other genes have also been implicated in the control of inflorescence structure (Kellogg, 2007). A full study of these genes in *Lolium* is required to investigate the contribution of these genes to *Lolium* inflorescence morphology but the results of this morphological study have helped determine basic patterns of morphological diversity and correlations on which these developmental genetic studies can be based.

While the relationship between rachis length and number of spikelets per spike, and also rachis length with numbers of florets per spikelet, may be important for breeding increased seed production, seed size is also an important factor. Elias *et al.* (2003) found that only 0.33% of caryopses which were less than one third the size of the palea had the ability to germinate, as opposed to 92% of seeds which were greater than one third the size of the palea being active. Seed size and viability would be valuable characters to assess in further studies.

Flowering time is important for forage and seed yield, and for forage quality and persistence in *L. perenne* (Humphreys & Eagles, 1988; Laidlaw, 2005). Swards which flower much later, or which have reduced numbers of flowering tillers, have better forage quality across the growing season because lignin content is increased in flowering stems, decreasing digestibility and voluntary animal intake (Laidlaw, 2005). This relationship between flowering date and yield potential was seen in this analysis where there was a low significant negative correlation between date of ear emergence and both spring and summer growth (Table 3.4.8). As date of ear emergence became later, spring and summer growth improved. Persistency is also associated with later flowering plants (Takasaki *et al.* 1989). However, later/reduced flowering results in lower seed yield and so for breeding a trade off must be made between earlier flowering with higher seed yield (necessary for cultivar development) and later flowering (necessary for agricultural quality) and lower seed yield. In this data set, many of the cultivars (with the exception of a number of early-flowering cultivars) were later-flowering than the ecotypes. Ecotypes had a mean date of ear emergence of May 17th with a range from May 5th for 3408 Italy to June 1st for IRL-OP-02018 Wicklow. Cultivars had a mean date of ear emergence of May 23rd and (with the exception of Aurora which had the extremely early date of ear emergence of April

23rd) ranged from May 12th for S24 to June 6th for Sarsfield. Van Treuren *et al.* (2005) found a difference of ten days in the mean date of ear emergence between Dutch ecotypes and cultivars. Loos (1994) also found that cultivars were generally later flowering than the Dutch ecotypes. This is an illustration of contrast between variation generated by adaptive variation in natural populations (where, for example, earlier flowering could convey a competitive advantage in terms of earlier seed set) and variation artificially selected by breeders (where, for example later flowering may be preferable).

When regression analysis was performed, strong positive relationships were found between numbers of spikelets per spike, numbers of florets per spikelet, glume length and rachis length. As spikelets per spike were also shown to be related to florets per spikelet in the stepwise regression analysis, rachis length has the potential to be used as a predictor for reproductive performance. As rachis length and spikelets per spike are characters which would be convenient to measure in the field, the prediction model could be used easily by breeders as a selection method for reproductive characters in breeding programmes. The character of rachis length is already used in DUS testing under UPOV guidelines. While such a model has not been proposed for *L.* perenne, panicle elongation was seen as the best estimate of seed number in sorghum (Gerik *et al.* 2004). While high numbers of spikelets per spike do not necessarily equate with higher seed yields, studies have shown that high seed yield come from plants with larger heads (Brown, 1980). Also, seed number per unit area was found to be closely associated with the number of floret sites per unit area in tall fescue by Young *et al.* (1998).

3.6 Conclusion

This study has quantified morphological and cpDNA variation in *Lolium perenne,* discussed the ability of these data to discriminate populations and groups of populations and discussed correlations between characters and the possible genetic control of such characters. These results will be highly valuable to botanists and breeders who need to understand and manipulate vegetative and reproductive characters in *Lolium*. Future studies should examine seed set and the genes involved in controlling inflorescence architecture.

Chapter 4

Variation in water soluble carbohydrate (WSC), dry matter and crude protein content in a collection of *Lolium perenne* L. ecotypes and commercial varieties.

4.1 Introduction

4.1.1 Water soluble carbohydrate: definition and structure

Water soluble carbohydrates (WSCs) are storage molecules which are soluble in cold water and include mono-, di-, oligo- and some polysaccharides (Jafari *et al.* 2003a). They mainly include, sucrose, reduced sugars (such as fructose and glucose, Figure 4.1.1), and fructans (Ding & Yang, 2007). Fructans are made by about 15% of flowering plants representing about 40,000 species (Cairns, 2003) and are the most prevalent type of WSC in the grasses (Pavis *et al.* 2001a). Fructans are fructose polymers derived from sucrose (Figure 4.1.2) and come in several different forms: (i) linear inulin: the simplest fructans, which consist of β(1-2)-linked fructose residues; present in the Asterales order of angiosperms, (ii) inulin neo-series: these have two β(1-2)-linked fructose chains attached to the sucrose starter unit and are present in members of the angiosperm Liliaceae family; (iii) Levan-type: a linear β(2-6)-linked fructose polymer, present in the Poaceae; and (iv) Graminans: β(2-6)-linked fructose residues with β(1-2) branches, also present in the Poaceae (Ritsema & Smeekens, 2003). *Lolium* species mainly accumulate fructans of the more complex graminan type (Pavis *et al.* 2001a). These fructans can be linear, branched and contain internal or terminal glucose residues (Pavis *et al.* 2001a).

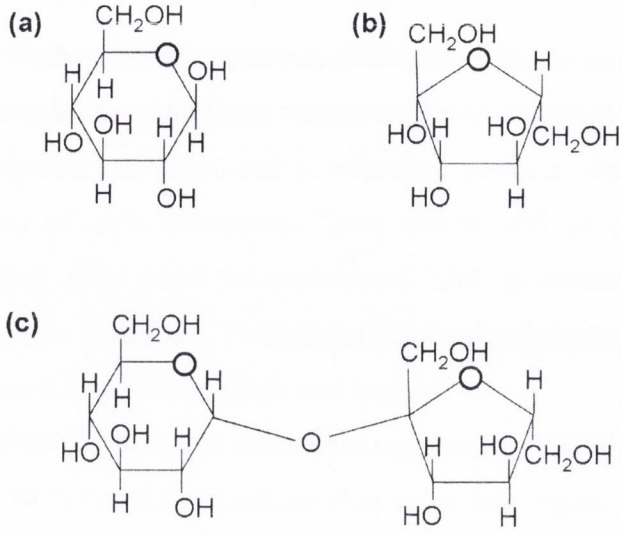

Figure 4.1.1 Chemical structure of carbohydrates. (a) glucose, (b) fructose, and (c) sucrose. (Hand drawn from Thain & Hickman, (2004).

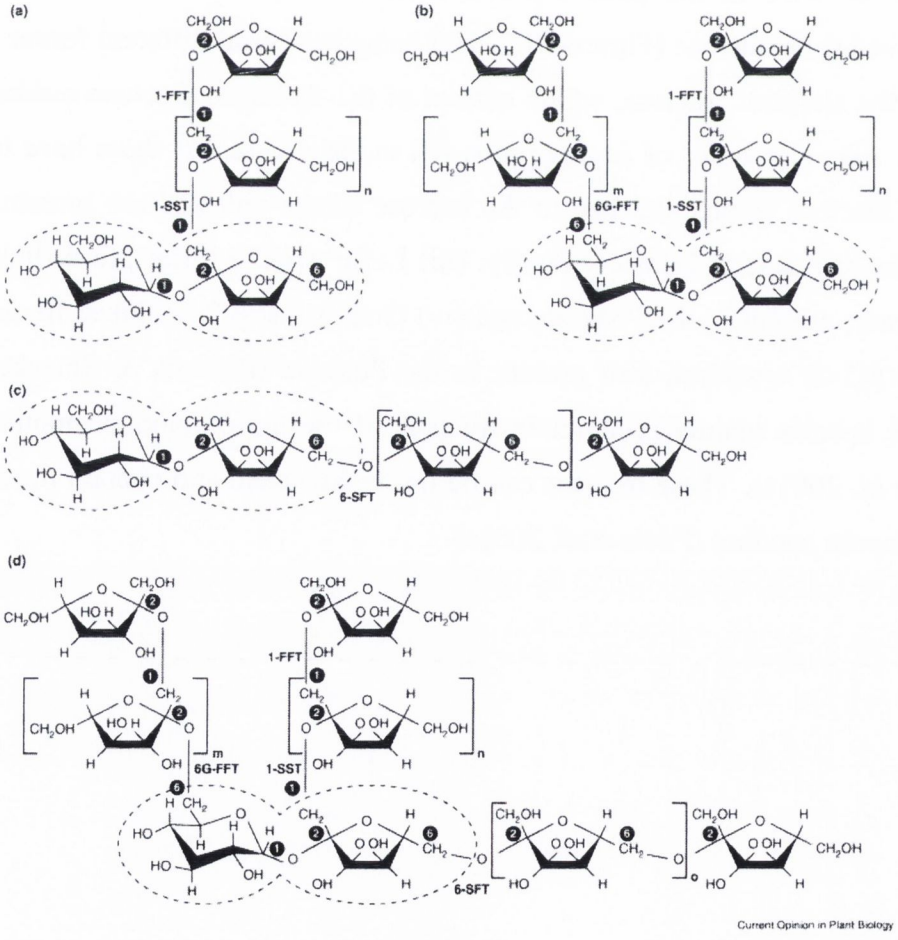

Figure 4.1.2 Examples of different types of fructans. The sucrose (a dimer of glucose and fructose) on which the fructans are built is encircled. Enzymes creating the

linkages are indicated. (a) Inulin (b) Neo-series inulin (c) Levan (phlein) (d) Graminan. Ritsema & Smeekens (2003). See Chalmers *et al.* (2005) for more examples.

WSCs are synthesised and stored in the vacuoles of both photosynthetic and storage cells (Cairns, 2003). They are usually found in the base of leaves (but can also be found in leaf blades) and are mobilized when plants are re-growing after defoliation (Chalmers *et al.* 2005). It has been suggested that fructans facilitate the uploading of sucrose from the phloem and thus maintain the appropriate osmotic potential to ensure cell enlargement in the base of the leaf during the cell elongation phase (Pavis *et al.* 2001b). They have also been associated with tolerance to abiotic stresses such as cold and drought (Chalmers *et al.* 2005).

4.1.2 Synthesis of WSC

Fixed carbon is produced by photosynthesis in the chloroplast from where it is exported to the cytoplasm for sucrose synthesis. Accumulated sucrose is exported to the apoplast or the vacuole for fructan synthesis, or hydrolized to produce fructose and glucose. At least eight enzymes are known to control the balance of fructan, fructose and glucose accumulation in the vacuole of perennial ryegrass, including fructosyltransferases, invertases and hydrolases (Francki *et al.* 2006). The addition of a fructose residue to any of the primary alcohol groups of sucrose by the enzyme 1-SST (sucrose:sucrose 1-fructosyltransferase) will form one of three possible trisaccharides (1-kestose, 6-kestose, and 6G-kestose). These trisaccharides are precursors for all fructans with a higher degree of polymerization. In *L. perenne*, only 1-kestose and 6G-kestose are present in significant amounts (Pavis *et al.* 2001a). The fructan profile of *L. perenne* is complex and so far only hypothetical pathways have been proposed. Chalmers *et al.* (2005) have proposed a possible metabolomic pathway for fructans in *L. perenne* (Figure 4.1.3).

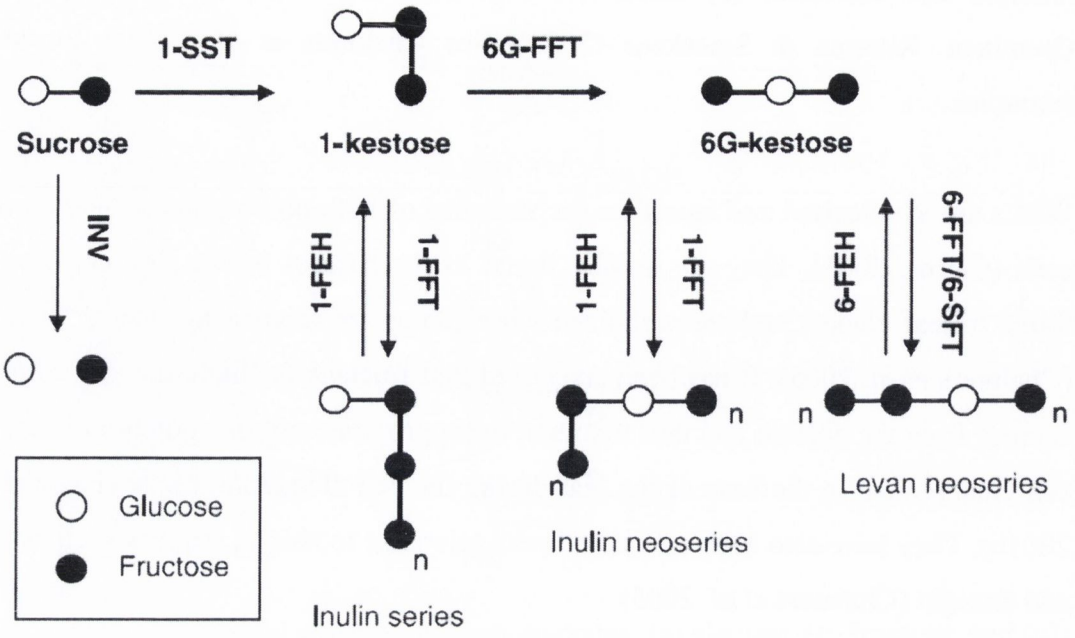

Figure 4.1.3 Proposed pathway of fructan metabolism in *L. perenne*. 1-SST: sucrose:sucrose 1-fructosyltransferase, 6G-FFT: 6-glucose fructosyltransferase, INV: invertase, 1-FEH: 1-fructan exohydrolase, 1-FFT: fructan:fructan 1-fructosyltransferase, 6-FEH: 6-fructan exohydrolase, 6-FFT: fructan:fructan 6-fructosyltransferase, 6-SFT: sucrose:fructan 6-fructosyltransferase. Chalmers *et al.* (2005).

According to this proposed pathway, members of the inulin series of fructans are produced by the addition of a fructose residue from sucrose to another sucrose molecule, catalysed by the enzyme 1-SST, forming 1-kestose. Then 1-FFT catalyses the addition of fructose units to 1-kestose to produce fructans of varying lengths. To produce fructans of the inulin neo-series, the enzyme 6G-FFT facilitates the transfer of a fructose unit from 1-kestose to the glucose unit of sucrose to form 6G-kestose (Shiomi, 1989). 6G-kestose is then elongated by the addition of fructose subunits, catalysed by 1-FFT at β(2-1) linkages. Where fructose units are added to 6G-kestose at β(2-6) linkages, levan neoseries are formed. This part of the pathway is catalysed by with 6-FFT or 6-SFT. However, the absence of bifurcase (whose presence is associated with the presence of 6-SFT) indicates that 6-FFT is the more likely candidate. Degradation of fructans occurs by the action of fructan exohydrolases, which cleave the β(2-1) and β(2-6) linkages, while the resulting sucrose molecules can then be further degraded to fructose and glucose by invertases (Chalmers *et al.*

2005). Fructosyltransferases have also been implicated in the degradation of fructans (Pavis *et al.* 2001b), while conversely, fructan exohydrolases have been implicated in fructan biosynthesis (Bancal *et al.* 1992). With respect to genetic control, genes involved in fructose metabolism, such as invertase genes, have been mapped using quantitative trait loci (QTL) mapping, and have been principally associated with linkage group 6 in *L. perenne* (Turner *et al.* 2006).

4.1.3 Uses of WSC: plants, humans and animals

WSCs have several uses in plants. They are accumulated during photosynthesis and are then utilised when photosynthesis levels are low (i.e. at night, or in the roots). They are also used for re-growth after cutting and for the growth of tillers and seeds (Humphreys, 2005). They facilitate the uploading of sucrose from the phloem and also help maintain osmotic potential. This maintenance of osmotic potential may protect the cell membranes under stress (such as cold or drought). They also ensure cell enlargement in the cell elongation zone during growth (Pavis *et al.* 2001b). WSC synthesis lowers sucrose concentration in the cell and prevents sugar-induced feedback inhibition of photosynthesis (Pollock, 1986).

Fructans have been used since the 1930's in tests of human kidney function (Vijn & Smeekens, 1999). More recently, fructans (specifically inulins) have been recognised as beneficial food ingredients. In food, fructans are soluble fibres which can not be digested by humans. However, they can be preferentially fermented by beneficial bowel bacteria, so that pathological bacteria become less abundant (Ritsema & Smeekens, 2003).

WSC is completely digestible by ruminants and is their primary source of readily available energy (Turner *et al.* 2001). As well as being a source of metabolomic energy, WSC also provides a source of carbon skeletons for general biosynthesis (Miller *et al.* 2001). WSC provides a source of readily fermentable sugar for the growth of rumen microbes, which can then efficiently convert nitrogen to protein in the gut. High WSC grasses have been shown to increase animal performance, increase growth rates, and boost milk and meat production (Lee *et al.* 2003). High WSC grass fed to ruminants also increases nitrogen secreted in the milk, while decreasing

nitrogen secreted in the urine and so can have positive environmental effects (Miller *et al.* 2001). As a result of the benefits to *L. perenne* itself and to ruminants of higher concentrations of WSC, this character is a target for breeding programmes. WSC content in *L. perenne* has been measured in populations of ecotypes and cultivars in several studies (e.g. in Ireland: Jafari *et al.* 2003a, and in Australia: Fulkerson *et al.* 2003).

4.1.4 Crude protein and dry matter

Crude protein and dry matter are also important characters in *L. perenne*, particularly in relation to WSC content. It has been shown previously that crude protein and dry matter tend to have an inverse relationship that varies seasonally (Pontes *et al.* 2007). During periods of vegetative growth, crude protein levels are low while dry matter contents are high (Pontes *et al.* 2007). During flowering time, crude protein content increases as the number of flowering stems increase, and dry matter contents increase. Crude protein is a source of amino acids for microorganisms in the ruminant gut that participate in digestion of WSC. Therefore, maintaining an advantageous balance of crude protein and dry matter is an important goal for grass breeders.

Dry matter is an important measure of yield in grass and has been an important character for grass breeders hoping to produce improved varieties of *L. perenne*. As with WSC content, it is important to characterise crude protein and dry matter in collections of ecotypic material in order to provide basic information of novel material for breeding programmes.

4.2 Aims

The aim of this chapter was to investigate diversity of Irish *Lolium perenne* accessions in comparison to cultivars with respect to a number of biochemical characters, over the growing season, including fructose, glucose, total WSC, crude protein and dry matter production. Specific objectives were to:

(1) measure the variability of various carbohydrates, dry matter and protein production in a broad range of ecotypes, cultivars and cultivars of different ploidy,

(2) test if variation in any of these characters are correlated and to seek explanation for such correlations,

(3) record changes in WSC and other variables over the season (5 cutting times),

(4) assess whether different categories of *Lolium* accessions (such as cultivars, or ecotypes) respond differentially during the growing season, and

(5) test if multivariate PCA analysis can separate cultivars, ecotypes and cultivars of differing ploidy based on biochemical data and whether such a separation is maintained over each cutting period. To investigate which factors contribute most to the PCA variation.

4.3 Materials and methods

4.3.1 Selection of samples for analysis

A total of 1,320 individuals from a selection of 33 *Lolium perenne* accessions were used to investigate WSC concentration (Appendix 8.1). These accessions were also used in the work reported in Chapters 2 and 3 to investigate chloroplast DNA diversity and population genetic structure and pattern, as well as morphological diversity. Forty individuals per accession were selected for analysis. Ten individuals of an accession were pooled together at a time, to reduce the large number of samples to test. This gave four samples per accession, a total of 132 samples per cutting time point.

4.3.2 Growth of plant material

Seeds were grown, and plants transferred to the field in Oak Park, Carlow in 2003 as described in Chapter 2. Plants were laid out in the field as spaced plants in 2m x 4.5m blocks with 5 plants in each row, 0.5m apart. There were a total of 10 plants per row. Blocks were spaced 1m apart from each other in rows of 17 blocks. After each cutting time point, nitrogen fertiliser at a rate of 80kg/ha was applied to the plants.

4.3.3 Collection of plant material

At five time points throughout the growing season in 2004 (May 2nd, June 9th, July 13th, August 31st and October 26th), four pooled samples of ten individuals each per population were collected. All samples were collected on one day at the same time for each cutting point. After collection, the fresh weight of samples was recorded, and the samples subsequently dried for 48 hours at 70°C. Dry weight of each sample was recorded. Dry matter contents in % were calculated from fresh and dry weight data. The following climate parameters were collected from the weather station at Oak Park for the two weeks before each cutting point: mean rainfall, irradiance, mean temperature.

4.3.4 Extraction and analysis of WSC and crude protein

Dried samples were ground to pass through a 1.0mm screen using a Retsch impeller-type mill. Approximately 1g of this material was placed overnight in a drying oven at 70°C to ensure that all moisture was removed from the samples. Water soluble carbohydrates were extracted from each sample using the method described by Jafari *et al.* (2003b). After extraction, samples were filtered through 0.45μm filters. Samples were analysed with high performance liquid chromatography (HPLC) using a two-stage pump (Waters 45M), an amino reverse phase column (250 x 4.6 mm) heated at 30 degree Celsius and a refractive index detector (Shimadzu RID6-A) at a flow rate of 1.5mL per minute. The sample injection volume was 10 μl and the mobile phase was a degassed 80% aqueous acetonitrile solution. External standards (0.25% fructose, 0.21% glucose) were included in the analysis. Peak heights were used to quantify detected carbohydrates (equations 4.3.1 and 4.3.2).

$$\text{Fructose concentration} = 10\left(\frac{\text{Sample peak}}{\text{Standard peak}}\frac{1}{\text{Weight of sample}}\right)$$

Equation 4.3.1 Fructose concentration (in %).

$$\text{Glucose concentration} = 4\left(\frac{\text{Sample peak}}{\text{Standard peak}}\frac{1}{\text{Weight of sample}}\right)$$

Equation 4.3.2 Glucose concentration (in %).

Water soluble carbohydrate was calculated by adding the fructose and glucose values. Nitrogen was estimated using a LECO 228 (LECO Corporation, St Joseph, MI, USA) nitrogen determinator by combustion at 1050 degrees Celsius and collection of gases which were expressed as a percentage. Crude protein was calculated as N x 6.25 (g/kg dry matter, Jafari *et al.* 2003b).

4.3.5 Data analysis

All data analysis for basic statistics, data transformations, correlation analysis and regression analyses were performed using Minitab® Version 15 Statistical Software

(Minitab Incorporated, 2000). All other analyses were performed using the Statistical Analysis System (2002-2003) (version 9.1, SAS Institute Inc., Cary, NC, USA).

Arithmetic means, standard deviations, season yields and ranges of yield across the season were calculated for each character for each population, overall, for ecotypes, and for commercial varieties. Season yields were calculated by converting percentage values of each character at each cut to their g/kg dry matter values and adding each amount to determine a season yield. Scatterplots were constructed to display the ranges at each cut for each character, as well as for the ratio fructose:glucose. The adjusted Tukey test (Equation 4.3.3; Tukey, 1953; Kramer, 1956) was used to test if the means of each type (cultivar, ecotype) at each cut were significantly different. The adjusted Tukey test was also used to test if the means of different types (ecotypes, cultivars, diploid cultivars, tetraploid cultivars) were significantly different from each other.

$$q = \frac{\bar{x}_i - \bar{x}_j}{\sqrt{\frac{s_w^2}{n}}}$$

Equation 4.3.3 Tukey test statistic, q, where $\bar{x}$ = group mean, n = number of samples, and s^2 = mean square error. The Tukey-Kramer adjustment is used for unbalanced comparisons. The two means are considered significantly different if $|E_{ij}| \geq q(\alpha; k, \nu)$, where $q(\alpha;k,\upsilon)$ is the α level critical value of a studentized range distribution of k independent normal random variables with υ degrees of freedom.

Normality tests

Histograms and probability plots were constructed and normality tests for each character at each cutting point and over all cuts were performed as described in section 3.3.4.

Data transformation for non-normal distributed characters

Where data were determined not to be normally distributed, data transformation was performed and the tests for normality as described in section 3.3.4 were repeated. The

following transformations were attempted: log transformation, square root transformation, reciprocal transformation and natural log transformation.

For the characters which were not normally distributed after data transformation, Johnson's transformations were performed as described in section 3.3.4. Equations 4.3.4 to Equation 4.3.8 (Box 4.3.1) were the specific Johnson transformation functions used in these analyses. Histograms and probability plots were constructed and the normality of the transformed data was then analysed as described in section 3.3.4.

Box 4.3.1

$$S_U = 0.474459 + 1.62974\left(ArcSine\left(\frac{x - 21.16}{1.89821} \right) \right)$$

Equation 4.3.4 Transformation function for dry matter (cut 1) of the type S_U.

$$S_U = 0.853917 + 1.52391\left(ArcSine\left(\frac{x - 7.86595}{2.24729} \right) \right)$$

Equation 4.3.5 Transformation function for fructose (cut 2) of the type S_U.

$$S_U = 1.01167 + 2.01051\left(ArcSine\left(\frac{x - 13.7326}{5.11533} \right) \right)$$

Equation 4.3.6 Transformation function for WSC (cut 2) of the type S_U.

$$S_B = -7.23381 + 2.05628\left(\log_{10}\left(\frac{x + 121.296}{28.4142 - x} \right) \right)$$

Equation 4.3.7 Transformation function for crude protein (cut 4) of the type S_B.

$$S_U = 0.514939 + 1.34727\left(ArcSine\left(\frac{x - 27.067}{1.30074} \right) \right)$$

Equation 4.3.8 Transformation function for crude protein (cut 5) of the type S_U.

Correlations between characters and cuts

Pearson correlation coefficients were calculated for each pair of normally distributed characters and cutting points as described in section 3.3.4. Spearman rank correlations were performed for those characters which were not normally transformed.

Principal components analysis

Principal components analysis (PCA) was performed for each cut on the population means data using NTSYSpc V2.2 software (Rohlf, 2005) and according to the procedure outlined in Chapter 3. Where a separation of accessions was seen, a canonical variates analysis was performed on the data in order to determine which characters influenced the separation of accessions in each dimension using the modules POOLVC and CVA.

ANOVA analysis

Data for each character (log transformed fructose, glucose, log transformed WSC, dry matter and crude protein) were analysed using PROC MIXED of the Statistical Analysis System (2002-2003) (version 9.1, SAS Institute Inc., Cary, NC, USA) in order to determine the influence of type (ecotype or cultivar), cut, or type*cut interactions on the variation in the dataset. Means of sub-samples for each accession were calculated. The type of accession (ecotype or cultivar) was distributed randomly across the site. Repeated measurements on individual accessions within each type were treated as correlated observations.

An unstructured covariance model was fitted to each character (Equation 4.3.9) with type, cut and type*cut as fixed effects. Weather variables (irradiance, rainfall, mean temperature) for each cutting point were added to the model as covariates. The fit of a set of different covariance models was tested using -2 residual log likelihood, Akaike's Information Criterion (AIC; Akaike, 1974), and Schwarz's Bayesian Criterion (BIC; Schwarz, 1978). The significance of the fit of the model was tested with the null model likelihood ratio test. The significance of each of the fixed effects was tested with Type 3 hypotheses. T-tests of differences of least squares means were

used to determine if the means were significantly different from each other using the Tukey-Kramer test (Kramer, 1956) to adjust for multiple comparisons.

$$Y = \mu + C + T + C*T + e$$

Equation 4.3.9 Model tested by ANOVA analysis, where Y: character of interest, μ: overall mean; C: cut, T: type (ecotype or cultivar), $C*T$: cut*type interaction, and e: error term.

4.4 Results

4.4.1 Data description

Dry matter (%), WSC (%) and crude protein values (%) were calculated for 132 pooled samples at each of the five cutting time points (Appendix 8.8). Summary statistics (mean, standard deviations, season yield and ranges of the yield) were calculated for each character (Tables 4.4.1, 4.4.2, 4.4.3 and 4.4.4, Figure 4.4.1). Tukey tests were performed to test if group means were significantly different from each other (Tables 4.4.4, 4.4.5).

Fructose

The third cutting point had the overall highest mean fructose content (8.92%; Table 4.4.4) with the fourth cut having the lowest overall mean fructose content (4.63% (Table 4.4.4). Standard deviations ranged from 1.25 in the fourth cut (Table 4.4.1) to 3.74 in the third cut. The first cut had a mean fructose content of 8.85% (Table 4.4.4), and values ranged from 4.8% (in ecotype IRL-OP-02483; Table 4.4.1, Figure 4.4.1) to 8.85% (in ecotype IRL-OP-02258). Standard deviations ranged from 0.39 (in ecotype IRL-OP-02337 Table 4.4.1) to 3.7 (in ecotype IRL-OP-02018). An individual sample with exceptional fructose content was a sample from ecotype IRL-OP-02538 (15.77%, Appendix 8.8). The second cut had a mean fructose content of 6.34% (Table 4.4.4) and values ranged from 4.3% (in cultivar Sarsfield; Table 4.4.1, Figure 4.4.1) to 6.34% (in cultivar Shandon). Standard deviations ranged from 0.53 (in ecotype IRL-OP-02068; Table 4.4.1, Figure 4.4.1) to 4.47 (in ecotype IRL-OP-02015). Individual samples with exceptional fructose contents were samples from ecotypes IRL-OP-2015 (10.76%, Appendix 8.8), IRL-OP-2018 (10.57%) and IRL-OP-0419 (11.29%) and from cultivar Shandon (10.70%). The third cut had a mean fructose content of 8.92% (Table 4.4.4) and values ranged from 5.42% (in ecotype IRL-OP-02059; Table 4.4.1, Figure 4.4.1) to 15.8% (in ecotype IRL-OP-02018). Standard deviations ranged from 0.6 (in cultivar Greengold; Table 4.4.1) to 5.5 (in ecotype IRL-OP-02274). Individual samples with exceptional fructose contents were two samples from ecotype IRL-OP-02018(18.97%, 17.55%, Appendix 8.8), and a sample each from ecotypes IRL-OP-02128 (16.82%) and IRL-OP-02419 (16.85%). The fourth cut had a mean fructose

content of 4.63% (Table 4.4.4) and values ranged from 3.43% (in ecotype IRL-OP-02258; Table 4.4.1, Figure 4.4.1) to 6.8% (in ecotype IRL-OP-02173). Standard deviations ranged from 0.13 (in cultivar Odenwaelder, Table 4.4.1) to 1.93 (in cultivar Greengold). An individual sample with exceptional fructose content was a sample from ecotype IRL-OP-02173 (7.91%, Appendix 8.8). Finally, the fifth cut had a mean fructose content of 7.53% (Table 4.4.4), with values ranging from 4.95% (in ecotype IRL-OP-02483; Table 4.4.1, Figure 4.4.1) to 11.5% (in cultivar Greengold). Standard deviations ranged from 0.12 (in ecotype IRL-OP-02241, Table 4.4.1) to 1.85 (in ecotype IRL-OP-02274). Individual samples with exceptional fructose contents were samples from ecotype IRL-OP-02274 (11.11%, Appendix 8.8) and from cultivar Greengold (11.80%). In general cultivars had higher mean fructose contents compared to ecotypes at the first, second and fourth cuts (Table 4.4.4), but the mean fructose contents were not significantly different from each other (Table 4.4.5). Cultivars had higher standard deviations than ecotypes in the first and fifth cut (Table 4.4.4). Within cultivars, tetraploid cultivars had higher mean fructose contents than diploid cultivars at cuts one, four and five (Table 4.4.4) but these means were not significantly different from each other (Table 4.4.5). Tetraploid cultivars had higher standard deviations than diploid cultivars in the fourth and fifth cuts (Table 4.4.4). Over the whole season, the mean season yield of fructose was 362.27g/kg (Table 4.4.4), ranging from 301.6g/kg (in ecotype IRL-OP-02483; Table 4.4.1) to 457.4g/kg (in ecotype IRL-OP-02018). Ecotypes had a mean season yield of fructose of 360.99g/kg (Table 4.4.4), while the mean season yield of cultivars was slightly higher at 365.22g/kg. Within cultivars, tetraploid cultivars had higher mean season yield (368.68g/kg; Table 4.4.4) than diploid cultivars (361.76g/kg). An individual with exceptional season yield for fructose was a sample from ecotype IRL-OP-02018 (551.48g/kg, Appendix 8.8).

Glucose

The fifth cutting point had the highest mean glucose content (4.50%; Table 4.4.4) with the fourth cut having the lowest overall mean glucose content (3.57%). Standard deviations ranged from 1.07 (Table 4.4.1) in the first cut to 1.81 in the third cut. The first cut had a mean glucose content of 3.98% (Table 4.4.4), and values ranged from 2.02% (in ecotype IRL-OP-02483; Table 4.4.1, Figure 4.4.1) to 5.47% (in cultivar

Millenium). Standard deviations ranged from 0.31 (in cultivar Cancan; Table 4.4.1) to 1.74 (in cultivar Navan). An individual sample with exceptional glucose content was a sample from cultivar Navan (6.89%, Appendix 8.8). The second cut had a mean glucose content of 4.39% (Table 4.4.4) and values ranged from 3.04% (in ecotype IRL-OP-02480; Table 4.4.1, Figure 4.4.1) to 5.94% (in IRL-OP-02018). Standard deviations ranged from 0.1 (in ecotype IRL-OP-02011; Table 4.4.1) to 2.57 (in ecotype IRL-OP-02015). The third cut had a mean glucose content of 4.40% (Table 4.4.4) and values ranged from 1.91% (in ecotype IRL-OP-02059, Table 4.4.1, Figure 4.4.1) to 6.4% (in ecotype IRL-OP-02018). Standard deviations ranged from 0.42 (in cultivar Portstewart, Table 4.4.1) to 4.57 (in ecotype IRL-OP-2007). Individual samples with exceptional glucose contents were samples from ecotypes IRL-OP-02007 (12.34%, Appendix 8.8) and IRL-OP-02258 (11.95%). The fourth cut had a mean glucose content of 3.57% (Table 4.4.4) and values ranged from 2.40% (in ecotype IRL-OP-02419; Table 4.4.1, Figure 4.4.1) to 6.55% (in cultivar Greengold). Standard deviations ranged from 0.14 (in ecotype IRL-OP-02480, Table 4.4.1) to 2.44 (in cultivar Greengold). Individual samples with exceptional glucose contents were two samples from cultivar Greengold (7.72%, 9.43%, Appendix 8.8). Finally, the fifth cut had a mean glucose content of 4.57% (Table 4.4.4), with values ranging from 2.26% (in ecotype IRL-OP-02483; Table 4.4.1, Figure 4.4.1) to 6.55% (in cultivar Greengold). Standard deviations ranged from 0.16 (in cultivar Portstewart, Table 4.4.1) to 1.34 (in ecotype IRL-OP-02064). Cultivars had higher mean glucose contents than ecotypes at all cuts except the second (Table 4.4.4) however, none of these differences were significant (Table 4.4.5). Cultivars had higher standard deviations than ecotypes in the first, fourth and fifth cut (Table 4.4.4). Within cultivars, tetraploid cultivars had higher mean glucose contents than diploid cultivars at cuts one, three and five and had equal mean glucose contents at cut three (Table 4.4.4) but again, none of these differences were significant. Tetraploid cultivars had higher standard deviations than diploid cultivars at all cuts (Table 4.4.4). Over the whole season, the mean season yield of glucose was 208.29g/kg (Table 4.4.4), ranging from 169g/kg (in ecotype IRL-OP-02483, Table 4.4.1) to 257g/kg (in cultivar Greengold; in Table 4.4.1). Ecotypes had a mean season yield of glucose of 206.56g/kg (Table 4.4.4), while the mean season yield of cultivars was slightly higher at 212.28g/kg (Table 4.4.4). Within cultivars, tetraploid cultivars had higher mean season yield (217.78g/kg; Table 4.4.4) than diploid cultivars (206.78g/kg; Table

4.4.4). Individuals with exceptional season yields for glucose were samples from ecotype IRL-OP-02258 (298.45g/kg, Appendix 8.8) and from cultivar Greengold (298.20).

WSC

The third cutting point had the highest mean WSC content (13.33%; Table 4.4.4) with the fourth cut having the lowest overall mean WSC content (8.20%). Standard deviations ranged from 2.19 (Table 4.4.4) in the fourth cut to 5.18 in the third cut. The first cut had a mean WSC content of 12.83% (Table 4.4.4), and values ranged from 6.81% (in ecotype IRL-OP-02483; Table 4.4.2, Figure 4.4.1) to 17% (in ecotype IRL-OP-02258). Standard deviations ranged from 0.52 (in ecotype IRL-OP-02337; Table 4.4.2) to 5.35 (in ecotype IRL-OP-02018). Individual samples with exceptional WSC contents were samples from ecotype IRL-OP-02538 (21.49%, Appendix 8.8) and from cultivar Navan (21.25). The second cut had a mean WSC content of 10.73% (Table 4.4.4) and values ranged from 7.54% (in cultivar Sarsfield; Table 4.4.2, Figure 4.4.1) to 14.42% (in IRL-OP-02018). Standard deviations ranged from 0.47 (in ecotype IRL-OP-02011; Table 4.4.2) to 7.03 (in ecotype IRL-OP-02015). Individual samples with exceptional WSC contents were samples from ecotypes IRL-OP-02015 (16.65%, 17.27%, Appendix 8.8), IRL-OP-02018 (17.23%), IRL-OP-02419 (17.35%) and from cultivar Shandon (16.92%). The third cut had a mean WSC content of 13.33% (Table 4.4.4) and values ranged from 7.52% (in ecotype IRL-OP-02059; Table 4.4.2, Figure 4.4.1) to 22.19% (in ecotype IRL-OP-02018). Standard deviations ranged from 1.1 (in ecotype IRL-OP-02059; Table 4.4.2) to 8.75 (in ecotype IRL-OP-02007). Individual samples with exceptional WSC contents were samples from ecotypes IRL-OP-02007 (27.19%, Appendix 8.8), IRL-OP-02018 (21.21%, 24.49%, 25.78%), IRL-OP-02128 (23.07%) and IRL-OP-02419 (22.57%). The fourth cut had a mean WSC content of 8.20% (Table 4.4.4) and values ranged from 6.05% (in ecotype IRL-OP-02128; Table 4.4.2, Figure 4.4.1) to 11.79% (in ecotype IRL-OP-02173). Standard deviations ranged from 0.3 (in ecotype IRL-OP-02048, Table 4.4.2) to 4.37 (in cultivar Greengold). Finally, the fifth cut had a mean WSC content of 12.03% (Table 4.4.4), with values ranging from 7.21% (in ecotype IRL-OP-02483; Table 4.4.2, Figure 4.4.1) to 18.05% (in cultivar Greengold). Standard deviations ranged from 0.26 (in cultivar Cashel; Table 4.4.2) to 3.09 (in ecotype IRL-OP-02274).

Cultivars had higher mean WSC contents than ecotypes at the first, third, fourth and fifth cuts (Table 4.4.4), however none of these differences were significant (Table 4.4.5). Cultivars had higher standard deviations than ecotypes in the first, fourth and fifth cut (Table 4.4.4). Within cultivars, tetraploid cultivars had higher mean WSC contents than diploid cultivars at cuts one, four and five (Table 4.4.4) but again these differences were not significant. Tetraploid cultivars had higher standard deviations than diploid cultivars at all cuts with the exception of the third cut (Table 4.4.4). Over the whole season, the mean season yield of WSC was 570.56g/kg (Table 4.4.4), ranging from 470.8g/kg (in ecotype IRL-OP-02483; Table 4.4.2) to 703.5g/kg (in ecotype IRL-OP-02018; Table 4.4.2). Ecotypes had a mean season yield of WSC of 567.53g/kg (Table 4.4.4), while the mean season yield of cultivars was higher at 577.53g/kg (Table 4.4.4). Within cultivars, tetraploid cultivars had higher mean season yield (586.48g/kg; Table 4.4.4) than diploid cultivars (568.58g/kg; Table 4.4.4). Individuals with exceptional season yields for WSC were samples from ecotypes IRL-OP-02018 (818.10g/kg, Appendix 8.8) and IRL-OP-02258 (752.27g/kg).

Dry matter

The third cutting point had the highest overall mean dry matter content (26.98g/kg; Table 4.4.4) with the fifth cut having the lowest overall mean dry matter content (18.56g/kg). Standard deviations ranged from 1.67 (Table 4.4.4) in the first cut to 2.18 in the fourth cut. The first cut had a mean dry matter content of 20.48g/kg (Table 4.4.4), and values ranged from 17.03g/kg (in cultivar Magician; Table 4.4.3, Figure 4.4.1) to 22.38g/kg (in ecotype IRL-OP-02241). Standard deviations ranged from 0.17 (in ecotype IRL-OP-02059, Table 4.4.3) to 5.66 (in ecotype IRL-OP-02538). The second cut had a mean dry matter content of 25.08g/kg (Table 4.4.4) and values ranged from 21.25g/kg (in cultivar Sarsfield; Table 4.4.3, Figure 4.4.1) to 28.73g/kg (in ecotype IRL-OP-02078). Standard deviations ranged from 0.21 (in cultivar Cancan; Table 4.4.3) to 1.63 (in ecotype IRL-OP-02258). The third cut had a mean dry matter content of 26.98g/kg (Table 4.4.4) and values ranged from 22.5g/kg (in cultivar Sarsfield; Table 4.4.3, Figure 4.4.1) to 29.5g/kg (in ecotype IRL-OP-02538). Standard deviations ranged from 0.22 (in cultivar Navan; Table 4.4.3) to 1.77 (in ecotype IRL-OP-02128). The fourth cut had a mean dry matter content of 22.51g/kg

(Table 4.4.4) and values ranged from 17.4g/kg (in cultivar Sarsfield; Table 4.4.3, Figure 4.4.1) to 26.43g/kg (in ecotype IRL-OP-02442). Standard deviations ranged from 0.22 (in ecotype IRL-OP-02230; Table 4.4.3) to 2.41 (in ecotype IRL-OP-02128). Finally, the fifth cut had a mean dry matter content of 18.56g/kg (Table 4.4.4), with values ranging from 15.33g/kg (in cultivar Sarsfield; Table 4.4.3, Figure 4.4.1) to 21.48g/kg (in ecotype IRL-OP-02258). Standard deviations ranged from 0.27 (in cultivar Cashel; Table 4.4.3) to 2.18 (in cultivar Cancan). Cultivars had lower mean dry matter contents than ecotypes at each cut (Table 4.4.4) and these differences were significant at each cut with the exception of the fifth (Table 4.4.5). Cultivars had higher standard deviations than ecotypes in the second, third and fourth cut (Table 4.4.4). Within cultivars, tetraploid cultivars had lower mean dry matter contents than diploid cultivars at each cut (Table 4.4.4) and these differences were significant at the third and fourth cuts (Table 4.4.5). Tetraploid cultivars had higher standard deviations than diploid cultivars at the first, fourth and fifth cut (Table 4.4.4). Over the whole season, the mean season yield of dry matter was 113.63g/kg (Table 4.4.4), ranging from 93.88g/kg (in cultivar Sarsfield; Table 4.4.3) to 125.14g/kg (in ecotype IRL-OP-02258). Ecotypes had a mean season yield of dry matter of 117.12g/kg (Table 4.4.4), while the mean season yield of cultivars was lower at 105.60g/kg (Table 4.4.4). Within cultivars, tetraploid cultivars had lower mean season yield (100.76g/kg, Table 4.4.4) than diploid cultivars (110.44g/kg).

Crude protein

Accessions at the fifth cutting point had the highest mean crude protein content (26.41g/kg; Table 4.4.4) with the third cut having the lowest overall mean crude protein content (19.28g/kg). Standard deviations ranged from 0.98 in the third cut (Table 4.4.4) to 2.59 in the fourth cut. The first cut had a mean crude protein content of 24.19g/kg (Table 4.4.4), and values ranged from 21.49g/kg (in ecotype IRL-OP-02015; Table 4.4.3, Figure 4.4.1) to 27.04g/kg (in ecotype IRL-OP-02272). Standard deviations ranged from 0.24 (in cultivar Shandon; Table 4.4.3) to 4.21 (in cultivar Magician). An individual sample with exceptional crude protein content was a sample from cultivar Magician (31.32g/kg, Appendix 8.8). The second cut had a mean crude protein content of 21.30g/kg (Table 4.4.4) and values ranged from 18.88g/kg (in ecotype IRL-OP-02442; Table 4.4.3, Figure 4.4.1) to 24.05g/kg (in cultivar Sarsfield).

Standard deviations ranged from 0.32 (in ecotype IRL-OP-02059; Table 4.4.3) to 2.91 (in ecotype IRL-OP-02011). An individual sample with exceptional crude protein content was a sample from ecotype IRL-OP-02011 (26.11g/kg, Appendix 8.8). The third cut had a mean crude protein content of 19.28g/kg (Table 4.4.4) and values ranged from 18.44g/kg (in IRL-OP-02538; Table 4.4.3, Figure 4.4.1) to 20.85g/kg (in ecotype IRL-OP-02064). Standard deviations ranged from 0.1 (in ecotype IRL-OP-02483; Table 4.4.3) to 2.07 (in cultivar Magician). Individual samples with exceptional crude protein contents were samples from ecotypes IRL-OP-02064 (23.27g/kg, Appendix 8.8) and IRL-OP-02480 (21.79g/kg) and from cultivars Magician (22.42g/kg) and Sarsfield (21.54g/kg). The fourth cut had a mean crude protein content of 23.69g/kg (Table 4.4.4) and values ranged from 16.67g/kg (in cultivar Navan; Table 4.4.3, Figure 4.4.1) to 25.64g/kg (in cultivar Cashel). Standard deviations ranged from 0.22 (in ecotype IRL-OP-02258; Table 4.4.3) to 9.36 (in cultivar Navan). Finally, the fifth cut had a mean crude protein content of 26.41g/kg, (Table 4.4.4) with values ranging from 24.44g/kg (in ecotype IRL-OP-02018; Table 4.4.3, Figure 4.4.1) to 28.35g/kg (in ecotype IRL-OP-02483). Standard deviations ranged from 0.29 (in ecotype IRL-OP-02230; Table 4.4.3) to 3.21 (in ecotype IRL-OP-02064). Individual samples with exceptional crude protein contents were samples from ecotype IRL-OP-02059 (29.79g/kg, Appendix 8.8) and from cultivar Odenwaelder (30.18g/kg). Cultivars had lower mean crude protein contents than ecotypes at the first, fourth and fifth cut (Table 4.4.4) but none of these differences were significant (Table 4.4.5). Cultivars had lower standard deviations than ecotypes at all cuts with the exception of the fourth cut (Table 4.4.4). Within cultivars, tetraploid cultivars had lower mean crude protein contents than diploid cultivars at each cut except the third cut (Table 4.4.4), but none of these differences were significant (Table 4.4.5). Tetraploid cultivars had higher standard deviations than diploid cultivars at all cuts (Table 4.4.4). Over the whole season, the mean season yield of crude protein was 114.90g/kg (Table 4.4.4), ranging from 105.2g/kg (in cultivar Navan; Table 4.4.3) to 120.84g/kg (in ecotype IRL-OP-02068). Ecotypes had a mean season yield of crude protein of 115.24g/kg (Table 4.4.4), while the mean season yield of cultivars was lower at 114.12g/kg (Table 4.4.4). Within cultivars, tetraploid cultivars had lower mean season yield (111.43g/kg; Table 4.4.4) than diploid cultivars (116.81g/kg).

Table 4.4.1 Means and standard deviations, season yield and minimum and maximum of yields across five cuts for each population for the characters fructose and glucose.

Accession name	Fructose							Glucose						
	Cut 1 (%)	Cut 2 (%)	Cut 3 (%)	Cut 4 (%)	Cut 5 (%)	Season Yield (g/kg)	Minimum and maximum of yield* (g/kg)	Cut 1 (%)	Cut 2 (%)	Cut 3 (%)	Cut 4 (%)	Cut 5 (%)	Season Yield (g/kg)	Minimum and maximum of yield* (g/kg)
IRL-OP-02337	6.69	5.72	8.85	5.02	7.44	337.20	29.04^{1}-132.72^{3}	3.28	3.82	4.55	3.02	2.54	172.10	16.65^{4}-55.97^{3}
Carlow	(0.39)	(2.07)	(3.24)	(1.46)	(0.87)			(0.42)	(1.30)	(0.93)	(0.91)	(0.75)		
IRL-OP-02059	7.91	4.98	5.42	4.95	7.99	312.50	27.70^{2}-112.11^{1}	3.38	3.41	1.91	3.76	5.90	183.60	10.67^{3}-67.17^{5}
Clare	(2.65)	(1.68)	(0.67)	(1.21)	(0.94)			(1.20)	(0.88)	(1.05)	(1.04)	(0.73)		
IRL-OP-02007	8.74	7.25	9.49	4.23	9.01	387.20	27.30^{4}-148.43^{3}	3.73	4.65	5.76	2.86	6.40	234.00	18.15^{1}-123.43^{3}
Cork	(3.28)	(0.90)	(4.91)	(1.08)	(0.30)			(1.39)	(0.72)	(4.57)	(0.78)	(0.43)		
IRL-OP-02011	9.21	6.50	7.36	3.90	6.38	333.50	35.21^{4}-124.38^{3}	4.51	4.34	3.70	3.03	4.80	203.80	24.23^{4}-51.95^{1}
Cork	(1.11)	(0.54)	(3.41)	(0.40)	(1.16)			(0.56)	(0.10)	(0.47)	(0.42)	(0.39)		
IRL-OP-02015	9.09	6.50	6.71	5.69	5.54	335.30	24.67^{2}-123.67^{1}	2.99	4.37	3.77	4.04	2.77	179.40	18.55^{5}-66.83^{2}
Cork	(2.20)	(4.47)	(3.59)	(1.42)	(0.42)			(0.43)	(2.57)	(1.46)	(1.21)	(0.76)		
IRL-OP-02048	8.10	6.00	9.32	5.20	5.76	343.80	36.27^{2}-144.51^{3}	4.11	5.14	4.01	3.34	3.49	200.90	26.03^{3}-62.26^{5}
Cork	(1.46)	(1.61)	(3.94)	(0.22)	(0.59)			(0.33)	(1.56)	(1.62)	(0.23)	(0.42)		
IRL-OP-02192	7.08	7.35	9.85	4.67	7.88	368.30	35.18^{4}-126.25^{3}	3.44	5.14	4.89	3.34	4.26	210.70	26.64^{4}-64.63^{3}
Cork	(1.84)	(0.57)	(1.94)	(1.06)	(0.81)			(0.46)	(0.68)	(1.45)	(0.51)	(1.00)		
IRL-OP-02064	6.04	5.64	7.49	5.03	8.18	323.80	22.47^{3}-140.75^{3}	3.66	4.50	3.17	3.65	4.56	195.40	22.47^{3}-65.76^{5}
Galway	(0.73)	(1.63)	(4.45)	(1.12)	(1.60)			(0.43)	(1.52)	(1.60)	(0.78)	(1.34)		
IRL-OP-02078	10.36	6.47	8.11	5.77	6.20	369.10	47.06^{3}-135.16^{1}	4.17	4.25	3.97	4.71	3.69	207.90	25.27^{1}-53.36^{4}
Galway	(3.23)	(0.77)	(3.71)	(0.77)	(0.40)			(1.14)	(0.44)	(1.25)	(0.62)	(0.22)		
IRL-OP-02230	9.70	5.66	8.25	5.06	7.22	358.90	34.59^{2}-108.15^{3}	4.75	3.76	4.08	4.15	4.39	211.30	22.81^{3}-59.00^{3}
Galway	(1.16)	(2.06)	(2.75)	(0.70)	(0.56)			(0.55)	(1.19)	(1.56)	(0.36)	(0.46)		
IRL-OP-02128	11.05	6.92	13.11	3.61	7.40	420.90	26.43^{4}-168.19^{3}	3.79	5.25	5.30	2.44	3.80	205.80	20.01^{4}-62.52^{3}
Kerry	(1.28)	(1.94)	(3.29)	(0.92)	(1.30)			(0.54)	(1.44)	(0.77)	(0.49)	(0.77)		
IRL-OP-02538	11.85	6.35	7.09	4.37	7.95	376.10	26.93^{4}-157.67^{1}	4.75	3.95	3.52	3.37	5.42	210.10	20.43^{3}-58.56^{5}
Laois	(2.77)	(2.94)	(3.26)	(1.80)	(1.04)			(0.90)	(1.68)	(1.55)	(1.12)	(0.31)		
IRL-OP-02274	10.23	6.31	6.36	4.50	8.43	358.30	16.49^{3}-141.87^{3}	3.76	4.50	3.25	3.26	5.09	198.60	19.13^{3}-69.44^{5}
Limerick	(2.98)	(1.86)	(5.45)	(1.23)	(1.85)			(1.09)	(1.32)	(1.51)	(1.01)	(1.25)		
IRL-OP-02480	10.39	4.73	10.14	4.45	6.87	365.80	28.74^{2}-12.37^{1}	4.91	3.04	4.96	3.76	4.62	212.90	22.12^{2}-62.82^{3}

Limerick	(1.40)	(2.13)	(3.18)	(0.38)	(0.49)			(0.35)	(0.86)	(1.31)	(0.14)	(0.17)			
IRL-OP-02442	10.71	4.59	8.58	4.52	6.58	349.80	29.43^{4}-148.57^{1}	3.91	3.58	4.11	3.47	2.70	177.70	21.63^{3}-60.87^{3}
Mayo	(3.33)	(1.38)	(4.47)	(1.10)	(1.24)			(0.59)	(1.56)	(1.72)	(0.77)	(0.57)		
IRL-OP-02068	6.42	7.94	7.44	4.77	6.70	332.70	28.19^{4}-122.57^{3}	3.58	5.23	3.51	4.22	4.52	210.60	22.41^{3}-59.99^{2}
Offaly	(1.21)	(0.53)	(3.55)	(1.31)	(1.10)			(0.81)	(0.63)	(1.68)	(1.29)	(0.36)		
IRL-OP-02241	10.02	6.69	10.60	4.69	7.29	392.90	22.61^{4}-130.72^{3}	4.35	4.80	5.52	3.58	4.44	226.90	18.78^{4}-64.49^{3}
Offaly	(2.85)	(1.28)	(3.83)	(1.74)	(0.12)			(1.48)	(0.98)	(1.36)	(1.16)	(0.27)		
IRL-OP-02419	9.58	7.42	12.91	3.79	6.72	404.20	22.01^{4}-168.47^{3}	4.24	4.70	5.03	2.40	4.54	209.10	18.39^{4}-60.62^{2}
Roscommon	(1.91)	(2.98)	(4.19)	(1.37)	(1.32)			(0.86)	(1.24)	(1.15)	(0.70)	(0.33)		
IRL-OP-02258	12.25	5.62	6.49	3.42	7.72	355.00	25.55^{4}-137.73^{1}	4.76	3.61	6.37	3.60	5.06	234.00	15.64^{3}-119.47^{3}
Tipperary	(1.15)	(1.87)	(4.06)	(1.25)	(0.41)			(0.60)	(1.09)	(4.37)	(1.50)	(0.63)		
IRL-OP-02272	5.34	6.37	9.39	5.41	7.13	336.40	23.60^{1}-136.73^{3}	3.38	4.42	4.16	4.26	4.33	205.50	17.52^{1}-64.52^{2}
Tipperary	(2.19)	(2.31)	(4.28)	(1.72)	(0.80)			(1.45)	(1.58)	(1.72)	(1.35)	(0.62)		
IRL-OP-02173	9.83	7.76	5.67	6.80	8.14	382.00	50.84^{3}-113.41^{1}	4.74	5.35	3.92	4.99	5.52	245.20	25.02^{3}-62.55^{2}
Waterford	(1.21)	(1.26)	(0.76)	(0.75)	(0.66)			(0.57)	(0.62)	(1.56)	(0.34)	(0.40)		
IRL-OP-02483	4.80	7.67	8.73	4.01	4.95	301.60	26.08^{4}-108.53^{3}	2.02	5.18	4.40	3.07	2.26	169.30	15.68^{1}-59.98^{3}
Wexford	(1.40)	(0.63)	(2.59)	(1.30)	(0.24)			(0.46)	(0.28)	(1.65)	(0.99)	(0.31)		
IRL-OP-02018	8.85	8.48	15.79	3.80	8.82	457.40	34.84^{4}-189.98^{3}	4.44	5.94	6.40	2.88	4.94	246.00	19.97^{4}-69.40^{3}
Wicklow	(3.70)	(1.41)	(3.50)	(0.32)	(0.84)			(1.65)	(0.50)	(0.62)	(0.65)	(0.24)		
cv. Cancan	7.69	7.09	7.77	5.32	7.47	353.40	42.81^{4}-135.04^{3}	3.27	4.53	3.69	3.32	3.51	183.20	18.46^{5}-65.64^{3}
	(0.73)	(1.97)	(3.87)	(1.27)	(1.53)			(0.31)	(0.84)	(1.92)	(0.67)	(1.22)		
cv. Cashel	5.29	5.93	9.15	3.50	6.79	306.60	23.72^{4}-111.66^{3}	2.96	4.24	4.42	2.99	4.62	192.30	19.99^{1}-50.09^{2}
	(1.99)	(2.05)	(2.26)	(0.92)	(0.19)			(1.00)	(0.71)	(0.80)	(0.69)	(0.16)		
cv. Greengold	11.56	5.54	5.56	4.70	11.50	388.60	23.53^{4}-126.91^{1}	5.01	4.06	3.41	6.71	6.55	257.40	17.34^{4}-94.29^{4}
	(1.16)	(1.16)	(0.61)	(1.93)	(0.30)			(0.42)	(0.86)	(1.51)	(2.44)	(0.56)		
cv. Magician	8.14	6.08	12.05	4.36	5.60	362.30	33.80^{2}-135.56^{3}	3.77	4.72	5.04	3.20	3.13	198.60	19.87^{5}-59.73^{2}
	(2.12)	(1.94)	(1.78)	(0.52)	(1.34)			(0.48)	(1.46)	(0.98)	(0.27)	(1.02)		
cv. Millenium	11.40	4.77	8.58	4.05	10.05	388.50	29.00^{2}-132.65^{1}	5.47	3.46	3.57	3.15	5.76	214.10	19.05^{3}-61.68^{1}
	(2.36)	(1.70)	(2.77)	(0.95)	(0.58)			(0.73)	(0.99)	(1.60)	(0.87)	(0.23)		
cv. Navan	11.41	5.68	7.54	4.70	8.93	382.60	28.63^{4}-143.58^{1}	4.91	4.43	5.48	3.41	5.68	239.10	22.90^{4}-79.59^{3}
	(3.36)	(1.83)	(1.57)	(1.55)	(1.77)			(1.74)	(1.57)	(1.74)	(0.97)	(0.82)		
cv. Odenwaelder	8.44	5.02	6.82	4.72	7.73	327.30	43.86^{1}-122.13^{1}	3.84	3.33	3.73	3.93	5.78	206.10	22.81^{1}-62.31^{5}
	(3.34)	(0.66)	(2.22)	(0.13)	(1.31)			(1.09)	(0.22)	(1.54)	(0.65)	(0.43)		
cv. Portstewart	10.17	7.10	12.50	3.46	9.67	429.00	31.29^{4}-138.84^{3}	4.65	4.45	5.80	3.07	5.59	235.60	26.68^{4}-60.77^{3}
	(1.49)	(1.23)	(1.02)	(0.55)	(0.86)			(0.66)	(0.73)	(0.42)	(0.55)	(0.16)		
cv. Sarsfield	7.93	4.33	8.16	5.17	6.55	321.40	31.10^{2}-109.48^{3}	3.60	3.20	4.69	3.50	2.98	179.70	22.09^{2}-71.35^{3}

	(1.18)	(1.62)	(3.19)	(1.07)	(1.44)			(0.59)	(1.45)	(1.88)	(0.43)	(0.42)		
cv. Shandon	5.72	8.83	11.59	5.28	7.83	392.50	33.25[1]-135.23[3]	3.25	5.50	4.81	3.32	4.79	216.70	19.74[1]-62.25[2]
	(1.88)	(2.04)	(3.03)	(1.26)	(0.65)			(1.17)	(1.06)	(1.03)	(0.57)	(0.49)		

*Numbers in superscript indicate which cut the minimum or maximum range value occurred.

Table 4.4.2 Summary statistics (mean and standard deviation), season yield and range of yield across cuts for each population for the character water soluble carbohydrate.

Accession name	WSC						
	Cut 1 (%)	Cut 2 (%)	Cut 3 (%)	Cut 4 (%)	Cut 5 (%)	Season Yield (g/kg)	Minimum and maximum of yield* (g/kg)
IRL-OP-02337	9.97	9.54	13.40	8.04	9.98	509.30	45.69^{4*}-183.10^{3}
Carlow	(0.52)	(3.33)	(3.93)	(2.34)	(1.18)		
IRL-OP-02059	11.29	8.39	7.32	8.71	13.88	495.90	49.94^{2}-161.64^{1}
Clare	(3.80)	(2.56)	(1.10)	(2.24)	(1.60)		
IRL-OP-02007	12.47	11.90	15.25	7.09	15.41	621.20	45.56^{4}-271.86^{3}
Cork	(4.66)	(1.53)	(8.75)	(1.73)	(0.64)		
IRL-OP-02011	13.72	10.85	11.06	6.93	11.18	537.40	59.44^{4}-163.43^{3}
Cork	(1.59)	(0.47)	(3.63)	(0.74)	(1.49)		
IRL-OP-02015	12.07	10.87	10.48	9.73	8.31	514.60	45.36^{2}-172.56^{4}
Cork	(2.57)	(7.03)	(4.42)	(2.63)	(1.03)		
IRL-OP-02048	12.21	11.15	13.33	8.54	9.26	544.90	65.38^{2}-201.56^{3}
Cork	(1.73)	(3.17)	(5.51)	(0.30)	(0.92)		
IRL-OP-02192	10.51	12.49	14.74	8.00	12.13	578.70	61.83^{4}-178.09^{3}
Cork	(2.26)	(1.19)	(2.82)	(1.56)	(1.72)		
IRL-OP-02064	9.70	10.14	10.66	8.69	12.75	519.40	60.03^{2}-196.43^{3}
Galway	(1.05)	(3.12)	(6.04)	(1.80)	(2.94)		
IRL-OP-02078	14.54	10.72	12.08	10.47	9.89	577.00	73.48^{3}-185.54^{1}
Galway	(4.36)	(1.03)	(4.91)	(1.38)	(0.58)		
IRL-OP-02230	14.45	9.42	12.33	9.20	11.62	570.20	57.48^{2}-167.15^{3}
Galway	(1.67)	(3.24)	(4.28)	(1.05)	(1.02)		
IRL-OP-02128	14.84	12.17	18.41	6.05	11.20	626.70	46.44^{4}-230.71^{3}
Kerry	(1.82)	(3.38)	(4.06)	(1.28)	(1.96)		
IRL-OP-02538	16.59	10.30	10.62	7.74	13.37	586.20	51.94^{4}-214.94^{1}
Laois	(3.61)	(4.59)	(4.75)	(2.91)	(1.33)		
IRL-OP-02274	13.99	10.81	9.61	7.75	13.53	556.90	41.81^{3}-195.73^{3}
Limerick	(4.04)	(3.11)	(6.89)	(2.24)	(3.09)		
IRL-OP-02480	15.30	7.76	15.10	8.21	11.48	578.50	50.86^{2}-180.39^{3}
Limerick	(1.74)	(2.97)	(4.41)	(0.40)	(0.54)		
IRL-OP-02442	14.62	8.17	12.69	7.99	9.28	527.50	54.24^{3}-195.58^{1}
Mayo	(3.92)	(2.90)	(6.14)	(1.80)	(1.77)		
IRL-OP-02068	10.00	13.17	10.95	8.99	11.22	543.30	51.41^{4}-181.50^{3}
Offaly	(2.00)	(0.90)	(5.22)	(2.57)	(1.40)		
IRL-OP-02241	14.37	11.49	16.12	8.28	11.73	619.90	41.38^{4}-191.19^{3}
Offaly	(4.25)	(2.23)	(5.16)	(2.88)	(0.36)		
IRL-OP-02419	13.82	12.12	17.95	6.19	11.26	613.40	40.40^{4}-225.80^{3}
Roscommon	(2.73)	(4.19)	(5.31)	(2.04)	(1.63)		
IRL-OP-02258	17.00	9.23	12.85	7.02	12.78	588.80	47.97^{4}-243.19^{3}
Tipperary	(1.67)	(2.93)	(8.32)	(2.36)	(0.84)		
IRL-OP-02272	8.72	10.79	13.55	9.67	11.46	541.90	41.12^{1}-192.36^{3}
Tipperary	(3.60)	(3.88)	(5.96)	(3.06)	(1.28)		
IRL-OP-02173	14.57	13.11	9.59	11.79	13.66	627.20	78.85^{3}-165.17^{1}
Waterford	(1.73)	(1.87)	(1.50)	(0.79)	(1.02)		
IRL-OP-02483	6.81	12.85	13.13	7.08	7.21	470.80	50.68^{4}-168.50^{3}
Wexford	(1.86)	(0.91)	(4.16)	(2.23)	(0.36)		
IRL-OP-02018	13.29	14.42	22.19	6.69	13.76	703.50	54.81^{4}-257.67^{3}
Wicklow	(5.35)	(1.90)	(3.79)	(0.83)	(1.01)		
cv. Cancan	10.95	11.62	11.46	8.65	10.99	536.70	69.44^{4}-200.68^{3}
	(1.01)	(2.75)	(5.79)	(1.91)	(2.70)		
cv. Cashel	8.25	10.18	13.57	6.49	11.41	499.00	44.86^{4}-161.11^{3}
	(2.97)	(2.57)	(2.98)	(1.60)	(0.26)		
cv. Greengold	16.57	9.60	8.97	11.42	18.05	646.10	60.68^{4}-187.53^{5}

	(1.49)	(1.99)	(2.00)	(4.37)	(0.81)		
cv. Magician	11.91	10.80	17.09	7.56	8.74	561.00	59.86^{2}-193.04^{3}
	(2.41)	(3.40)	(2.53)	(0.73)	(2.31)		
cv. Millenium	16.87	8.23	12.15	7.20	15.81	602.60	55.05^{2}-192.48^{1}
	(2.81)	(2.66)	(4.11)	(1.64)	(0.66)		
cv. Navan	16.31	10.10	13.02	8.11	14.61	621.50	51.53^{4}-212.53^{1}
	(5.07)	(3.37)	(1.91)	(2.52)	(2.54)		
cv. Odenwaelder	12.28	8.35	10.55	8.65	13.50	533.30	66.67^{1}-170.09^{1}
	(4.39)	(0.88)	(3.72)	(0.61)	(1.72)		
cv. Portstewart	14.82	11.54	18.31	6.53	15.27	664.70	59.55^{4}-197.63^{3}
	(2.05)	(1.42)	(1.19)	(1.09)	(0.98)		
cv. Sarsfield	11.53	7.54	12.85	8.67	9.53	501.20	53.19^{2}-179.45^{3}
	(1.71)	(3.07)	(4.94)	(1.32)	(1.85)		
cv. Shandon	8.97	14.33	16.40	8.60	12.62	609.20	52.99^{1}-187.05^{1}
	(3.02)	(3.06)	(4.04)	(1.78)	(0.95)		

*Numbers in superscript indicate which cut the minimum or maximum range value occurred.

Table 4.4.3 Means and standard deviations, season yield and minimum and maximum of yield across five cuts for each population for the characters dry matter and crude protein.

Accession name	Dry matter							Crude protein						
	Cut 1 (g/kg)	Cut 2 (g/kg)	Cut 3 (g/kg)	Cut 4 (g/kg)	Cut 5 (g/kg)	Overall mean (g/kg)	Minimum and maximum of yield* (g/kg)	Cut 1 (g/kg)	Cut 2 (g/kg)	Cut 3 (g/kg)	Cut 4 (g/kg)	Cut 5 (g/kg)	Season yield (g/kg)	Minimum and maximum of yield* (g/kg)
IRL-OP-02337	20.20	24.28	26.48	23.40	16.60	22.19	15.9^{5}-27.9^{3}	26.65	22.37	19.88	22.39	26.36	117.65	19.32^{3}-27.67^{1}
Carlow	(0.75)	(1.20)	(1.64)	(2.05)	(0.58)	(3.74)		(0.72)	(1.71)	(0.76)	(2.05)	(0.74)		
IRL-OP-02059	21.65	26.13	27.28	23.28	21.10	23.89	20.4^{5}-28^{3}	24.03	19.72	18.80	24.69	26.54	113.78	16.82^{3}-29.79^{5}
Clare	(0.17)	(0.96)	(0.81)	(0.83)	(0.90)	(2.59)		(2.28)	(0.32)	(1.59)	(1.29)	(2.27)		
IRL-OP-02007	20.48	25.63	27.40	23.00	20.65	23.43	19.3^{5}-28.5^{3}	24.20	20.02	18.69	24.83	25.98	113.72	17.35^{3}-26.53^{5}
Cork	(0.51)	(1.07)	(1.06)	(0.85)	(1.19)	(2.93)		(0.91)	(0.45)	(1.31)	(0.68)	(0.42)		
IRL-OP-02011	19.83	25.65	26.58	21.65	18.85	22.51	18.2^{5}-28.2^{3}	24.58	21.77	19.70	25.02	26.44	117.51	19.11^{3}-28.06^{5}
Cork	(0.51)	(1.13)	(1.60)	(1.06)	(0.58)	(3.31)		(0.67)	(2.91)	(0.63)	(1.28)	(1.36)		
IRL-OP-02015	20.28	27.40	28.98	23.05	16.00	23.14	15.4^{5}-29.7^{3}	21.49	22.29	19.80	24.66	27.71	115.95	18.92^{3}-28.19^{5}
Cork	(0.85)	(1.20)	(0.55)	(0.62)	(0.45)	(4.89)		(0.68)	(0.46)	(0.60)	(0.44)	(0.48)		
IRL-OP-02048	20.05	26.25	26.73	22.70	17.60	22.67	15.4^{5}-28.3^{3}	26.03	21.10	19.64	24.69	26.63	118.09	18.71^{3}-28.50^{5}
Cork	(0.73)	(1.32)	(1.73)	(0.96)	(1.53)	(3.79)		(1.17)	(1.31)	(1.00)	(1.85)	(2.28)		
IRL-OP-02192	20.43	24.90	27.00	25.18	17.70	23.04	16.8^{5}-29.1^{3}	26.31	20.36	18.85	23.16	26.04	114.72	18.12^{3}-27.90^{5}
Cork	(1.17)	(1.49)	(1.73)	(1.21)	(0.70)	(3.71)		(1.10)	(1.04)	(0.55)	(0.67)	(1.96)		
IRL-OP-02064	20.60	24.90	26.20	23.98	16.85	22.51	15.8^{5}-26.9^{3}	25.03	22.77	20.85	24.50	24.91	118.06	19.26^{3}-28.04^{5}
Galway	(0.47)	(0.35)	(0.50)	(1.80)	(0.93)	(3.57)		(3.11)	(0.64)	(1.71)	(1.22)	(3.21)		
IRL-OP-02078	21.80	28.73	28.58	24.18	19.28	24.51	18.8^{5}-29.5^{3}	21.98	20.27	19.79	24.54	26.57	113.15	17.32^{2}-26.96^{5}
Galway	(0.77)	(0.54)	(0.91)	(0.72)	(0.59)	(3.87)		(0.77)	(1.97)	(0.53)	(0.80)	(0.39)		
IRL-OP-02230	22.00	26.58	28.35	22.70	19.00	23.73	18.6^{5}-29.6^{3}	23.15	19.72	18.61	24.97	26.37	112.82	18.02^{3}-26.66^{5}
Galway	(0.27)	(0.75)	(1.11)	(0.22)	(0.32)	(3.48)		(0.69)	(0.71)	(0.99)	(1.59)	(0.29)		
IRL-OP-02128	21.78	26.68	28.28	23.33	18.05	23.62	17^{5}-30.2^{3}	24.02	22.04	18.60	24.32	25.80	114.78	17.81^{3}-26.90^{5}
Kerry	(0.68)	(0.81)	(1.77)	(2.41)	(0.82)	(3.94)		(0.42)	(1.09)	(1.01)	(1.37)	(0.88)		
IRL-OP-02538	18.85	27.00	29.60	23.53	20.63	23.92	10.4^{1}-30.9^{3}	22.19	19.39	18.44	23.73	26.37	110.12	18.09^{2}-26.76^{5}
Laois	(5.66)	(1.54)	(0.88)	(0.57)	(0.73)	(4.71)		(1.08)	(1.48)	(0.43)	(1.15)	(0.48)		
IRL-OP-02274	21.03	25.53	27.80	22.50	19.95	23.36	19^{5}-29.6^{3}	22.86	19.32	18.98	23.86	25.77	110.79	18.35^{3}-26.54^{5}
Limerick	(0.49)	(0.79)	(1.58)	(0.93)	(0.68)	(3.10)		(1.31)	(0.70)	(0.42)	(2.46)	(0.94)		

IRL-OP-02480	20.95	26.18	26.53	22.20	18.80	22.93	18.5^{5}-27.6^{2}	23.48	20.77	20.52	23.63	26.72	115.12	19.14^{2}-27.52^{5}
Limerick	(0.76)	(1.49)	(0.54)	(0.69)	(0.29)	(3.17)		(1.02)	(1.35)	(0.95)	(1.54)	(0.64)		
IRL-OP-02442	21.88	27.88	29.05	26.43	17.30	24.51	16.8^{5}-29.9^{3}	24.03	18.88	18.76	24.31	26.12	112.1	17.64^{2}-29.96^{5}
Mayo	(0.60)	(1.60)	(0.77)	(0.62)	(0.53)	(4.53)		(1.88)	(0.85)	(0.43)	(1.77)	(0.66)		
IRL-OP-02068	20.80	24.68	26.15	24.18	18.30	22.82	17.9^{5}-26.5^{3}	26.30	22.66	19.60	24.81	27.47	120.84	18.90^{3}-28.56^{5}
Offaly	(1.81)	(0.81)	(0.26)	(0.39)	(0.29)	(3.05)		(1.55)	(1.91)	(0.54)	(2.06)	(0.98)		
IRL-OP-02241	22.38	26.45	27.35	23.10	20.30	23.92	19.1^{5}-29.4^{3}	23.16	20.19	19.74	24.30	26.59	113.98	17.10^{2}-27.59^{5}
Offaly	(1.25)	(1.61)	(1.50)	(1.27)	(1.22)	(2.95)		(0.83)	(2.49)	(0.30)	(1.07)	(0.76)		
IRL-OP-02419	22.18	27.48	28.63	25.10	19.33	24.54	17.7^{5}-29.9^{3}	25.30	21.04	19.37	23.24	26.66	115.61	18.91^{3}-28.49^{5}
Roscommon	(0.75)	(0.69)	(1.15)	(0.70)	(1.39)	(3.61)		(0.80)	(0.73)	(0.41)	(1.94)	(2.14)		
IRL-OP-02258	22.03	27.15	29.43	25.05	21.48	25.03	19.8^{5}-29.9^{3}	23.43	18.92	18.48	25.33	25.81	111.97	17.76^{3}-26.60^{5}
Tipperary	(1.30)	(1.63)	(0.49)	(1.30)	(1.14)	(3.28)		(0.67)	(0.64)	(0.50)	(0.22)	(0.63)		
IRL-OP-02272	21.25	24.60	27.05	23.50	19.70	23.22	18.4^{5}-27.9^{3}	27.04	23.32	18.63	23.82	27.16	119.97	17.40^{3}-28.55^{1}
Tipperary	(0.34)	(0.23)	(0.70)	(1.00)	(1.20)	(2.72)		(1.05)	(1.10)	(0.97)	(2.12)	(0.75)		
IRL-OP-02173	21.08	24.80	27.83	22.90	20.78	23.48	19.8^{5}-28.8^{3}	25.21	21.69	18.64	24.58	27.05	117.17	18.04^{3}-27.73^{5}
Waterford	(0.32)	(0.67)	(0.77)	(1.18)	(0.87)	(2.77)		(1.23)	(1.38)	(0.40)	(1.63)	(0.80)		
IRL-OP-02483	20.13	24.15	26.28	21.73	16.43	21.74	15.4^{5}-26.9^{3}	25.85	23.57	19.44	23.20	28.35	120.41	19.32^{3}-29.07^{5}
Wexford	(0.53)	(0.89)	(0.54)	(0.66)	(0.76)	(3.52)		(1.19)	(1.94)	(0.10)	(2.33)	(0.69)		
IRL-OP-02018	21.78	26.35	28.45	24.58	18.95	24.02	18.5^{5}-29.6^{3}	24.98	20.82	18.96	23.08	24.44	112.28	17.79^{3}-27.72^{5}
Wicklow	(1.56)	(1.04)	(1.18)	(1.00)	(0.53)	(3.58)		(0.89)	(1.28)	(0.93)	(1.35)	(2.94)		
cv. Cancan	19.03	21.43	26.10	20.90	18.95	21.28	17.6^{5}–26.6^{3}	24.12	23.53	18.62	22.10	26.47	114.84	17.62^{3}-27.16^{5}
	(0.36)	(0.21)	(0.47)	(1.41)	(2.18)	(2.87		(1.19)	(0.94)	(0.77)	(2.22)	(0.69)		
cv. Cashel	19.98	24.03	26.45	20.33	17.50	21.66	17.3^{5}-27.2^{3}	26.12	22.84	19.18	25.64	27.05	120.83	17.43^{3}-27.61^{5}
	(0.51)	(0.50)	(1.12)	(0.72)	(0.27)	(3.32)		(0.40)	(0.93)	(1.21)	(2.31)	(0.54)		
cv. Greengold	18.95	22.75	25.38	19.78	18.28	21.03	17.6^{5}-26.3^{3}	23.33	20.70	19.39	21.92	25.13	110.47	18.95^{3}-26.59^{5}
	(0.61)	(0.65)	(0.68)	(0.59)	(0.48)	(2.78)		(0.86)	(0.52)	(0.49)	(1.25)	(1.06)		
cv. Magician	17.03	22.28	24.70	18.63	15.93	19.71	15.2^{5}-25.2^{3}	25.01	21.65	19.37	23.58	27.41	117.02	18.03^{3}-31.32^{1}
	(0.70)	(0.35)	(0.53)	(0.31)	(0.61)	(3.41)		(4.21)	(0.74)	(2.07)	(2.27)	(0.89)		
cv. Millenium	19.80	21.28	24.15	18.80	17.30	20.27	16.8^{5}-24.7^{3}	21.99	20.71	19.12	21.70	24.49	108.01	18.54^{3}-26.00^{5}
	(0.90)	(0.55)	(0.39)	(0.45)	(0.39)	(2.45)		(0.75)	(0.49)	(0.72)	(2.24)	(1.10)		
cv. Navan	20.00	22.23	23.90	20.00	18.73	20.97	17.9^{5}-24.2^{3}	23.61	20.25	19.30	16.67	25.37	105.2	18.84^{3}-26.52^{5}
	(0.37)	(0.28)	(0.22)	(0.26)	(0.96)	(1.95)		(1.01)	(0.93)	(0.41)	(9.33)	(1.08)		
cv. Odenwaelder	20.23	25.38	26.98	22.00	20.40	23.00	19.4^{1}-27.8^{3}	23.02	21.81	20.35	25.19	28.17	118.54	19.55^{2}-30.18^{5}
	(0.68)	(0.75)	(0.95)	(0.50)	(0.68)	(2.86)		(1.33)	(2.46)	(0.46)	(2.22)	(1.51)		
cv. Portstewart	19.98	22.95	25.43	21.83	18.23	21.68	17.9^{5}-26.3^{3}	22.68	22.08	19.44	23.60	26.03	113.83	18.84^{3}-26.56^{5}
	(0.19)	(1.35)	(0.85)	(0.24)	(0.28)	(2.62)		(0.53)	(1.04)	(0.46)	(1.09)	(0.66)		

cv. Sarsfield	17.40 (0.64)	21.25 (0.84)	22.50 (0.42)	17.40 (0.27)	15.33 (0.62)	18.78 (2.79)	14.8^{5}-22.9^{3}	24.33 (1.02)	24.05 (1.03)	20.01 (1.08)	21.36 (1.02)	26.68 (0.56)	116.43	19.05^{3}-27.14^{5}
cv. Shandon	20.03 (0.56)	24.78 (1.28)	28.83 (0.54)	22.05 (0.57)	18.40 (0.24)	22.82 (3.83)	18.1^{5}-29.4^{3}	23.25 (0.24)	22.45 (0.64)	18.88 (0.44)	24.36 (1.56)	27.05 (0.59)	115.99	18.24^{3}-27.84^{5}

*Numbers in superscript indicate which cut the minimum or maximum range value occurred.

Table 4.4.4 Overall and group means, standard deviations and season yields for the characters: dry matter, fructose, glucose, WSC and crude protein. Values with common superscript letters are not significantly different at $p<0.05$.

Type	Cut	Dry matter (g/kg)		Fructose (%)		Glucose (%)		WSC (%)		Crude protein (g/kg)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Overall	1	20.48	1.67	8.85	2.75	3.98	1.07	12.83	3.73	24.19	1.88
	2	25.08	2.14	6.34	1.96	4.39	1.24	10.73	3.12	21.30	1.82
	3	26.98	1.87	8.92	3.74	4.40	1.81	13.33	5.18	19.28	0.98
	4	22.51	2.18	4.63	1.25	3.57	1.12	8.20	2.19	23.69	2.59
	5	18.56	1.75	7.53	1.61	4.50	1.25	12.03	2.69	26.41	1.45
	Season yield	113.63	7.24	362.27	36.08	208.29	22.41	570.56	54.27	114.90	3.65
Ecotype	1	21.02^{d}	1.55	8.88^{abcd}	2.76	3.94^{abc}	1.04	12.82^{abcd}	3.65	24.39^{adfi}	1.89
	2	26.06^{c}	1.54	6.48^{abcd}	1.95	4.48^{abc}	1.27	10.95^{abcd}	3.16	21.00^{bg}	1.85
	3	27.65	1.46	8.87^{abcd}	4.00	4.36^{abc}	1.94	13.23^{acd}	5.55	19.25^{ch}	0.98
	4	23.53^{b}	1.51	4.68^{abcd}	1.27	3.53^{ac}	1.00	8.17^{d}	2.14	24.16^{adfi}	1.54
	5	18.85^{ade}	1.74	7.23^{abcd}	1.31	4.35^{b}	1.19	11.75^{abc}	3.15	26.43^{ej}	1.50
	Season yield	117.12	4.11	360.99	35.92	206.56	21.31	567.53	53.00	115.24	3.04
Cultivar	1	19.24^{a}	1.22	8.77^{bd}	2.89	4.07^{abc}	1.16	12.85^{abcd}	3.97	23.74^{adfi}	1.80
	2	22.83^{b}	1.56	6.04^{a}	1.95	4.19^{abc}	1.15	10.23^{abcd}	3.01	22.01^{bdg}	1.54
	3	25.44^{c}	1.81	9.06^{abd}	3.09	4.49^{abc}	1.48	13.55^{abcd}	4.24	19.36^{ch}	0.96
	4	20.17^{ad}	1.61	4.53^{abc}	1.19	3.66^{abc}	1.37	8.19^{abcd}	2.30	22.61^{abdfi}	3.90
	5	17.90^{ae}	1.61	8.21^{abcd}	2.00	4.84^{ab}	1.33	12.17^{abcd}	2.78	26.38^{ej}	1.35
	Season yield	105.60	6.51	365.22	38.22	212.28	25.50	577.53	59.40	114.12	4.86
Diploid cultivar	1	19.85	0.61	7.46	2.60	3.59	1.01	11.05	3.54	23.84	2.00
	2	23.71	1.66	6.80	1.99	4.41	0.98	11.21	2.87	22.54	1.36
	3	26.76	1.39	9.57	3.25	4.49	1.38	14.05	4.55	19.29	0.89
	4	21.42	1.00	4.46	1.18	3.33	0.65	7.79	1.69	24.18	2.15

	5	18.70	1.36	7.90	1.34	4.86	1.00	12.76	2.10	26.95	1.07
	Season yield	110.44	3.84	361.76	49.41	206.78	20.59	568.58	67.07	116.81	2.85
Tetraploid cultivar	1	18.64	1.38	10.09	2.60	4.55	1.12	14.64	3.61	23.65	2.10
	2	21.96	0.79	5.28	1.62	3.97	1.29	9.26	2.89	21.47	1.56
	3	24.13	1.07	8.53	2.90	4.49	1.62	13.02	3.95	19.44	1.05
	4	18.92	1.02	4.60	1.22	3.99	1.79	8.59	2.68	21.04	4.63
	5	17.11	1.46	8.52	2.48	4.82	1.62	13.35	4.05	25.81	1.39
	Season yield	100.76	4.70	368.68	28.55	217.78	31.05	586.48	56.93	111.43	5.19

Table 4.4.5 Significance of t-tests between groups of observations (cultivars *versus* ecotypes, diploid cultivars *versus* tetraploid cultivars) for the characters: dry matter, fructose, glucose, WSC and crude protein content.

	Cut	Dry matter	Fructose	Glucose	WSC	Crude protein
Cultivar vs. ecotype	1	0.0015	N/S*	N/S	N/S	N/S
Cultivar vs. ecotype	2	<0.0001	N/S	N/S	N/S	N/S
Cultivar vs. ecotype	3	0.0040	N/S	N/S	N/S	N/S
Cultivar vs. ecotype	4	<0.0001	N/S	N/S	N/S	N/S
Cultivar vs. ecotype	5	N/S	N/S	N/S	N/S	N/S
Diploid cultivar vs. tetraploid cultivar	1	N/S	N/S	N/S	N/S	N/S
Diploid cultivar vs. tetraploid cultivar	2	N/S	N/S	N/S	N/S	N/S
Diploid cultivar vs. tetraploid cultivar	3	0.0103	N/S	N/S	N/S	N/S
Diploid cultivar vs. tetraploid cultivar	4	0.0212	N/S	N/S	N/S	N/S
Diploid cultivar vs. tetraploid cultivar	5	N/S	N/S	N/S	N/S	N/S

*N/S: non significant

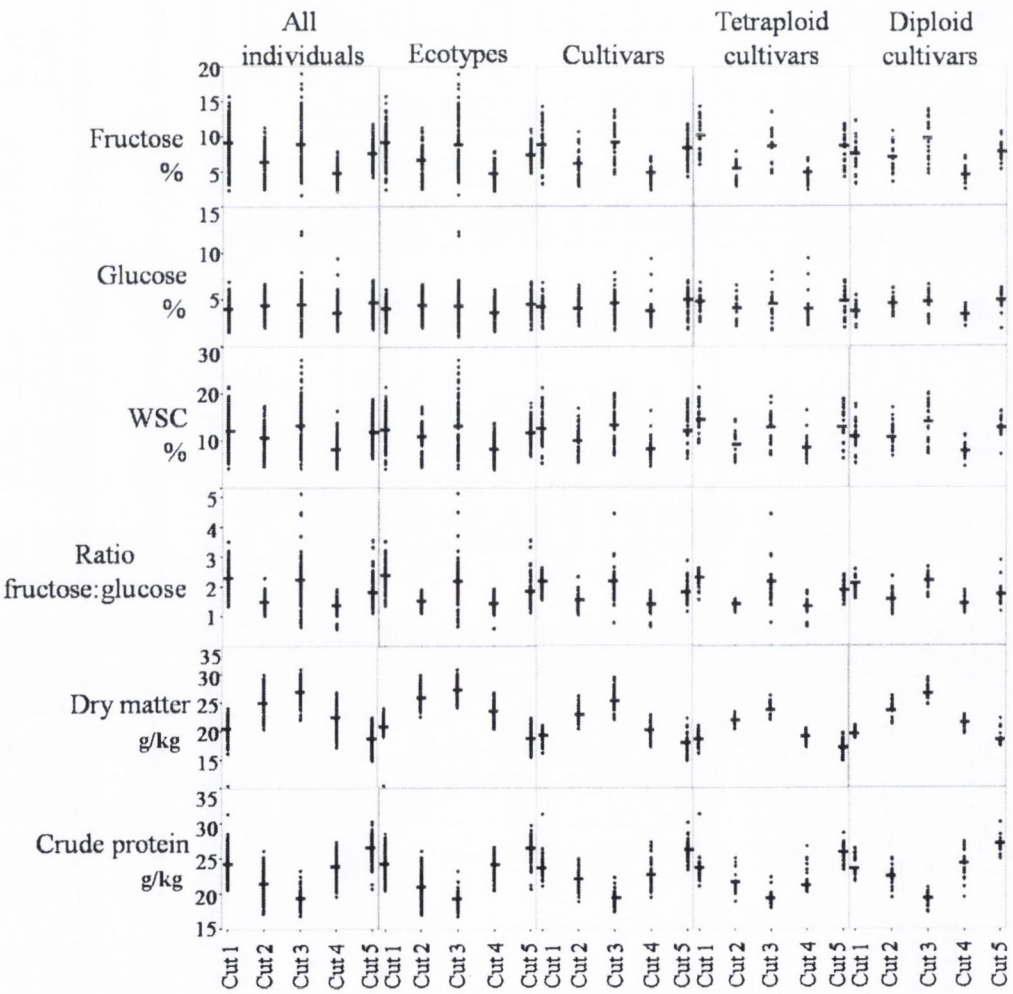

Figure 4.4.1 Scatterplots for the characters: fructose, glucose, WSC, ratio fructose/glucose, dry matter and crude protein, showing character values (y-axis)

overall, for ecotypes, cultivars, tetraploid cultivars, and diploid cultivars, at each cut (x-axis).

4.4.2 Data analysis

Normality tests for characters at each cutting point

Histograms to display the normal or non-normal distribution of the characters were constructed for each character at each cutting point, as well as over all cuts (Figure 4.4.2). The histograms for the majority of characters had a normal distribution appearance (in a bell-shaped curve). The histograms for the other characters were skewed, either to the left (dry matter, cut 1; WSC, cut 2; crude protein, cut 4; crude protein, cut 5; fructose overall, and crude protein overall) or to the right of the bell curve (crude protein, cut 1; fructose, cut 2; fructose, glucose and WSC, cut 3; glucose, cut 4; and fructose, cut 5).

Probability plots were constructed for each character at each cutting point and over all cuts using the Kolmogorov-Smirnov test statistic (Figure 4.4.3). Fructose, glucose and WSC (cut 1), glucose, dry matter and crude protein (cut 2), dry matter and crude protein (cut 3), fructose, WSC and dry matter (cut 4) glucose, WSC, dry matter (cut 5), glucose overall, WSC overall and dry matter overall followed a straight line in these plots indicating normal distribution. The other characters deviated from a straight line in the tails of the distributions, indicating that these characters may not have been normally distributed. The indications from the histograms and probability plots were confirmed using the Kolmogorov-Smirnov test statistics (Table 4.4.6). A character was deemed to be normally distributed if the value of the Kolmogorov test statistics is smaller than the corresponding p-value.

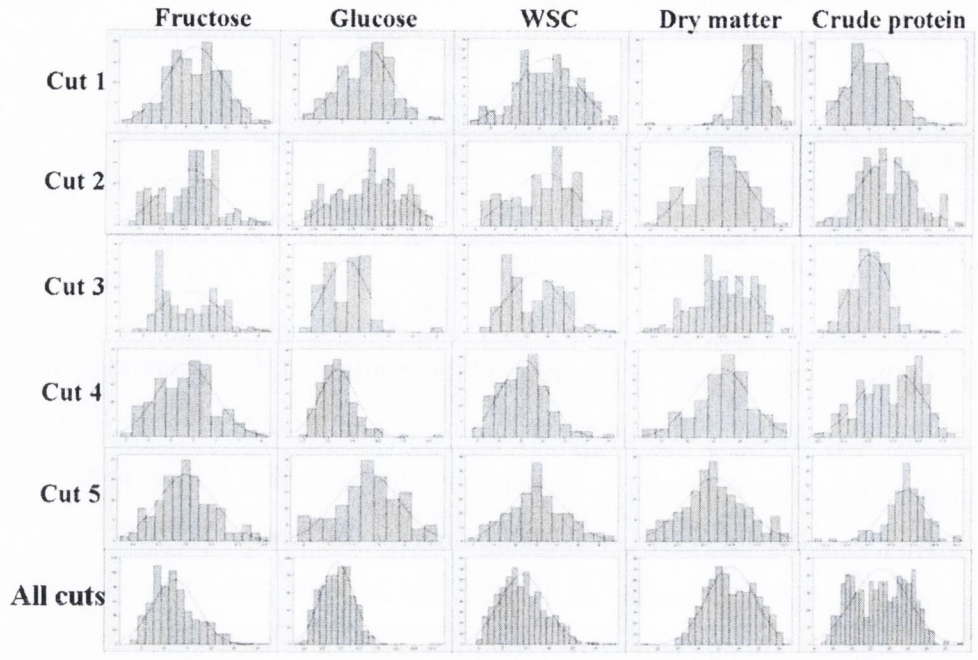

Figure 4.4.2 Histograms with fitted normal distribution curves for the characters fructose, glucose, WSC, dry matter and crude protein at each cutting point and over all cutting points. Y-axis: Frequency. X-axis: value of character of interest.

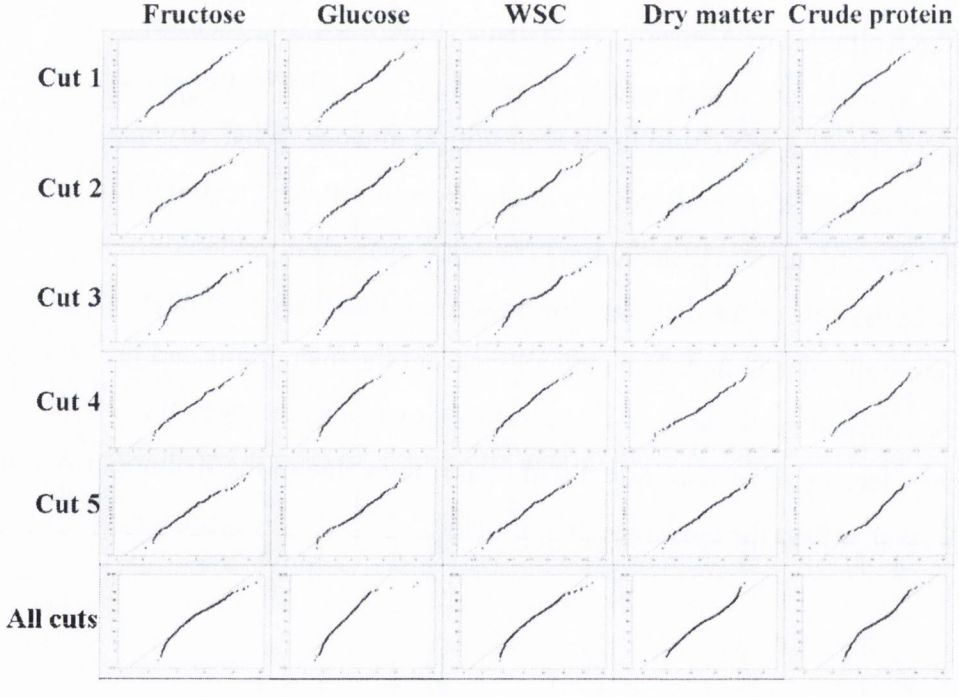

Figure 4.4.3 Probability plots using the Kolmogorov-Smirnov test for the characters fructose, glucose, WSC, dry matter and crude protein at each cutting point and over all cutting points. Y-axis: Percentage. X-axis: value of character of interest.

Table 4.4.6 Kolmogorov-Smirnov statistics and p-values for each character at each cutting point and over all cutting points.

Character	Kolmogorov-Smirnov statistic	p-value
Fructose cut 1	0.042	>0.150
Glucose cut 1	0.058	>0.150
WSC cut 1	0.037	>0.150
Dry matter cut 1*	0.096	<0.010
Crude protein cut 1*	0.079	0.047
Fructose cut 2*	0.087	0.023
Glucose cut 2	0.060	>0.150
WSC cut 2*	0.089	0.013
Dry matter cut 2	0.064	>0.150
Crude protein cut 2	0.054	>0.150
Fructose cut 3*	0.147	<0.010
Glucose cut 3*	0.085	0.031
WSC cut 3*	0.126	<0.010
Dry matter cut 3	0.049	>0.150
Crude protein cut 3	0.058	>0.150
Fructose cut 4	0.064	>0.150
Glucose cut 4*	0.084	0.031
WSC cut 4	0.055	>0.150
Dry matter cut 4	0.063	>0.150
Crude protein cut 4*	0.093	<0.010
Fructose cut 5*	0.080	0.042
Glucose cut 5	0.070	0.113
WSC cut 5	0.072	0.095
Dry matter cut 5	0.038	>0.150
Crude protein cut 5*	0.093	<0.010
Fructose overall*	0.084	<0.01
Glucose overall	0.031	0.113
WSC overall	0.049	0.050
Dry matter overall	0.053	0.061
Crude protein overall*	0.067	<0.01

*Non-normally distributed characters

Data transformation for non-normal distributed characters

(1) Log transformation

Data from the non-normally distributed characters were transformed using a log transformation. Histograms with fitted normal distributions were constructed (Figure 4.4.4). The histograms for the characters crude protein (cut 1), glucose (cut 4), fructose (cut 5), fructose overall and crude protein overall were normally distributed.

The data of the other characters were all skewed to the left. Probability plots were constructed for each character using the Kolmogorov-Smirnov test statistic (Figure 4.4.5). Log transformed data for the characters crude protein (cut 1), glucose (cut 4), fructose (cut 5), fructose overall and crude protein overall followed a straight line in the probability plots. The other characters were still deviating from a straight line in the tails of the distributions. Indications of normality in the plots were confirmed with the Kolmogorov-Smirnov test statistic (Table 4.4.7).

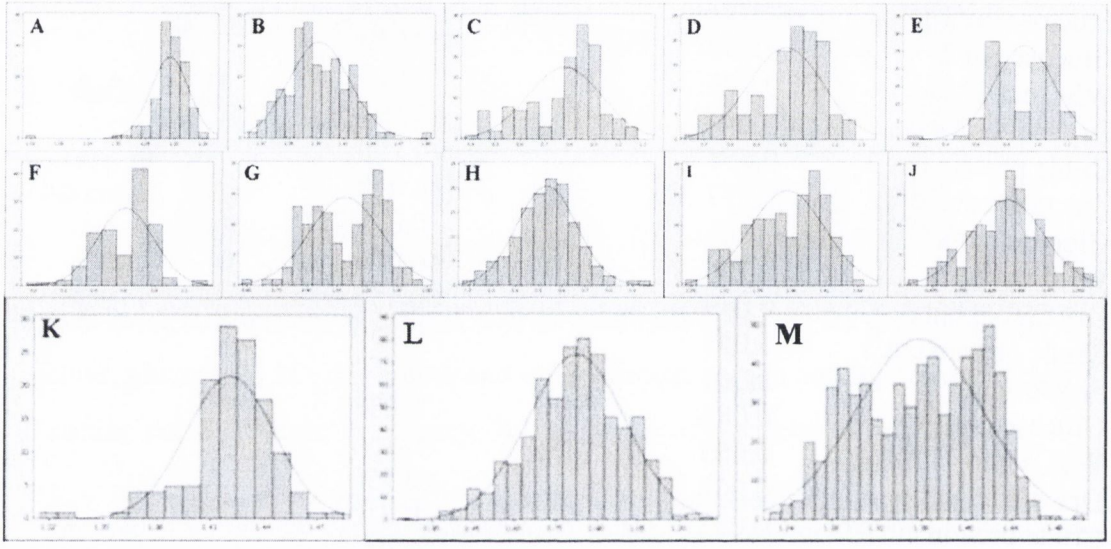

Figure 4.4.4 Histograms with fitted normal distribution curves for the following log transformed data of characters: A: Dry matter cut 1, B: Crude protein cut 1, C: Fructose cut 2, D: WSC cut 2, E: Fructose cut 3, F: Glucose cut 3, G: WSC cut 3, H: Glucose cut 4, I: Crude protein cut 4, J: Fructose cut 5, K: Crude protein cut 5, L: Fructose overall, M: Crude protein overall. Y-axis: Frequency. X-axis: Value of log transformed character of interest.

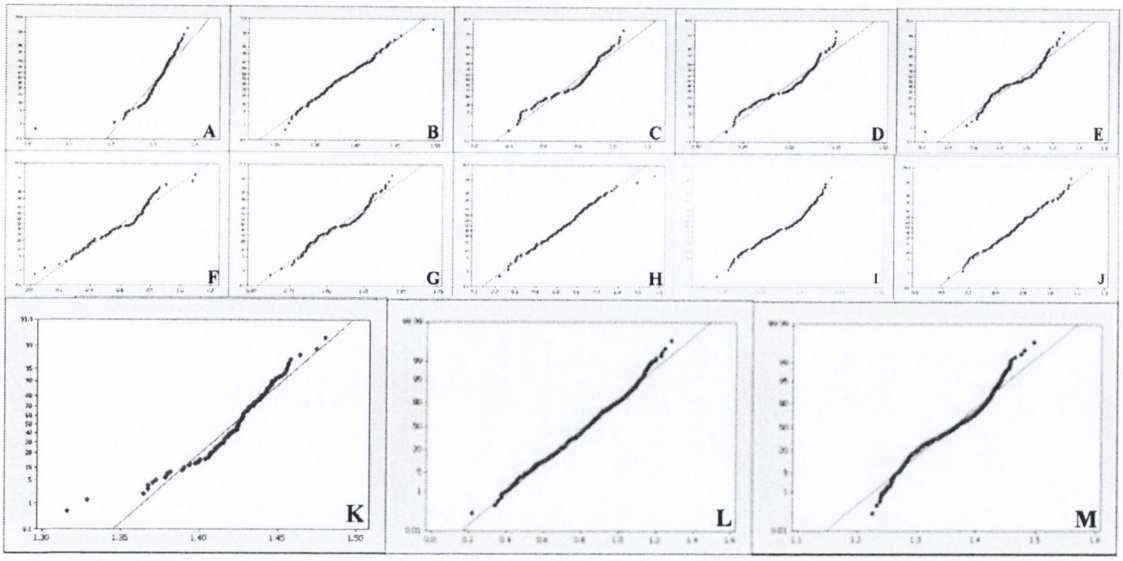

Figure 4.4.5 Probability plots using the Kolmogorov-Smirnov test for the log transformed data of characters: A: Dry matter cut 1, B: Crude protein cut 1, C: Fructose cut 2, D: WSC cut 2, E: Fructose cut 3, F: Glucose cut 3, G: WSC cut 3, H: Glucose cut 4, I: Crude protein cut 4, J: Fructose cut 5, K: Crude protein cut 5, L: Fructose overall, M: Crude protein overall. Y-axis: percentage. X-axis: value of character of interest.

Table 4.4.7 Kolmogorov-Smirnov statistics and p-values for each log transformed character.

Character	Kolmogorov-Smirnov statistic	p-value
Dry matter cut 1*	0.129	<0.010
Crude protein cut 1	0.066	>0.150
Fructose cut 2*	0.150	<0.010
WSC cut 2*	0.146	<0.010
Fructose cut 3*	0.130	<0.010
Glucose cut 3*	0.151	<0.010
WSC cut 3*	0.126	<0.010
Glucose cut 4	0.040	>0.150
Crude protein cut 4*	0.107	<0.010
Fructose cut 5	0.056	>0.150
Crude protein cut 5*	0.105	<0.010
Fructose	0.033	0.079
CP	0.068	0.070

*Non-normal characters

Square root, reciprocal or natural log transformations did not transform the eight remaining characters to normality (Appendix 8.8).

(2) Johnson transformation

Johnson's transformations did not transform the following characters to normality: fructose cut 3, glucose cut 3 and WSC cut 3. Johnson's transformation functions were successfully determined for the five remaining non-normally distributed characters (dry matter cut 1, fructose cut 2, WSC cut 2, crude protein cut 4, crude protein cut 5) and the data were transformed according to Equations 4.3.4 to Equation 4.3.8. Histograms for the five Johnson transformed characters were constructed (Figure 4.4.6). All characters followed an approximately normal distribution. Probability plots were constructed for each transformed character using the Anderson-Darling statistic (Figure 4.4.6). Each character followed an approximately straight line. The normality of the Johnson transformed characters was confirmed by the low Anderson-Darling statistics (Table 4.4.8).

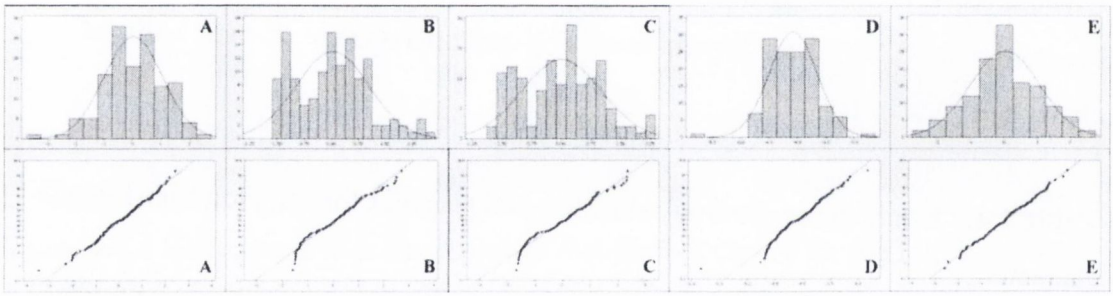

Figure 4.4.6 Histograms and probability plots with fitted normal distribution curves for the following Johnson transformed data of characters: A: Dry matter cut 1, B: Fructose cut 2, C: WSC cut 2, D: Crude protein cut 4, E: Crude protein cut 5. Y-axis: Frequency (histograms)/percentage (probability plots). X-axis: Data for Johnson transformed character of interest.

Table 4.4.8 Anderson-Darling statistics and p-values for each Johnson transformed character.

Character	Anderson-Darling statistic	p-value
Dry matter cut 1	0.323	0.524
Fructose cut 2	0.919	0.019
WSC cut 2	0.927	0.019
Crude protein cut 4	0.757	0.048
Crude protein cut 5	0.260	0.708

Correlations between characters and cuts

Pearson and Spearman rank correlation coefficients were calculated for each pair of characters (Table 4.4.9). For data which were originally normally distributed, or transformed to normality, Pearson's correlation was used, while for data which could not be transformed to normality, Spearman's rank correlation was used (indicated in Table 4.4.9). Correlation coefficients between either fructose or glucose and WSC in the same cut were not performed, as the data were not independent. Within cut 1, all correlations were significant, with the single exception of the correlation of crude protein and dry matter. Fructose and glucose showed a strong positive and highly significant correlation of 0.84 ($p<0.0001$). Dry matter showed weak but highly significant positive correlations with fructose, glucose and WSC (0.29, $p<0.001$; 0.19, $p<0.05$; 0.27, $p<0.001$). Crude protein had moderate negative but highly significant correlations with fructose, glucose and WSC (-0.53, -0.31, -0.48; all $p<0.0001$). Within cuts 2, 3 and 4, only two correlations in each were significant, strong positive correlations between fructose and glucose (0.88, 0.77, 0.76, all $p<0.0001$), and moderate negative correlations between dry matter and crude protein (-0.49, $p<0.0001$; -0.35, $p<0.0001$; -0.25, $p<0.001$). Within cut five, all correlations were significant, with positive correlations between fructose and glucose (0.78, $p<0.0001$), between dry matter and fructose, glucose and WSC (0.37, 0.6, 0.47, all $p<0.0001$), and negative correlations between crude protein and dry matter, fructose, glucose and WSC (-0.56, $p<0.0001$; -0.35, $p<0.0001$; -0.5, $p<0.0001$, -0.2, $p<0.05$). Between different cuts, most correlations are non-significant. Values recorded within cuts 1 at the beginning of the vegetation period and cut 5 at the end of the vegetation period showed the most significant correlations, with positive correlations between fructose, glucose, WSC and dry matter, and negative correlations between crude protein and the other characters.

Table 4.4.9 Correlations (Pearson and Spearman rank) and their significance levels between each pair of characters at each cut.

		Cut 1					Cut 2					Cut 3					Cut 4					Cut 5				
		F[a]	G[b]	WSC[c]	DM[d]	CP[e]	F	G	WSC	DM	CP	F†	G†	WSC†	DM	CP	F	G	WSC	DM	CP	F	G	WSC	DM	CP
1	F																									
	G	0.84 ***																								
	WSC	N/A*	N/A																							
	DM	0.29 **	0.19 *	0.27 **																						
	CP	-0.53 ***	-0.31 ***	-0.48 ***	N/S‡																					
2	F	N/S	N/S	N/S	N/S	N/S																				
	G	N/S	N/S	N/S	N/S	0.20 *	0.88 ***																			
	WSC	N/S	N/S	N/S	N/S	N/S	N/A	N/A																		
	DM	0.22 *	N/S	0.18 *	0.72 ***	N/S	N/S	N/S	N/S																	
	CP	-0.48 ***	-0.34 ***	-0.45 ***	-0.38 ***	0.28 **	N/S	N/S	N/S	-0.49 ***																
3	F†	N/S	N/S	N/S	N/S	N/S	0.19 *	N/S	0.19 *	N/S	N/S															
	G†	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	0.77 ***														
	WSC†	N/S	N/S	N/S	N/S	N/S	0.18 *	N/S	0.18 *	N/S	N/S	N/A	N/A													
	DM	N/S	N/S	N/S	0.62 ***	N/S	0.27 **	0.17 *	0.24 **	0.80 ***	-0.38 ***	N/S	N/S	N/S												
	CP	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	0.26 **	N/S	N/S	N/S	-0.35 ***											
4	F	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S										
	G	N/S	0.20 *	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	-0.17 *	N/S	N/S	N/S	N/S	0.76 ***									
	WSC	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/A	N/A								
	DM	N/S	N/S	N/S	0.65 ***	N/S	0.22 **	0.19 *	0.22 *	0.75 ***	-0.34 ***	N/S	N/S	N/S	0.74 ***	N/S	N/S	N/S	N/S							
	CP	-0.18 *	-0.18 *	-0.18 *	0.23 **	N/S	N/S	N/S	N/S	0.35 ***	N/S	N/S	N/S	N/S	0.27 **	N/S	N/S	N/S	N/S	-0.25 **						
5	F	0.25 **	0.36 ***	0.29 **	N/S	N/S	N/S	N/S	N/S	-0.23 **	-0.20 *	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S					
	G	0.24 **	0.36 ***	0.28 **	N/S	N/S	N/S	N/S	N/S	N/S	-0.29 **	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	0.78 ***				

WSC	0.27 **	0.39 ***	0.31 ***	N/S	N/S	N/S	N/S	N/S	-0.20 *	-0.25 **	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/A	N/A	
DM	0.27 **	0.26 **	0.27 **	0.42 ***	N/S	N/S	N/S	N/S	0.36 ***	-0.43 ***	N/S	N/S	N/S	0.44 ***	-0.32 ***	N/S	N/S	N/S	0.36 ***	0.21 *	0.37 ***	0.60 ***	0.47 ***	
CP	-0.40 ***	-0.38 ***	-0.41 ***	-0.20 *	N/S	N/S	N/S	N/S	N/S	0.30 ***	N/S	N/S	N/S	N/S	0.19 *	N/S	N/S	N/S	N/S	0.20 *	-0.56 ***	0.35 ***	-0.50 ***	-0.20 *

[a]F: Fructose; [b]G: Glucose; [c]WSC: Water soluble carbohydrate; [d]DM: Dry matter; [e]CP: Crude protein; †: for non-normally distributed characters Spearman correlations were used; [*]N/A: not applicable, correlation not performed due to lack of independence among characters; ‡N/S: not significant; *p<0.05, **p<0.01, ***p<0.001.

Principal components analysis

The first eigenvalue (Table 4.4.10) of the principal components analysis (PCA) on all data for the first cut explained 81.33% of the variation of the dataset, while the remainder of the variation was explained by the next two eigenvalues. When the eigenvectors were plotted for the first two dimensions (Figure 4.4.7) a separation was seen between the cultivars on the right hand side of the diagram, with the majority of the ecotypes (with the exception of IRL-OP-02007, IRL-OP-02011, IRL-OP-0 2048, IRL-OP-02064, IRL-OP-02068, IRL-OP-02192, IRL-OP-02272, IRL-OP-02337, IRL-OP-02483) being found in the other quadrants. After canonical variates analysis, the scores for each character (Table 4.4.11) showed the relative importance of each character to the separation seen in the PCA in the first axis and indicated that fructose and dry matter content were the main characters influencing the split between ecotypes and cultivars.

The first eigenvalue (Table 4.4.10) of the principal components analysis for the second cut explained 97.47% of the variation of the dataset and the remainder of the variation was explained by the next two eigenvalues. When the eigenvectors were plotted for the first two dimensions (Figure 4.4.7) a separation was seen between the tetraploid cultivars on the left hand side of the diagram, with the majority of the ecotypes (with the exception of IRL-OP-02337, IRL-OP-02064, IRL-OP-02068, IRL-OP-2272 and IRL-OP-2483) found on the right hand side of the diagram. The canonical variates analysis (Table 4.4.11) showed that the dry matter and crude protein contents were the main characters influencing the split between ecotypes and cultivars at the second cutting time point.

The first eigenvalue (Table 4.4.10) of the principal components analysis for the third cut explained 97.98% of the variation of the dataset and the remainder of the variation was explained by the next three eigenvalues. When the eigenvectors were plotted for the first two dimensions (Figure 4.4.7) a separation was seen between the tetraploid cultivars on the left hand side of the diagram, with the majority of the diploid accessions (with the exception of IRL-OP-02064 and IRL-OP-02480) being found on the right hand side of the diagram. The canonical variates analysis (Table 4.4.11)

showed that dry matter and crude protein were the main characters influencing the split between tetraploid and diploid accessions.

The first eigenvalue (Table 4.4.10) of the principal components analysis for the fourth cut explained 91.30%. of the variation of the dataset, the remainder of the variation was explained by the next two eigenvalues. When the eigenvectors were plotted for the first two dimensions (Figure 4.4.7 a separation was seen between the tetraploid cultivars on the bottom right hand side of the diagram, with the rest of the accessions being found in the rest of the diagram. The canonical variates analysis (Table 4.4.11) showed that dry matter, and to a lesser extent, fructose, and crude protein were the main characters influencing the split between ecotypes and cultivars.

The first two eigenvalues (Table 4.4.10) of the principal components analysis for the fifth cut explained nearly all of the variation of the dataset, with the first eigenvalue explaining 89.09% and the second eigenvalue explaining a further 10.66% of the variation. The remainder of the variation was explained by the final eigenvalue. When the eigenvectors were plotted for the first two dimensions (Figure 4.4.7) no separation was seen between any groups of accessions.

Overall, there is no unifying pattern to the data in the PCA. Within the first four cuts, accessions with high dry matter content had low crude protein contents. Within cuts one, three and four, cultivars (particularly tetraploid cultivars) had high dry matter contents and low crude protein contents, and vice versa for cut two. Fructose and glucose only appeared to influence the PCA in cut one.

Table 4.4.10 Eigenvalues from principal components analysis and percentage of the variation explained by each dimension for each cut.

	Cut 1			Cut 2			Cut 3			Cut 4			Cut 5		
	Axis			Axis			Axis			Axis			Axis		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Eigenvalue	1.30	0.30	0.01	0.86	0.20	0.01	0.23	0.01	0.01	0.19	0.02	0.01	0.46	0.06	0.01
Percentage variation explained	81.34	18.43	0.23	97.47	2.28	0.25	97.98	1.92	0.09	91.30	8.62	0.08	89.09	10.66	0.25
Cumulative percentage variation explained	81.34	99.77	100.00	97.47	99.75	100	97.98	99.91	100.00	91.3	99.92	100.00	89.09	99.75	100.00

Table 4.4.11 Scores for each character for the first dimension of the principal components analysis (cut 5 not analysed).

	Cut 1	Cut 2	Cut 3	Cut 4	Cut 5
Fructose	0.663	0.031	0.020	-0.140	N/A
Glucose	0.118	0.030	0.004	-0.034	N/A
Dry matter	0.375	0.971	1.130	1.196	N/A
Crude protein	-0.006	-0.584	-0.259	0.138	N/A

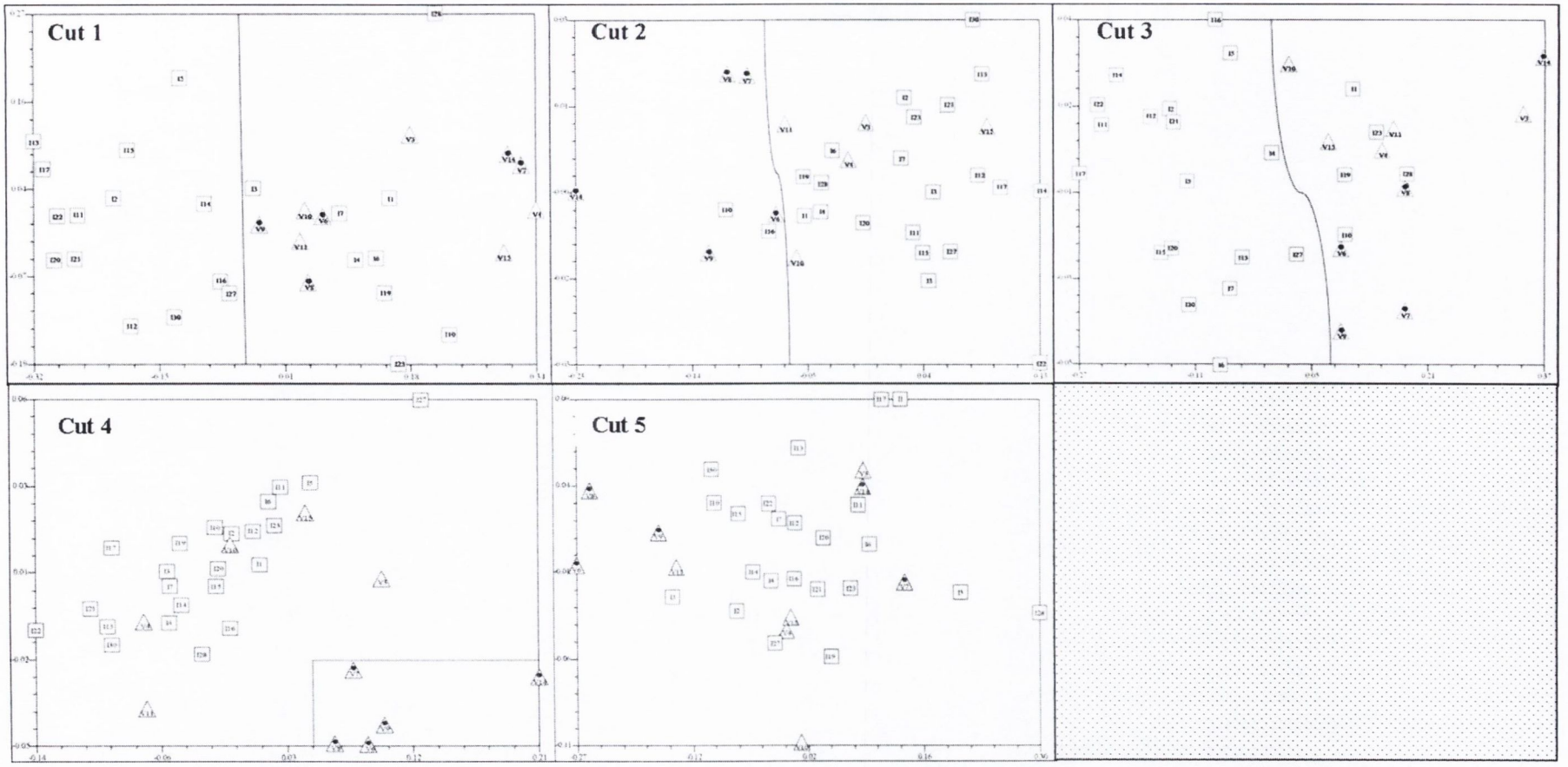

Figure 4.4.7 Principal components diagram in two dimensions for data in each cut showing assumed groups. X axis: Dimension 1, Y axis: Dimension 2, □: Irish ecotype, Δ: Cultivar, ▲: Tetraploid cultivar. Numbers of accessions are given in Appendix 8.1.

ANOVA analysis

The model given in Equation 4.3.9 best fitted all characters with a significance level of at least $p<0.01$ in the comparisons with the null hypothesis model (Table 4.4.12). Climate parameters did not affect the model and so were removed from the analysis.

(1) Fructose

Only cutting time (cut) was a significant factor for fructose content. (Table 4.4.13, $p<0.0001$). Cuts one, three and five had high fructose contents, while cuts two and four had significantly lower fructose contents (Figure 4.4.8).

(2) Glucose

Again, only cutting time had a significant effect on variation in the character glucose (Table 4.4.13, $p<0.001$). Glucose content increased from the early May cutting point to the mid growing season (cut three). Glucose content had significantly decreased by late growing season (cut four) and increased again by late October (cut 5, Figure 4.4.8).

(3) WSC

Like glucose and fructose content, only the cutting point had a significant effect on the variation in WSC content (Table 4.4.13, $p<0.0001$). Similar patterns across the growing season to fructose were seen in WSC content, with high WSC content in cuts one, three and five, lower content in cut two and significantly lower in cut four (Figure 4.4.8).

(4) Dry matter

All three effects (cut, type of accession and cut*type interaction) were significant factors within the model for dry matter content. All type*cut interactions were significant at $p<0.0001$ (Table 4.4.13). Differences between cultivars and ecotypes at different cuts, and between the ecotypes at different cuts contributed most to the

significant type*cut interactions. With the exception of the significant differences between cultivars at cut 3 and cultivars at cuts 4 and 5, differences between cultivars at different cuts did not contribute to the variation. Dry matter tended to rise from cuts 1 to 3 and then decreased again through cut 4 to cut 5 (Figure 4.4.8).

(5) Crude protein

Cut and type*cut interaction were significant effects in the model for crude protein content (Table 4.4.13). There was significant variation between all types and cuts at $p<0.0001$. Crude protein first decreased over time and then increased, in an inverse pattern to dry matter content (Figure 4.4.8).

Table 4.4.12 Statistics for the fit of the mixed model for each character and significance levels for the fit of the model.

	Fructose	**Glucose**	**WSC**	**Dry matter**	**Crude protein**
d.f[a].	14	14	14	14	14
χ^2	29.76	40.20	38.22	75.50	55.52
$p> \chi^2$	<0.01	<0.001	<0.0001	<0.0001	<0.0001

[a]d.f.: degrees of freedom

Table 4.4.13 Significance levels for each effect in the model and the percentage variation explained by each effect.

Factors	[1]N. d.f.	[2]Den. d.f.	Fructose		Glucose		WSC		Dry matter		Crude protein	
			F[3]	p	F	p	F	p	F	p	F	p
Type	1	31	0.01	[4]N/S	0.45	N/S	0.08	N/S	38.11	<0.0001	0.17	N/S
Cut	4	31	36.00	0.0001	6.32	<0.001	19.42	<0.0001	272.41	<0.0001	362.75	<0.0001
Type x cut	4	31	1.90	N/S	0.88	N/S	1.90	N/S	8.09	0.0001	3.65	<0.05

[1]N. d.f.: numerator degrees of freedom; [2]Den d.f.: denominator degrees of freedom; [3]F: F ratio; [4]N/S: Non-significant

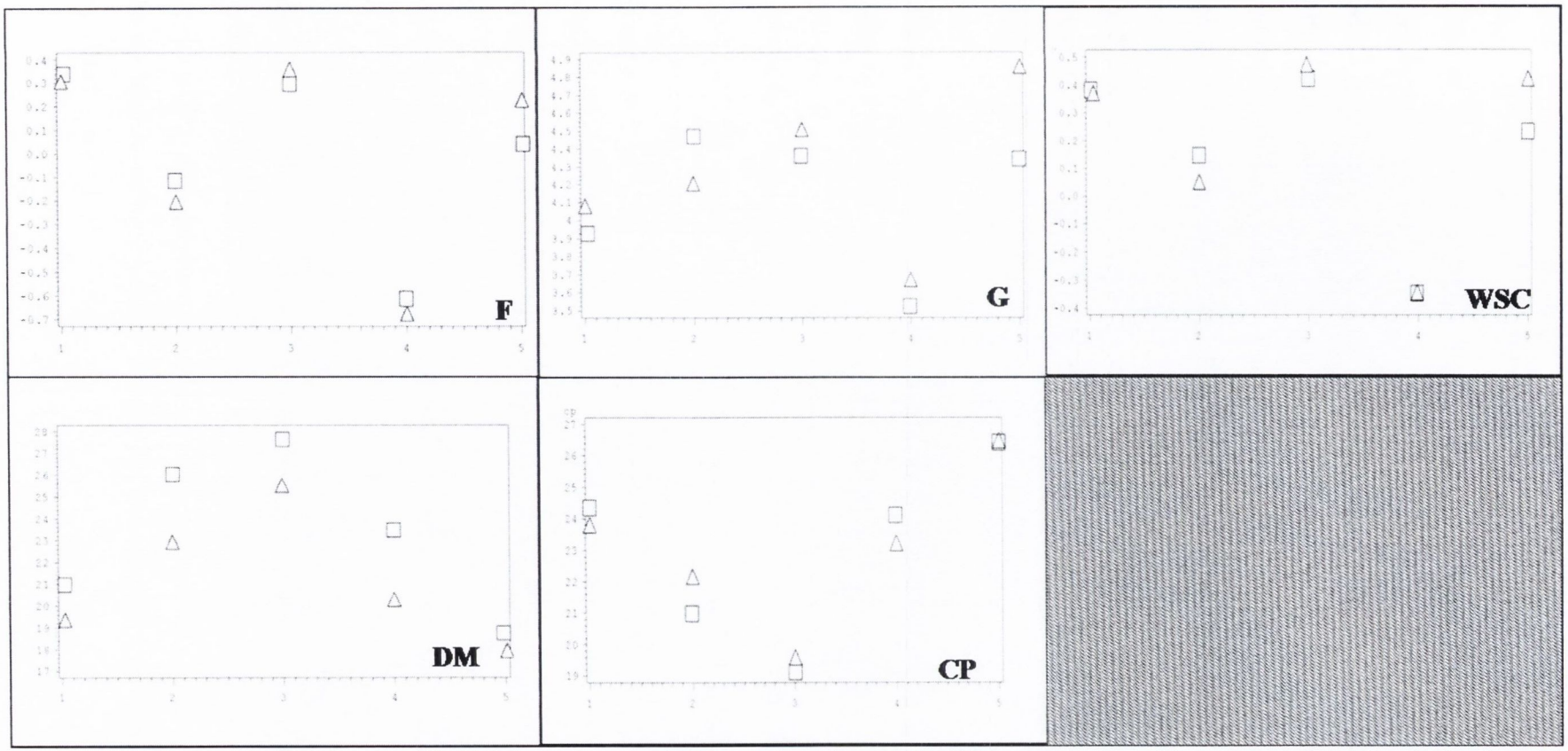

Figure 4.4.8 Fructose (F), glucose (G), WSC, dry matter (DM), and crude protein (CP) content (y-axis) across each cutting point (x-axis) for ecotypes (square) and cultivars (triangle).

4.5 Discussion

4.5.1 Biochemical diversity

Variation within the collection of *L. perenne* accessions ranged widely across characters, across cuts and between groups of populations. Fructose and WSC contents showed the widest range of variation across cuts (301.6 - 457.6g/kg DM, 470.8 – 703.5 g/kg DM, respectively; Table 4.4.1, Table 4.4.2). Fructose values for ecotypes, cultivars, tetraploid cultivars and diploid cultivars all varied in the same way according to the cut. They showed an oscillating pattern, decreasing in cut 2, increasing in cut 3, and decreasing in cut 4. The ranges of variation in glucose, crude protein and dry matter were much lower (169.3 – 257.4 g/kg DM, 105.2 – 120.84 g/kg DM, and 18.78 – 25.03 g, respectively; Tables 4.4.1 and 4.4.3). Mean contents of each character at each cutting point were highly variable, with broad ranges of values also within each cut. The wide variability of biochemical characters within the collection is another indication of the high genetic diversity within the collection. Such high variation of WSC, dry matter and crude protein was seen in other studies across cuts in cultivars (Gilliland *et al.* 2002; Smit *et al.* 2006; Tas *et al.* 2006) and across ecotypes (Skot *et al.* 2007). High variation was found in morphological characters, and chloroplast SSR markers (Chapter 2 and Chapter 3) and this suggests that there is a wide potential for using the ecotypic material in breeding programmes in general but also to improve biochemical characters such as WSC or crude protein. While such high levels of diversity were detected in morphological characters and chloroplast SSR markers, none of the exceptional populations seen in the WSC analysis were exceptional in the other analyses. Increased WSC content is preferred by ruminants (Jones & Roberts, 1991; Smit *et al.* 2006). It has been suggested that improvements in herbage quality characters have the potential to increase live weight gain by 20% (Marley *et al.* 2005) and milk production by 25% (Smith *et al.* 1997). An improved balance of sugar and protein content is also essential to ensure efficient crude protein degradation and reduced excretion of urea into the environment (Tas *et al.* 2006). The high biochemical diversity could be due to the fact that *L. perenne* is an outbreeding species, which could give rise to high levels of genetic variation within nuclear genes responsible for such phenotypic variation. The high biochemical diversity between populations could reflect local adaptation of populations.

While significant differences in mean character values between groups of populations were seen only within the character dry matter (Table 4.4.5), individual ecotypes showed higher mean season yields. In fructose, ecotype IRL-OP-02018 had a higher season yield than all the cultivars, and ecotype IRL-OP-02128 had a higher mean season yield than all cultivars with the exception of cultivar Portstewart. In glucose, ecotypes IRL-OP-02173 and IRL-OP-02018 had higher season yields than all cultivars with the exception of cultivar Greengold. Again, in character WSC, ecotype IRL-OP-02018 had a higher mean season yield than all cultivars. So while in general, cultivars show higher mean WSC contents than ecotypes, individual ecotypes in the collection, such as IRL-OP-02018, IRL-OP-02173, and IRL-OP-02128 show potential to be used to improve varieties in breeding programmes. Such broad ranges of WSC within ecotypes (as well as the superior levels of WSC in individual, locally adapted, ecotypes) could also be exploited to increase stress tolerance within new varieties. It has been shown that high WSC genotypes maintain high plant reserves, which can be important for persistence and stress tolerance (Turner *et al.* 2006) through membrane stabilisation (Hincha *et al.* 2002; Vereyken *et al.* 2003). It has been found for *Agrostis*, bentgrass, that exploration and collection of plant material in stressful environments has provided useful germplasm for stress tolerance improvement in that species (Casler, 2006). So there is a proven use of such adapted germplasm for improved variety creation. Characters such as WSC, crude protein and dry matter have already been shown to have moderate to high heritabilities (Jafari *et al.* 2003a; Turner *et al.* 2006; Xiong *et al.* 2006) and so are useful characters for QTL analysis. QTL analysis has already suggested chromosomal linkage group locations for these characters. For example, QTLs for crude protein have been founded on linkage group two (Xiong *et al.* 2006), and linkage groups three and four (Cogan *et al.* 2005). QTLs for WSC have been found on linkage group three (Cogan *et al.* 2005) and a highly significant QTL (explaining 38.7% of variation in the character) on linkage group six (Turner *et al.* 2006). Molecular markers linked to such QTLs could then be used for marker assisted selection (MAS) which makes targeted breeding strategies possible (Humphreys, 2005) and more efficient.

4.5.2 Relationships between characters

Relationships between characters were determined using correlation analysis. In general, fructose and glucose showed strong positive correlations, which was to be expected, considering that both are the products of hydrolysis of fructans. Early in the growing season, and at the end of the growing season, significant positive correlations were seen between dry matter and both fructose and glucose. Early in the growing season, there are more leaves than stem, also, late in the growing season, all the populations would have completed flowering and so returned to vegetative growth and higher dry matter and sugar contents. During flowering time, saccharides are used as an energy source by the plant to create flowering stems, which contain more crude protein than vegetative leaves. Within most cuts, there were negative correlations between dry matter and crude protein. It has already been reported that increasing crude protein content reduces the amount of dry matter produced per unit nitrogen (Wilkins & Humphreys, 2003). Also, crude protein levels decrease in late May or early June in *L. perenne* when much of the plant is stem (Wilkins & Humphreys, 2003), as seen with these data also (Figure 4.4.8). Xiong *et al.* (2006) has suggested that negative correlations between fibre and crude protein contents comes from QTLs with opposing effects and it could be a similar relationship between gene regions causing the negative correlations. Increasing content of either of these characters could be achieved by targeting one of the gene regions by MAS at a time.

4.5.3 Relationships between accessions

In cutting points one to four, separations in the PCA were seen for the characters fructose, glucose, dry matter and crude protein between ecotypes and cultivars (cutting point one and three) or between tetraploid cultivars and the other accessions (cuts two and four). In general, high dry matter content was the most important character separating the accessions in the first four cuts. Tetraploid varieties of *L. perenne* have been shown to have higher sugar contents than diploid varieties (Wilkins, 1991). However other studies have shown that, when comparing diploid varieties with other diploid varieties which had been bred for higher WSC content and with tetraploid varieties, that tetraploid superiority depends on the genetic background of the variety in question. Diploid varieties bred for higher WSC concentration were

superior to tetraploid varieties in some cases (Smith *et al.* 2001). This could explain the fact that the tetraploid varieties were not always separated from the other populations. Another factor could be the high variation between populations. Low crude protein contents were also important in separating the accessions in cutting points two to four. This agrees with the correlation results discussed earlier. Interestingly, fructose content was only important in the first cutting point, while glucose content was not contributing significantly to the separation in any cut.

4.5.4. Response of groups of accessions over the growing season and to environmental influences

For all characters, the cutting point was the most significant factor influencing the variation in the characters. In other studies cutting time point was also more of an influence over the variation than genotype, both across years (Turner *et al.* 2006; Skot *et al.* 2007) and across seasons (Tas *et al.* 2006). This suggests that grassland management and environmental conditions may have more control over the different characters than genotype. However, in this study, the weather conditions (rainfall, irradiance, mean temperature) had no significant effect on the model for each character. This may be the result of several factors, that is, the high variability within characters caused by the cutting point, which masked any small level of variation caused by weather effects. Additionally, the plants were only analysed in one site and so environmental effects could have been expected to affect all populations in the same way.

4.6 Conclusion

Water soluble carbohydrate, crude protein, and dry matter contents were recorded for 33 *L. perenne* ecotypes and cultivars at five different harvest time points across the 2004 growing season. While, in general, the cultivars had higher WSC contents than the ecotypes, individual ecotypes did show potential to be used in breeding programmes, as they showed higher values than all other accessions at particular cutting points. In correlation analyses, positive relationships were shown between dry matter and glucose both early and late in the growing season, and this was in agreement with the amount of leaves compared to stem at these times in the growing season. PCA analysis allowed the separation either between cultivars and ecotypes, or between tetraploid cultivars and the rest of the accessions at four out of five cutting points. In the ANOVA analysis, cutting point was the most significant factor influencing the variation in the traits.

Chapter 5

Characterisation of genetic diversity and population structure in a collection of *Lolium perenne* L. accessions using nuclear DNA microsatellite markers

5.1 Introduction

5.1.1 Nuclear DNA markers

Several types of nuclear markers have been developed and used in plant species, including random amplified polymorphic (RAPD) markers (Williams *et al.* 1990), amplified fragment length polymorphism (AFLP) markers (Vos *et al.* 1995), restriction fragment length polymorphism (RFLP) markers (Botstein *et al.* 1980), cleaved amplified polymorphic sequence (CAPS) markers (Konieczny & Ausubel, 1993), sequence-tagged site (STS) markers (Beckmann & Soller, 1990), single nucleotide polymorphism (SNP) markers (Fischer & Lerman, 1983), and simple sequence repeat (SSR) markers (Jones *et al.* 2001; Kubik *et al.* 2001). These markers can be divided into dominant (RAPD, AFLP) and co-dominant (RFLP, CAPS, STS, SNP, SSR) systems. RAPD and AFLP markers have the advantage that there is no need for previous knowledge of the genome as universal primers are applied. In the case of AFLP, the analysis is of high resolution, a large number of easily generated markers can be produced, and the analysis is reliable and reproducible (Cresswell *et al.* 2001). AFLP markers are often preferred to RAPD markers because of reproducibility issues with the latter and because AFLP marker systems generally detect higher number of alleles per reaction. However, both of these systems have the disadvantage of dominant marker systems, that is, that heterozygotes cannot be detected.

RFLP profiling generates codominant DNA markers by the selective hybridization of labelled DNA probes to endonuclease-fragmented nucleic acids that were previously separated by electrophoresis and bound to membranes (Helentjaris *et al.* 1985). RFLPs are powerful markers (Faville *et al.* 2004) but their detection is labour intensive, time consuming, and can detect multiple paralagous sequences and may fail to give locus-specific positions on genetic maps (Caetano-Anolles, 1998). SSR

markers are tandem repeated sequences made of one to six base pair repeats (Asp *et al.* 2007). They are useful markers because of various characteristics, including their abundance across plant genomes (Wang *et al.* 1994), their multi-allelism, extensive genome coverage, high reproducibility, high levels of polymorphism and simple PCR-based detection (Powell *et al.* 1996). Some disadvantages are that their detection can be time consuming and costly and that there are a limited number of publicly available SSR markers (Jones *et al.* 2001; Kubik *et al.* 2001; Warnke *et al.* 2004). SSR markers have many uses such as the construction of genetic linkage maps (Taramino *et al.* 1997), population genetics (Ram *et al.* 2007), genetic diversity analysis (Kubik *et al.* 2001), cultivar fingerprinting, marker assisted selection, and genotype assignment (Waser & Strobeck, 1998). SNP markers are single nucleotide differences between individuals. They are highly abundant within the genomes of higher plant species and are a fundamental source of variation for molecular genetic marker development (Cho *et al.* 1999), and they allow the creation of high density molecular maps (Simko *et al.* 2004). A disadvantage of SNP markers is that often a low number of alleles are present (Butler *et al.* 2007).

5.1.2 Use of nuclear markers to characterise genetic diversity in *Lolium perenne*

Nuclear markers have been used in many studies to characterise genetic diversity and population structure in *L. perenne* and related species (e.g. Cresswell *et al.* 2001; Kubik *et al.* 2001; Bolaric *et al.* 2005a). For example, RAPD analysis has been used to study diversity within and among *L. perenne* cultivars and ecotypes (Bolaric *et al.* 2005a and 2005b) and in comparisons of several forage grass species including *L. perenne* (Kolliker *et al.* 1999). AFLP markers have also been used to characterise genetic diversity in *L. perenne* cultivars (Roldan-Ruiz *et al.* 2000; Guthridge *et al.* 2001), in *L. perenne* ecotypes (Skot *et al.* 2002), for comparisons of genetic diversity between ecotypes and cultivars (Van Treuren *et al.* 2005) and for the characterisation of genetic diversity between different forage species (Cresswell *et al.* 2001). STS markers have been used to characterise genetic diversity in *L. perenne* cultivars (Roldan-Ruiz *et al.* 2001; Lem & Lallemand, 2003; Auzanneau *et al.* 2007) but their application has been limited (Kubik *et al.* 2001; Momotaz *et al.* 2004) probably because of the relative lack, until recently, of publicly available markers (Jones *et al.* 2001; Kubik *et al.* 2001; Warnke *et al.* 2004). Genetic diversity has been

characterised using SSRs in cultivars (Kubik *et al.* 2001) and between different closely related forage species (Momotaz *et al.* 2004). Nuclear SSR markers have not, so far, been used to characterise genetic diversity in Irish ecotypes or applied to the collections housed by Teagasc at Oak Park for plant breeding applications such as linkage mapping, QTL studies or MAS application.

5.1.3 Linkage disequilibrium (LD)

Linked genes are genes found on the same chromosome. Linkage disequilibrium (LD) is therefore the non-random occurrence of alleles at different loci (Flint-Garcia *et al.* 2003). LD is said to occur if two alleles from different genes/markers on the same chromosomes tend to be associated in different individuals at a greater frequency than would be expected due to random association. The level of LD in plants is affected by the breeding system of the particular plant (Flint-Garcia *et al.* 2003) because high levels of recombination seen in outbreeding species results in lower levels of linkage disequilibrium, and *vice versa* in inbreeding species (Charlesworth & Wright, 2001). Several other factors can also affect linkage disequilibrium, such as population structure, which occurs when the frequency of the character of interest varies across subpopulations (Gupta *et al.* 2005) causing spurious associations between genotype and phenotype (Pritchard *et al.* 2000a). Other factors include epistasis, gene conversion, and ascertainment bias (Gupta *et al.* 2005). The study of LD can also be used to characterise populations or used in association mapping studies. Association mapping uses the LD occurring in natural and breeding populations. Marker-trait associations can be detected in collections of unrelated genotypes when the LD stemming from the linkage between a marker and a gene underlying the character has not been completely broken by recombination events. LD/association mapping has several applications, for example the identification and mapping of QTLs (Meuwissen & Goddard, 2000), which has been performed on a number of plant systems (e.g. maize, Labate *et al.* 2000; *L. perenne,* Skot *et al.* 2005). LD/association mapping can be used to identify genes which are responsible for the difference in two alternative phenotypes (Palaisa *et al.* 2004) and this approach has also been applied in various plant systems (e.g. maize, Thornsberry *et al.* 2001; *Arabidopsis*, Nordborg & Tavare, 2002; and potato, Simko *et al.* 2004). As well as gene identification, LD/association mapping can be used in population genetics to determine the effect of selection and

domestication events (Peng *et al.* 2003), and to determine the extent of LD across genomes (Remington *et al.* 2001) including *L. perenne* (e.g. Auzanneau *et al.* 2007; Ponting *et al.* 2007; Xing *et al.* 2007). Several association mapping studies have been performed on *L. perenne*, both whole genome association mapping (Skot *et al.* 2002; Skot *et al.* 2005) and candidate gene association mapping (Skot *et al.* 2007). Whole genome association involves the use of many markers distributed across the genome to evaluate all genes simultaneously, while the candidate gene approach focuses on limited numbers of gene regions suspected to have an association with a particular character (Flint-Garcia *et al.* 2003). Associations between markers and cold tolerance genes were detected by Skot *et al.* (2002, 2005). Associations between markers and flowering time and WSC were detected by Skot *et al.* (2007). It is also important to assess LD between pairs of loci to determine whether population structure can be reliably assessed using these loci. If they are not independent then they have to be applied with caution for population genetic assessments.

5.2 Aims

The aims of this chapter are to characterise nuclear DNA variation in a collection of *Lolium perenne* that had also been characterised for plastid DNA, morphological, phenological and biochemical (WSC and protein) variation. One associated aim was to develop reliable markers for future plant breeding applications. The specific objectives of the nuclear DNA marker work were to:

(1) describe nuclear DNA allelic and genotypic diversity in natural and breeding populations of *Lolium perenne* including Irish and other European ecotypes and bred cultivars,

(2) develop and select suitable markers for plant breeding applications,

(3) determine the partitioning of the variation between and among the accessions and groups of accessions, and evaluate population structure in the collection, and

(4) determine the extent of linkage disequilibrium so that the potential for LD/association mapping application could be assessed, and to determine if population structure can be reliably assessed.

5.3 Materials and Methods

5.3.1 Selection of samples for analysis

A total of 928 individuals from a selection of 40 diploid *Lolium perenne* accessions were used (Appendix 8.1). These 40 accessions have been previously used in the work reported to investigate chloroplast DNA diversity and population genetic structure and pattern (Chapter 2), and morphological (Chapter 3) and biochemical diversity (Chapter 4). Approximately 24 individuals from each accession were selected for analysis. DNA was extracted in the manner described in section 2.3.18. No tetraploid accessions were used because of genotyping difficulties and time constraints.

5.3.2 Amplification of nuclear microsatellite markers

Microsatellite primers were chosen from a private set developed and obtained under licence from IGER (Turner *et al.* 2006) and from a private set obtained under licence from ViaLactia Biosciences (Gill *et al.* 2006; Table 5.3.1). Microsatellite loci were selected based on previously published associations with genes for characters of interest (Jensen *et al.* 2005; Turner *et al.* 2006, Table 5.3.1). Samples were prepared for amplification as described in section 2.3.15 and under the master mix and thermalcycling conditions outlined in the source publications.

Table 5.3.1 Microsatellite primers, source, characteristics, associated characters and details of analysis.

Primer	Source	Associated characters	Linkage group
LpHCA18F11	Turner *et al.* (2006)	Heading date[1]	7
LpACT26D12	Turner *et al.* (2006)	Heading date[1], WSC[2]	6
LpACT13H2	Turner *et al.* (2006)	Heading date[1]; glucose, fructose and WSC[2]	6
rv0264	Gill *et al.* 2006	Heading date[3]	7
rv0908	Gill *et al.* 2006	Heading date[1]	7
rv0449	Gill *et al.* 2006	Heading date[1], WSC[2]	6
rv1239	Gill *et al.* 2006	Heading date[1]	2
rv1423	Gill *et al.* 2006	WSC[2]	6

[1]Heading date, Jensen *et al.* 2005; Turner *et al.* 2006; Heading date[3], unpublished .

5.3.3 Analysis of microsatellite amplification products

Amplified PCR products were prepared for analysis on the ABI3100 automated DNA sequencer according to section 2.3.14 (without the dilution step).

5.3.4 Data analysis

Allelic variation

Allele numbers and size ranges of alleles per locus were calculated over all accessions and for each group of accessions (ecotypes, cultivars, Irish ecotypes and European ecotypes).

Observed and expected heterozygosities were calculated for each population at each locus and over all loci, as well as for each group of accessions according to Equation 5.3.1 and Equation 5.3.2 (Nei, 1973) using POPGENE (Yeh & Boyle, 1997).

$$H_O = \frac{N_H}{N_g}$$

Equation 5.3.1 Observed heterozygosity, H_O, where N_H is the number of heterozygotes and N_g is the number of genotypes.

$$H_E = 1 - \sum_{i=1-k} p_i^2$$

Equation 5.3.2 Expected heterozygosity, H_E, where p_i is the frequency of the ith allele. This statistic is also known as gene diversity (Nei, 1973).

Tests for Hardy-Weinberg equilibrium were performed on each population at each locus and for each group of accessions using a modified Markov-chain random walk algorithm (Guo & Thompson, 1992), and were tested for significance using 1000 permutations in ARLEQUIN 2.0 software (Schneider *et al.* 2000).

Polymorphic information content (PIC) values were calculated by hand for each locus over all accessions and for each group of accessions, from allele frequency data

calculated in POPGENE (Yeh & Boyle, 1997), according to Equation 5.3.3 (Botstein *et al.* 1980).

$$PIC = \sum_{i=1}^{n} p_i^2 - 2\sum_{i=1}^{k-1}\sum_{j=1}^{k} p_i^2 p_j^2$$

Equation 5.3.3 Polymorphic information content (*PIC*), where p_i is the frequency of the ith allele.

Genetic distance between populations

A genetic distance matrix (Appendix 8.9) was calculated based on Nei's standard genetic distance measure (Nei, 1972), using allele data (characters) without size information, and calculated according to Equation 2.3.2 (Nei, 1972) using POGENE (Yeh and Boyle, 1997). Nei's genetic distance measure was used, in preference to other distances, because it was used in Chapter 2 and because it is a very robust distance measure used in population studies.

From this matrix, a dendrogram showing the similarities between populations was constructed using the unweighted pair group method using arithmetic means (UPGMA) method (Sneath and Sokal, 1973) as implemented in POPGENE (Yeh and Boyle, 1997). Bootstrapping analysis was performed on the UPGMA data with 1,000 replicates as implemented in NTSYSpc V2.2 software (Rohlf, 2005).

Mantel test

A geographic distance matrix was constructed (Appendix 8.4), using the 23 accessions used in this analysis where an exact geographical origin was known. A Mantel test was used to correlate the pairwise comparisons in the geographic distance matrix and the genetic distance matrix as described in section 2.3.19 and according to equation 2.3.3. Further Mantel tests were used to correlate the pairwise comparisons in the nuclear genetic distance matrix and the chloroplast genetic distance matrix (Appendix 8.3) and to compare the nuclear genetic matrix and the morphological distance matrix (Appendix 8.6).

Principal components analysis

PCA was performed on the genetic distance matrix (Appendix 8.9) which was based on Nei's genetic distance (Nei, 1972), according to section 3.3.4 and according to equations 3.3.20, 3.3.21 and 3.3.22. Graphs of the eigenvectors and eigenvalues for two dimensions were constructed.

AMOVA analyses

An analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) was carried out with ARLEQUIN 2.0 software (Schneider *et al.* 2000). The level of significance for variance component estimates was calculated by non-parametric permutation procedures using 1,000 permutations. The data were partitioned in several combinations to display among and within population variation of Irish and European *L. perenne* accessions and cultivars, and to assess groupings found in PCA analysis.

Nei's coefficient of differentiation (G_{ST}) was calculated for all populations and for subgroups of accessions (cultivars, all ecotypes, Irish ecotypes, and European ecotypes) according to equation 2.3.10.

F statistics were calculated over all populations, for the groups cultivars, Irish ecotypes and European ecotypes, according to Weir & Cockerham (1984) and equation 5.3.4. Weir's *F* statistics (based on variance values of allele frequencies) were used in preference to standard F statistics (based on allele frequencies) because it has been shown that they are more reliable when sample size for each population is lower than 30 (Berg & Hamrick, 1995).

$$\theta_{ST} = \frac{\sum_i \sum_u \sigma_B^2}{\sum_i \sum_u \sigma_T^2}$$

Equation 5.3.4 θ_{ST}, where $\sigma_T^2 = \sigma_B^2 + \sigma_W^2 + \sigma_I^2$, and σ_T^2 = total variance of allele frequency within a population, σ_B^2 = between subpopulation variance in allele

frequency, σ_W^2 = between individuals within population variance in allele frequency, and σ_I^2 = between gametes within individuals variance in allele frequency.

Population structure

Genetic structure among individuals over the whole data-set was investigated with a model-based clustering approach using the software package STRUCTURE. The basic algorithm in STRUCTURE was described by Pritchard *et al.* (2000b). Extensions to the method were published by Falush *et al.* (2003, 2007). The number of subpopulations (K) was set from 1 to 40. Each run started with 20,000 burn-ins, followed by 20,000 iterations, employing an admixture model. Each run was performed independently ten times. In order to determine the ideal value of K, K was plotted against the mean lnPr(x|K), and the final K was chosen based on the highest values of lnPr(x|K). Proportions of each population assigned to each subpopulation were determined and plotted on a line graph.

Linkage disequilibrium

Linkage disequilibrium between all pairs of loci over all populations was calculated using a likelihood-ratio test according to Equation 5.3.2 (Slatkin & Excoffier, 1996) with 10,000 permutations and according to the statistic r^2 according to equation 5.3.3 (Flint-Garcia *et al.* 2003). Levels of r^2 between loci were visualised using disequilibrium matrices between each pair of loci.

$$S = -2log(L_H^*/L_H)$$

Equation 5.3.5 *S*, likelihood ratio statistic, where L_H^* is the likelihood of the data assuming linkage equilibrium and L_H is the likelihood of the data assuming linkage disequilibrium.

$$r^2 = \frac{(D_{ab})^2}{\pi_A \pi_a \pi_B \pi_b}$$

Equation 5.3.6 r^2, the square of the correlation coefficient between two loci, where π_A, π_a, π_B, and π_b are the allele frequencies at each locus and D_{ab} is the difference between the observed and expected haplotype frequencies (π_{AB}-$\pi_A\pi_B$).

5.4 Results

5.4.1 Data analysis

Heterozygosity and allelic variation

Expected heterozygosity (gene diversity) values varied considerably between accessions and between loci (a full range between 0.04 in Irish ecotype IRL-OP-02007 at locus LpHCA18F11 and 0.91 in ecotypes IRL-OP-02059, IRL-OP-02538 and IV-51-161 Hungary at locus rv1239 and IV-51-16 Hungary at locus LpACT13H2 in the entire collection; Table 5.4.1). When calculated over all loci, gene diversity values ranged from 0.57 in cultivar Cancan, to 0.8 in ecotype IV-51-161 Hungary. Accessions also showed significant deviations from Hardy-Weinberg equilibrium for each locus (Table 5.4.1). The majority of populations at each locus were less heterozygous than would be expected under conditions of Hardy-Weinberg equilibrium. At locus LpHCA18F11, the Irish ecotypes IRL-OP-02059, IRL-OP-02007, IRL-OP-02538, IRL-OP-02274, and IRL-OP-02241, the European ecotypes 3408 Italy, 920 Bulgaria, and IV-51-161 Hungary and the cultivar Cashel did not deviate significantly from Hardy-Weinberg equilibrium. Only Irish ecotypes IRL-OP-02048 and IRL-OP-02128 were more heterozygous than would be expected under Hardy-Weinberg equilibrium. Observed heterozygosity ranged from 0.04 in Irish ecotype IRL-OP-02007 to 0.88 in European ecotype IV-51-161 Hungary. At locus LpACT26D12, the Irish ecotypes IRL-OP-02337, IRL-OP-02048, IRL-OP-02192, IRL-OP-02064, IRL-OP-02128, IRL-OP-02274, IRL-OP-02272 and IRL-OP-02173 and the cultivars Portstewart and S24 did not deviate significantly from Hardy-Weinberg equilibrium. None of the accessions were more heterozygous than would be expected under Hardy-Weinberg equilibrium. Observed heterozygosity ranged from 0.17 in cultivar Aurora to 0.96 in Irish ecotype IRL-OP-02192. At locus rv0264, the Irish ecotypes IRL-OP-02337, IRL-OP-02015, IRL-OP-02230, IRL-OP-02274, IRL-OP-02442 and IRL-OP-02173 and the cultivar Cancan did not deviate significantly from Hardy-Weinberg equilibrium. The Irish ecotypes IRL-OP-02059, IRL-OP-02007, IRL-OP-02272, IRL-OP-02483, and IRL-OP-02018, the European ecotypes 16-7-62-2 Nordic, 3199 Romania Podoloni, IV-51-161 Hungary and the cultivars Aurora, Barlenna and Fennema were more heterozygous than would be expected

under Hardy-Weinberg equilibrium. Observed heterozygosity ranged from 0.09 in Irish ecotype IRL-OP-02258 to 1 in cultivar Aurora. At locus rv0908, Irish ecotype IRL-OP-02011, European ecotype No. 10 Spain, and the cultivars Cashel and Odenwaelder did not deviate significantly from Hardy Weinberg equilibrium. Irish ecotype IRL-OP-02337 was the only accession at this locus that was more heterozygous than would be expected under Hardy-Weinberg equilibrium. Observed heterozygosity ranged from 0 in Irish ecotype IRL-OP-02064 to 0.88 in Irish ecotype IRL-OP-02337. At locus LpACT13H2, only Irish ecotype IRL-OP-02064 did not deviate from Hardy-Weinberg equilibrium, and none of the accessions were more heterozygous than would be expected under Hardy-Weinberg equilibrium. Observed heterozygosity ranged from 0 in Irish ecotype IRL-OP-02018 to 0.73 in European ecotype 3199 Romania Podoloni. At locus rv0449, the Irish ecotypes IRL-OP-02007 and IRL-OP-02538 and the European ecotype IV-51-161 Hungary did not deviate from Hardy-Weinberg equilibrium. The Irish ecotype IRL-OP-02230 and the cultivar Fennema were more heterozygous than would be expected under Hardy-Weinberg equilibrium. Observed heterozygosity ranged from 0.09 in Irish ecotype IRL-OP-02128 to 0.84 in cultivar Fennema. At locus rv1239, the cultivars Aurora, Barlenna and Talbot did not deviate from Hardy-Weinberg equilibrium and only the Irish ecotypes IRL-OP-02538 and IRL-OP-02068 were more heterozygous that expected under Hardy-Weinberg equilibrium. Observed heterozygosity ranged from 0.12 in European ecotype 920 Bulgaria to 0.92 in Irish ecotype IRL-OP-02538. Finally, at locus rv1423, the Irish ecotypes IRL-OP-02078, IRL-OP-02128, IRL-OP-02274, IRL-OP-02419, IRL-OP-02272, IRL-OP-02173, IRL-OP-02483, and IRL-OP-02018 and all the European ecotypes (with the exception of IV-51-161 Hungary) and the cultivars Barlenna, Cancan, Fennema, Portstewart and Shandon did not deviate significantly from Hardy-Weinberg equilibrium. None of the accessions were more heterozygous than expected under Hardy Weinberg equilibrium. Observed heterozygosity ranged from 0.38 in Irish ecotype IRL-OP-02064 to 0.78 in Irish ecotype IRL-OP-02272.

Observed and expected heterozygosities (gene diversity) were calculated for each locus, over all accessions, and for each group of accessions (cultivars, ecotypes, Irish ecotypes, and European ecotypes; Table 5.4.2). All loci, both over all accessions and for each group of accessions were significantly less heterozygous than would be

expected if the accessions were under Hardy-Weinberg equilibrium, with a significance level of $p < 0.001$. Over all loci, across all subpopulations, gene diversity ranged from 0.61 in the European ecotypes at locus rv0908 to 0.90 in cultivars at locus rv0449. At locus LpHCA18F11, gene diversity ranged from 0.69 in Irish ecotypes to 0.83 in European ecotypes. At locus LpACT26D12, they ranged from 0.75 in cultivars to 0.83 in ecotypes. At locus rv0264, gene diversity was 0.86 in European ecotypes, while in all other subpopulations it was 0.87. At locus rv0908, they ranged from 0.61 in European ecotypes to 0.75 in Irish ecotypes and cultivars. At locus LpHCA13H2, they ranged from 0.78 in Irish ecotypes to 0.87 in cultivars. At locus rv0449, they ranged from 0.88 in Irish ecotypes to 0.90 in cultivars. At locus rv1423, they ranged from 0.77 in all ecotypes to 0.82 in cultivars. When calculated over all loci, gene diversity values ranged from 0.80 in European ecotypes to 0.82 in cultivars. The coefficient of differentiation (G_{ST}), was 0.23 over all populations and ranged from 0.18 in European ecotypes to 0.23 in cultivars.

Table 5.4.1 Observed and expected heterozygosity for each population at each locus and over all loci. Significant deviations from Hardy-Weinberg equilibrium are indication in superscript (N/S: not significant, *p<0.05, **p<0.01, ***p<0.001).

Accession number	Group[a]	N[b]	Loci																Overall	
			LpHCA18F11		LpACT26D12		rv0264		rv0908		LpACT13H2		rv449		rv1239		rv1423			
			H_O[c]	H_E[d]	H_O	H_E	H_O	H_E	H_O	H_E	H_O[c]	H_E[d]	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E
IRL-OP-02337	I 1	24	0.35	0.57^{***}	0.76	$0.79^{N/S}$	0.76	$0.75^{N/S}$	0.88	0.74^{*}	0.08	0.66^{***}	0.33	0.80^{***}	0.46	0.77^{***}	0.52	0.74^{***}	0.52	0.73
IRL-OP-02059	I 2	24	0.50	$0.57^{N/S}$	0.67	0.87^{*}	0.92	0.85^{**}	0.57	0.64^{**}	0.67	0.83^{**}	0.73	0.81^{**}	0.65	0.91^{***}	0.74	0.75^{*}	0.68	0.78
IRL-OP-02007	I 3	24	0.04	$0.04^{N/S}$	0.50	0.74^{***}	0.75	0.73^{***}	0.57	0.74^{***}	0.58	0.89^{***}	0.65	$0.75^{N/S}$	0.71	0.90^{**}	0.75	0.78^{*}	0.57	0.70
IRL-OP-02011	I 4	24	0.75	0.81^{***}	0.71	0.82^{***}	0.71	0.81^{**}	0.71	$0.62^{N/S}$	0.67	0.87^{***}	0.50	0.81^{***}	0.67	0.85^{***}	0.71	0.74^{**}	0.68	0.79
IRL-OP-02015	I 5	24	0.65	0.72^{*}	0.54	0.79^{**}	0.75	$0.77^{N/S}$	0.71	0.73^{*}	0.33	0.69^{***}	0.27	0.69^{***}	0.25	0.71^{***}	0.63	0.85^{***}	0.52	0.74
IRL-OP-02048	I 6	21	0.86	$0.77^{N/S}$	0.65	$0.84^{N/S}$	0.24	0.65^{***}	0.37	0.68^{***}	0.00	0.52^{***}	0.33	0.82^{***}	0.33	0.82^{***}	0.42	0.59^{**}	0.40	0.71
IRL-OP-02192	I 7	24	0.21	0.39^{**}	0.96	$0.90^{N/S}$	0.35	0.70^{***}	0.42	0.70^{***}	0.13	0.33^{***}	0.30	0.71^{***}	0.42	0.85^{***}	0.57	0.77^{***}	0.42	0.67
IRL-OP-02064	I 10	12	0.67	0.72^{*}	0.75	$0.87^{N/S}$	0.36	0.67^{*}	0.00	0.49^{***}	0.08	$0.23^{N/S}$	0.50	0.66^{***}	0.17	0.63^{***}	0.38	0.84^{***}	0.36	0.64
IRL-OP-02078	I 11	24	0.25	0.55^{***}	0.58	0.68^{***}	0.67	0.82^{*}	0.38	0.56^{**}	0.08	0.46^{***}	0.17	0.68^{***}	0.32	0.88^{***}	0.67	$0.70^{N/S}$	0.39	0.67
IRL-OP-02230	I 12	24	0.25	0.77^{***}	0.54	0.78^{**}	0.79	$0.78^{N/S}$	0.71	0.78^{***}	0.71	0.91^{***}	0.75	0.73^{**}	0.65	0.88^{**}	0.39	0.63^{**}	0.60	0.78
IRL-OP-02128	I 13	24	0.61	0.53^{**}	0.63	$0.72^{N/S}$	0.71	0.80*	0.54	0.56^{*}	0.30	0.67^{***}	0.09	0.49^{***}	0.18	0.73^{***}	0.75	$0.65^{N/S}$	0.48	0.64
IRL-OP-02538	I 14	24	0.52	$0.59^{N/S}$	0.58	0.81^{**}	0.71	0.79^{**}	0.46	0.62^{***}	0.42	0.85^{***}	0.71	$0.65^{N/S}$	0.92	0.91^{*}	0.57	0.85^{**}	0.61	0.76
IRL-OP-02274	I 15	24	0.50	$0.57^{N/S}$	0.58	$0.76^{N/S}$	0.74	$0.80^{N/S}$	0.38	0.56^{**}	0.33	0.57^{***}	0.75	0.86^{***}	0.13	0.71^{***}	0.67	$0.57^{N/S}$	0.51	0.68
IRL-OP-02442	I 17	24	0.50	0.61^{*}	0.46	0.75^{**}	1.00	$0.81^{N/S}$	0.74	0.78^{**}	0.04	0.54^{***}	0.14	0.54^{***}	0.50	0.82^{***}	0.59	0.82^{**}	0.50	0.71
IRL-OP-	I 19	24	0.21	0.46^{***}	0.75	0.79^{**}	0.83	0.89^{**}	0.46	0.73^{***}	0.00	0.64^{***}	0.29	0.65^{***}	0.79	0.76^{***}	0.38	0.55^{**}	0.46	0.68

02068																				
IRL-OP-02241	I 20	24	0.46	0.50$^{N/S}$	0.54	0.72**	0.71	0.84**	0.67	0.68*	0.58	0.86***	0.74	0.75*	0.88	0.89**	0.48	0.80***	0.63	0.76
IRL-OP-02419	I 21	22	0.32	0.67***	0.59	0.83***	0.60	0.84***	0.36	0.74***	0.14	0.52***	0.41	0.85***	0.35	0.75***	0.68	0.69$^{N/S}$	0.43	0.74
IRL-OP-02258	I 22	24	0.25	0.66***	0.63	0.84**	0.09	0.82***	0.58	0.75***	0.29	0.50***	0.21	0.72***	0.54	0.88***	0.59	0.76***	0.40	0.74
IRL-OP-02272	I 23	18	0.33	0.63***	0.33	0.38$^{N/S}$	0.83	0.80**	0.33	0.61***	0.00	0.74***	0.33	0.85***	0.27	0.74***	0.78	0.72$^{N/S}$	0.40	0.68
IRL-OP-02173	I 27	24	0.75	0.81***	0.67	0.72$^{N/S}$	0.63	0.73$^{N/S}$	0.46	0.58*	0.17	0.53***	0.36	0.75***	0.17	0.58***	0.54	0.66$^{N/S}$	0.47	0.67
IRL-OP-02483	I 28	24	0.63	0.78*	0.50	0.82***	0.88	0.80*	0.71	0.74**	0.04	0.56***	0.23	0.76***	0.61	0.80***	0.42	0.51$^{N/S}$	0.50	0.72
IRL-OP-02018	I 30	18	0.56	0.84***	0.22	0.60***	0.94	0.76***	0.18	0.48***	0.00	0.76***	0.28	0.79***	0.33	0.64**	0.72	0.73$^{N/S}$	0.40	0.70
16-7-62-2 Nordic	Δ5	24	0.58	0.67***	0.43	0.67***	0.87	0.77**	0.42	0.71***	0.46	0.89***	0.65	0.76**	0.71	0.87***	0.67	0.75$^{N/S}$	0.60	0.76
No 10 Spain	■15	22	0.62	0.73**	0.29	0.72***	0.75	0.82***	0.23	0.21$^{N/S}$	0.00	0.57***	0.29	0.82***	0.36	0.65**	0.77	0.73$^{N/S}$	0.41	0.65
3408 Italy	■16	24	0.54	0.54$^{N/S}$	0.70	0.86*	0.46	0.85***	0.30	0.70***	0.04	0.74***	0.22	0.59***	0.17	0.47***	0.71	0.66$^{N/S}$	0.39	0.68
3013 Romania	■18	24	0.46	0.77***	0.29	0.80***	0.54	0.76**	0.25	0.44*	0.04	0.59***	0.50	0.79***	0.21	0.78***	0.67	0.57$^{N/S}$	0.37	0.69
3199 Romania Podoloni	■19	24	0.17	0.70***	0.46	0.73***	0.87	0.81***	0.63	0.64*	0.73	0.85***	0.58	0.81***	0.58	0.89***	0.67	0.73$^{N/S}$	0.59	0.77
920 Bulgaria	■20	24	0.83	0.83$^{N/S}$	0.62	0.81*	0.58	0.88**	0.27	0.36**	0.04	0.65***	0.77	0.80***	0.12	0.68***	0.62	0.57$^{N/S}$	0.48	0.70
IV-51-161 Hungary	●32	24	0.88	0.82$^{N/S}$	0.42	0.68***	0.88	0.81***	0.46	0.74***	0.71	0.91**	0.67	0.79$^{N/S}$	0.78	0.91**	0.67	0.78*	0.68	0.80
cv. Aurora	V 1	23	0.52	0.58***	0.17	0.59***	1.00	0.79***	0.22	0.47**	0.65	0.83***	0.63	0.78**	0.78	0.90$^{N/S}$	0.61	0.85***	0.57	0.72
cv. Barlenna	V 2	24	0.29	0.49***	0.46	0.55**	0.83	0.75***	0.71	0.65**	0.43	0.83***	0.52	0.80***	0.77	0.89$^{N/S}$	0.61	0.67$^{N/S}$	0.58	0.70
cv. Cancan	V 3	24	0.46	0.70**	0.63	0.70***	0.67	0.67$^{N/S}$	0.13	0.33***	0.13	0.37***	0.21	0.64***	0.17	0.33**	0.71	0.81$^{N/S}$	0.39	0.57
cv. Cashel	V 4	24	0.79	0.81$^{N/S}$	0.42	0.76***	0.63	0.88***	0.67	0.57$^{N/S}$	0.00	0.52***	0.38	0.77***	0.41	0.63***	0.52	0.58**	0.48	0.69
cv.	V 5	24	0.60	0.79***	0.56	0.74***	0.84	0.81***	0.68	0.73***	0.36	0.74***	0.84	0.78***	0.64	0.89***	0.71	0.76$^{N/S}$	0.65	0.78

Fennema																				
cv. Odenwaelder	V 10	24	0.42	0.77^{***}	0.46	0.67^{***}	0.77	0.86^{*}	0.25	$0.22^{N/S}$	0.52	0.82^{***}	0.27	0.84^{***}	0.47	0.76^{**}	0.57	0.85^{*}	0.47	0.72
cv. Portstewart	V 11	24	0.50	0.66^{*}	0.46	$0.52^{N/S}$	0.83	0.89^{*}	0.79	0.85^{***}	0.17	0.51^{***}	0.42	0.77^{***}	0.39	0.70^{***}	0.75	$0.72^{N/S}$	0.54	0.70
cv. Premo	V 12	24	0.29	0.58^{***}	0.54	0.76^{**}	0.70	0.85^{**}	0.42	0.59^{**}	0.57	0.87^{***}	0.43	0.88^{***}	0.71	0.85^{***}	0.52	0.80^{***}	0.52	0.77
cv. S24	V 13	20	0.30	0.57^{**}	0.40	$0.50^{N/S}$	0.50	0.82^{***}	0.20	0.55^{***}	0.40	0.87^{***}	0.70	0.72^{**}	0.68	0.77^{**}	0.50	0.61^{*}	0.46	0.68
cv. Shandon	V 15	24	0.35	0.69^{***}	0.63	0.73^{*}	0.78	0.81^{***}	0.67	0.75^{**}	0.21	0.49^{***}	0.30	0.68^{***}	0.25	0.78^{***}	0.63	$0.70^{N/S}$	0.48	0.70
cv. Talbot	V 16	24	0.43	0.69^{*}	0.38	0.83^{***}	0.61	0.80^{**}	0.30	0.46^{**}	0.61	0.88^{***}	0.28	0.89^{***}	0.86	$0.89^{N/S}$	0.57	0.84^{***}	0.50	0.78

[a]Group: I = Irish ecotype, △= Northern Europe, ■ = Southern Europe, ● = Eastern Europe, V = *Lolium perenne* variety; [b]N = number of individuals, [c]H_O = observed heterozygosity, [d]H_E = expected heterozygosity.

Table 5.4.2 Observed and expected heterozygosity at each locus and over all loci and G_{ST} for each group of populations (overall, cultivars, ecotypes, Irish ecotypes, and European ecotypes). Significant deviations from Hardy-Weinberg equilibrium are indication in superscript (N/S: not significant, *p<0.05, **p<0.01, ***p<0.001).

		Locus																		
		LpHCA18F11		LpACT26D12		rv0264		rv0908		LpACT13H2		rv449		rv1239		rv1423		Overall		G_{ST}
G[a]	N[b]	H_O[c]	H_E[d]	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	
Overall	928	0.48	0.74^{***}	0.54	0.81^{***}	0.71	0.87^{***}	0.48	0.74^{***}	0.30	0.83^{***}	0.45	0.89^{***}	0.49	0.88^{***}	0.61	0.79^{***}	0.51	0.82	0.23
Cultivars	260	0.45	0.73^{***}	0.47	0.75^{***}	0.75	0.87^{***}	0.46	0.75^{***}	0.36	0.87^{***}	0.46	0.90^{***}	0.56	0.87^{***}	0.61	0.82^{***}	0.51	0.82	0.23
Ecotypes	668	0.49	0.74^{***}	0.57	0.83^{***}	0.69	0.87^{***}	0.49	0.74^{***}	0.27	0.81^{***}	0.45	0.89^{***}	0.47	0.88^{***}	0.61	0.77^{***}	0.50	0.81	0.22
Irish ecotypes	500	0.45	0.69^{***}	0.60	0.82^{***}	0.69	0.87^{***}	0.53	0.75^{***}	0.27	0.78^{***}	0.42	0.88^{***}	0.49	0.89^{***}	0.61	0.79^{***}	0.50	0.81	0.21
European ecotypes	168	0.58	0.83^{***}	0.46	0.82^{***}	0.70	0.86^{***}	0.37	0.61^{***}	0.30	0.83^{***}	0.45	0.89^{***}	0.49	0.83^{***}	0.61	0.82^{***}	0.50	0.80	0.18

[a]G = Group; [b]N = number of individuals, [c]H_O = observed heterozygosity, [d]H_E = expected heterozygosity.

Numbers of alleles, size ranges, and PIC values were calculated for each locus over all accessions, for cultivars, ecotypes overall, Irish ecotypes and European ecotypes (Table 5.4.3). Loci rv1239 and rv1423 had the highest number of alleles (26), while locus LpACT26D12 had the lowest number of alleles (16). The widest range of allele sizes was seen in locus LpACT26D12 (61bp) and the shortest size range seen in locus rv0908 (25bp). PIC contents over all accessions ranged from 0.70 in locus rv0908 to 0.88 in loci rv0449 and rv1239. In cultivars, the highest numbers of alleles were seen in loci rv1239 and rv1423 (25) and the lowest number of alleles were seen in loci LpHCA18F11 and rv0264 (15). As in all accessions, the widest size range of alleles was seen in locus LpACT26D12 (47bp) and the lowest in locus rv0908 (24bp). PIC values ranged from 0.71 in locus LpHCA18F11 to 0.89 in locus rv0449. Similarly to all accessions, in ecotypes the lowest numbers of alleles were seen in locus rv0264 (16) and the highest in loci rv1239 and rv1423 (26). Similar minimum and maximums were found for size ranges in ecotypes as over all accessions. PIC values in ecotypes ranged from 0.72 at loci LpHCA18F11 and rv0908 to 0.88 at loci rv0449 and rv1239. Irish and European ecotypes when analysed separately were very similar to ecotypes as a whole.

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Table 5.4.3 Number of alleles, size ranges (in base pairs) and polymorphic information content at each locus for each group of populations (overall, cultivars, ecotypes, Irish ecotypes, and European ecotypes).

G[a]	N[b]	LpHCA18F11			LpACT26D12			rv0264			rv0908			LpACT13H2			rv449			rv1239			rv1423	
		NA[c]	Size (bp)	PIC[d]	NA	Size (bp)	PIC	NA	Size (bp)	PIC	NA	Size (bp)	PIC	NA	Size (bp)	PIC	NA	Size (bp)	PIC	NA	Size (bp)	PIC	NA[b]	Size (bp)	PIC
O[e]	928	25	228-261	0.74	24	87-148	0.81	16	131-177	0.87	20	193-218	0.74	20	117-150	0.83	21	106-149	0.89	26	110-145	0.88	26	117-152	0.71
C[f]	260	15	228-259	0.73	16	101-148	0.75	15	131-177	0.87	18	193-217	0.75	20	117-150	0.87	21	106-149	0.90	25	110-145	0.87	25	118-152	0.82
E[g]	668	25	231-261	0.74	24	87-148	0.83	16	131-177	0.87	19	193-218	0.74	20	117-150	0.81	21	106-149	0.89	26	110-145	0.88	26	117-152	0.77
Ie[h]	500	23	231-261	0.69	23	87-148	0.82	16	131-177	0.87	19	193-218	0.75	20	117-150	0.78	21	106-149	0.88	26	110-145	0.89	26	117-152	0.78
Ee[i]	168	18	232-261	0.83	17	87-116	0.81	15	132-177	0.86	10	206-218	0.61	16	117-150	0.85	19	106-149	0.90	22	110-145	0.84	18	117-152	0.72

[a]G = Group; [b]N = number of individuals, [c]NA = number of alleles, [d]PIC = polymorphic information content, [e]O = overall, [f]C = cultivars, [g]E = ecotypes, [h]Ie = Irish ecotypes, [i]Ee = European ecotypes.

Linkage disequilibrium

Levels of linkage disequilibrium were calculated for all sites at each pair of loci (Figure 5.4.1). Levels of r^2 ranged from 0 to 0.8. The majority of pairs of sites were in linkage equilibrium. Levels of linkage disequilibrium were calculated for each pair of loci overall using an exact test (Table 5.4.4). Linkage disequilibrium was only found at the pairs of loci which were shown in previous studies to be on the same linkage group.

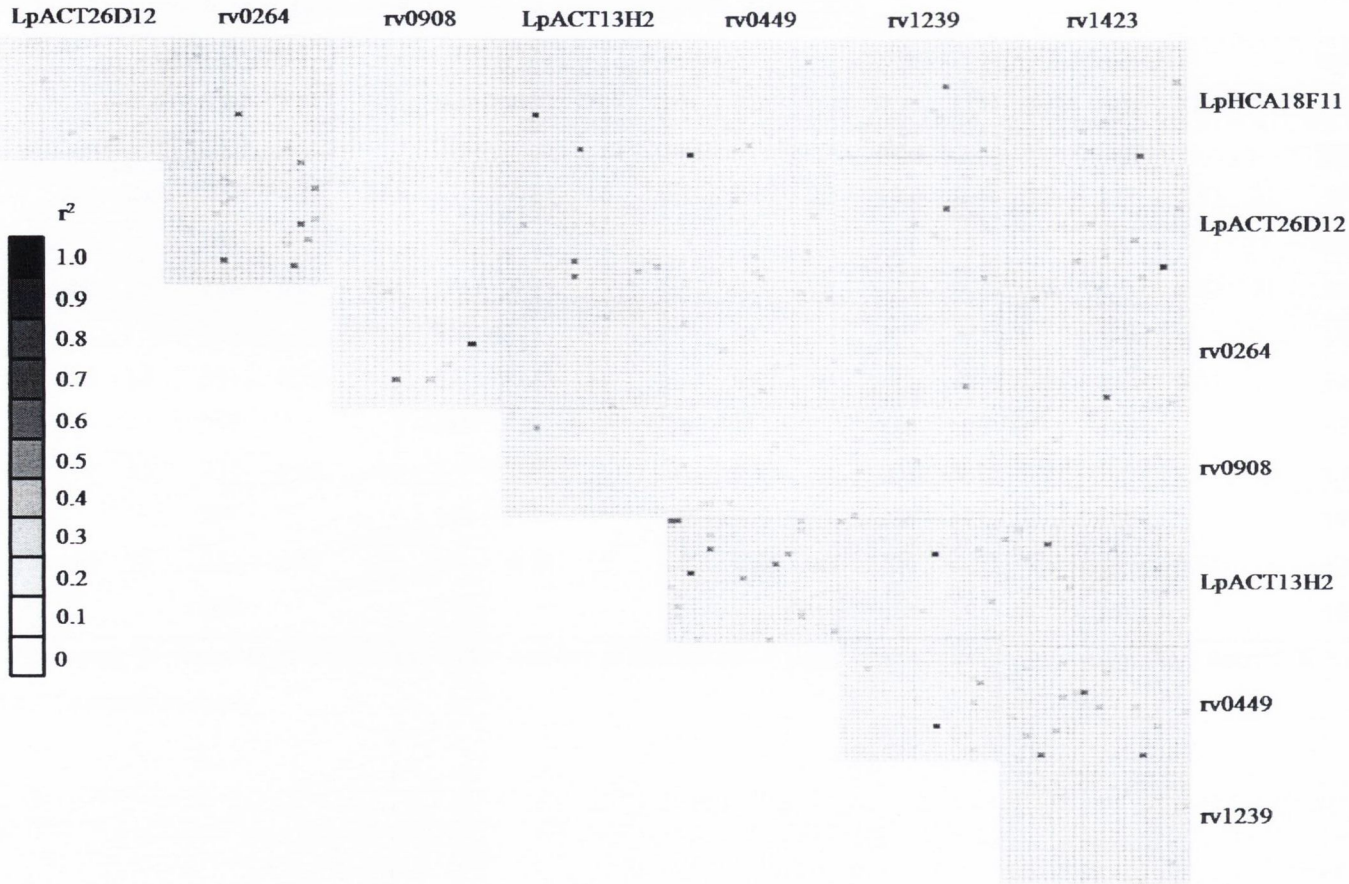

Figure 5.4.1 Linkage disequilibrium (r^2) between each pair of alleles at each locus, with levels of r^2 indicated by different shading, with lighter shading indicating lower r^2.and darker shading indicating higher r^2.

Table 5.4.4 Significant linkage disequilibrium between loci ($p < 0.05$).

	LpHCA18F11	**LpACT26D12**	**rv0264**	**rv0908**	**LpACT13H2**	**rv449**	**rv1239**	**rv1423**
LpHCA18F11	N/A[a]							
LpACT26D12	N/S[b]	N/A						
rv0264	$p < 0.05$	N/S	N/A					
rv0908	$p < 0.05$	N/S	$p < 0.05$	N/A				
LpACT13H2	N/S	$p < 0.05$	N/S	N/S	N/A			
rv449	N/S	$p < 0.05$	N/S	N/S	$p < 0.05$	N/A		
rv1239	N/S	N/S	N/S	N/S	N/S	N/S	N/A	
rv1423	N/S	$p < 0.05$	N/S	N/S	$p < 0.05$	$p < 0.05$	N/S	N/A

[a]N/A: not applicable, [b]N/S: not significant

Genetic distance between populations

The genetic distance matrix between the populations is shown in Appendix 8.9. The UPGMA dendrogram constructed from this matrix is shown in Figure 5.4.2. The dendrogram could be divided into two major groups I and 2. Group I had weak bootstrap support of 41%, and contained all the Irish ecotypes, with the exception of IRL-OP-02007 (I3), IRL-OP-02011 (I4), IRL-OP-02059 (I2), IRL-OP-02230 (I12), IRL-OP-02241 (I20) and IRL-OP-02538 (I14). It also contained the European ecotypes No 10 Spain (■15), 3408 Italy (■16), 3013 Romania (■18) and 920 Bulgaria (■20) and the commercial varieties Cancan (V3), Cashel (V4), Portstewart (V11), and Shandon (V15). Group I could be divided into three subgroups I(a), I(b) and I(c). Group I(a) included all four cultivars in Group I and which had been bred recently by Irish and Northern Irish breeding groups. Cashel and Shandon clustered together with a bootstrap value of 26%. Group I(b) contained all the European ecotypes found in Group I and several other Irish ecotypes. Group I(c) contained only Irish ecotypes. Ecotypes IRL-OP-02048 (I6) and IRL-OP-02192 (I7), both from Cork, clustered together with bootstrap support of 26%. Group II had weak bootstrap support of 56% and could be divided into two subgroups 2(a) and 2(b). Group 2 contained the Irish ecotypes IRL-OP-02007 (I3), IRL-OP-02011 (I4), IRL-OP-02059 (I2), IRL-OP-02230 (I12), IRL-OP-02241 (I20) and IRL-OP-02538 (I14), the European ecotypes 16-7-62-2 Nordic (Δ5), 3199 Romania Podoloni (■19) and IV-51-161 Hungary (●32) and the cultivars Aurora (V1), Barlenna (V2), Fennema (V5), Odenwaelder (V10), Premo (V12), S24 (V13) and Talbot (V16). Within subgroup 2(a) the European ecotypes 3199 Romania Podoloni (■19) and IV-51-161 Hungary (●32) clustered together with bootstrap support of 48%. Group 2(b) only contained cultivars which had been developed in the earlier European breeding programmes and individual clusters within this group had moderate to good bootstrap support.

When this dendrogram was compared with those for chloroplast and morphological data for the same populations, by overlaying groups found in the chloroplast and morphology dendrogram onto the nuclear dendrogram, similar patterns were not seen.

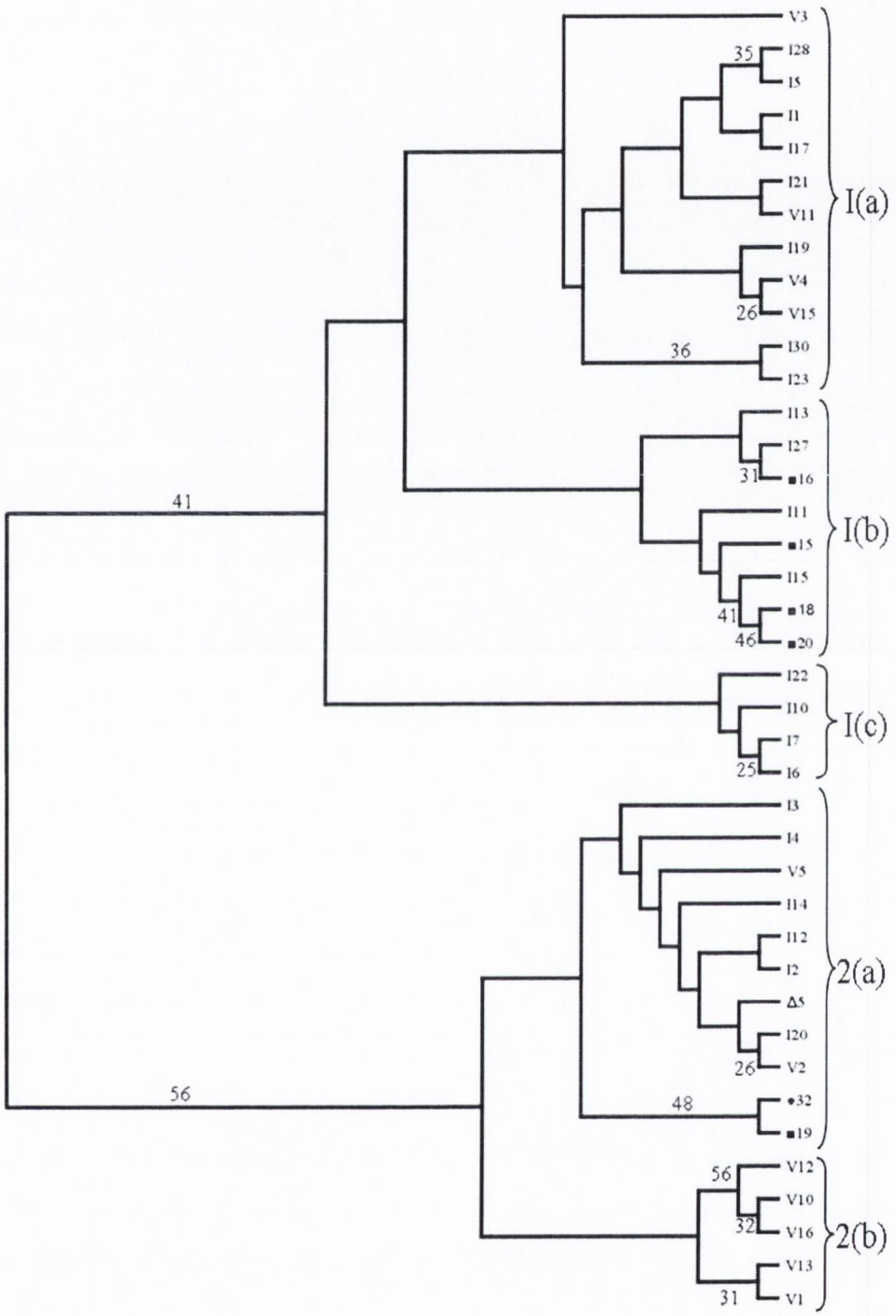

Figure 5.4.2 Unrooted dendrogram showing similarities between populations, constructed using UPGMA (Sneath & Sokal, 1973) as implemented in POPGENE

(Yeh & Boyle, 1997), based on Nei's genetic distance measures (Nei, 1972). Subgroups are indicated by parentheses. Numbers on the branches are percentage bootstrap values generated in NTSYSpc V2.2 (Rohlf, 2005). Different symbols represent a geographical group: △ = Northern Europe ■ = Southern Europe ● = Eastern Europe, I = Ireland, V = Cultivar.

Principal components analysis

Eigenvectors and eigenvalues were calculated for the genetic distance matrix. The percentage accounted for by each eigenvalue was determined (Table 5.4.5) and the first dimension was plotted against the second dimension (Figure 5.4.3). All the variation in the dataset was explained by three eigenvalues. The first eigenvalue explained 57.60% of the variation, the second explained 31.49% and the third eigenvalue explained the final 10.91% of the variation. When the eigenvectors were plotted for the first two dimensions (Figure 5.4.3), several patterns could be seen in the diagram. These patterns were broadly congruent with the groupings found in the UPGMA analysis. Firstly a split was seen between two groups of accessions (indication in the diagram by a dashed line). On the left hand side of the diagram were seven of the eleven cultivars (these were part of historic breeding material from Europe), as well as three European ecotypes and six Irish ecotypes, two of which were collected in Cork (IRL-OP-02007, I3; and IRL-OP-02011, I4) and grouped closely together. On the right hand side of the diagram were most of the Irish ecotypes, four cultivars (which are more recent cultivars from Irish and Northern Irish breeding programs), and four European ecotypes. Two of the European ecotypes (3013 Romania, ■18 and 920 Bulgaria, ■20) were relatively close geographically and grouped together in the diagram. Within the Irish ecotypes on this side of the diagram, there were several geographically close ecotypes which grouped together in the diagram. For example, IRL-OP-02128 (I13) and IRL-OP-02274 (I15) were collected in Kerry and Limerick, respectively. IRL-OP-02015 (I5), IRL-OP-02048 (I6) and IRL-OP-02192 (I7) were all collected in Cork. IRL-OP-02483 (I28) and IRL-OP-02018 (I30) were collected in Wexford and Waterford respectively. Other groupings could be visualised on the diagram and were indicated by dashed circles. These groupings did not appear to have any underlying pattern, nonetheless, they were further tested by AMOVA analysis.

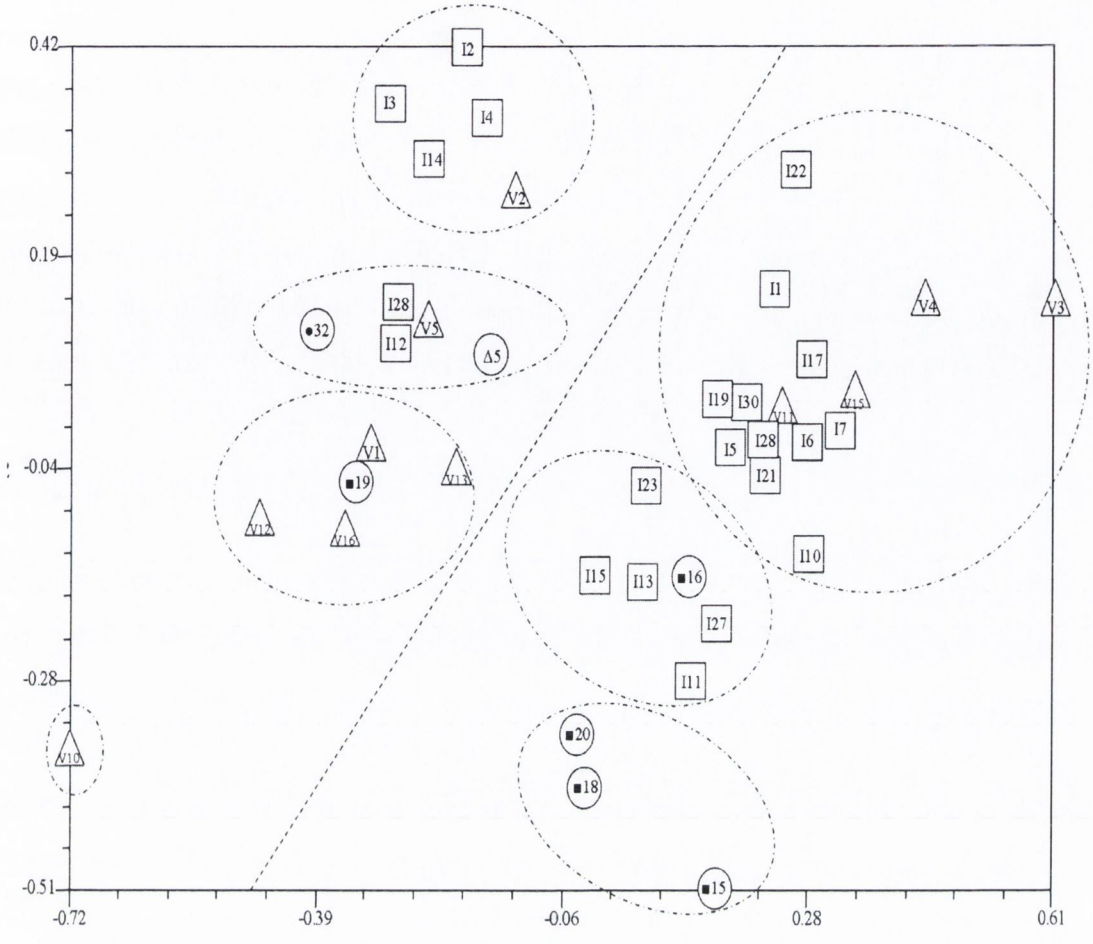

Figure 5.4.3 Principal components analysis diagram in two dimensions for nuclear SSR data. X axis: Dimension 1, Y axis: Dimension 2. □: Irish ecotype, ○: European ecotype, Δ: Cultivar, Numbers of the populations are given in Appendix 8.1. Dimension 1 explained 57.60% of the variation and dimension 2 explained 31.49% of the variation. Ellipses represent groups to be analysed further via PCA.

Table 5.4.5 Eigenvalues and percentage of the variation in the nuclear SSR dataset explained by each axis.

Axis	Eigenvalue	Percentage variance explained	Cumulative percentage variance explained
1	3.13	57.60	57.60
2	1.71	31.49	89.09
3	0.86	10.91	100

Mantel test

An extremely weak non-significant correlation between geographic distance and genetic distance was found for 23 populations ($r = -0.093$, $p = 0.224$). An extremely weak correlation between nuclear genetic distance and chloroplast genetic distance was found for all 40 populations ($r = 0.020$, $p = 0.626$). Another extremely weak non-significant correlation between nuclear genetic distance and Euclidean distance for morphology data was found for all 40 populations ($r = 0.084$, $p = 0.876$).

AMOVA analysis

AMOVA analysis was carried out on ten different subgroups of accessions (Table 5.4.6) to test for differences in genetic structure between and within Irish and European ecotypes and commercial varieties and to test the genetic structure of groups of populations observed in the PCA (Figure 5.4.3). For all subgroups tested within population variation accounted for most variation. For example, for all ecotypes, within population variation accounted for 90.35% of the total variation, with a significance level of $p<0.001$. Within population variation was higher for ecotypes (90.35% over all ecotypes, 91.1% in Irish ecotypes and 91.44% in European ecotypes) than for commercial varieties (87.57%). In any case, levels of variation in each subgroup were similar to each other. When groups of accessions were compared to each other, levels of within population variation were very similar to each other (89.50%, 89.07% and 89.17% respectively). Among group variation was highest between Irish and European ecotypes (2.31%) and lowest between commercial varieties and all ecotypes (0.11%). When the two groups observed in the PCA analysis (Figure 5.4.3, split indicated by a dashed line) were tested, most variation was again seen within populations (88.14%). When historic breeding material was tested against newer commercial varieties from Ireland and Northern Ireland, much more variation was seen between groups (11.6%) than in the other partitions, but within population variation was still significant and high (82.05%). When the seven groups observed in the PCA analysis (Figure 5.4.3, indicated by ellipses) were tested against each other, 4.36% of the variation was between groups, and 88.84% was within populations and the remainder (6.80%) was among populations within groups.

Table 5.4.6 Analysis of molecular variance (AMOVA) for Irish and European *Lolium perenne* accessions, and commercial *L. perenne* varieties and for subgroups determined in other analyses.

Source of Variation	d.f.[a]	SSD[b]	Variance component	Variance (%)	P[c]
Commercial varieties					
Among populations	10	184.23	0.34	12.43	***
Within populations	509	1216.63	2.39	87.57	***
All ecotypes					
Among populations	28	407.06	0.26	9.65	***
Within populations	1307	3210.16	2.46	90.35	***
Irish ecotypes					
Among populations	21	279.61	0.24	8.90	***
Within populations	978	2395.55	2.45	91.10	***
European ecotypes					
Among populations	6	81.59	0.23	8.56	***
Within populations	329	814.6	2.48	91.44	***
Commercial varieties vs. ecotypes					
Among groups (Commercial varieties vs. ecotypes)	1	18.00	0.01	0.11	***
Among populations/within groups	38	591.29	0.28	10.39	***
Within populations	1816	4426.79	2.44	89.50	N/S
Irish ecotypes vs. European ecotypes					
Among groups (Irish ecotypes vs. European ecotypes)	1	45.86	0.06	2.31	***
Among populations/within groups	27	361.20	0.24	8.61	***
Within populations	1307	3210.16	2.46	89.07	***
Irish ecotypes vs. European ecotypes vs. commercial varieties					
Among groups (Irish ecotypes vs. European ecotypes vs. commercial varieties)	2	63.85	0.03	1.11	***
Among populations/within groups	37	545.43	0.27	9.71	***
Within populations	1816	4426.79	2.44	89.17	**
Groups determined from PCA/UPGMA[d]					
Among groups	1	91.85	0.09	3.14	***
Among populations/within groups	38	517.43	0.24	8.72	***
Within populations	1816	4426.79	2.44	88.14	***
Groups determined from PCA/UPGMA[e]					
Among groups	1	80.62	0.33	11.6	***
Among populations/within groups	7	76.12	0.18	6.34	***
Within populations	413	972.28	2.35	82.05	**
Groups determined from PCA/UPGMA[f]					
Among groups	6	244.06	0.12	4.36	***
Among populations/within groups	33	365.23	0.19	6.80	***
Within populations	1816	4426.79	2.44	88.84	***

[a]d.f.: degrees of freedom, [b]SSD: Sum of squared differences, [c]p: ** indicates significance value p = <0.01, *** indicates significance value p = <0.001, N/S = not significant; [d]All accessions on left hand side of PCA Figure 5.4.3 *vs.* all other accessions, indicated by a dashed line; [e]Cultivars Cancan (V3), Cashel (V4), Portstewart (V11), and Shandon (V15) *vs.* all other cultivars; [f]Accessions in groups indicated by ellipses in Figure 5.4.3 against each other

F-statistics

Weir and Cockerham (1984) *F* statistics were calculated for each locus and over all loci (Table 5.4.7), to test the level of variation within and between Irish and European ecotypes and cultivars, and similarly for the three separate groupings on the PCA diagram. For the Irish ecotypes *vs.* European ecotypes *vs.* cultivars, F_{IT}, which is the proportion of variation among all individuals, ranged from 0.19 at locus rv0264 to 0.64 at locus LpHCA13H2, and was 0.39 over all loci. F_{ST}, which is the proportion of variation among all populations, ranged from 0.07 at locus rv1423 to 0.16 at loci rv0908 and LpHCA13H2, and was 0.11 over all loci. F_{PT}, which is the proportion of variation among groups ranged from 0 at four loci to 0.03 at locus rv0908, and over all loci was 0.01. F_{IS}, which is the proportion of variation between individuals among populations, ranged from 0.14 at locus rv0264 to 0.58 at locus LpHCA13H2 and over all loci was 0.31. F_{SP}, which is the proportion of variation between populations among groups ranged from 0.06 at loci LpACT26D12 and rv0264 to 0.15 at locus LpHCA13H2, and over all loci was 0.10. F_{IP}, which is the proportion of variation between individuals among groups, ranged from 0.19 at locus rv0264 to 0.64 at locus LpHCA13H2, and over all loci was 0.38. For the other tests, results were very similar to those seen in the AMOVA analysis.

Table 5.4.7 *F*-statistics for a three-level sampling hierarchy (with Irish ecotypes, European ecotypes and cultivars as groups) at each locus and over all loci.

	Irish and European ecotypes, cultivars						Two groups seen on PCA and dendrogram						Historic *vs.* new breeding material						Seven minor groups on PCA					
	F_{IT}[a]	F_{ST}	F_{PT}	F_{IS}	F_{SP}	F_{IP}	F_{IT}[a]	F_{ST}	F_{PT}	F_{IS}	F_{SP}	F_{IP}	F_{IT}[a]	F_{ST}	F_{PT}	F_{IS}	F_{SP}	F_{IP}	F_{IT}[a]	F_{ST}	F_{PT}	F_{IS}	F_{SP}	F_{IP}
LpHCA18F11	0.36	0.11	0.02	0.28	0.09	0.35	0.36	0.10	0	0.28	0.10	0.36	0.39	0.07	0	0.34	0.07	0.38	0.36	0.11	0.02	0.28	0.09	0.34
LpACT26D12	0.35	0.08	0.02	0.29	0.06	0.33	0.34	0.07	0.01	0.29	0.06	0.34	0.38	0.09	0	0.33	0.09	0.39	0.34	0.07	0.02	0.29	0.05	0.33
rv0264	0.19	0.06	0	0.14	0.06	0.19	0.19	0.06	0	0.14	0.06	0.19	0.15	0.05	0.01	0.10	0.05	0.15	0.19	0.06	0	0.14	0.06	0.19
rv0908	0.36	0.16	0.03	0.24	0.14	0.35	0.35	0.15	0	0.24	0.16	0.36	0.42	0.28	0.07	0.20	0.22	0.37	0.37	0.18	0.14	0.24	0.05	0.27
LpHCA13H2	0.64	0.16	0.01	0.58	0.15	0.64	0.67	0.22	0.16	0.58	0.07	0.61	0.60	0.22	0.07	0.49	0.16	0.57	0.65	0.17	0.11	0.58	0.07	0.61
rv0449	0.50	0.13	0	0.42	0.14	0.50	0.52	0.18	0.10	0.42	0.09	0.47	0.51	0.14	0.06	0.43	0.09	0.48	0.50	0.15	0.07	0.42	0.08	0.47
rv1239	0.44	0.09	0	0.39	0.09	0.44	0.45	0.10	0.03	0.39	0.07	0.43	0.38	0.12	0.03	0.29	0.09	0.36	0.44	0.09	0.01	0.39	0.08	0.44
rv1423	0.22	0.07	0	0.16	0.07	0.22	0.22	0.07	0	0.16	0.07	0.22	0.26	0.08	0	0.20	0.08	0.26	0.22	0.07	0	0.16	0.07	0.22
Over all loci	0.39	0.11	0.01	0.31	0.10	0.38	0.36	0.10	0	0.28	0.10	0.36	0.39	0.13	0.03	0.30	0.10	0.37	0.36	0.11	0.02	0.28	0.09	0.34

[a] $(1-F_{IT}) = (1- F_{IS})(1- F_{SP})(1- F_{PT}) = (1- F_{IS})(1-F_{ST}) = (1-F_{IP})(1- F_{PT})$

Structure

Genetic structure among individuals over the whole data-set was investigated. While number of assumed populations (K) was set from one to 40. Each run was repeated 10 times to ensure the STRUCTURE was consistent across runs and values of K. When K was plotted against the lnPr(x|K) values, it could be seen that the lnPr(x|K) values increased consistently until K=18 (Figure 5.4.4). After this point, levels of lnPr(x|K) rose and fell intermittently. 18 was chosen as the number of K because it was the highest reliable lnPr(x|K), and because the structuring results achieved at this value of K made biological sense (as described by Heuertz *et al.* 2004). The alpha level at this K was 0.042. The proportion of individuals from each of the 40 accessions assigned to the 18 clusters is given in Table 5.4.8 and visually in Figure 5.4.5. In cultivars, the majority of individuals were assigned to clusters three, five, seven, ten, twelve, fifteen and eighteen. In Irish ecotypes, the majority of individuals were assigned to clusters one, two, five, seven, eight and seventeen. The majority of individuals from European ecotypes were assigned to clusters one, three, six and thirteen. In the visualisation of the eighteen clusters (Figure 5.4.5), patterns in each cluster were found in the UPGMA dendrogram (Figure 5.4.2). For example, cluster one was prominent in three of six European ecotypes (■15, ■18, and ■20), I15 and I11 which all clustered together in subgroup I(b) on the UPGMA dendrogram. Similar patterns were seen in cluster three. Cluster two was prominent in I6, I7, I10 and I22, which corresponds to cluster I(c) on the UPGMA dendrogram. The patterns for the remaining clusters also agreed with the clustering on the UPGMA dendrogram.

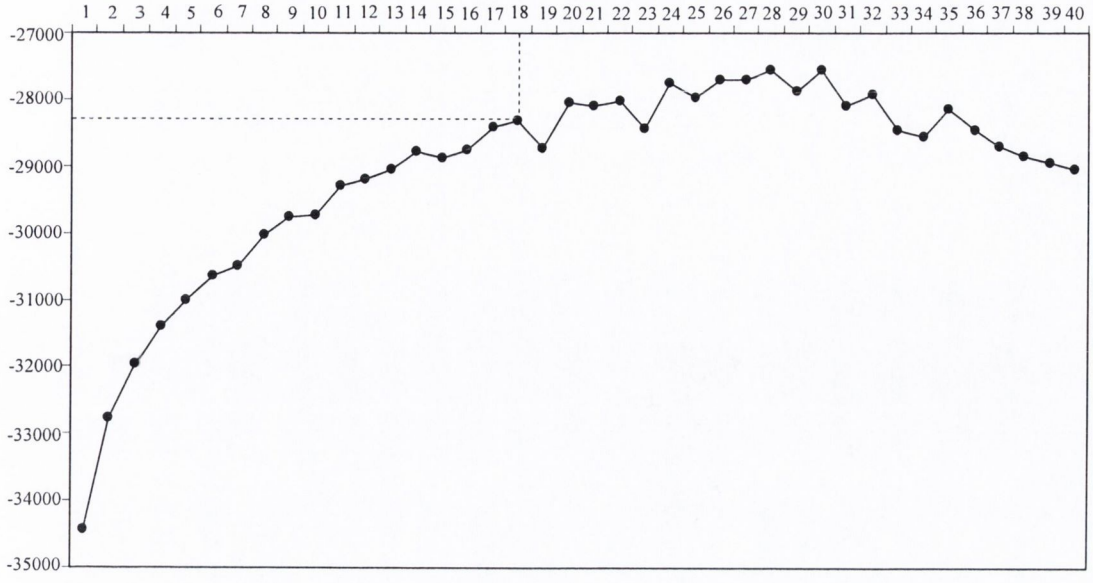

Figure 5.4.4 K (number of subpopulations, x-axis) *versus* estimated lnPr(x|K) values. Dashed line indicates the final level of K chosen.

Table 5.4.8 Proportion of individuals in each population that were assigned to each cluster following STRUCTURE analysis.

Population	Code	Cluster																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
cv. Aurora	**V1**	0.01	0.02	0.01	0.44	0.01	0.07	0.03	0.03	0.01	0.01	0.01	0.03	0.12	0.02	0.01	0.13	0.03	0.02
cv. Barlenna	**V2**	0.01	0.03	0.01	0.19	0.02	0.21	0.03	0.02	0.03	0.07	0.02	0.02	0.03	0.05	0.01	0.06	0.17	0.04
cv. Cancan	**V3**	0.02	0.01	0.01	0.01	0.01	0.01	0.36	0.05	0.01	0.01	0.05	0.02	0.01	0.02	0.04	0.01	0.01	0.36
cv.Cashel	**V4**	0.02	0.02	0.01	0.05	0.02	0.02	0.02	0.06	0.02	0.07	0.15	0.04	0.01	0.04	0.07	0.01	0.01	0.36
cv.Fennema	**V5**	0.04	0.03	0.01	0.05	0.02	0.40	0.03	0.03	0.03	0.02	0.02	0.02	0.11	0.01	0.01	0.04	0.12	0.02
cv. Odenwaelder	**V10**	0.02	0.01	0.04	0.01	0.01	0.06	0.01	0.01	0.42	0.02	0.01	0.01	0.09	0.07	0.02	0.17	0.01	0.01
cv. Portstewart	**V11**	0.07	0.02	0.03	0.01	0.13	0.02	0.07	0.08	0.03	0.11	0.05	0.05	0.02	0.04	0.10	0.01	0.01	0.16
cv.Premo	**V12**	0.03	0.01	0.04	0.06	0.01	0.03	0.01	0.01	0.03	0.01	0.04	0.03	0.16	0.07	0.01	0.42	0.02	0.01
cv.S24	**V13**	0.02	0.02	0.01	0.11	0.02	0.03	0.02	0.01	0.05	0.02	0.01	0.03	0.10	0.38	0.01	0.06	0.08	0.03
cv.Shandon	**V15**	0.12	0.01	0.02	0.04	0.02	0.02	0.14	0.06	0.01	0.14	0.12	0.03	0.01	0.02	0.02	0.01	0.01	0.20
cv. Talbot	**V16**	0.02	0.02	0.04	0.06	0.04	0.06	0.02	0.02	0.12	0.02	0.06	0.01	0.09	0.02	0.01	0.35	0.02	0.01
IRL-OP-02337	**I1**	0.02	0.01	0.02	0.03	0.04	0.02	0.35	0.07	0.03	0.10	0.08	0.01	0.01	0.04	0.03	0.02	0.06	0.07
IRL-OP-02059	**I2**	0.01	0.03	0.02	0.13	0.02	0.13	0.03	0.02	0.05	0.03	0.05	0.02	0.15	0.01	0.04	0.05	0.20	0.03
IRL-OP-02007	**I3**	0.02	0.01	0.11	0.04	0.01	0.08	0.01	0.01	0.01	0.03	0.02	0.01	0.28	0.02	0.01	0.04	0.26	0.02
IRL-OP-02011	**I4**	0.01	0.01	0.03	0.09	0.02	0.08	0.01	0.02	0.04	0.04	0.03	0.02	0.04	0.01	0.01	0.02	0.52	0.01
IRL-OP-02015	**I5**	0.06	0.01	0.03	0.02	0.09	0.02	0.11	0.08	0.01	0.04	0.17	0.01	0.02	0.16	0.03	0.02	0.01	0.13
IRL-OP-02048	**I6**	0.02	0.27	0.02	0.01	0.08	0.01	0.01	0.04	0.01	0.26	0.09	0.01	0.03	0.03	0.04	0.02	0.03	0.03
IRL-OP-02192	**I7**	0.01	0.22	0.07	0.01	0.15	0.01	0.04	0.03	0.01	0.27	0.06	0.01	0.03	0.01	0.03	0.02	0.02	0.02
IRL-OP-02064	**I10**	0.03	0.42	0.06	0.01	0.01	0.01	0.02	0.02	0.01	0.26	0.01	0.01	0.02	0.02	0.01	0.02	0.04	0.04
IRL-OP-02078	**I11**	0.23	0.05	0.03	0.03	0.08	0.01	0.06	0.11	0.06	0.06	0.03	0.02	0.01	0.07	0.07	0.03	0.02	0.04

IRL-OP-02230	**I12**	0.03	0.01	0.02	0.08	0.02	0.21	0.01	0.02	0.05	0.01	0.01	0.04	0.12	0.05	0.04	0.09	0.19	0.01
IRL-OP-02128	**I13**	0.07	0.01	0.05	0.02	0.28	0.01	0.03	0.15	0.04	0.04	0.12	0.04	0.01	0.02	0.05	0.03	0.01	0.01
IRL-OP-02538	**I14**	0.01	0.02	0.02	0.20	0.01	0.11	0.02	0.02	0.04	0.02	0.03	0.03	0.08	0.02	0.05	0.05	0.26	0.02
IRL-OP-02274	**I15**	0.41	0.02	0.11	0.01	0.06	0.02	0.01	0.07	0.03	0.02	0.02	0.07	0.01	0.04	0.07	0.01	0.02	0.03
IRL-OP-02442	**I17**	0.04	0.04	0.02	0.03	0.10	0.02	0.34	0.09	0.01	0.02	0.05	0.02	0.01	0.01	0.06	0.02	0.04	0.09
IRL-OP-02068	**I19**	0.06	0.06	0.01	0.01	0.04	0.01	0.01	0.07	0.01	0.09	0.25	0.01	0.01	0.09	0.05	0.01	0.01	0.20
IRL-OP-02241	**I20**	0.01	0.03	0.02	0.26	0.02	0.11	0.01	0.02	0.03	0.01	0.01	0.02	0.15	0.04	0.02	0.11	0.14	0.02
IRL-OP-02419	**I21**	0.03	0.04	0.05	0.02	0.15	0.01	0.05	0.03	0.08	0.20	0.08	0.02	0.02	0.02	0.07	0.02	0.03	0.09
IRL-OP-02258	**I22**	0.01	0.71	0.03	0.01	0.02	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.07	0.03	0.03	0.01	0.01	0.01
IRL-OP-02272	**I23**	0.05	0.01	0.02	0.03	0.11	0.02	0.01	0.09	0.05	0.07	0.10	0.04	0.01	0.12	0.10	0.02	0.02	0.12
IRL-OP-02173	**I27**	0.07	0.01	0.06	0.01	0.05	0.01	0.02	0.49	0.03	0.01	0.01	0.05	0.01	0.06	0.04	0.03	0.02	0.03
IRL-OP-02483	**I28**	0.05	0.01	0.01	0.04	0.11	0.02	0.11	0.07	0.03	0.06	0.14	0.03	0.02	0.08	0.03	0.01	0.08	0.10
IRL-OP-02018	**I30**	0.04	0.01	0.09	0.01	0.08	0.02	0.02	0.04	0.02	0.05	0.14	0.03	0.01	0.05	0.21	0.01	0.01	0.16
16-7-62-2 Nordic	**Δ5**	0.03	0.02	0.04	0.08	0.04	0.17	0.03	0.02	0.04	0.01	0.02	0.02	0.10	0.06	0.13	0.06	0.10	0.03
No 10 Spain	**■15**	0.09	0.01	0.07	0.01	0.11	0.01	0.01	0.08	0.01	0.03	0.01	0.35	0.01	0.05	0.07	0.03	0.01	0.04
3408 Italy	**■16**	0.02	0.03	0.20	0.01	0.06	0.01	0.03	0.22	0.04	0.03	0.03	0.07	0.02	0.03	0.09	0.04	0.01	0.06
3013 Romania	**■18**	0.34	0.02	0.06	0.02	0.02	0.02	0.01	0.02	0.03	0.02	0.01	0.33	0.01	0.01	0.06	0.01	0.01	0.01
3199 Romania Podoloni	**■19**	0.03	0.02	0.02	0.12	0.03	0.38	0.02	0.02	0.01	0.04	0.02	0.04	0.12	0.05	0.01	0.03	0.04	0.02
920 Bulgaria	**■20**	0.28	0.01	0.28	0.01	0.03	0.02	0.01	0.05	0.02	0.03	0.01	0.08	0.02	0.05	0.07	0.01	0.01	0.01
IV-51-161 Hungary	**●32**	0.01	0.02	0.02	0.04	0.02	0.35	0.05	0.01	0.03	0.01	0.02	0.03	0.22	0.02	0.02	0.07	0.07	0.01

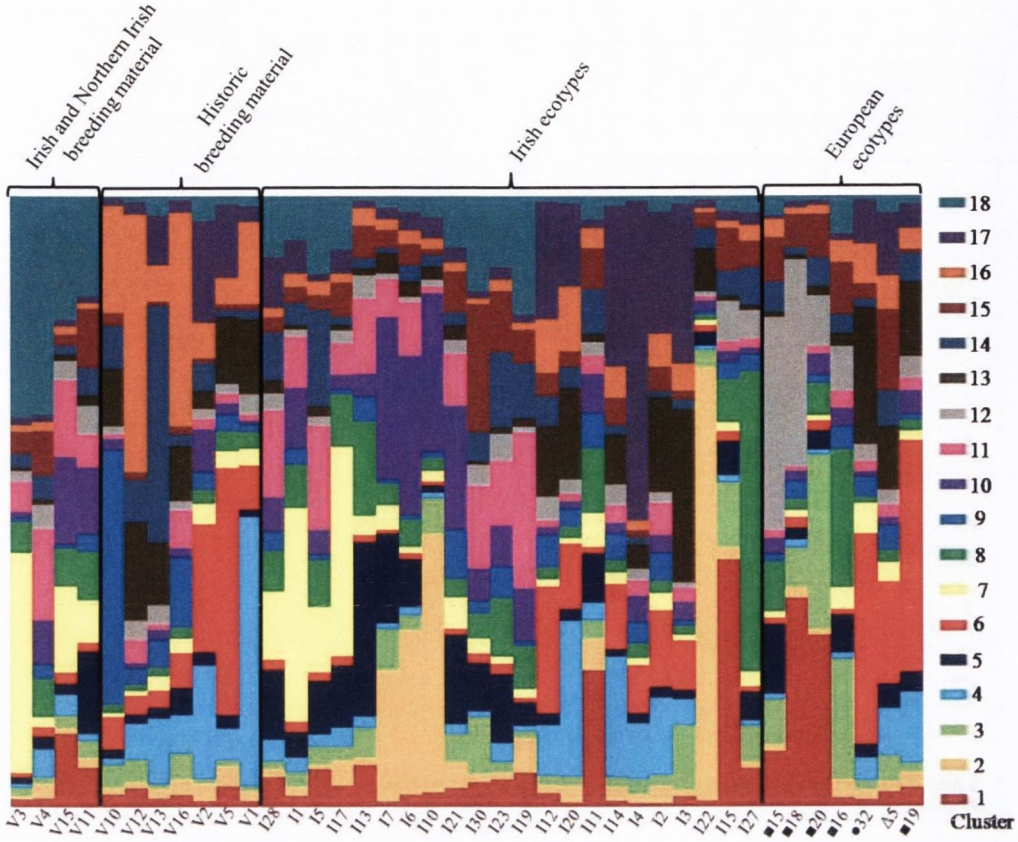

Figure 5.4.5 Proportion of individuals in each accession assigned to each cluster.

5.5 Discussion

5.5.1 Allelic and genotypic variation in *Lolium perenne*

Eight SSR markers were used to characterise genetic diversity in a collection of 40 diploid *L. perenne* accessions. Markers were highly polymorphic both overall and within individual populations. Allele numbers, observed and expected heterozygosities had wide variability both over groups within a marker and over all markers. The average number of alleles per locus over all accessions was 22.25 (calculated from Table 5.4.3), which is higher than that found in other studies using SSR markers (2 to 7 alleles per locus, Jones *et al.* 2001; 19.41 per locus, Kubik *et al.* 2001; 3.25 alleles per locus, Studer *et al.* 2006; 9.37 alleles per locus, Auzanneau *et al.* 2007). However, these studies had a smaller number of individuals and so a smaller number of alleles would be expected to be generated with less individuals. In subgroups of accessions (Irish ecotypes, European ecotypes and cultivars), allele numbers across loci ranged from an average of 16.88 in European ecotypes to 21.75 in Irish ecotypes, with cultivars having an intermediate average number of alleles (19.38, calculated from Table 5.4.3). Gene diversity values (H_E, Tables 5.4.1 and 5.4.2) ranged from 0.57 to 0.8 across populations, and had a value of 0.82 over all accessions. This value was much higher than values calculated in studies using SSR markers (0.59 – 0.64, Kubik *et al.* 2001; 0.56, Studer *et al.* 2006; 0.60, Auzanneau *et al.* 2007) and also much higher than values calculated for AFLP markers (0.17, Skot *et al.* 2005; 0.18 – 0.2, VanTreuren *et al.* 2005). Gene diversity values calculated using chloroplast SSR markers (Chapter 2, Tables 2.4.4 and 2.4.6) were much lower than the nuclear values (0 to 0.33 over all populations and 0.30 in cultivars, 0.31 in Irish ecotypes, and 0.33 in European ecotypes). Furthermore, PIC values ranged from 0.71 to 0.89 with an average of 0.81 over all markers. This was much higher than PIC values found in previous studies, both of cultivars (0.28, Roldan-Ruiz *et al.* 2000; 0.41, Bolaric *et al.* 2005a) and of ecotypes (0.33 – 0.40, Bolaric *et al.* 2005b). This may be due to the different marker system (SSR) used or to the fact that the sample size employed was bigger compared to previous studies.

An excess of homozygotes was found in many accessions or groups of accessions (Tables 5.4.1 and 5.4.2). This is in agreement with a previous study of *L. perenne*

using SSRs (Kubik *et al.* 2001), which also showed an excess of homozygotes. While small numbers of individual populations either agreed with Hardy-Weinberg equilibrium, or were slightly in excess of heterozygotes, the vast majority of accessions were less heterozygous than would be expected over Hardy Weinberg equilibrium. So while allele numbers were high, both in individual accessions and over all accessions, individuals were generally more homozygous than would be expected under Hardy-Weinberg equilibrium. Reasons why populations might deviate for Hardy-Weinberg equilibrium are non-random mating, due to inbreeding, or small population size, directional selection, mutation and migration. While the cultivars in this study could be expected to deviate from Hardy-Weinberg equilibrium because of inbreeding caused by selective breeding, the ecotypic material would be normally expected to be more heterozygous, which is not the case. F_{IS}, which is the proportion of variation between individuals among populations, is also known as the inbreeding coefficient (the higher the *F*is, the lower the heterozygosity). In the Weir and Cockerham (1984) *F*-statistics estimates for the partition of Irish ecotypes, European ecotypes and cultivars, *F*is ranged from 0.14 at locus rv0264 to 0.58 at locus LpHCA13H2 and over all loci was 0.31. Within the ecotypic material, the populations may have been isolated from each other or breeding may not have been random at the scale of sampling used. (eg increased mating between closely related plants, relative to the entire population, would occur because they lie in close proximity to each other).

5.5.2 Linkage disequilibrium

Linkage disequilibrium between all pairs of loci was only significant between pairs of loci previously shown to be on the same linkage group (Jensen *et al.* 2005; Turner *et al.* 2006). For example, between loci LpHCA18F11 and rv0264, which are both on linkage group seven, linkage disequilibrium was significant at $p<0.05$, Table 5.4.4. Linkage disequilibrium levels were low and non-significant for pairs of loci not on the same linkage group. Similar low levels of linkage disequilibrium were found in other studies of linkage disequilibrium in *L. perenne* (e.g. Xing *et al.* 2007). Such low levels of linkage disequilibrium mean that it may be possible to use these markers in the future in association mapping studies testing for association of the markers with characters measured in Chapter 3 and Chapter 4.

5.5.3 Partitioning of variation in *Lolium perenne*

AMOVA analysis was used to analyse the partitioning of variation within the collection of accessions. In every case, most variation was explained by within-population variation, which ranged from 87.57% in cultivars to 91.44% in European ecotypes. This is in agreement with many other studies of *L. perenne,* both cultivars and ecotypes, and irrespective of marker system (66%, Bolaric *et al.* 2005a; 71%, Bolaric *et al.* 2005b and 82%, Kolliker *et al.* 1999, 85.35%, Kubik *et al.* 2001, 89.6%, Guthridge *et al.* 2001; VanTreuren *et al.* 2005). Equally, when analysed by Weir and Cockerham (1984) F-statistics, the majority of variation over all accessions is explained by within population variation (F_{IT}: 0.39, F_{IS}: 0.31, F_{IP}: 0.38, Table 5.4.7). When different groups of accessions were compared to each other, within population variation was again high, and much lower variation was seen between groups than in the chloroplast SSR analysis which showed clearer distinctions between groups of accessions (e.g. Irish ecotypes *vs.* European ecotypes between group variation: 11%, Chapter 2, Table 2.4.7). When groups seen in PCA analysis were compared to each other by AMOVA and by F-statistics, similar results were seen, with the exception of the comparison between historic breeding material and Irish/Northern Irish breeding material, where between group variation was higher than in other comparisons. This would be expected from the different breeding histories of the groups. G_{ST} values were 0.23 overall (Table 5.4.2) and ranged from 0.18 in European ecotypes to 0.23 in cultivars. The higher value of G_{ST} in cultivars is in agreement with the higher level of between population variation seen in the AMOVA analysis for cultivars, as the G_{ST} value increase as the populations become more different from each other. G_{ST} values calculated with chloroplast SSR markers (Chapter 2, Table 2.4.6) were higher (0.24 in Irish ecotypes, 0.43 in European ecotypes and 0.29 in cultivars). This would be expected given the non-recombining nature of the plastid DNA molecule, and its transmission over geographical space solely via seed and not via pollen (a factor that will reduce geneflow and increase G_{ST}).

5.5.4 Relationships between accessions and genetic distance

Two groups of accessions could be defined in both the UPGMA dendrogram and the PCA. While Mantel tests did not show any correlation between geographical distance and genetic distance, several clusters of geographically close accession were found in both, and with reasonable bootstrap support in the UPGMA dendrogram. So perhaps in the overall group of populations there was no geographical links, close geographical populations may have bred together or have adapted to the same stresses. In the PCA diagram, the division of the populations would seem to be reliable given that 89.09% of the variation was explained by the first two dimensions. This was in agreement with a study using RAPD markers (Kolliker *et al.* 1999) where 88% of variation within *L. perenne* was explained by the first three dimensions. This is in comparison to previously more commonly used markers (AFLP) where there are often high levels of dimensionality. That is, the major axes only explained small amounts of the variation seen (Cresswell *et al.* 2001; Guthridge *et al,.* 2001; Skot *et al,.* 2002; Van Treuren *et al,.* 2005) indicating the many of the AFLP markers generated were not contributing to the variation in the populations.

The STRUCTURE analysis assigns individual multi-locus genotypes probalistically to a number (K) of user defined clusters or gene pools, achieving linkage equilibrium within clusters. The STRUCTURE analysis predicted 18 clusters (gene pools), and these were similar to the groups found in the UPGMA and PCA. When individuals were assigned to clusters, some individuals were assigned partially to more than one cluster, this might reflect continuous gradations in allele frequencies or admixture of neighbouring groups (Rosenberg *et al.* 2002). This is expected because of the outbreeding nature of *L. perenne*, as well as the easy spread of pollen (by wind) and seeds (by multiple means). It could also explain the general lack of geographic differentiation between accessions (with the exception of a number of accessions, in Cork for example, which could be explained by adaptation to similar environments). No geographic structuring, either within Ireland, or across Europe, was seen in this structuring analysis.

5.6 Conclusion

This study assessed the genetic diversity in 40 diploid populations of *L. perenne* using nuclear SSR markers. High levels of allelic and genetic diversity were found, with within population variation accounting for the majority of the variation. The majority of accessions deviated from Hardy Weinberg equilibrium and had relatively high inbreeding coefficients. Population structure and differentiation analyses confirmed the results found in the UPGMA and PCA analyses. These results will be useful for breeders wishing to exploit ecotype collections. Further analysis to determine possible associations between markers and quality characters should be carried out given that relatively few of the loci showed significant levels of LD.

Chapter 6

General discussion on the characterisation of genetic diversity of a collection of perennial ryegrass (*Lolium perenne* L.)

6.1 Introduction and overview of the findings

The overall aim of this work was to characterise molecular, morphological and biochemical diversity in a collection of *L. perenne* ecotypes and cultivars. The collection consisted mainly of a selection of accessions from around Ireland and a number of accessions from across Europe, and cultivars which were developed in Ireland and elsewhere. The characterisation was performed using a combination of DNA markers (nuclear and chloroplast SSRs), morphological characters, and biochemical characters. Diversity levels, differentiation of populations, and partitioning of variation were different for each marker system. This is likely to be a result of the different genetic basis of each of the different marker systems and is in agreement with studies comparing different marker systems (Powell *et al.* 1996; Roldan-Ruiz *et al.* 2001; Petit *et al.* 2005). However, the results in combination have permitted a detailed characerization of the collection and allowed us to draw a number of important conclusions, the details of which are summarized below:

6.1.1 Characterisation of *L. perenne* and related species accessions using chloroplast SSR markers

Ten novel chloroplast SSR primers were designed via sequencing of chloroplast genes and intergenic spacer regions and GenBank data mining, and were shown to amplify in members of nine out of 13 grass subfamilies, showing their cross species potential. The primer development paper, published in the journal Molecular Ecology Notes, resulting from this work (McGrath *et al.* 2006) can be found in the appendices.

The primers were applied successfully to our collection to make a detailed evaluation of the cytoplasmic gene pools in *Lolium*. The results of which have been published in the journal *Heredity* (McGrath *et al.* 2007; Appendix 8.10). Allelic and haplotypic variation was extremely high between and within Irish and European *L. perenne*

ecotypes. A total of 511 haplotypes were detected, with an average of 10.4 haplotypes per accession found. The breeding system and cultivation history of *L. perenne* are contributing factors to the high levels of diversity, as well as possible rapid evolution of SSR loci.

Some of the markers were more variable than others. This may be a result of different markers coming from gene regions with different rates of evolution, in accordance with expectations (Wolfe *et al.* 1987). It could also be a result of the varying lengths of the different SSR regions, where longer cpSSR loci have been shown to be more variable than shorter ones (Provan *et al.* 1999b).

Individual accessions showed a wide range of variation, particularly in the ecotypes, with genetic diversity (Nei's gene diversity values) in Irish *L. perenne* ecotypes ranging from 0.122 to 0.133 (Table 2.4.4). Isolation of ecotypes could have caused a lowering of diversity in some populations, while high movement of seeds between some ecotypes could have resulted in increased levels of diversity in others. While a suspected centre of origin for *L. perenne* in Europe would lead to an expectation of lower levels of genetic diversity in Irish ecotypes than several European ecotypes, in this study, this was not the case. This could be as a result of the thorough selection strategy of the Irish team in the ECPGR collection.

The cpSSR markers were useful for identifying genepools. 71% of haplotypes were unique to individual populations, much higher than in other studies of *L. perenne* (e.g. 27%, in Balfourier *et al.* 2000). While haplotypes could not assign individuals to populations, haplotypes could be used to assign individuals to groups of populations, and had the potential to distinguish geographic ecotypes and accessions. The markers were also able to distinguish between Irish and European ecotypes in the UPGMA dendrograms (Figures 2.4.10 and 2.4.12). AMOVA analyses (Table 2.4.7) were useful in supporting possible biogeographic patterns of variation. These included, a Mediterannean route of migration across Europe, migration from Southern Europe to Northern Europe including Ireland, as well as a partitioning consistent with post-glacial recolonization.

These markers were also shown to be useful for the study of introgression and hybridisation. On the UPGMA dendrogram (Figure 2.4.10), *L. temulentum* grouped with two *Festuca* species. This could be an indication of introgression of the plastid genome from *Festuca* species into *Lolium*. Six out of nine ×*Festulolium* cultivars grouped with the European *L. perenne* ecotypes, which also could indicate introgression from European and Near Eastern *L. perenne* ecotypes into ×*Festulolium*.

These markers were also useful for phylogenetic analysis. The separation of narrow and broad leaved *Festuca* species was seen, and this is in agreement with other studies (Darbyshire & Warwick, 1992; Catalan *et al.* 1997; Charmet *et al.* 1997; Catalan *et al.* 2004; Torrecilla *et al.* 2004). However, no separation between allogamous and autogamous *Lolium* species was seen, possibly as a result of homoplasy.

6.1.2 Morphological diversity of a collection of *Lolium perenne* ecotypes and varieties

Morphological variation was characterised for 2,481 individuals from 50 *L. perenne* accessions, a mixture of Irish and European ecotypes and cultivars. This represents, by far, the largest scale morphological analysis of this genus undertaken to date on this genus in Ireland. Levels of among and within population variation varied considerably across traits. For example, the characters height at ear emergence, height 30 days after ear emergence, length of flag leaf and width of flag leaf had the least amount of among population variation, while these characters had the highest amount of within population variation. Conversely, very high levels of among population variation were found for date of ear emergence, while within population variation was very low for this character. Such variation in ecotypes could be a result of adaptation to environmental factors which influence ear emergence, while in cultivars, it may be a result of selection for optimal forage potential during breeding.

Morphological characters were able to separate ecotypes from cultivars in both PCA and UPGMA dendrograms, much like other studies of *L. perenne* with morphological characters (Loos, 1994; Van Treuren *et al.* 2005). Cultivars generally had later dates of ear emergence, better spring and summer growth, longer rachis length and more spikelets per spike than ecotypes, which is in agreement with the breeding history of

cultivars and local adaptations of ecotypes. Like chloroplast SSRs and other studies of morphological variation in *L. perenne* (Loos, 1994; Fernando *et al.* 1997; Van Treuren *et al.* 2005), no obvious broadscale geographic structuring was seen in this part of the study.

Strong positive relationships (correlations) were seen between reproductive characters, i.e. rachis length with spikelets per spike, florets per spikelet and glume length. Relationships between rachis length, spikelets per spike and florets per spikelet is expected because these characters are directly related to inflorescence branching processes. Their relationships with glume length may be a result of genes for glume length being localised to branching area (Bortiri *et al.* 2006). Further studies would be required to investigate floral development in *L. perenne*. For example, studies on the heritability of floral traits, QTL analysis and genetic marker analysis of the genes responsible for floral architecture would be useful. In any case, the strong relationship between rachis length and the other reproductive characters mean that rachis length could be used as a predictor for reproductive performance (e.g. seed yield, which is important for breeding) in breeding programmes. In this study, later flowering was correlated with improved spring and summer growth. However later flowering is associated with lower seed yield. A trade off must be made between higher seed yield (important for cultivar development) and later flowering (important for agronomic quality). Using rachis length as a predictor for seed set could be a way to combine these two objectives. The results of the morphological study are currently being written up for publication.

6.1.3 Variation in water soluble carbohydrate, dry matter and crude protein content.

Water soluble carbohydrate, crude protein, and dry matter contents were determined for 1,320 individuals pooled into 132 samples from 33 *L. perenne* ecotypes and cultivars at five different points across the 2004 growing season. Variation in the biochemical characters varied widely across traits, cuts and between groups of populations. While high levels of variation were seen with the other systems in this study, none of the exceptional populations seen in the WSC analysis were exceptional in the other analyses. While, in general, the cultivars had higher WSC contents than the ecotypes, individual ecotypes did show potential to be used in breeding

programmes, as they were higher than all other accessions at particular cutting points. For example, ecotypes IRL-OP-02018 and IRL-OP-02419 look highly valuable. Such high levels of WSC could be used to increase stress tolerance in *L. perenne,* as high levels of WSC have been associated with cold stress tolerance (Turner *et al.* 2006).

Positive relationships were shown between dry matter and glucose both early and late in the growing season, and this is in agreement with the amount of leaves compared to stem at these times in the growing season. Negative correlations were seen between crude protein and dry matter, again in agreement with other studies (Wilkins & Humphreys, 2003).

Populations could be separated using the biochemical characters and PCA at the first four cuts, either between cultivars and ecotypes, or between tetraploid cultivars and the rest of the accessions. In general, dry matter was the character causing the split. Crude protein was also causing a certain amount of the separation seen in the PCA. For all traits, cutting point was the most significant factor influencing the variation in the traits. This is in agreement with other studies on *Lolium* (Tas *et al.* 2005; Turner *et al.* 2006; Skot *et al.* 2007). It seems therefore that either the management or environment has a larger effect on the characters than the genotype of the plants. This may limit the prospect of manipulating these traits in a breeding programme. However, it will be important to determine the heritability of these traits further and investigate the potential of using these traits for QTL mapping. The results of this work could therefore help select appropriate plant material for such studies. It is anticipated that the results of this work will be submitted for publication. However, it may also be possible and most profitable to discuss their variation in the context of the nuclear DNA variation and the association mapping of these traits.

6.1.4 Characterisation of genetic diversity and population structure in a collection of *Lolium perenne* accessions using nuclear microsatellite markers

Eight nuclear SSR markers were used to characterize genetic diversity in 928 individuals from 40 diploid ecotypes and cultivars of *L. perenne.* High levels of genetic diversity (Nei's gene diversity value of 0.82 over all accessions) and high numbers of alleles (22.25 number of alleles per locus) were found. This was higher

than that found in other studies (Jones *et al.* 2001; Kubik *et al.* 2001; Studer *et al.* 2006; Auzanneau *et al.* 2007). PIC values in this study were also higher than those seen in other studies, perhaps due to the higher numbers of samples used in this analysis, but also because the collection shows high diversity. The majority of populations had an excess of homozygotes. This excess of homozygotes in cultivars could be due to inbreeding caused by selective breeding. In ecotypes it could also be due to inbreeding and possible isolation of populations. Therefore even though genetic diversity within populations was high, heterozygosity was lower than expected under HW equilibrium. Even though *Lolium* is outbreeding and self incompatible with good gene flow potential, it is highly likely that the geographic space in which random mating occurs is small. Plants are more likely to interbreed with close neighbours and these close neighbours are more likely to be closely related.

Very low levels of linkage disequilibrium were found between pairs of loci tested, with the exception of those pairs of loci previously shown to have been located on the same linkage group. Similar results were seen in other studies (Xing *et al.* 2007). The set of markers which are not in linkage disequilibrium with each other can be further used in association studies, to determine if there are any links between them and the phenotypic data, using a candidate gene approach. If any associations are found, the markers could then be used for MAS in breeding programs.

In terms of genetic differentiation of populations, most variation was found within populations, (87.57% in cultivars, 90.35% in ecotypes). Similar levels of differentiation were found in other studies (e.g. Kolliker *et al.* 1999; Bolaric *et al.* 2005a; Bolaric *et al.* 2005b) when analysed by both AMOVA and *F* statistics. These results were also consistent in groups of populations. The only exception was a comparison of recent Irish breeding material and historic European breeding material, where between group variation was higher than in other comparisons. This was an indication of the differing breeding strategies and shows that the markers could be used to distinguish different gene pools. UPGMA, PCA and population structure analysis all gave similar patterns of relationships among populations. It is anticipated that the journal Annals of Botany will be the target for the nuclear SSR diversity/differentiation study work undertaken here.

6.2 The Irish ecotype collection and its potential for breeding

Lolium perenne is the most important forage species in Ireland. It is a major component of the grasslands of temperate climate zones. While it is a common species in these zones, in pasture it is mainly sown as cultivars. The processes of selection which occur in the development of cultivars cause the loss of rare alleles which are not involved in the trait of interest, but may be linked to important characteristics such as resistance to stress, disease tolerance, or high seed yield. Therefore it is important to maintain collections of germplasm containing such rare alleles. As well as simply keeping such collections, they also need to be characterized. The Irish collection of ecotypes needed to be characterized in such a manner in order to determine the amount of genetic diversity in the collection and to assess its potential for use in the Teagasc grass breeding programme. Any accessions which show good potential for use in the Teagasc grass breeding programme, could be used either as parental genotypes in half-sib selection, or as one of the source populations in full-sib selection.

Genetic diversity, irrespective of the marker system used, was high across all populations, whether cultivar or ecotype. Also, in traits of interest, such as WSC, several ecotypes had significantly and substantially higher levels of the character. This indicates that such ecotypes have the potential to be added to the breeding programme for high WSC grasses.

The following further studies would be recommended to continue the work undertaken in this thesis:

(1) further accessions from Europe could be assessed using chloroplast SSR markers, in order to clarify migration routes of *L. perenne* across Europe. While AMOVA analysis gave support to the idea of a Mediterranean migration route, higher numbers of European populations could give a clearer result.

(2) interesting correlations were found between rachis length and other reproductive traits, which could be linked to seed yield, an important target for

breeders. A detailed study of the genetics of inflorescence architecture and seed development would clarify these relationships. Furthermore QTLs for important agronomic traits could be assessed. If markers (the SSR markers used in this thesis; and possible new markers created via, for example, SNPs or AFLP), could be found to be closely linked to QTL then this offers the potential to use the markers for MAS strategies.

(3) the low levels of linkage disequilibrium seen for the eight nuclear SSR markers indicate that these could be used in association/LD candidate gene association mapping of the biochemical and morphological characters. If close associations were found, these markers could then be used for MAS.

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8.0 Appendices

8.1 Name, source, original location, and number of samples in each analysis of the *Lolium perenne* L. accessions used in this study (Chapters 2, 3, 4 and 5)

Species	Accession Number	Country of Origin	Location	Latitude	Longitude	Seed Source	Ploidy level	Cp[a]	N[b]	M[c]	B[d]
L. perenne	IRL-OP-02337	Ireland	Kellistown Farm, Carlow	N 52.47.60	W 06.49.73	Teagasc Oak Park	2n	15 (I 1)	24	46	40
L. perenne	IRL-OP-02059	Ireland	Moyneroe, Scarrif, Clare	N 52.54.35	W 08.30.32	Teagasc Oak Park	2n	16 (I 2)	24	48	40
L. perenne	IRL-OP-02007	Ireland	Bromcloc, Bantry, Cork	N 51.39.95	W 09.31.07	Teagasc Oak Park	2n	16 (I 3)	24	50	40
L. perenne	IRL-OP-02011	Ireland	Crowleys Pub, The Square, Bantry, Cork	N 51.42.15	W 09.27.67	Teagasc Oak Park	2n	14 (I 4)	24	50	40
L. perenne	IRL-OP-02015	Ireland	South Ring, Clonakilty, Cork	N 51.37.10	W 08.53.71	Teagasc Oak Park	2n	16 (I 5)	24	46	40
L. perenne	IRL-OP-02048	Ireland	Carrigeen, Conna, Old Kents, Fermoy, Cork	N 52.05.88	W 08.03.50	Teagasc Oak Park	2n	16 (I 6)	21	48	40
L. perenne	IRL-OP-02192	Ireland	Horse Island, Roaring Water Bay, West Cork	N 51.30.80	W 09.29.03	Teagasc Oak Park	2n	16 (I 7)	24	49	40
L. perenne	IRL-OP-02312	Ireland	Fortlands House, Charleville, Cork	N 52.20.86	W 08.42.27	Teagasc Oak Park	2n	16 (I 8)	0	50	0
L. perenne	IRL-OP-02320	Ireland	Clonakilty, Co. Cork	N 51.37.10	W 08.53.71	Teagasc Oak Park	2n	16 (I 9)	0	0	0
L. perenne	IRL-OP-02064	Ireland	Kilreekill, Loughrea, Galway	N 53.13.23	W 08.28.75	Teagasc Oak Park	2n	16 (I 10)	12	49	40
L. perenne	IRL-OP-02078	Ireland	Ballycahalan, Peterswell, Galway	N 53.05.64	W 08.36.72	Teagasc Oak Park	2n	18 (I 11)	24	49	40
L. perenne	IRL-OP-02230	Ireland	Clough, Cummer, Tuam, Galway	N 53.27.11	W 08.53.29	Teagasc Oak Park	2n	16 (I 12)	24	50	40
L. perenne	IRL-OP-02128	Ireland	Mahera Beg, Commonage North, Castlegregory, Kerry	N 52.17.79	W 10.01.38	Teagasc Oak Park	2n	16 (I 13)	24	50	40

L. perenne	IRL-OP-02538	Ireland	Colt, Ballyroan, Laois	N 52.58.08	W 07.20.70	Teagasc Oak Park	2n	16 (I 14)	24	49	40
L. perenne	IRL-OP-02274	Ireland	Buffanoka, Cappamore, Limerick	N 52.39.29	W 08.18.62	Teagasc Oak Park	2n	16 (I 15)	24	50	40
L. perenne	IRL-OP-02480	Ireland	Inch St, Lawrence, Caherconlish, Limerick	N 52.35.47	W 08.30.99	Teagasc Oak Park	2n	16 (I 16)	0	0	40
L. perenne	IRL-OP-02442	Ireland	Doughmakean, Roonagh, Westport, Mayo	N 53.44.74	W 09.53.69	Teagasc Oak Park	2n	16 (I 17)	24	50	40
L. perenne	IRL-OP-02444	Ireland	Barnabawn, Killadoon, Westport, Mayo	N 53.41.48	W 09.54.91	Teagasc Oak Park	2n	16 (I 18)	0	50	0
L. perenne	IRL-OP-02068	Ireland	Ballycommon, Tullamore, Offaly	N 53.17.50	W 07.23.11	Teagasc Oak Park	2n	15 (I 19)	24	50	40
L. perenne	IRL-OP-02241	Ireland	Clonohill, Birr, Offaly	N 53.05.20	W 07.53.73	Teagasc Oak Park	2n	16 (I 20)	24	48	40
L. perenne	IRL-OP-02419	Ireland	Johnstown,Cornafulla, Athlone, Roscommon	N 53.38.05	W 09.30.71	Teagasc Oak Park	2n	16 (I 21)	22	50	40
L. perenne	IRL-OP-02258	Ireland	The Lawn, Drum, Tipperary	N 52.46.32	W 07.52.89	Teagasc Oak Park	2n	16 (I 22)	24	50	40
L. perenne	IRL-OP-02272	Ireland	Ballycrana, Kilross, Tipperary	N 52.25.28	W 08.15.88	Teagasc Oak Park	2n	16 (I 23)	18	50	40
L. perenne	IRL-OP-02250	Ireland	Glown, Upperchurch, Tipperary	N 52.42.55	W 08.07.99	Teagasc Oak Park	2n	17 (I 24)	0	50	0
L. perenne	IRL-OP-02267	Ireland	Ballyhoulihan, Emly, Tipperary	N 52.26.88	W 08.22.06	Teagasc Oak Park	2n	16 (I 25)	0	50	0
L. perenne	IRL-OP-02269	Ireland	Ballycurrane, Emly, Tipperary	N 52.27.42	W 08.22.95	Teagasc Oak Park	2n	17 (I 26)	0	50	0
L. perenne	IRL-OP-02173	Ireland	Deerpark, Lismore, Waterford	N 52.08.04	W 07.55.62	Teagasc Oak Park	2n	16 (I 27)	24	50	40
L. perenne	IRL-OP-02483	Ireland	Edwardstown, Cleriestown, Wexford	N 52.16.20	W 06.38.25	Teagasc Oak Park	2n	16 (I 28)	24	50	40
L. perenne	IRL-OP-02491	Ireland	Heath Park, Newbawn, Wexford	N 52.23.32	W 06.48.61	Teagasc Oak Park	2n	16 (I 29)	0	0	0
L. perenne	IRL-OP-02018	Ireland	Ballynure Demesne, Grangecon, Wicklow	N 52.59.95	W 06.44.92	Teagasc Oak Park	2n	16 (I 30)	18	50	40
L. perenne	GR 5092	Germany	Malchow/Poel	N 54.00.00	E 11.28.00	IPK Gatersleben	2n	16 (△1)	0	0	0

L. perenne	PI 598445	Netherlands	Unknown	N 53.07.00	E 07.02.00	GRIN	2n	12 (△2)	0	0	0
L. perenne	ABY-Ba 12896	Denmark	Unknown	N 55.00.00	E 09.46.59	IGER	2n	16 (△3)	0	0	0
L. perenne	NGB14250	Sweden	Unknown	N 57.45.50	E 14.51.25	Nordic Gene Bank	2n	12 (△4)	0	0	0
L. perenne	16-7-62-2 Nordic	Norway	Sola	N 58.54.00	W 05.34.99	Teagasc Oak Park	2n	16 (△5)	24	50	0
L. perenne	PI 619024	England	Unknown	N 53.17.00	W 01.46.00	GRIN	2n	12 (△6)	0	0	0
L. perenne	W6 9339	Wales	Unknown	N 51.57.00	W 03.03.00	GRIN	2n	16 (△7)	0	0	0
L. perenne	PI 610958	Tunisia	Unknown	N 36.53.42	E 09.11.13	GRIN	2n	16 (□8)	0	0	0
L. perenne	ABY-Ba 11315	Morocco	Unknown	N 31.30.00	W 09.48.00	IGER	2n	16 (□9)	0	0	0
L. perenne	E1	Egypt	Unknown	Unknown	Unknown	PGG-Wrightson	2n	16 (□10)	0	0	0
L. perenne	W6 11325	Turkey	Karabuk, Ankara	N 41.07.12	E 32.22.12	GRIN	2n	16 (▲11)	0	0	0
L. perenne	PI 598512	Turkey	Antalya	N 36.54.45	E 30.41.23	GRIN	2n	16 (▲12)	0	0	0
L. perenne	PI 547390	Iran	Karaj	N 35.28.48	E 51.00.00	GRIN	2n	12 (▲13)	0	0	0
L. perenne	PI 317452	Afghanistan	North of Hari Rud River, 4 miles west of Besha	N 34.46.00	E 63.46.00	GRIN	2n	16 (▲14)	0	0	0
L. perenne	No 10 Spain	Spain	Unknown	Unknown	Unknown	Teagasc Oak Park	2n	16 (■15)	22	50	0
L. perenne	3408 Italy	Italy	Unknown	Unknown	Unknown	Teagasc Oak Park	2n	16 (■16)	24	50	0
L. perenne	W6 16127	Italy	Sardinia	N 40.34.37	E 09.12.18	GRIN	2n	15 (■17)	0	0	0
L. perenne	3013 Romania	Romania	Unknown	Unknown	Unknown	Teagasc Oak Park	2n	17 (■18)	24	49	0
L. perenne	3199 Romania Podoloni	Romania	Unknown	Unknown	Unknown	Teagasc Oak Park	2n	16 (■19)	24	50	0

L. perenne	920 Bulgaria	Bulgaria	Unknown	Unknown	Unknown	Teagasc Oak Park	2n	16 (■20)	24	50	0
L. perenne	PI 418701	Yugoslavia	Prizren	N 42.13.00	E 22.44.00	GRIN	2n	16 (■21)	0	0	0
L. perenne	ABY-Ba 11478	Greece	Unknown	N 38.00.00	E 22.10.00	IGER	2n	16 (■22)	0	0	0
L. perenne	W6 9286	France	Unknown	N 47.33.00	E 04.28.00	GRIN	2n	16 (❍23)	0	0	0
L. perenne	ABY-Ba 11514	France	Unknown	N 49.57.00	E 02.46.00	IGER	2n	16 (❍24)	0	0	0
L. perenne	CPI 44924	France	Arles	N 43.40.01	E 04.37.58	PGG-Wrightson	2n	16 (❍25)	0	0	0
L. perenne	GR 5095	Germany	Kempten	N 47.49.00	E 10.19.59	IPK Gatersleben	2n	16 (❍26)	0	0	0
L. perenne	GR 5105	Germany	Kempten	N 47.49.59	E 10.15.00	IPK Gatersleben	2n	16 (❍27)	0	0	0
L. perenne	PI 274637	Poland	Lublin	N 51.13.48	E 22.33.00	GRIN	2n	16 (●28)	0	0	0
L. perenne	PI 267058	Poland	Warszawa	N 52.35.00	E 21.05.00	GRIN	2n	16 (●29)	0	0	0
L. perenne	PI 182857	Czech Republic	Central Bohemia	Unknown	Unknown	GRIN	2n	16 (●30)	0	0	0
L. perenne	PI 321397	Czech Republic	Central Bohemia	Unknown	Unknown	GRIN	2n	16 (●31)	0	0	0
L. perenne	IV-51-161 Hungary	Hungary	Unknown	Unknown	Unknown	Teagasc Oak Park	2n	16 (●32)	24	50	0
L. perenne	cv. Aurora	N/A	N/A[d]	N/A	N/A	IGER	2n	17 (V 1)	23	50	0
L. perenne	cv. Barlenna	N/A	N/A	N/A	N/A	Barenbrug Holland BV	2n	16 (V 2)	24	50	0
L. perenne	cv. Cancan	N/A	N/A	N/A	N/A	DLF-Trifolium	2n	16 (V 3)	24	50	40
L. perenne	cv. Cashel	N/A	N/A	N/A	N/A	Teagasc	2n	16 (V 4)	24	50	40
L. perenne	cv. Fennema	N/A	N/A	N/A	N/A	Norddeutsche Pflanzenzucht	2n	16 (V 5)	24	50	0

L. perenne	cv. Greengold	N/A	N/A	N/A	N/A	Teagasc	4n	17 (V 6)	0	50	40
L. perenne	cv. Magician	N/A	N/A	N/A	N/A	Teagasc	4n	16 (V 7)	0	50	40
L. perenne	cv. Millenium	N/A	N/A	N/A	N/A	Teagasc	4n	16 (V 8)	0	50	40
L. perenne	cv. Navan	N/A	N/A	N/A	N/A	DARDNI	4n	16 (V 9)	0	50	40
L. perenne	cv. Odenwaelder	N/A	N/A	N/A	N/A	IPK Gatersleben	2n	16 (V 10)	24	50	40
L. perenne	cv. Portstewart	N/A	N/A	N/A	N/A	DARDNI	2n	16 (V 11)	24	50	40
L. perenne	cv. Premo	N/A	N/A	N/A	N/A	Mommersteeg International BV	2n	16 (V 12)	24	50	0
L. perenne	cv. S24	N/A	N/A	N/A	N/A	IGER	2n	17 (V 13)	20	50	0
L. perenne	cv. Sarsfield	N/A	N/A	N/A	N/A	Teagasc	4n	16 (V 14)	0	50	40
L. perenne	cv. Shandon	N/A	N/A	N/A	N/A	Teagasc	2n	16 (V 15)	24	50	40
L. perenne	cv. Talbot	N/A	N/A	N/A	N/A	Van der Have Grasses BV	2n	16 (V 16)	24	50	0

[a]Cp: chloroplast analysis. Characters in parentheses indicate geographical group for the populations.(I = Irish ecotype, △= Northern Europe group 1, □ = North Africa group 2, ▲= Near East group 3, ■ = Southern Europe group 4, ❍ = Western Europe group 5, ● = Eastern Europe group 6, V = *Lolium perenne* variety), [b]N: Nuclear analysis; [c]M: Morphological analysis; [d]B: Biochemical analysis; [e]N/A: Not applicable

8.2 Name, source, original location, and number of samples in each analysis of the non-*Lolium perenne* accessions used in this study (chapter 2)

Species	Subfamily	Accession Number	Country of Origin	Latitude	Longitude	Seed Source	Cp[a]
L. canariense	Poaceaea	PI 320544	Canary Islands	N 110.36.00	E 12.00.29	GRIN	16 (L1)
L. hybridum	"	ABY-Ba 13122	Portugal	N 40.56.00	W. 07.33.00	IGER	16 (L2)
L. hybridum	"	GR11849/94	N/A	Unknown	Unknown	IPK Gatersleben	8 (L3)
L. multiflorum	"	GR11855/98	N/A	Unknown	Unknown	IPK Gatersleben	8 (L4)
L. persicum	"	PI 229764	Iran	Unknown	Unknown	GRIN	16 (L5)
L. remotum	"	GR11839/99a	Germany	Unknown	Unknown	IPK Gatersleben	8 (L6)
L. rigidum	"	GR11848/91	Iran	Unknown	Unknown	IPK Gatersleben	8 (L7)
L. subulatum	"	PI 197310	Argentina	Unknown	Unknown	GRIN	16 (L8)
L. temulentum	"	ABY-Ba 13643	Morocco	N 35..34.00	W 05.22.00	IGER	16 (L9)
L. temulentum	"	ABY-Ba 8917	Iran	N 52.19.00	E 36.25.59	IGER	16 (L10)
L. temulentum	"	GR11880/82	Italy	Unknown	Unknown	IPK Gatersleben	8 (L11)
xFestulolium braunii	"	*cv.* Perun	N/A[b]	N/A	N/A	Plant Breeding Station Hladke Zivotice	16 (F1)
xFestulolium braunii	"	*cv.* HD 14 DK	N/A	N/A	N/A	Plant Breeding Station Hladke Zivotice	16 (F2)
xFestulolium braunii	"	*cv.* Paulita	N/A	N/A	N/A	Plant Breeding Station Hladke Zivotice	16 (F3)
xFestulolium braunii	"	*cv.* Achilles	N/A	N/A	N/A	Plant Breeding Station Hladke Zivotice	16 (F4)
xFestulolium Lolium multiflorum x Festuca arundinacea	"	*cv.* Lesana	N/A	N/A	N/A	Plant Breeding Station Hladke Zivotice	16 (F5)

xFestulolium Lolium multiflorum x Festuca arundinacea	"	*cv.* Becva	N/A	N/A	N/A	Plant Breeding Station Hladke Zivotice	16 (F6)
xFestulolium Lolium multiflorum x Festuca arundinacea	"	*cv.* Lofa	N/A	N/A	N/A	Plant Breeding Station Hladke Zivotice	16 (F7)
xFestulolium Lolium multiflorum x Festuca arundinacea	"	*cv.* Korina	N/A	N/A	N/A	Plant Breeding Station Hladke Zivotice	16 (F8)
xFestulolium Lolium multiflorum x Festuca arundinacea	"	*cv.* Felina	N/A	N/A	N/A	Plant Breeding Station Hladke Zivotice	12 (F9)
Festuca arundinacea	"	*cv.* Dovey	N/A	N/A	N/A	Barenbrug Holland BV	8 (NL1)
Festuca gigantea	"	PI 440362	Kazakhstan	Unknown	Unknown	GRIN	8 (NL2)/c.a[c].
Festuca ovina	"	PI 634304	China	N 43.28.05	E 81.06.39	GRIN	16 (NL3)/c.a.
Festuca pratensis	"	*cv.* Northland	N/A	N/A	N/A	PGG-Wrightson	8 (NL4)/c.a.
Festuca rubra	"	IRL-OP-02174	Ireland	Unknown	Unknown	Teagasc Oak Park	8 (NL5)/c.a.
Festuca vivipara	"	PI 251118	Yugoslavia	Unknown	Unknown	GRIN	16 (NL6)/c.a.
Aegilops speltoides	"	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a.
Agrostis canina	"	PI 290707	Unknown	Unknown	Unknown	GRIN	8/c.a.
Agrostis capillaris	"	PI 628720	Bulgaria	N 42.44.15	E 24.37.10	GRIN	8/c.a
Agrostis stolonifera	"	PI 439027	Uzbekhistan	Unknown	Unknown	GRIN	8/c.a
Alopecurus pratensis	"	PI 598718	Argentina	Unknown	Unknown	GRIN	8/c.a
Avena sativa	"	*cv.* Barra	N/A	N/A	N/A	Svalöf Weibull AB	1/c.a
Avena sativa	"	*cv.* Evita	N/A	N/A	N/A	Lochow-Petkus	1/c.a
Briza media	"	PI 378956	Unknown	Unknown	Unknown	GRIN	8/c.a
Bromus erectus	"	PI 619490	Hungary	Unknown	Unknown	GRIN	8/c.a

Cynosurus cristatus	"	PI 509441	Romania	N 46.57.00	E 25.34.00	GRIN	8/c.a
Dactylis glomerata	"	IRL-OP-02553	Ireland	Unknown	Unknown	Teagasc Oak Park	8/c.a
Holcus lanatus	"	W6 13845	Chile	S 53.09.00	W 70.55.00	GRIN	8/c.a
Hordeum vulgare	"	*cv.* Ludine	N/A	N/A	N/A	Joseph Breun, Germany	1/c.a
Hordeum vulgare	"	*cv.* Pewter	N/A	N/A	N/A	Cebeco Zaden BV	1/c.a
Hordeum vulgare	"	*cv.* Regina	N/A	N/A	N/A	Cebeco Zaden BV	1/c.a
Koeleria macrantha	„	PI 619546	Mongolia	N 49.27.28	E 90.06.05	GRIN	8/c.a
Phleum pratense	„	IRL-OP-02461	Ireland	N/A	N/A	Teagasc Oak Park	8/c.a
Poa palustris	„	PI 442546	Belgium	N 51.13.00	E 04.25.00	GRIN	8/c.a
Poa pratensis	„	PI 539060	Siberia	Unknown	Unknown	GRIN	8/c.a
Secale cereale	„	*cv.* Protector	N/A	N/A	N/A	Cebeco Zaden BV	1/c.a
Triticum aestivum	„	*cv.* Istabraq	N/A	N/A	N/A	Nickerson UK Ltd.	1/c.a
Triticum aestivum	„	*cv.* Robicum	N/A	N/A	N/A	CPB Twyford UK	1/c.a
x*Triticosecale*	„	*cv.* Benetto	N/A	N/A	N/A	DANKO Howdowla	1/c.a
x*Triticosecale*	„	*cv.* Ego	N/A	N/A	N/A	Semundo BV	1/c.a
x*Triticosecale*	„	*cv.* Fidelio	N/A	N/A	N/A	DANKO Howdowla	1/c.a
x*Triticosecale*	„	*cv* .Lamberto	N/A	N/A	N/A	DANKO Howdowla	1/c.a
x*Triticosecale*	„	*cv.* Lupus	N/A	N/A	N/A	Nordsaat Saatzuch GmbH	1/c.a
x*Triticosecale*	„	*cv.* SW Fargo	N/A	N/A	N/A	Svalöf Weibull AB	1/c.a
x*Triticosecale*	„	*cv.* Tricolor	N/A	N/A	N/A	Florimund Desprez	1/c.a

x*Triticosecale*	„	*cv.* Trigantus	N/A	N/A	N/A	Saatzucht Dr. Hege GbRmbH	1/c.a
Streptochaeta spicata	Anomochlooideae	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Arundo donax	Arundinoideae	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Phyllostachys flexuosa	Bambusoideae	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Phyllostacys nuda	"	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Chasmanthium latifolium	Centothecoideae	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Orthoclada laxa	"	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Chloris sp	Chloridoideae	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Eleusine coricana	"	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Eragrostis chloromatus	"	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Danthonia decumbens	Danthonioideae	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Oryza sativa	Ehrhartoideae	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Miscanthus sinensis	Panicoideae	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Pharus latifolius	"	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Saccharum arundinaceum	"	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Saccharum spontaneum	"	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Zea diploperennis	"	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a
Zea mays	"	Unknown	Unknown	Unknown	Unknown	School of Botany, TCD	1/c.a

[a]Cp: chloroplast analysis. Characters in parentheses indicate geographical group for the populations. F: x*Festulolium* variety, N/L: Non-*Lolium* species; [b]N/A: Not applicable; [c]/c.a: Population used in cross-amplification test.

8.3 Nei's (1973) chloroplast genetic identity and genetic distance matrix between all populations. Accession codes given in Appendix 8.1

	V3[a]	I28	V14	I1	I5	I17	V7	I13	I7	V4	I6	I10	V15	I21	I30	I23	I19	V8	I16	I12	V11	I20
V3	N/A[b]	0.97	0.94	0.86	0.96	0.93	0.93	0.92	0.89	0.97	0.88	0.88	0.90	0.92	0.90	0.92	0.93	0.95	0.95	0.95	0.94	0.94
I28	0.03	N/A	0.96	0.86	0.96	0.97	0.93	0.88	0.93	0.92	0.91	0.92	0.90	0.95	0.91	0.93	0.94	0.89	0.96	0.94	0.93	0.95
V14	0.07	0.04	N/A	0.82	0.97	0.92	0.88	0.85	0.88	0.93	0.85	0.86	0.85	0.87	0.85	0.89	0.91	0.87	0.92	0.89	0.88	0.95
I1	0.15	0.15	0.20	N/A	0.85	0.93	0.96	0.82	0.95	0.87	0.97	0.97	0.96	0.90	0.93	0.88	0.84	0.88	0.85	0.81	0.79	0.73
I5	0.04	0.04	0.03	0.16	N/A	0.93	0.89	0.87	0.90	0.94	0.87	0.87	0.89	0.89	0.87	0.93	0.96	0.91	0.96	0.94	0.91	0.95
I17	0.07	0.03	0.08	0.07	0.07	N/A	0.96	0.89	0.98	0.89	0.98	0.97	0.97	0.98	0.97	0.96	0.93	0.89	0.96	0.93	0.92	0.90
V7	0.08	0.07	0.13	0.04	0.11	0.04	N/A	0.83	0.94	0.91	0.95	0.96	0.94	0.94	0.93	0.87	0.88	0.90	0.88	0.86	0.83	0.81
I13	0.09	0.13	0.17	0.20	0.14	0.11	0.19	N/A	0.87	0.90	0.87	0.86	0.91	0.91	0.92	0.94	0.87	0.96	0.92	0.94	0.96	0.87
I7	0.12	0.07	0.13	0.05	0.10	0.02	0.07	0.14	N/A	0.85	0.99	0.99	0.98	0.97	0.97	0.95	0.90	0.86	0.93	0.89	0.88	0.84
V4	0.03	0.08	0.08	0.14	0.06	0.11	0.09	0.10	0.17	N/A	0.85	0.86	0.87	0.86	0.86	0.87	0.90	0.97	0.90	0.90	0.88	0.89
I6	0.13	0.09	0.16	0.03	0.14	0.02	0.05	0.13	0.01	0.16	N/A	0.99	0.98	0.97	0.98	0.93	0.88	0.88	0.90	0.87	0.87	0.80
I10	0.12	0.09	0.15	0.03	0.13	0.03	0.04	0.15	0.01	0.15	0.01	N/A	0.97	0.96	0.96	0.91	0.86	0.87	0.89	0.85	0.85	0.80
V15	0.11	0.11	0.16	0.04	0.12	0.03	0.06	0.10	0.02	0.14	0.02	0.03	N/A	0.96	0.98	0.95	0.89	0.91	0.92	0.90	0.89	0.81
I21	0.08	0.05	0.14	0.10	0.11	0.02	0.06	0.10	0.03	0.15	0.03	0.04	0.04	N/A	0.98	0.95	0.91	0.88	0.95	0.93	0.94	0.87
I30	0.10	0.09	0.17	0.07	0.14	0.03	0.07	0.08	0.03	0.15	0.02	0.04	0.02	0.02	N/A	0.96	0.90	0.91	0.93	0.92	0.92	0.84
I23	0.08	0.08	0.12	0.13	0.08	0.05	0.14	0.06	0.06	0.13	0.07	0.09	0.05	0.05	0.04	N/A	0.96	0.91	0.98	0.97	0.96	0.90
I19	0.07	0.06	0.09	0.17	0.04	0.07	0.13	0.14	0.10	0.11	0.13	0.15	0.12	0.10	0.11	0.04	N/A	0.89	0.98	0.97	0.93	0.93
V8	0.05	0.12	0.14	0.12	0.10	0.11	0.11	0.04	0.15	0.03	0.13	0.14	0.09	0.13	0.10	0.09	0.12	N/A	0.91	0.92	0.92	0.86
I16	0.05	0.04	0.09	0.17	0.04	0.05	0.13	0.09	0.07	0.11	0.10	0.11	0.08	0.05	0.07	0.02	0.02	0.10	N/A	0.98	0.97	0.95
I12	0.05	0.07	0.11	0.21	0.06	0.08	0.16	0.06	0.12	0.11	0.13	0.16	0.10	0.07	0.08	0.03	0.03	0.08	0.02	N/A	0.98	0.94
V11	0.07	0.07	0.13	0.24	0.09	0.08	0.19	0.04	0.12	0.13	0.14	0.16	0.11	0.07	0.08	0.04	0.07	0.09	0.03	0.02	N/A	0.94
I20	0.06	0.05	0.05	0.31	0.06	0.11	0.21	0.14	0.18	0.12	0.23	0.22	0.21	0.14	0.18	0.11	0.07	0.15	0.05	0.06	0.06	N/A
I11	0.04	0.08	0.07	0.10	0.06	0.09	0.06	0.13	0.13	0.03	0.12	0.12	0.11	0.13	0.12	0.11	0.09	0.05	0.10	0.10	0.15	0.13
I14	0.16	0.09	0.10	0.09	0.11	0.03	0.11	0.19	0.02	0.19	0.04	0.05	0.06	0.08	0.07	0.08	0.11	0.20	0.10	0.15	0.16	0.17
V10	0.05	0.01	0.05	0.16	0.04	0.03	0.09	0.13	0.07	0.10	0.09	0.09	0.10	0.05	0.08	0.06	0.04	0.13	0.02	0.05	0.06	0.05
I4	0.09	0.04	0.03	0.18	0.06	0.04	0.13	0.14	0.08	0.13	0.11	0.11	0.11	0.09	0.11	0.07	0.07	0.16	0.05	0.09	0.09	0.05
I2	0.16	0.08	0.09	0.16	0.11	0.04	0.13	0.21	0.05	0.22	0.08	0.09	0.10	0.07	0.09	0.08	0.08	0.24	0.07	0.12	0.13	0.12
I3	0.17	0.13	0.23	0.12	0.16	0.09	0.09	0.32	0.11	0.23	0.11	0.12	0.14	0.10	0.12	0.14	0.09	0.24	0.11	0.16	0.22	0.24
V6	0.08	0.14	0.14	0.07	0.13	0.12	0.06	0.14	0.16	0.03	0.12	0.11	0.11	0.16	0.12	0.17	0.18	0.04	0.18	0.18	0.21	0.22
I22	0.08	0.14	0.11	0.18	0.12	0.15	0.14	0.08	0.21	0.03	0.19	0.19	0.15	0.19	0.15	0.14	0.16	0.04	0.15	0.13	0.14	0.14
V9	0.07	0.11	0.07	0.24	0.11	0.13	0.18	0.08	0.21	0.06	0.21	0.22	0.17	0.18	0.16	0.12	0.13	0.07	0.12	0.09	0.10	0.07
I15	0.04	0.12	0.13	0.14	0.09	0.12	0.12	0.04	0.16	0.02	0.15	0.16	0.10	0.14	0.11	0.09	0.12	0.00	0.10	0.08	0.09	0.14

[a]V3 = Population codes given in Appendix 8.1, [b]N/A = Not applicable

	V3	I28	V14	I1	I5	I17	V7	I13	I7	V4	I6	I10	V15	I21	I30	I23	I19	V8	I16	I12	V11	I20
I27	0.01	0.05	0.09	0.13	0.07	0.07	0.06	0.10	0.13	0.03	0.12	0.12	0.11	0.09	0.10	0.10	0.09	0.05	0.07	0.08	0.09	0.09
V12	0.11	0.15	0.08	0.21	0.12	0.15	0.18	0.12	0.21	0.07	0.21	0.21	0.16	0.22	0.17	0.15	0.16	0.08	0.15	0.14	0.16	0.12
I24	0.05	0.01	0.06	0.16	0.07	0.03	0.08	0.12	0.08	0.11	0.09	0.08	0.10	0.03	0.08	0.08	0.09	0.13	0.05	0.07	0.07	0.06
■15	0.11	0.06	0.11	0.08	0.10	0.02	0.07	0.16	0.01	0.17	0.03	0.02	0.04	0.04	0.04	0.07	0.10	0.17	0.07	0.12	0.12	0.15
I18	0.19	0.13	0.15	0.13	0.23	0.08	0.08	0.26	0.11	0.21	0.08	0.08	0.13	0.11	0.10	0.20	0.24	0.25	0.21	0.25	0.26	0.24
V13	0.13	0.14	0.07	0.29	0.14	0.17	0.19	0.24	0.27	0.11	0.28	0.27	0.27	0.25	0.24	0.23	0.17	0.19	0.19	0.19	0.22	0.09
■18	0.09	0.05	0.12	0.07	0.11	0.01	0.04	0.14	0.02	0.14	0.02	0.02	0.04	0.01	0.03	0.08	0.12	0.14	0.08	0.12	0.12	0.17
I25	0.08	0.07	0.12	0.03	0.09	0.03	0.02	0.19	0.03	0.12	0.03	0.03	0.04	0.06	0.06	0.09	0.09	0.13	0.09	0.13	0.17	0.18
V16	0.09	0.19	0.20	0.17	0.15	0.20	0.14	0.14	0.24	0.09	0.21	0.21	0.15	0.20	0.18	0.18	0.21	0.06	0.19	0.16	0.20	0.24
I9	0.12	0.16	0.17	0.20	0.15	0.17	0.19	0.10	0.20	0.13	0.17	0.18	0.15	0.17	0.14	0.13	0.21	0.09	0.17	0.14	0.16	0.22
■20	0.14	0.13	0.17	0.07	0.15	0.08	0.11	0.11	0.07	0.17	0.05	0.06	0.05	0.08	0.06	0.08	0.18	0.12	0.14	0.15	0.16	0.25
■16	0.10	0.18	0.20	0.20	0.13	0.20	0.18	0.14	0.24	0.11	0.23	0.23	0.16	0.20	0.19	0.16	0.18	0.08	0.17	0.14	0.18	0.22
V2	0.16	0.25	0.23	0.22	0.20	0.26	0.22	0.21	0.29	0.15	0.26	0.25	0.22	0.28	0.24	0.24	0.28	0.13	0.26	0.24	0.28	0.29
●32	0.18	0.22	0.20	0.21	0.19	0.18	0.24	0.13	0.19	0.21	0.18	0.19	0.14	0.19	0.15	0.14	0.25	0.15	0.20	0.18	0.19	0.25
V5	0.13	0.10	0.14	0.06	0.14	0.03	0.09	0.09	0.03	0.15	0.02	0.03	0.02	0.05	0.02	0.06	0.15	0.11	0.10	0.12	0.12	0.20
△5	0.15	0.21	0.20	0.16	0.20	0.20	0.17	0.16	0.23	0.14	0.18	0.18	0.16	0.22	0.17	0.20	0.27	0.11	0.24	0.22	0.25	0.29
■19	0.11	0.08	0.06	0.09	0.09	0.03	0.10	0.11	0.05	0.12	0.05	0.06	0.05	0.08	0.06	0.06	0.12	0.12	0.09	0.12	0.12	0.13
I26	0.11	0.10	0.05	0.16	0.11	0.08	0.13	0.12	0.12	0.10	0.13	0.13	0.11	0.13	0.11	0.11	0.14	0.13	0.12	0.13	0.13	0.10
I8	0.05	0.07	0.09	0.07	0.06	0.04	0.08	0.06	0.05	0.05	0.06	0.06	0.03	0.07	0.05	0.04	0.07	0.04	0.06	0.07	0.08	0.12
V1	0.10	0.12	0.12	0.15	0.16	0.09	0.12	0.06	0.13	0.10	0.11	0.12	0.10	0.10	0.07	0.10	0.18	0.08	0.13	0.12	0.11	0.14
I29	0.06	0.04	0.07	0.08	0.09	0.02	0.05	0.07	0.04	0.08	0.04	0.04	0.04	0.04	0.03	0.06	0.11	0.08	0.07	0.09	0.08	0.11
▲12	0.08	0.13	0.12	0.16	0.09	0.18	0.12	0.20	0.23	0.04	0.22	0.20	0.20	0.22	0.22	0.21	0.17	0.08	0.18	0.18	0.23	0.20
■21	0.15	0.21	0.17	0.38	0.13	0.26	0.37	0.11	0.31	0.16	0.34	0.35	0.26	0.29	0.27	0.15	0.19	0.13	0.17	0.13	0.14	0.16
●28	0.16	0.22	0.19	0.30	0.15	0.25	0.31	0.15	0.28	0.19	0.29	0.30	0.23	0.27	0.25	0.16	0.22	0.15	0.20	0.17	0.19	0.22
▲14	0.09	0.15	0.13	0.26	0.09	0.21	0.23	0.11	0.26	0.08	0.26	0.26	0.21	0.23	0.22	0.15	0.17	0.07	0.15	0.13	0.15	0.16
△7	0.10	0.11	0.12	0.17	0.09	0.10	0.18	0.06	0.11	0.14	0.12	0.13	0.09	0.11	0.10	0.06	0.14	0.09	0.09	0.09	0.09	0.15
●29	0.15	0.20	0.16	0.35	0.12	0.25	0.35	0.11	0.29	0.16	0.31	0.33	0.24	0.28	0.25	0.14	0.18	0.12	0.16	0.13	0.14	0.16
●30	0.33	0.38	0.33	0.34	0.29	0.36	0.42	0.30	0.35	0.34	0.36	0.37	0.30	0.41	0.33	0.27	0.36	0.28	0.34	0.33	0.38	0.43
▲11	0.28	0.34	0.29	0.30	0.23	0.29	0.38	0.23	0.28	0.32	0.29	0.32	0.22	0.33	0.27	0.18	0.28	0.23	0.26	0.24	0.29	0.34
△2	0.23	0.29	0.25	0.28	0.19	0.28	0.34	0.24	0.28	0.26	0.30	0.32	0.23	0.33	0.27	0.19	0.24	0.21	0.24	0.23	0.28	0.31
△6	0.32	0.37	0.36	0.25	0.30	0.28	0.35	0.26	0.26	0.37	0.25	0.28	0.19	0.31	0.23	0.20	0.33	0.27	0.30	0.29	0.33	0.44
▲13	0.30	0.36	0.31	0.29	0.26	0.33	0.38	0.26	0.31	0.31	0.31	0.33	0.25	0.37	0.29	0.23	0.33	0.24	0.31	0.29	0.34	0.41
■17	0.55	0.67	0.63	0.46	0.54	0.64	0.58	0.56	0.60	0.51	0.56	0.58	0.51	0.69	0.57	0.51	0.61	0.45	0.64	0.59	0.71	0.83

	V3	I28	V14	I1	I5	I17	V7	I13	I7	V4	I6	I10	V15	I21	I30	I23	I19	V8	I16	I12	V11	I20
●31	0.19	0.24	0.17	0.37	0.17	0.28	0.32	0.33	0.35	0.24	0.39	0.40	0.32	0.36	0.35	0.25	0.21	0.26	0.24	0.23	0.30	0.19
○23	0.23	0.31	0.21	0.37	0.18	0.35	0.39	0.27	0.39	0.22	0.41	0.42	0.32	0.42	0.37	0.25	0.26	0.21	0.28	0.24	0.31	0.26
□8	0.20	0.22	0.20	0.37	0.17	0.27	0.35	0.25	0.30	0.28	0.33	0.35	0.28	0.31	0.29	0.19	0.23	0.25	0.21	0.19	0.25	0.23
△4	0.20	0.21	0.22	0.16	0.21	0.14	0.21	0.14	0.14	0.24	0.12	0.15	0.10	0.16	0.11	0.10	0.23	0.17	0.19	0.17	0.19	0.28
■22	0.17	0.23	0.14	0.26	0.13	0.25	0.27	0.25	0.30	0.16	0.31	0.31	0.25	0.34	0.29	0.21	0.20	0.17	0.23	0.21	0.28	0.22
L9	0.25	0.28	0.24	0.30	0.26	0.29	0.32	0.25	0.32	0.26	0.30	0.31	0.26	0.34	0.26	0.26	0.33	0.24	0.31	0.28	0.33	0.33
○27	0.17	0.24	0.19	0.16	0.16	0.21	0.21	0.18	0.22	0.14	0.21	0.22	0.15	0.27	0.19	0.16	0.22	0.11	0.22	0.20	0.26	0.29
△1	0.13	0.17	0.14	0.21	0.13	0.14	0.24	0.05	0.16	0.14	0.17	0.19	0.11	0.17	0.12	0.07	0.16	0.09	0.12	0.10	0.10	0.15
○26	0.16	0.18	0.17	0.22	0.15	0.15	0.26	0.06	0.16	0.18	0.17	0.19	0.12	0.17	0.12	0.08	0.18	0.12	0.13	0.11	0.11	0.19
□9	0.21	0.24	0.21	0.20	0.18	0.18	0.28	0.11	0.18	0.21	0.18	0.20	0.12	0.22	0.15	0.10	0.20	0.14	0.17	0.16	0.17	0.25
□10	0.38	0.41	0.31	0.41	0.31	0.39	0.49	0.31	0.39	0.37	0.41	0.43	0.33	0.46	0.36	0.29	0.38	0.32	0.36	0.34	0.39	0.40
○24	0.19	0.20	0.19	0.20	0.19	0.14	0.23	0.10	0.15	0.19	0.16	0.18	0.11	0.17	0.11	0.10	0.19	0.14	0.15	0.14	0.14	0.21
L10	0.44	0.35	0.31	0.49	0.45	0.37	0.41	0.51	0.43	0.43	0.43	0.41	0.47	0.44	0.42	0.50	0.51	0.53	0.48	0.51	0.52	0.40
△3	0.42	0.32	0.32	0.58	0.46	0.31	0.46	0.38	0.37	0.51	0.38	0.38	0.43	0.32	0.35	0.39	0.48	0.54	0.39	0.41	0.34	0.32
○25	0.42	0.31	0.31	0.52	0.46	0.30	0.42	0.41	0.35	0.50	0.35	0.34	0.41	0.31	0.33	0.39	0.48	0.54	0.40	0.43	0.37	0.34
L2	0.46	0.38	0.33	0.58	0.47	0.39	0.50	0.47	0.46	0.48	0.47	0.46	0.49	0.45	0.44	0.48	0.52	0.54	0.46	0.48	0.47	0.37
F1	0.21	0.18	0.17	0.32	0.21	0.21	0.28	0.21	0.25	0.25	0.25	0.25	0.25	0.23	0.22	0.21	0.28	0.25	0.23	0.22	0.23	0.22
F5	0.15	0.15	0.07	0.36	0.14	0.23	0.25	0.25	0.33	0.15	0.33	0.32	0.32	0.30	0.31	0.26	0.24	0.22	0.23	0.22	0.25	0.13
F6	0.23	0.20	0.16	0.16	0.25	0.15	0.15	0.25	0.18	0.22	0.15	0.15	0.17	0.20	0.15	0.23	0.31	0.24	0.28	0.28	0.31	0.29
F7	0.19	0.20	0.16	0.29	0.23	0.21	0.24	0.18	0.26	0.20	0.24	0.25	0.23	0.24	0.20	0.22	0.30	0.20	0.25	0.22	0.23	0.23
F8	0.11	0.11	0.04	0.31	0.12	0.18	0.19	0.21	0.27	0.10	0.28	0.26	0.28	0.24	0.25	0.23	0.20	0.18	0.19	0.18	0.20	0.09
F2	0.22	0.20	0.15	0.31	0.22	0.21	0.27	0.22	0.26	0.23	0.25	0.26	0.25	0.26	0.22	0.23	0.29	0.25	0.25	0.23	0.26	0.23
F9	0.15	0.14	0.18	0.21	0.16	0.16	0.20	0.16	0.18	0.18	0.18	0.18	0.17	0.17	0.17	0.16	0.21	0.17	0.17	0.17	0.19	0.23
F3	0.21	0.21	0.21	0.25	0.20	0.23	0.24	0.23	0.24	0.22	0.23	0.23	0.23	0.25	0.23	0.22	0.28	0.22	0.24	0.24	0.28	0.30
F4	0.30	0.31	0.26	0.31	0.34	0.33	0.26	0.39	0.39	0.26	0.34	0.33	0.36	0.38	0.34	0.42	0.46	0.31	0.44	0.41	0.48	0.41
L11	0.46	0.44	0.39	0.42	0.41	0.41	0.49	0.43	0.40	0.41	0.42	0.41	0.40	0.48	0.44	0.42	0.49	0.43	0.46	0.48	0.50	0.50
L1	0.14	0.09	0.03	0.32	0.11	0.14	0.20	0.27	0.22	0.15	0.26	0.24	0.27	0.22	0.26	0.22	0.16	0.26	0.16	0.20	0.21	0.07
L3	0.25	0.22	0.26	0.36	0.27	0.24	0.32	0.21	0.27	0.27	0.28	0.28	0.27	0.24	0.23	0.25	0.29	0.26	0.24	0.24	0.22	0.26
L4	0.48	0.45	0.40	0.47	0.38	0.43	0.57	0.41	0.39	0.48	0.44	0.45	0.40	0.50	0.46	0.37	0.46	0.46	0.42	0.44	0.46	0.50
L5	0.31	0.23	0.25	0.36	0.25	0.23	0.36	0.33	0.22	0.37	0.27	0.25	0.28	0.26	0.28	0.25	0.27	0.39	0.24	0.30	0.28	0.29
L6	0.34	0.24	0.23	0.62	0.36	0.30	0.45	0.38	0.38	0.43	0.42	0.41	0.47	0.33	0.37	0.38	0.40	0.51	0.33	0.36	0.31	0.22
L7	0.30	0.28	0.30	0.40	0.28	0.27	0.41	0.23	0.27	0.34	0.30	0.30	0.28	0.28	0.27	0.24	0.31	0.30	0.25	0.27	0.24	0.31
L8	0.17	0.13	0.25	0.26	0.24	0.17	0.14	0.31	0.22	0.23	0.20	0.19	0.26	0.14	0.20	0.27	0.24	0.27	0.21	0.24	0.25	0.26

	V3	I28	V14	I1	I5	I17	V7	I13	I7	V4	I6	I10	V15	I21	I30	I23	I19	V8	I16	I12	V11	I20
NL2	0.30	0.27	0.20	0.32	0.24	0.24	0.35	0.28	0.25	0.30	0.28	0.28	0.25	0.31	0.27	0.24	0.29	0.31	0.27	0.27	0.30	0.28
NL3	0.39	0.39	0.42	0.63	0.39	0.44	0.60	0.33	0.46	0.42	0.50	0.53	0.49	0.45	0.47	0.33	0.39	0.42	0.35	0.33	0.31	0.38
NL4	0.73	0.67	0.50	0.69	0.66	0.60	0.74	0.67	0.63	0.64	0.66	0.65	0.63	0.74	0.65	0.66	0.73	0.72	0.71	0.72	0.75	0.62
NL5	0.43	0.45	0.40	0.55	0.37	0.46	0.59	0.43	0.46	0.41	0.51	0.53	0.48	0.54	0.53	0.36	0.38	0.43	0.39	0.39	0.41	0.43
NL6	0.57	0.54	0.60	0.79	0.59	0.59	0.79	0.45	0.59	0.65	0.61	0.65	0.62	0.56	0.58	0.45	0.59	0.60	0.51	0.48	0.45	0.58

	I11	I14	V10	I4	I2	I3	V6	I22	V9	I15	I27	V12	I24	■15	I18	V13	■18	I25	V16	I9	■20	■16
V3	0.96	0.85	0.95	0.91	0.86	0.84	0.92	0.92	0.93	0.96	0.99	0.90	0.95	0.89	0.82	0.87	0.92	0.92	0.91	0.89	0.87	0.91
I28	0.93	0.91	0.99	0.96	0.93	0.87	0.87	0.87	0.89	0.89	0.95	0.86	0.99	0.94	0.88	0.87	0.95	0.93	0.83	0.85	0.87	0.83
V14	0.93	0.90	0.95	0.97	0.91	0.79	0.87	0.90	0.93	0.88	0.92	0.92	0.94	0.90	0.86	0.93	0.88	0.89	0.82	0.84	0.84	0.82
I1	0.91	0.92	0.86	0.83	0.85	0.89	0.93	0.84	0.78	0.87	0.88	0.81	0.85	0.93	0.88	0.75	0.93	0.97	0.85	0.82	0.93	0.82
I5	0.94	0.90	0.96	0.95	0.90	0.85	0.87	0.88	0.90	0.91	0.93	0.89	0.93	0.91	0.79	0.87	0.89	0.92	0.86	0.86	0.86	0.88
I17	0.91	0.97	0.97	0.96	0.96	0.91	0.89	0.86	0.87	0.89	0.93	0.86	0.97	0.98	0.92	0.84	0.99	0.97	0.82	0.84	0.93	0.82
V7	0.94	0.90	0.92	0.87	0.87	0.91	0.94	0.87	0.83	0.89	0.94	0.84	0.93	0.93	0.92	0.83	0.97	0.98	0.87	0.83	0.90	0.84
I13	0.88	0.83	0.87	0.87	0.81	0.73	0.87	0.92	0.92	0.96	0.91	0.89	0.89	0.85	0.77	0.78	0.87	0.83	0.87	0.91	0.90	0.87
I7	0.88	0.98	0.93	0.93	0.95	0.90	0.86	0.81	0.81	0.85	0.88	0.81	0.93	0.99	0.90	0.76	0.98	0.97	0.79	0.82	0.94	0.79
V4	0.97	0.82	0.90	0.88	0.80	0.79	0.97	0.97	0.94	0.98	0.97	0.94	0.90	0.85	0.81	0.89	0.87	0.89	0.92	0.88	0.85	0.89
I6	0.89	0.96	0.91	0.89	0.92	0.90	0.89	0.83	0.81	0.86	0.89	0.81	0.92	0.97	0.92	0.76	0.98	0.97	0.81	0.84	0.95	0.80
I10	0.89	0.96	0.91	0.90	0.91	0.89	0.89	0.82	0.80	0.85	0.89	0.81	0.92	0.98	0.92	0.77	0.98	0.97	0.81	0.83	0.94	0.80
V15	0.90	0.95	0.90	0.89	0.91	0.87	0.90	0.86	0.84	0.90	0.90	0.85	0.90	0.96	0.88	0.76	0.96	0.96	0.86	0.86	0.96	0.85
I21	0.88	0.93	0.95	0.91	0.93	0.91	0.85	0.83	0.84	0.87	0.92	0.80	0.97	0.97	0.90	0.78	0.99	0.95	0.82	0.84	0.92	0.82
I30	0.89	0.93	0.92	0.90	0.91	0.89	0.88	0.86	0.86	0.90	0.91	0.84	0.93	0.96	0.90	0.78	0.97	0.95	0.84	0.87	0.94	0.83
I23	0.90	0.93	0.94	0.93	0.92	0.87	0.84	0.87	0.89	0.91	0.90	0.86	0.92	0.93	0.82	0.79	0.92	0.92	0.83	0.87	0.92	0.85
I19	0.91	0.89	0.97	0.93	0.92	0.91	0.84	0.85	0.88	0.89	0.92	0.86	0.91	0.90	0.79	0.84	0.89	0.92	0.81	0.81	0.84	0.83
V8	0.95	0.82	0.88	0.85	0.78	0.79	0.96	0.96	0.93	1.00	0.96	0.92	0.88	0.84	0.78	0.83	0.87	0.88	0.94	0.91	0.89	0.92
I16	0.91	0.91	0.98	0.95	0.93	0.89	0.84	0.86	0.89	0.90	0.93	0.86	0.95	0.93	0.81	0.83	0.92	0.91	0.82	0.84	0.87	0.84
I12	0.90	0.86	0.95	0.92	0.89	0.85	0.84	0.88	0.91	0.92	0.93	0.87	0.93	0.89	0.78	0.83	0.89	0.88	0.85	0.87	0.86	0.87
V11	0.86	0.85	0.94	0.91	0.88	0.80	0.81	0.87	0.91	0.92	0.91	0.85	0.93	0.88	0.77	0.80	0.89	0.85	0.82	0.85	0.86	0.83
I20	0.88	0.84	0.95	0.95	0.89	0.79	0.80	0.87	0.93	0.87	0.91	0.89	0.94	0.86	0.79	0.91	0.85	0.83	0.78	0.80	0.78	0.81
I11	N/A	0.86	0.91	0.89	0.84	0.85	0.97	0.95	0.92	0.96	0.96	0.93	0.91	0.87	0.85	0.89	0.89	0.94	0.94	0.91	0.90	0.93
I14	0.15	N/A	0.93	0.95	0.98	0.87	0.83	0.81	0.82	0.82	0.84	0.84	0.90	0.97	0.90	0.80	0.94	0.94	0.74	0.78	0.91	0.74
V10	0.10	0.08	N/A	0.97	0.95	0.91	0.85	0.85	0.88	0.88	0.93	0.86	0.98	0.95	0.87	0.87	0.95	0.93	0.79	0.81	0.85	0.80
I4	0.12	0.05	0.03	N/A	0.97	0.83	0.83	0.87	0.92	0.86	0.90	0.91	0.95	0.94	0.89	0.90	0.91	0.90	0.75	0.79	0.85	0.76

	I11	I14	V10	I4	I2	I3	V6	I22	V9	I15	I27	V12	I24	■15	I18	V13	■18	I25	V16	I9	■20	■16
I2	0.18	0.02	0.05	0.03	N/A	0.89	0.78	0.78	0.83	0.79	0.85	0.83	0.92	0.96	0.90	0.84	0.92	0.92	0.69	0.74	0.85	0.71
I3	0.16	0.14	0.09	0.19	0.12	N/A	0.80	0.72	0.71	0.77	0.85	0.71	0.86	0.90	0.83	0.73	0.90	0.94	0.74	0.71	0.81	0.75
V6	0.03	0.19	0.16	0.19	0.25	0.22	N/A	0.96	0.90	0.96	0.94	0.92	0.86	0.85	0.86	0.86	0.88	0.91	0.93	0.87	0.88	0.89
I22	0.05	0.21	0.16	0.14	0.24	0.33	0.04	N/A	0.98	0.98	0.94	0.98	0.86	0.81	0.82	0.92	0.83	0.84	0.90	0.88	0.84	0.88
V9	0.08	0.19	0.12	0.09	0.18	0.34	0.11	0.02	N/A	0.95	0.93	0.98	0.89	0.82	0.82	0.94	0.82	0.82	0.85	0.85	0.81	0.84
I15	0.05	0.20	0.13	0.15	0.24	0.26	0.05	0.02	0.05	N/A	0.96	0.94	0.88	0.84	0.78	0.85	0.86	0.87	0.93	0.90	0.88	0.92
I27	0.04	0.17	0.07	0.11	0.17	0.16	0.06	0.07	0.07	0.04	N/A	0.90	0.94	0.88	0.85	0.88	0.92	0.93	0.90	0.87	0.86	0.88
V12	0.08	0.17	0.16	0.10	0.19	0.35	0.09	0.02	0.02	0.06	0.10	N/A	0.85	0.82	0.83	0.95	0.80	0.83	0.85	0.83	0.82	0.83
I24	0.10	0.10	0.02	0.05	0.08	0.15	0.15	0.15	0.12	0.13	0.06	0.17	N/A	0.94	0.91	0.87	0.97	0.92	0.82	0.85	0.88	0.82
■15	0.14	0.03	0.05	0.06	0.04	0.11	0.17	0.21	0.20	0.18	0.12	0.20	0.06	N/A	0.91	0.80	0.97	0.96	0.78	0.81	0.91	0.78
I18	0.16	0.10	0.13	0.12	0.11	0.19	0.15	0.19	0.20	0.25	0.16	0.19	0.09	0.10	N/A	0.86	0.93	0.90	0.73	0.76	0.85	0.70
V13	0.11	0.22	0.14	0.10	0.18	0.31	0.15	0.08	0.06	0.16	0.12	0.05	0.14	0.23	0.15	N/A	0.79	0.81	0.77	0.74	0.72	0.75
■18	0.11	0.06	0.05	0.09	0.08	0.10	0.13	0.19	0.20	0.15	0.09	0.22	0.03	0.03	0.07	0.23	N/A	0.96	0.81	0.83	0.92	0.80
I25	0.07	0.06	0.07	0.10	0.08	0.06	0.10	0.17	0.19	0.14	0.08	0.19	0.08	0.04	0.11	0.21	0.04	N/A	0.85	0.83	0.92	0.84
V16	0.06	0.31	0.23	0.29	0.37	0.30	0.07	0.10	0.16	0.07	0.11	0.17	0.19	0.25	0.31	0.26	0.21	0.16	N/A	0.95	0.89	0.99
I9	0.10	0.24	0.21	0.23	0.30	0.34	0.13	0.13	0.17	0.10	0.14	0.18	0.16	0.21	0.28	0.30	0.18	0.18	0.05	N/A	0.94	0.95
■20	0.11	0.10	0.16	0.16	0.16	0.21	0.13	0.17	0.21	0.13	0.15	0.20	0.13	0.09	0.16	0.33	0.08	0.08	0.12	0.06	N/A	0.89
■16	0.08	0.30	0.22	0.27	0.34	0.29	0.12	0.13	0.18	0.09	0.13	0.19	0.19	0.25	0.36	0.29	0.22	0.17	0.01	0.05	0.12	N/A
V2	0.10	0.34	0.31	0.34	0.42	0.38	0.13	0.15	0.21	0.13	0.18	0.20	0.26	0.30	0.35	0.30	0.28	0.21	0.03	0.05	0.12	0.03
●32	0.14	0.21	0.25	0.23	0.27	0.38	0.18	0.16	0.18	0.15	0.21	0.18	0.20	0.21	0.26	0.30	0.21	0.20	0.10	0.04	0.05	0.08
V5	0.12	0.04	0.11	0.09	0.08	0.18	0.12	0.14	0.15	0.12	0.13	0.15	0.09	0.05	0.09	0.24	0.04	0.07	0.20	0.14	0.03	0.21
△5	0.09	0.27	0.27	0.28	0.35	0.35	0.10	0.13	0.19	0.12	0.16	0.18	0.20	0.24	0.24	0.28	0.21	0.18	0.04	0.03	0.07	0.05
■19	0.09	0.03	0.08	0.04	0.05	0.20	0.11	0.10	0.09	0.12	0.11	0.08	0.08	0.05	0.08	0.13	0.07	0.07	0.20	0.15	0.07	0.20
I26	0.10	0.08	0.10	0.04	0.09	0.27	0.12	0.06	0.04	0.11	0.11	0.03	0.09	0.11	0.09	0.06	0.13	0.13	0.23	0.19	0.15	0.24
I8	0.05	0.07	0.07	0.07	0.11	0.17	0.07	0.07	0.08	0.04	0.06	0.08	0.09	0.07	0.17	0.18	0.07	0.06	0.12	0.12	0.07	0.13
V1	0.10	0.14	0.13	0.10	0.16	0.29	0.09	0.05	0.05	0.07	0.09	0.06	0.09	0.14	0.09	0.12	0.11	0.15	0.15	0.12	0.10	0.18
I29	0.07	0.06	0.05	0.05	0.08	0.17	0.08	0.09	0.09	0.08	0.06	0.10	0.03	0.04	0.06	0.14	0.03	0.06	0.16	0.13	0.07	0.18
▲12	0.03	0.27	0.18	0.22	0.32	0.26	0.06	0.10	0.16	0.08	0.08	0.15	0.16	0.23	0.27	0.19	0.19	0.14	0.05	0.09	0.16	0.07
■21	0.16	0.32	0.24	0.22	0.34	0.50	0.25	0.15	0.13	0.12	0.21	0.16	0.24	0.33	0.50	0.30	0.34	0.32	0.13	0.08	0.18	0.10
●28	0.14	0.30	0.27	0.26	0.34	0.43	0.23	0.18	0.19	0.14	0.21	0.20	0.25	0.30	0.44	0.34	0.30	0.25	0.09	0.04	0.12	0.06
▲14	0.07	0.29	0.20	0.22	0.33	0.37	0.13	0.10	0.12	0.07	0.12	0.14	0.18	0.27	0.38	0.24	0.25	0.22	0.05	0.04	0.14	0.04
△7	0.11	0.13	0.14	0.13	0.17	0.28	0.16	0.13	0.13	0.09	0.14	0.15	0.12	0.13	0.25	0.27	0.13	0.14	0.11	0.04	0.04	0.09

	I11	I14	V10	I4	I2	I3	V6	I22	V9	I15	I27	V12	I24	■15	I18	V13	■18	I25	V16	I9	■20	■16
●29	0.15	0.29	0.23	0.21	0.32	0.47	0.24	0.14	0.13	0.11	0.20	0.15	0.24	0.31	0.48	0.29	0.32	0.29	0.12	0.07	0.16	0.09
●30	0.26	0.35	0.43	0.39	0.44	0.53	0.34	0.32	0.35	0.29	0.37	0.33	0.43	0.34	0.54	0.52	0.43	0.33	0.21	0.15	0.19	0.19
▲11	0.22	0.27	0.36	0.31	0.33	0.46	0.32	0.28	0.28	0.23	0.32	0.26	0.38	0.32	0.50	0.46	0.37	0.27	0.18	0.12	0.13	0.13
△2	0.17	0.28	0.33	0.30	0.33	0.41	0.28	0.25	0.26	0.20	0.27	0.24	0.35	0.32	0.49	0.41	0.36	0.24	0.15	0.11	0.14	0.10
△6	0.25	0.26	0.40	0.36	0.34	0.43	0.32	0.33	0.35	0.27	0.35	0.32	0.40	0.30	0.44	0.55	0.34	0.24	0.20	0.13	0.10	0.16
▲13	0.22	0.31	0.40	0.36	0.40	0.48	0.29	0.28	0.31	0.24	0.34	0.28	0.40	0.34	0.50	0.48	0.39	0.29	0.17	0.12	0.14	0.14
■17	0.40	0.65	0.75	0.76	0.81	0.73	0.45	0.52	0.64	0.47	0.58	0.57	0.73	0.65	0.79	0.83	0.66	0.51	0.33	0.32	0.36	0.33
●31	0.16	0.30	0.27	0.22	0.28	0.39	0.30	0.23	0.18	0.23	0.20	0.18	0.30	0.35	0.41	0.21	0.39	0.24	0.22	0.23	0.30	0.18
○23	0.16	0.36	0.35	0.30	0.39	0.52	0.27	0.20	0.19	0.19	0.26	0.18	0.37	0.41	0.53	0.30	0.45	0.31	0.14	0.13	0.22	0.11
□8	0.19	0.30	0.27	0.26	0.31	0.41	0.34	0.29	0.25	0.24	0.24	0.27	0.28	0.30	0.47	0.37	0.34	0.26	0.19	0.11	0.19	0.14
△4	0.15	0.15	0.24	0.20	0.20	0.32	0.20	0.19	0.20	0.17	0.21	0.19	0.20	0.17	0.22	0.33	0.18	0.15	0.15	0.08	0.04	0.14
■22	0.09	0.26	0.27	0.22	0.30	0.39	0.18	0.15	0.15	0.15	0.19	0.12	0.29	0.31	0.37	0.21	0.34	0.21	0.12	0.12	0.18	0.10
L9	0.20	0.30	0.34	0.29	0.34	0.48	0.24	0.20	0.22	0.23	0.27	0.20	0.31	0.31	0.33	0.30	0.34	0.28	0.19	0.14	0.18	0.19
○27	0.09	0.22	0.27	0.25	0.30	0.35	0.12	0.12	0.17	0.11	0.18	0.13	0.28	0.25	0.32	0.27	0.27	0.17	0.07	0.07	0.10	0.07
△1	0.13	0.15	0.18	0.13	0.19	0.37	0.16	0.08	0.07	0.08	0.15	0.07	0.18	0.18	0.27	0.21	0.20	0.20	0.15	0.09	0.10	0.14
○26	0.16	0.17	0.19	0.16	0.20	0.37	0.20	0.13	0.12	0.11	0.19	0.13	0.19	0.17	0.29	0.29	0.20	0.21	0.16	0.08	0.09	0.14
□9	0.17	0.16	0.25	0.19	0.22	0.38	0.20	0.15	0.16	0.13	0.23	0.14	0.26	0.20	0.33	0.31	0.25	0.21	0.18	0.12	0.10	0.16
□10	0.29	0.35	0.45	0.36	0.41	0.61	0.38	0.29	0.30	0.31	0.42	0.27	0.45	0.40	0.52	0.44	0.49	0.39	0.27	0.18	0.22	0.23
○24	0.19	0.14	0.19	0.14	0.16	0.33	0.20	0.13	0.12	0.13	0.19	0.11	0.21	0.15	0.24	0.24	0.20	0.19	0.25	0.20	0.15	0.25
L10	0.43	0.36	0.37	0.31	0.35	0.57	0.43	0.40	0.36	0.50	0.42	0.34	0.36	0.37	0.28	0.29	0.40	0.43	0.64	0.50	0.51	0.67
△3	0.51	0.33	0.31	0.25	0.28	0.60	0.55	0.43	0.34	0.51	0.43	0.38	0.27	0.34	0.26	0.33	0.33	0.48	0.73	0.53	0.46	0.71
○25	0.49	0.30	0.31	0.25	0.27	0.56	0.52	0.44	0.36	0.52	0.43	0.39	0.26	0.32	0.22	0.33	0.31	0.43	0.71	0.52	0.43	0.70
L2	0.50	0.37	0.38	0.30	0.35	0.65	0.51	0.41	0.34	0.51	0.46	0.34	0.37	0.40	0.33	0.31	0.44	0.50	0.72	0.59	0.54	0.72
F1	0.21	0.25	0.22	0.20	0.26	0.41	0.28	0.23	0.21	0.25	0.24	0.24	0.18	0.23	0.25	0.28	0.23	0.26	0.23	0.13	0.17	0.23
F5	0.11	0.27	0.20	0.15	0.26	0.44	0.20	0.12	0.10	0.19	0.17	0.10	0.16	0.30	0.22	0.08	0.28	0.26	0.19	0.16	0.25	0.19
F6	0.14	0.15	0.23	0.18	0.19	0.32	0.15	0.16	0.18	0.23	0.21	0.15	0.17	0.17	0.07	0.17	0.16	0.16	0.21	0.16	0.11	0.24
F7	0.17	0.25	0.24	0.20	0.27	0.42	0.19	0.14	0.14	0.18	0.20	0.15	0.18	0.22	0.19	0.19	0.23	0.25	0.19	0.13	0.17	0.21
F8	0.09	0.23	0.14	0.11	0.21	0.37	0.15	0.09	0.06	0.15	0.12	0.07	0.11	0.24	0.16	0.03	0.22	0.22	0.20	0.18	0.25	0.21
F2	0.20	0.23	0.23	0.18	0.24	0.41	0.24	0.19	0.17	0.24	0.23	0.17	0.20	0.23	0.21	0.20	0.25	0.25	0.26	0.18	0.20	0.27
F9	0.16	0.21	0.18	0.21	0.25	0.29	0.20	0.22	0.23	0.18	0.18	0.25	0.16	0.17	0.28	0.34	0.17	0.18	0.17	0.11	0.12	0.17
F3	0.17	0.26	0.26	0.27	0.31	0.35	0.23	0.25	0.27	0.22	0.23	0.28	0.23	0.23	0.31	0.36	0.24	0.22	0.17	0.11	0.14	0.17
F4	0.20	0.39	0.38	0.37	0.44	0.46	0.21	0.24	0.29	0.31	0.28	0.27	0.30	0.36	0.23	0.26	0.33	0.30	0.22	0.20	0.25	0.26

	I11	I14	V10	I4	I2	I3	V6	I22	V9	I15	I27	V12	I24	■15	I18	V13	■18	I25	V16	I9	■20	■16
L11	0.46	0.35	0.44	0.37	0.42	0.61	0.44	0.42	0.42	0.43	0.49	0.38	0.50	0.40	0.51	0.51	0.48	0.45	0.58	0.50	0.43	0.59
L1	0.16	0.16	0.10	0.05	0.12	0.31	0.23	0.16	0.10	0.23	0.15	0.11	0.10	0.18	0.15	0.04	0.19	0.20	0.37	0.34	0.33	0.38
L3	0.31	0.27	0.22	0.23	0.28	0.41	0.32	0.27	0.24	0.26	0.26	0.28	0.24	0.24	0.35	0.34	0.27	0.32	0.41	0.29	0.32	0.41
L4	0.49	0.36	0.47	0.39	0.42	0.65	0.54	0.50	0.47	0.46	0.55	0.45	0.51	0.41	0.64	0.64	0.50	0.47	0.54	0.41	0.36	0.51
L5	0.39	0.20	0.22	0.20	0.21	0.38	0.45	0.43	0.38	0.39	0.35	0.39	0.27	0.20	0.39	0.45	0.26	0.29	0.56	0.43	0.33	0.53
L6	0.46	0.33	0.25	0.20	0.26	0.57	0.54	0.40	0.29	0.48	0.37	0.35	0.22	0.33	0.29	0.26	0.34	0.46	0.70	0.53	0.51	0.69
L7	0.38	0.27	0.27	0.25	0.30	0.49	0.41	0.35	0.31	0.31	0.35	0.34	0.31	0.27	0.47	0.49	0.32	0.36	0.44	0.32	0.29	0.42
L8	0.23	0.29	0.14	0.26	0.27	0.17	0.25	0.34	0.35	0.29	0.17	0.42	0.12	0.19	0.21	0.33	0.13	0.19	0.33	0.32	0.30	0.36
NL1	0.61	0.38	0.51	0.34	0.36	0.71	0.67	0.60	0.51	0.74	0.68	0.44	0.56	0.46	0.43	0.41	0.57	0.56	1.05	0.89	0.69	1.07
NL2	0.28	0.20	0.27	0.18	0.22	0.44	0.32	0.26	0.22	0.29	0.32	0.20	0.30	0.23	0.31	0.28	0.31	0.29	0.40	0.31	0.26	0.39
NL3	0.51	0.50	0.40	0.39	0.49	0.69	0.57	0.47	0.41	0.41	0.45	0.48	0.41	0.46	0.68	0.59	0.48	0.57	0.63	0.53	0.53	0.62
NL4	0.69	0.47	0.64	0.44	0.49	0.93	0.65	0.53	0.47	0.67	0.73	0.40	0.70	0.59	0.53	0.45	0.73	0.70	0.98	0.84	0.72	1.01
NL5	0.46	0.44	0.44	0.37	0.46	0.67	0.52	0.45	0.41	0.42	0.49	0.40	0.50	0.49	0.68	0.52	0.54	0.53	0.64	0.60	0.55	0.62
NL6	0.71	0.65	0.57	0.56	0.64	0.87	0.79	0.68	0.61	0.61	0.65	0.71	0.54	0.57	0.82	0.86	0.59	0.74	0.78	0.59	0.60	0.76

	V2	●32	V5	△5	■19	I26	I8	V1	I29	▲12	■21	●28	▲14	△7	●29	●30	▲11	△2	△6	▲13	■17	●31
V3	0.85	0.83	0.88	0.86	0.90	0.90	0.95	0.90	0.94	0.93	0.86	0.85	0.92	0.90	0.86	0.72	0.76	0.79	0.72	0.74	0.57	0.83
I28	0.78	0.81	0.90	0.81	0.92	0.91	0.93	0.89	0.96	0.88	0.81	0.80	0.86	0.89	0.82	0.68	0.71	0.75	0.69	0.70	0.51	0.79
V14	0.79	0.82	0.87	0.82	0.94	0.95	0.92	0.89	0.93	0.89	0.85	0.83	0.88	0.89	0.85	0.72	0.75	0.78	0.70	0.73	0.53	0.84
I1	0.81	0.81	0.94	0.85	0.91	0.85	0.94	0.86	0.92	0.85	0.68	0.74	0.77	0.85	0.70	0.71	0.74	0.76	0.78	0.75	0.63	0.69
I5	0.82	0.82	0.87	0.82	0.91	0.90	0.95	0.85	0.92	0.91	0.88	0.86	0.91	0.91	0.89	0.75	0.79	0.83	0.74	0.77	0.58	0.85
I17	0.77	0.83	0.97	0.82	0.97	0.93	0.96	0.92	0.98	0.83	0.77	0.78	0.81	0.90	0.78	0.70	0.75	0.76	0.75	0.72	0.53	0.75
V7	0.80	0.79	0.92	0.85	0.91	0.87	0.93	0.88	0.95	0.89	0.69	0.73	0.80	0.83	0.71	0.66	0.68	0.72	0.71	0.69	0.56	0.72
I13	0.81	0.88	0.92	0.85	0.89	0.89	0.94	0.94	0.93	0.82	0.89	0.86	0.89	0.94	0.90	0.74	0.79	0.78	0.77	0.77	0.57	0.72
I7	0.75	0.83	0.97	0.80	0.95	0.89	0.95	0.88	0.96	0.79	0.73	0.76	0.77	0.89	0.75	0.70	0.76	0.75	0.77	0.73	0.55	0.70
V4	0.86	0.81	0.86	0.87	0.89	0.90	0.95	0.90	0.92	0.96	0.85	0.83	0.92	0.87	0.85	0.71	0.73	0.77	0.69	0.74	0.60	0.79
I6	0.77	0.84	0.98	0.83	0.95	0.88	0.94	0.90	0.97	0.81	0.71	0.75	0.77	0.89	0.73	0.70	0.75	0.74	0.78	0.73	0.57	0.68
I10	0.78	0.83	0.97	0.83	0.94	0.88	0.94	0.89	0.96	0.82	0.71	0.74	0.77	0.88	0.72	0.69	0.73	0.73	0.76	0.72	0.56	0.67
V15	0.80	0.87	0.98	0.85	0.95	0.89	0.97	0.91	0.96	0.82	0.77	0.80	0.81	0.92	0.79	0.74	0.80	0.80	0.82	0.78	0.60	0.73
I21	0.75	0.82	0.95	0.81	0.92	0.88	0.93	0.90	0.96	0.80	0.75	0.76	0.79	0.89	0.76	0.66	0.72	0.72	0.74	0.69	0.50	0.70
I30	0.79	0.86	0.98	0.84	0.94	0.90	0.95	0.93	0.97	0.80	0.76	0.78	0.80	0.91	0.78	0.72	0.77	0.76	0.80	0.75	0.57	0.71
I23	0.79	0.87	0.95	0.82	0.94	0.90	0.96	0.90	0.94	0.81	0.86	0.85	0.86	0.94	0.87	0.76	0.83	0.83	0.82	0.79	0.60	0.78

	V2	●32	V5	△5	■19	I26	I8	V1	I29	▲12	■21	●28	▲14	△7	●29	●30	▲11	△2	△6	▲13	■17	●31
I19	0.76	0.78	0.86	0.76	0.89	0.87	0.93	0.83	0.90	0.84	0.82	0.80	0.85	0.87	0.83	0.69	0.76	0.78	0.72	0.72	0.54	0.81
V8	0.88	0.86	0.89	0.90	0.89	0.88	0.96	0.92	0.92	0.92	0.88	0.86	0.93	0.91	0.88	0.75	0.79	0.81	0.77	0.79	0.64	0.77
I16	0.77	0.82	0.90	0.78	0.91	0.89	0.95	0.87	0.93	0.83	0.85	0.82	0.86	0.91	0.85	0.71	0.77	0.79	0.74	0.73	0.53	0.79
I12	0.79	0.84	0.88	0.80	0.89	0.88	0.93	0.89	0.92	0.83	0.88	0.85	0.88	0.91	0.88	0.72	0.78	0.80	0.75	0.75	0.56	0.80
V11	0.76	0.83	0.89	0.78	0.88	0.87	0.92	0.90	0.92	0.79	0.87	0.82	0.86	0.91	0.87	0.69	0.75	0.75	0.72	0.71	0.49	0.74
I20	0.75	0.78	0.82	0.75	0.88	0.91	0.88	0.87	0.89	0.82	0.85	0.80	0.85	0.86	0.85	0.65	0.71	0.73	0.64	0.67	0.44	0.82
I11	0.91	0.87	0.88	0.92	0.91	0.91	0.95	0.91	0.93	0.97	0.85	0.87	0.93	0.90	0.86	0.77	0.80	0.84	0.78	0.80	0.67	0.85
I14	0.71	0.81	0.96	0.77	0.97	0.92	0.94	0.87	0.95	0.76	0.73	0.74	0.74	0.88	0.75	0.70	0.77	0.76	0.77	0.73	0.52	0.74
V10	0.74	0.78	0.90	0.77	0.92	0.91	0.93	0.88	0.95	0.84	0.78	0.77	0.82	0.87	0.79	0.65	0.70	0.72	0.67	0.67	0.47	0.76
I4	0.71	0.80	0.91	0.75	0.96	0.96	0.93	0.90	0.95	0.80	0.80	0.77	0.81	0.88	0.81	0.68	0.74	0.74	0.70	0.70	0.47	0.80
I2	0.66	0.77	0.92	0.71	0.95	0.92	0.90	0.85	0.93	0.73	0.71	0.71	0.72	0.84	0.73	0.65	0.72	0.72	0.71	0.67	0.44	0.76
I3	0.69	0.68	0.83	0.71	0.82	0.76	0.84	0.75	0.85	0.77	0.61	0.65	0.69	0.75	0.62	0.59	0.63	0.66	0.65	0.62	0.48	0.68
V6	0.88	0.83	0.89	0.91	0.89	0.89	0.94	0.92	0.92	0.94	0.78	0.80	0.88	0.85	0.79	0.71	0.73	0.76	0.72	0.75	0.64	0.74
I22	0.86	0.85	0.87	0.88	0.90	0.94	0.93	0.95	0.92	0.90	0.86	0.83	0.90	0.87	0.87	0.73	0.76	0.78	0.72	0.76	0.59	0.80
V9	0.81	0.84	0.86	0.83	0.91	0.96	0.92	0.95	0.92	0.86	0.87	0.83	0.89	0.88	0.88	0.70	0.76	0.77	0.71	0.73	0.53	0.84
I15	0.88	0.86	0.88	0.89	0.89	0.90	0.96	0.93	0.92	0.92	0.89	0.87	0.93	0.91	0.89	0.75	0.79	0.82	0.76	0.78	0.63	0.79
I27	0.84	0.81	0.88	0.85	0.89	0.90	0.94	0.91	0.94	0.92	0.81	0.81	0.89	0.87	0.82	0.69	0.72	0.77	0.71	0.71	0.56	0.82
V12	0.82	0.84	0.86	0.84	0.93	0.97	0.92	0.94	0.91	0.86	0.85	0.82	0.87	0.86	0.86	0.72	0.77	0.79	0.72	0.75	0.56	0.84
I24	0.77	0.81	0.91	0.81	0.92	0.91	0.91	0.91	0.97	0.85	0.79	0.78	0.84	0.89	0.79	0.65	0.68	0.70	0.67	0.67	0.48	0.74
■15	0.74	0.81	0.96	0.79	0.95	0.90	0.94	0.87	0.96	0.79	0.72	0.74	0.76	0.87	0.74	0.71	0.72	0.73	0.74	0.71	0.52	0.71
I18	0.71	0.77	0.91	0.79	0.93	0.92	0.85	0.91	0.94	0.76	0.61	0.64	0.68	0.78	0.62	0.58	0.61	0.61	0.65	0.61	0.45	0.66
V13	0.74	0.74	0.79	0.76	0.88	0.94	0.84	0.89	0.87	0.83	0.74	0.71	0.79	0.76	0.75	0.59	0.63	0.66	0.58	0.62	0.44	0.81
■18	0.75	0.81	0.96	0.81	0.93	0.88	0.93	0.90	0.97	0.83	0.71	0.74	0.78	0.87	0.72	0.65	0.69	0.70	0.71	0.68	0.52	0.68
I25	0.81	0.82	0.93	0.84	0.93	0.88	0.94	0.86	0.94	0.87	0.73	0.78	0.80	0.87	0.75	0.72	0.76	0.79	0.78	0.75	0.60	0.79
V16	0.97	0.91	0.82	0.96	0.82	0.80	0.88	0.86	0.85	0.95	0.88	0.91	0.95	0.90	0.88	0.81	0.83	0.86	0.82	0.84	0.72	0.80
I9	0.95	0.96	0.87	0.97	0.86	0.82	0.89	0.89	0.88	0.91	0.92	0.96	0.96	0.96	0.93	0.86	0.89	0.90	0.88	0.89	0.73	0.79
■20	0.88	0.95	0.97	0.93	0.93	0.86	0.94	0.90	0.94	0.86	0.84	0.89	0.87	0.96	0.85	0.83	0.88	0.87	0.90	0.87	0.70	0.74
■16	0.97	0.92	0.81	0.95	0.81	0.79	0.88	0.84	0.83	0.94	0.91	0.95	0.96	0.92	0.92	0.83	0.87	0.90	0.85	0.87	0.72	0.83
V2	N/A	0.94	0.79	0.98	0.80	0.77	0.84	0.82	0.80	0.93	0.88	0.94	0.94	0.89	0.89	0.84	0.88	0.90	0.87	0.89	0.75	0.82
●32	0.06	N/A	0.89	0.96	0.89	0.84	0.88	0.89	0.87	0.84	0.91	0.95	0.91	0.96	0.92	0.88	0.94	0.93	0.94	0.91	0.72	0.82
V5	0.23	0.12	N/A	0.86	0.98	0.92	0.96	0.94	0.98	0.80	0.78	0.80	0.81	0.93	0.80	0.75	0.80	0.79	0.82	0.78	0.59	0.71
△5	0.02	0.05	0.15	N/A	0.85	0.82	0.87	0.88	0.86	0.92	0.87	0.93	0.93	0.92	0.88	0.85	0.87	0.88	0.87	0.88	0.75	0.78

	V2	●32	V5	△5	■19	I26	I8	V1	I29	▲12	■21	●28	▲14	△7	●29	●30	▲11	△2	△6	▲13	■17	●31
■19	0.22	0.12	0.02	0.16	N/A	0.98	0.96	0.95	0.98	0.83	0.81	0.82	0.83	0.93	0.83	0.76	0.82	0.81	0.81	0.79	0.58	0.80
I26	0.26	0.17	0.08	0.20	0.02	N/A	0.93	0.96	0.96	0.82	0.81	0.78	0.82	0.88	0.82	0.70	0.75	0.76	0.73	0.73	0.52	0.80
I8	0.18	0.13	0.04	0.14	0.04	0.07	N/A	0.93	0.97	0.89	0.86	0.85	0.89	0.94	0.87	0.78	0.83	0.84	0.81	0.81	0.63	0.79
V1	0.19	0.12	0.06	0.12	0.05	0.04	0.07	N/A	0.97	0.83	0.82	0.81	0.85	0.91	0.83	0.71	0.76	0.75	0.76	0.74	0.55	0.75
I29	0.22	0.14	0.02	0.15	0.02	0.04	0.03	0.04	N/A	0.86	0.80	0.80	0.85	0.92	0.81	0.71	0.75	0.75	0.75	0.74	0.55	0.74
▲12	0.08	0.18	0.22	0.08	0.19	0.20	0.12	0.18	0.15	N/A	0.85	0.88	0.95	0.87	0.85	0.76	0.77	0.82	0.74	0.79	0.68	0.81
■21	0.13	0.10	0.25	0.14	0.21	0.22	0.15	0.20	0.22	0.16	N/A	0.97	0.97	0.94	1.00	0.84	0.91	0.91	0.84	0.87	0.66	0.85
●28	0.06	0.05	0.22	0.08	0.20	0.24	0.16	0.21	0.22	0.13	0.03	N/A	0.97	0.95	0.98	0.89	0.95	0.96	0.91	0.92	0.74	0.87
▲14	0.06	0.09	0.21	0.07	0.19	0.20	0.12	0.16	0.17	0.05	0.03	0.03	N/A	0.94	0.97	0.84	0.87	0.90	0.82	0.86	0.70	0.84
△7	0.11	0.04	0.07	0.08	0.08	0.13	0.06	0.10	0.08	0.14	0.06	0.05	0.06	N/A	0.95	0.86	0.92	0.91	0.90	0.89	0.68	0.81
●29	0.12	0.08	0.23	0.13	0.19	0.20	0.14	0.19	0.21	0.16	0.00	0.02	0.03	0.05	N/A	0.86	0.92	0.93	0.85	0.89	0.68	0.86
●30	0.17	0.13	0.29	0.16	0.28	0.36	0.25	0.34	0.34	0.27	0.17	0.12	0.18	0.15	0.15	N/A	0.90	0.91	0.89	0.92	0.75	0.78
▲11	0.13	0.07	0.22	0.14	0.20	0.28	0.19	0.27	0.29	0.26	0.10	0.05	0.14	0.08	0.08	0.10	N/A	0.99	0.98	0.95	0.74	0.87
△2	0.10	0.08	0.24	0.13	0.21	0.28	0.18	0.28	0.28	0.20	0.09	0.04	0.11	0.09	0.08	0.10	0.01	N/A	0.97	0.95	0.76	0.91
△6	0.14	0.06	0.19	0.14	0.21	0.32	0.21	0.28	0.29	0.31	0.18	0.09	0.20	0.11	0.16	0.11	0.02	0.04	N/A	0.94	0.75	0.82
▲13	0.12	0.09	0.25	0.13	0.24	0.32	0.21	0.30	0.30	0.24	0.14	0.08	0.15	0.12	0.11	0.09	0.06	0.06	0.06	N/A	0.80	0.81
■17	0.28	0.33	0.53	0.29	0.54	0.66	0.47	0.60	0.59	0.39	0.41	0.30	0.35	0.39	0.39	0.29	0.30	0.28	0.28	0.22	N/A	0.61
●31	0.19	0.20	0.34	0.25	0.23	0.22	0.23	0.29	0.30	0.22	0.17	0.14	0.17	0.21	0.15	0.25	0.14	0.09	0.20	0.21	0.49	N/A
❍23	0.10	0.11	0.33	0.14	0.24	0.25	0.22	0.28	0.32	0.17	0.07	0.04	0.08	0.13	0.06	0.14	0.05	0.03	0.12	0.10	0.32	0.06
□8	0.15	0.12	0.28	0.18	0.23	0.28	0.22	0.30	0.28	0.22	0.10	0.06	0.12	0.11	0.09	0.14	0.08	0.06	0.12	0.12	0.36	0.09
△4	0.13	0.03	0.08	0.09	0.09	0.16	0.12	0.12	0.13	0.23	0.16	0.09	0.16	0.05	0.14	0.15	0.07	0.08	0.04	0.10	0.33	0.20
■22	0.09	0.11	0.25	0.11	0.17	0.18	0.16	0.22	0.24	0.11	0.09	0.06	0.08	0.12	0.08	0.15	0.07	0.04	0.13	0.11	0.32	0.05
L9	0.16	0.11	0.22	0.13	0.19	0.20	0.22	0.20	0.23	0.23	0.20	0.15	0.18	0.16	0.17	0.17	0.16	0.15	0.17	0.13	0.36	0.22
❍27	0.06	0.06	0.16	0.06	0.14	0.18	0.11	0.17	0.18	0.11	0.11	0.06	0.08	0.08	0.09	0.11	0.06	0.04	0.07	0.06	0.24	0.14
△1	0.16	0.07	0.09	0.13	0.07	0.09	0.06	0.07	0.10	0.20	0.07	0.09	0.10	0.04	0.06	0.17	0.10	0.12	0.14	0.13	0.43	0.19
❍26	0.17	0.06	0.10	0.13	0.10	0.13	0.08	0.10	0.12	0.23	0.08	0.09	0.11	0.04	0.07	0.14	0.10	0.11	0.11	0.11	0.40	0.23
□9	0.17	0.07	0.11	0.15	0.10	0.15	0.09	0.14	0.16	0.25	0.11	0.10	0.14	0.06	0.09	0.15	0.07	0.09	0.09	0.10	0.36	0.22
□10	0.20	0.13	0.30	0.20	0.25	0.30	0.27	0.31	0.35	0.33	0.16	0.13	0.21	0.17	0.14	0.13	0.10	0.11	0.12	0.09	0.33	0.23
❍24	0.29	0.16	0.10	0.23	0.09	0.10	0.09	0.11	0.12	0.31	0.21	0.23	0.23	0.13	0.19	0.26	0.21	0.22	0.21	0.23	0.54	0.28
L10	0.67	0.57	0.39	0.50	0.31	0.25	0.43	0.32	0.33	0.53	0.66	0.70	0.61	0.48	0.66	0.73	0.73	0.76	0.80	0.73	1.14	0.59
△3	0.78	0.51	0.32	0.56	0.29	0.24	0.42	0.25	0.27	0.69	0.59	0.67	0.63	0.39	0.60	0.85	0.73	0.82	0.79	0.85	1.50	0.67
❍25	0.74	0.49	0.30	0.53	0.27	0.23	0.41	0.25	0.26	0.65	0.62	0.67	0.64	0.39	0.63	0.81	0.71	0.80	0.75	0.81	1.38	0.67

	V2	●32	V5	△5	■19	I26	I8	V1	I29	▲12	■21	●28	▲14	△7	●29	●30	▲11	△2	△6	▲13	■17	●31
L2	0.75	0.57	0.40	0.57	0.32	0.25	0.44	0.32	0.34	0.63	0.61	0.70	0.63	0.46	0.61	0.75	0.71	0.77	0.80	0.75	1.35	0.60
F1	0.22	0.15	0.20	0.17	0.17	0.19	0.21	0.18	0.17	0.24	0.20	0.18	0.18	0.14	0.19	0.25	0.26	0.24	0.25	0.23	0.50	0.29
F5	0.17	0.17	0.27	0.16	0.16	0.11	0.21	0.14	0.17	0.13	0.16	0.16	0.12	0.17	0.15	0.32	0.28	0.24	0.36	0.30	0.58	0.14
F6	0.19	0.12	0.11	0.11	0.08	0.09	0.18	0.08	0.11	0.22	0.33	0.25	0.26	0.17	0.30	0.27	0.27	0.27	0.23	0.26	0.49	0.28
F7	0.19	0.13	0.17	0.13	0.14	0.13	0.19	0.10	0.14	0.21	0.21	0.20	0.18	0.15	0.20	0.15	0.27	0.27	0.28	0.24	0.49	0.28
F8	0.21	0.21	0.23	0.18	0.13	0.07	0.17	0.11	0.12	0.13	0.20	0.22	0.14	0.19	0.19	0.40	0.36	0.31	0.44	0.37	0.67	0.18
F2	0.25	0.17	0.19	0.19	0.14	0.14	0.20	0.15	0.16	0.25	0.24	0.23	0.23	0.18	0.23	0.27	0.29	0.28	0.30	0.26	0.54	0.27
F9	0.19	0.16	0.16	0.15	0.17	0.22	0.14	0.20	0.14	0.17	0.20	0.17	0.15	0.12	0.18	0.22	0.25	0.23	0.25	0.20	0.43	0.33
F3	0.16	0.14	0.21	0.12	0.20	0.26	0.19	0.23	0.20	0.17	0.20	0.15	0.15	0.14	0.19	0.19	0.22	0.19	0.22	0.17	0.37	0.30
F4	0.19	0.23	0.31	0.14	0.26	0.26	0.32	0.23	0.26	0.20	0.39	0.29	0.26	0.30	0.37	0.33	0.40	0.35	0.37	0.31	0.46	0.35
L11	0.63	0.51	0.36	0.51	0.33	0.34	0.33	0.41	0.38	0.51	0.48	0.54	0.50	0.39	0.48	0.52	0.50	0.53	0.56	0.50	0.80	0.62
L1	0.41	0.36	0.23	0.37	0.12	0.06	0.18	0.16	0.13	0.24	0.32	0.38	0.28	0.27	0.31	0.56	0.50	0.45	0.60	0.54	0.95	0.25
L3	0.49	0.39	0.24	0.39	0.24	0.22	0.22	0.23	0.21	0.38	0.36	0.42	0.35	0.26	0.35	0.50	0.49	0.51	0.55	0.50	0.97	0.53
L4	0.52	0.39	0.37	0.48	0.35	0.43	0.33	0.49	0.42	0.51	0.35	0.37	0.41	0.31	0.33	0.37	0.35	0.36	0.38	0.23	0.54	0.52
L5	0.64	0.47	0.26	0.52	0.24	0.28	0.24	0.36	0.25	0.48	0.43	0.49	0.45	0.29	0.42	0.51	0.49	0.51	0.55	0.51	0.97	0.55
L6	0.77	0.55	0.36	0.61	0.29	0.21	0.40	0.28	0.26	0.59	0.53	0.64	0.55	0.42	0.53	0.81	0.79	0.81	0.90	0.84	1.60	0.58
L7	0.50	0.36	0.25	0.40	0.26	0.28	0.22	0.30	0.26	0.44	0.31	0.36	0.34	0.22	0.29	0.40	0.38	0.41	0.43	0.35	0.82	0.53
L8	0.42	0.47	0.27	0.35	0.30	0.32	0.26	0.28	0.18	0.26	0.55	0.52	0.37	0.35	0.53	0.67	0.76	0.67	0.72	0.57	0.84	0.62
NL1	1.05	0.78	0.50	0.90	0.37	0.35	0.54	0.54	0.48	0.83	0.84	0.93	0.90	0.69	0.82	0.87	0.86	0.86	0.91	0.85	0.96	0.67
NL2	0.40	0.27	0.21	0.34	0.16	0.17	0.20	0.24	0.22	0.37	0.31	0.32	0.33	0.23	0.28	0.32	0.30	0.31	0.33	0.30	0.65	0.33
NL3	0.74	0.60	0.46	0.69	0.46	0.46	0.39	0.47	0.41	0.58	0.42	0.55	0.46	0.42	0.43	0.66	0.69	0.69	0.80	0.72	0.71	0.75
NL4	0.98	0.73	0.53	0.81	0.42	0.35	0.55	0.48	0.54	0.86	0.76	0.88	0.86	0.66	0.75	0.80	0.76	0.82	0.84	0.79	1.26	0.70
NL5	0.72	0.61	0.47	0.70	0.41	0.42	0.37	0.51	0.45	0.55	0.44	0.55	0.48	0.45	0.44	0.71	0.59	0.59	0.71	0.67	0.62	0.61
NL6	0.85	0.66	0.56	0.76	0.60	0.65	0.55	0.61	0.55	0.78	0.55	0.64	0.61	0.51	0.55	0.64	0.78	0.80	0.82	0.70	0.67	0.97

	○23	□8	△4	■22	L9	○27	△1	○26	□9	□10	○24	L10	△3	○25	L2	F1	F5	F6	F7	F8	F2	F9
V3	0.80	0.82	0.82	0.85	0.78	0.84	0.87	0.85	0.81	0.68	0.83	0.64	0.66	0.65	0.63	0.81	0.86	0.79	0.82	0.90	0.80	0.86
I28	0.73	0.80	0.81	0.79	0.75	0.79	0.85	0.84	0.78	0.66	0.82	0.70	0.73	0.73	0.68	0.84	0.86	0.82	0.82	0.90	0.82	0.87
V14	0.81	0.82	0.81	0.87	0.79	0.83	0.87	0.85	0.81	0.73	0.83	0.73	0.73	0.73	0.72	0.85	0.94	0.85	0.85	0.96	0.86	0.84
I1	0.69	0.69	0.85	0.77	0.74	0.85	0.81	0.80	0.82	0.66	0.82	0.61	0.56	0.60	0.56	0.72	0.70	0.85	0.75	0.73	0.74	0.81
I5	0.83	0.84	0.81	0.88	0.77	0.85	0.88	0.86	0.84	0.73	0.83	0.64	0.63	0.63	0.62	0.81	0.87	0.78	0.79	0.89	0.80	0.85
I17	0.71	0.76	0.87	0.78	0.75	0.81	0.87	0.86	0.84	0.68	0.87	0.69	0.73	0.74	0.68	0.81	0.79	0.86	0.81	0.84	0.81	0.85
V7	0.68	0.70	0.81	0.77	0.73	0.81	0.79	0.77	0.76	0.61	0.79	0.66	0.63	0.66	0.61	0.76	0.78	0.86	0.79	0.82	0.77	0.82
I13	0.77	0.78	0.87	0.78	0.78	0.84	0.95	0.94	0.90	0.73	0.90	0.60	0.68	0.67	0.63	0.81	0.78	0.78	0.84	0.81	0.80	0.85
I7	0.68	0.74	0.87	0.74	0.73	0.80	0.85	0.85	0.84	0.67	0.86	0.65	0.69	0.71	0.63	0.78	0.72	0.83	0.77	0.76	0.77	0.84
V4	0.80	0.76	0.78	0.86	0.77	0.87	0.87	0.84	0.81	0.69	0.82	0.65	0.60	0.61	0.62	0.78	0.86	0.80	0.82	0.90	0.79	0.84
I6	0.66	0.72	0.88	0.73	0.74	0.81	0.84	0.85	0.83	0.67	0.86	0.65	0.68	0.71	0.62	0.78	0.72	0.86	0.79	0.76	0.78	0.84
I10	0.65	0.71	0.86	0.73	0.73	0.80	0.83	0.83	0.82	0.65	0.84	0.66	0.68	0.71	0.63	0.78	0.73	0.86	0.78	0.77	0.77	0.84
V15	0.73	0.75	0.90	0.78	0.77	0.86	0.89	0.89	0.89	0.72	0.90	0.62	0.65	0.67	0.61	0.78	0.72	0.85	0.79	0.76	0.78	0.84
I21	0.65	0.73	0.85	0.71	0.71	0.77	0.84	0.84	0.80	0.63	0.84	0.64	0.73	0.74	0.64	0.80	0.74	0.82	0.79	0.79	0.77	0.84
I30	0.69	0.75	0.90	0.75	0.77	0.82	0.89	0.89	0.86	0.70	0.89	0.66	0.71	0.72	0.65	0.80	0.74	0.86	0.82	0.78	0.80	0.85
I23	0.78	0.82	0.90	0.81	0.77	0.85	0.93	0.92	0.90	0.75	0.90	0.61	0.68	0.68	0.62	0.81	0.77	0.80	0.80	0.80	0.80	0.85
I19	0.77	0.80	0.79	0.82	0.72	0.81	0.85	0.83	0.82	0.68	0.83	0.60	0.62	0.62	0.60	0.76	0.79	0.73	0.74	0.82	0.75	0.81
V8	0.81	0.78	0.85	0.85	0.79	0.89	0.92	0.89	0.87	0.73	0.87	0.59	0.59	0.58	0.58	0.78	0.80	0.79	0.82	0.84	0.78	0.84
I16	0.76	0.81	0.83	0.80	0.74	0.81	0.89	0.88	0.84	0.70	0.86	0.62	0.68	0.67	0.63	0.80	0.79	0.76	0.78	0.83	0.78	0.84
I12	0.78	0.82	0.84	0.81	0.75	0.82	0.91	0.89	0.85	0.71	0.87	0.60	0.66	0.65	0.62	0.80	0.80	0.75	0.80	0.83	0.79	0.85
V11	0.73	0.78	0.83	0.75	0.72	0.77	0.91	0.89	0.84	0.68	0.87	0.59	0.71	0.69	0.63	0.80	0.78	0.73	0.79	0.82	0.77	0.83
I20	0.77	0.80	0.76	0.80	0.72	0.75	0.86	0.83	0.78	0.67	0.81	0.67	0.73	0.71	0.69	0.80	0.88	0.75	0.80	0.91	0.80	0.79
I11	0.85	0.82	0.86	0.91	0.82	0.92	0.88	0.85	0.84	0.75	0.83	0.65	0.60	0.61	0.61	0.81	0.89	0.87	0.85	0.91	0.82	0.85
I14	0.70	0.74	0.86	0.77	0.74	0.80	0.86	0.85	0.85	0.71	0.87	0.70	0.72	0.74	0.69	0.78	0.76	0.86	0.78	0.79	0.80	0.81
V10	0.70	0.76	0.79	0.76	0.71	0.76	0.84	0.82	0.78	0.64	0.83	0.69	0.73	0.73	0.68	0.80	0.82	0.79	0.79	0.87	0.79	0.83
I4	0.74	0.77	0.82	0.80	0.75	0.78	0.88	0.86	0.83	0.70	0.87	0.73	0.78	0.78	0.74	0.82	0.86	0.84	0.82	0.90	0.83	0.81
I2	0.67	0.74	0.82	0.74	0.71	0.74	0.83	0.82	0.80	0.66	0.85	0.70	0.75	0.76	0.70	0.77	0.77	0.83	0.76	0.81	0.79	0.78
I3	0.59	0.66	0.73	0.68	0.62	0.71	0.69	0.69	0.68	0.54	0.72	0.56	0.55	0.57	0.52	0.66	0.65	0.72	0.66	0.69	0.66	0.75
V6	0.77	0.71	0.82	0.84	0.79	0.89	0.85	0.82	0.82	0.69	0.82	0.65	0.58	0.59	0.60	0.76	0.82	0.86	0.83	0.86	0.79	0.82
I22	0.82	0.75	0.83	0.87	0.82	0.89	0.92	0.88	0.86	0.75	0.87	0.67	0.65	0.65	0.66	0.79	0.88	0.85	0.87	0.92	0.83	0.81
V9	0.82	0.78	0.82	0.86	0.80	0.85	0.93	0.88	0.85	0.74	0.89	0.70	0.71	0.70	0.71	0.81	0.91	0.83	0.87	0.94	0.84	0.79
I15	0.83	0.78	0.84	0.86	0.80	0.90	0.93	0.89	0.87	0.73	0.88	0.60	0.60	0.59	0.60	0.78	0.83	0.80	0.83	0.86	0.79	0.84

	❍23	□8	△4	■22	L9	❍27	△1	❍26	□9	□10	❍24	L10	△3	❍25	L2	F1	F5	F6	F7	F8	F2	F9
I27	0.77	0.79	0.81	0.83	0.76	0.83	0.86	0.83	0.79	0.66	0.83	0.66	0.65	0.65	0.63	0.79	0.84	0.81	0.82	0.89	0.79	0.84
V12	0.84	0.76	0.82	0.88	0.82	0.88	0.93	0.87	0.87	0.76	0.89	0.71	0.68	0.68	0.71	0.79	0.90	0.86	0.86	0.93	0.84	0.78
I24	0.69	0.76	0.81	0.75	0.73	0.76	0.84	0.83	0.77	0.63	0.81	0.70	0.77	0.77	0.69	0.84	0.85	0.84	0.83	0.90	0.82	0.85
■15	0.66	0.74	0.84	0.73	0.73	0.78	0.84	0.84	0.82	0.67	0.86	0.69	0.71	0.73	0.67	0.80	0.74	0.84	0.81	0.79	0.80	0.84
I18	0.59	0.62	0.80	0.69	0.72	0.72	0.77	0.75	0.72	0.59	0.79	0.76	0.77	0.80	0.72	0.78	0.81	0.94	0.83	0.85	0.81	0.76
V13	0.74	0.69	0.72	0.81	0.74	0.76	0.81	0.75	0.73	0.65	0.79	0.75	0.72	0.72	0.73	0.76	0.93	0.85	0.83	0.97	0.82	0.71
■18	0.64	0.71	0.84	0.71	0.71	0.77	0.81	0.82	0.78	0.62	0.82	0.67	0.72	0.74	0.64	0.79	0.75	0.85	0.79	0.80	0.78	0.84
I25	0.73	0.77	0.86	0.81	0.76	0.84	0.82	0.81	0.81	0.68	0.83	0.65	0.62	0.65	0.60	0.77	0.77	0.85	0.78	0.80	0.78	0.84
V16	0.87	0.83	0.86	0.89	0.82	0.93	0.86	0.85	0.84	0.77	0.78	0.53	0.48	0.49	0.49	0.79	0.83	0.81	0.82	0.82	0.77	0.84
I9	0.88	0.90	0.93	0.89	0.87	0.93	0.91	0.92	0.89	0.83	0.82	0.60	0.59	0.59	0.56	0.88	0.85	0.85	0.88	0.83	0.84	0.89
■20	0.80	0.83	0.96	0.83	0.83	0.91	0.91	0.92	0.91	0.80	0.86	0.60	0.63	0.65	0.58	0.85	0.78	0.89	0.84	0.78	0.82	0.88
■16	0.90	0.87	0.87	0.91	0.83	0.93	0.87	0.87	0.85	0.79	0.78	0.51	0.49	0.50	0.49	0.80	0.83	0.79	0.81	0.81	0.77	0.84
V2	0.90	0.86	0.88	0.92	0.85	0.94	0.85	0.85	0.84	0.82	0.75	0.51	0.46	0.48	0.47	0.80	0.85	0.83	0.83	0.81	0.78	0.82
●32	0.90	0.89	0.97	0.90	0.89	0.94	0.94	0.94	0.93	0.88	0.85	0.57	0.60	0.61	0.57	0.86	0.84	0.89	0.88	0.81	0.84	0.85
V5	0.72	0.75	0.92	0.78	0.80	0.85	0.91	0.91	0.90	0.74	0.91	0.68	0.72	0.74	0.67	0.82	0.77	0.90	0.84	0.80	0.83	0.85
△5	0.87	0.84	0.92	0.89	0.88	0.94	0.88	0.88	0.86	0.82	0.79	0.61	0.57	0.59	0.57	0.85	0.85	0.89	0.88	0.83	0.83	0.86
■19	0.79	0.79	0.91	0.84	0.82	0.87	0.93	0.91	0.90	0.78	0.92	0.73	0.75	0.77	0.73	0.84	0.86	0.93	0.87	0.88	0.87	0.84
I26	0.78	0.75	0.85	0.84	0.82	0.84	0.92	0.88	0.86	0.74	0.90	0.78	0.79	0.79	0.78	0.83	0.90	0.91	0.88	0.93	0.87	0.80
I8	0.81	0.80	0.89	0.85	0.80	0.90	0.94	0.92	0.91	0.76	0.92	0.65	0.66	0.66	0.65	0.81	0.81	0.84	0.83	0.84	0.82	0.87
V1	0.76	0.74	0.89	0.80	0.82	0.85	0.93	0.90	0.87	0.73	0.90	0.72	0.78	0.78	0.73	0.84	0.87	0.92	0.90	0.90	0.86	0.82
I29	0.73	0.76	0.88	0.79	0.80	0.83	0.90	0.89	0.86	0.70	0.89	0.72	0.76	0.77	0.71	0.84	0.84	0.90	0.87	0.88	0.85	0.87
▲12	0.85	0.81	0.79	0.90	0.79	0.90	0.82	0.80	0.78	0.72	0.73	0.59	0.50	0.52	0.53	0.79	0.88	0.81	0.81	0.88	0.78	0.84
■21	0.94	0.90	0.86	0.91	0.82	0.90	0.93	0.92	0.90	0.85	0.81	0.52	0.56	0.54	0.54	0.82	0.85	0.72	0.81	0.82	0.78	0.82
●28	0.96	0.94	0.91	0.94	0.86	0.94	0.91	0.92	0.90	0.88	0.79	0.50	0.51	0.51	0.50	0.84	0.86	0.78	0.82	0.81	0.80	0.84
▲14	0.92	0.89	0.86	0.92	0.84	0.93	0.91	0.89	0.87	0.81	0.79	0.54	0.53	0.53	0.53	0.83	0.88	0.77	0.83	0.87	0.80	0.86
△7	0.88	0.89	0.95	0.89	0.85	0.92	0.96	0.96	0.94	0.85	0.88	0.62	0.68	0.68	0.63	0.87	0.84	0.84	0.86	0.83	0.84	0.89
●29	0.95	0.91	0.87	0.92	0.84	0.91	0.94	0.93	0.91	0.87	0.83	0.52	0.55	0.54	0.54	0.83	0.86	0.74	0.82	0.82	0.80	0.83
●30	0.87	0.87	0.86	0.86	0.85	0.90	0.84	0.87	0.86	0.88	0.77	0.48	0.43	0.45	0.47	0.78	0.72	0.76	0.86	0.67	0.76	0.80
▲11	0.95	0.92	0.93	0.93	0.85	0.94	0.90	0.91	0.93	0.91	0.81	0.48	0.48	0.49	0.49	0.77	0.76	0.77	0.76	0.70	0.75	0.78
△2	0.97	0.95	0.92	0.96	0.86	0.96	0.89	0.89	0.91	0.90	0.81	0.47	0.44	0.45	0.46	0.79	0.79	0.76	0.77	0.73	0.76	0.79
△6	0.89	0.89	0.96	0.88	0.84	0.93	0.87	0.89	0.92	0.88	0.81	0.45	0.46	0.47	0.45	0.78	0.70	0.79	0.76	0.64	0.74	0.78
▲13	0.91	0.89	0.90	0.90	0.87	0.94	0.88	0.89	0.90	0.91	0.80	0.48	0.43	0.44	0.47	0.79	0.74	0.77	0.79	0.69	0.77	0.82

	❍23	◘8	△4	■22	L9	❍27	△1	❍26	◘9	◘10	❍24	L10	△3	❍25	L2	F1	F5	F6	F7	F8	F2	F9
■17	0.72	0.70	0.72	0.72	0.70	0.78	0.65	0.67	0.70	0.72	0.58	0.32	0.22	0.25	0.26	0.61	0.56	0.61	0.61	0.51	0.58	0.65
●31	0.94	0.92	0.82	0.95	0.81	0.87	0.82	0.79	0.80	0.80	0.76	0.55	0.51	0.51	0.55	0.75	0.87	0.76	0.76	0.84	0.76	0.72
❍23	N/A	0.93	0.86	0.98	0.85	0.94	0.88	0.86	0.87	0.89	0.77	0.50	0.45	0.45	0.49	0.77	0.86	0.76	0.78	0.81	0.77	0.76
◘8	0.07	N/A	0.88	0.92	0.84	0.89	0.86	0.87	0.85	0.86	0.77	0.57	0.51	0.51	0.51	0.83	0.83	0.75	0.79	0.78	0.79	0.82
△4	0.15	0.13	N/A	0.87	0.87	0.93	0.93	0.95	0.94	0.86	0.89	0.58	0.63	0.64	0.58	0.87	0.80	0.90	0.86	0.77	0.83	0.84
■22	0.02	0.08	0.14	N/A	0.87	0.96	0.89	0.86	0.88	0.87	0.80	0.58	0.50	0.52	0.56	0.79	0.90	0.82	0.81	0.86	0.80	0.79
L9	0.16	0.17	0.13	0.14	N/A	0.90	0.87	0.87	0.86	0.86	0.82	0.61	0.56	0.57	0.60	0.84	0.83	0.85	0.87	0.80	0.85	0.81
❍27	0.06	0.12	0.07	0.04	0.11	N/A	0.93	0.92	0.94	0.90	0.87	0.56	0.49	0.51	0.54	0.84	0.83	0.85	0.84	0.80	0.82	0.84
△1	0.13	0.15	0.07	0.12	0.14	0.08	N/A	0.99	0.97	0.87	0.96	0.63	0.67	0.66	0.66	0.87	0.84	0.84	0.88	0.84	0.86	0.85
❍26	0.15	0.14	0.06	0.15	0.14	0.08	0.01	N/A	0.98	0.89	0.96	0.61	0.66	0.65	0.64	0.91	0.81	0.83	0.88	0.80	0.85	0.87
◘9	0.13	0.17	0.06	0.13	0.15	0.06	0.03	0.02	N/A	0.88	0.95	0.57	0.59	0.59	0.59	0.85	0.77	0.82	0.83	0.75	0.81	0.82
◘10	0.12	0.15	0.15	0.14	0.15	0.11	0.14	0.12	0.13	N/A	0.79	0.52	0.47	0.50	0.53	0.79	0.76	0.77	0.80	0.71	0.79	0.76
❍24	0.26	0.26	0.11	0.23	0.20	0.14	0.04	0.04	0.05	0.23	N/A	0.65	0.69	0.68	0.68	0.87	0.75	0.82	0.83	0.77	0.82	0.80
L10	0.70	0.55	0.54	0.55	0.49	0.58	0.46	0.50	0.57	0.65	0.43	N/A	0.85	0.87	0.88	0.66	0.70	0.74	0.70	0.74	0.72	0.61
△3	0.80	0.68	0.46	0.69	0.58	0.71	0.40	0.41	0.52	0.75	0.37	0.16	N/A	0.99	0.90	0.70	0.68	0.71	0.70	0.72	0.69	0.60
❍25	0.80	0.67	0.45	0.66	0.56	0.67	0.42	0.43	0.53	0.70	0.39	0.14	0.01	N/A	0.90	0.69	0.69	0.74	0.71	0.73	0.70	0.61
L2	0.71	0.67	0.54	0.59	0.51	0.62	0.42	0.45	0.53	0.64	0.38	0.13	0.11	0.10	N/A	0.66	0.68	0.71	0.73	0.72	0.81	0.69
F1	0.26	0.19	0.14	0.23	0.18	0.18	0.14	0.10	0.16	0.24	0.14	0.42	0.35	0.37	0.41	N/A	0.85	0.85	0.88	0.84	0.87	0.85
F5	0.15	0.19	0.23	0.11	0.19	0.18	0.17	0.22	0.26	0.27	0.29	0.36	0.38	0.38	0.38	0.16	N/A	0.88	0.88	0.99	0.87	0.78
F6	0.28	0.29	0.11	0.20	0.16	0.16	0.17	0.18	0.20	0.26	0.20	0.31	0.34	0.30	0.35	0.17	0.13	N/A	0.92	0.88	0.89	0.80
F7	0.25	0.23	0.15	0.21	0.14	0.17	0.13	0.13	0.19	0.23	0.18	0.35	0.36	0.35	0.32	0.13	0.13	0.08	N/A	0.88	0.93	0.87
F8	0.22	0.25	0.26	0.15	0.23	0.22	0.18	0.23	0.28	0.35	0.26	0.30	0.32	0.32	0.33	0.18	0.01	0.13	0.13	N/A	0.86	0.78
F2	0.26	0.23	0.18	0.22	0.16	0.20	0.15	0.16	0.21	0.24	0.19	0.33	0.37	0.35	0.21	0.14	0.14	0.12	0.08	0.15	N/A	0.93
F9	0.28	0.20	0.17	0.24	0.21	0.18	0.16	0.14	0.20	0.27	0.22	0.49	0.51	0.50	0.37	0.16	0.25	0.22	0.14	0.25	0.07	N/A
F3	0.23	0.18	0.17	0.20	0.18	0.15	0.19	0.16	0.20	0.22	0.26	0.52	0.60	0.57	0.40	0.16	0.23	0.20	0.14	0.25	0.06	0.01
F4	0.32	0.33	0.26	0.25	0.20	0.22	0.31	0.32	0.36	0.33	0.38	0.41	0.58	0.53	0.37	0.21	0.17	0.12	0.12	0.19	0.07	0.14
L11	0.56	0.60	0.49	0.49	0.49	0.44	0.36	0.38	0.37	0.50	0.36	0.48	0.56	0.53	0.30	0.51	0.55	0.47	0.43	0.51	0.29	0.29
L1	0.37	0.36	0.37	0.26	0.36	0.36	0.24	0.30	0.35	0.49	0.27	0.26	0.26	0.26	0.27	0.26	0.09	0.20	0.23	0.05	0.21	0.31
L3	0.52	0.38	0.38	0.46	0.42	0.41	0.25	0.26	0.33	0.51	0.26	0.21	0.30	0.31	0.31	0.34	0.40	0.39	0.32	0.34	0.32	0.30
L4	0.43	0.40	0.38	0.42	0.40	0.37	0.31	0.29	0.29	0.36	0.35	0.84	0.89	0.86	0.77	0.43	0.54	0.51	0.47	0.56	0.43	0.36
L5	0.58	0.46	0.42	0.51	0.50	0.48	0.33	0.31	0.36	0.53	0.30	0.39	0.35	0.35	0.36	0.38	0.50	0.47	0.44	0.44	0.39	0.31
L6	0.73	0.60	0.55	0.63	0.41	0.71	0.41	0.43	0.57	0.72	0.40	0.25	0.14	0.15	0.21	0.35	0.31	0.38	0.34	0.25	0.32	0.45

	○23	□8	△4	■22	L9	○27	△1	○26	□9	□10	○24	L10	△3	○25	L2	F1	F5	F6	F7	F8	F2	F9
L7	0.46	0.40	0.34	0.44	0.41	0.38	0.23	0.22	0.27	0.41	0.25	0.45	0.38	0.39	0.38	0.34	0.48	0.46	0.37	0.44	0.36	0.28
L8	0.73	0.53	0.46	0.58	0.52	0.51	0.46	0.45	0.57	0.80	0.46	0.46	0.45	0.43	0.53	0.34	0.39	0.36	0.35	0.31	0.38	0.25
NL1	0.82	0.79	0.66	0.67	0.65	0.72	0.56	0.62	0.60	0.71	0.48	0.51	0.58	0.55	0.36	0.61	0.53	0.45	0.50	0.47	0.30	0.50
NL2	0.32	0.31	0.25	0.28	0.25	0.25	0.17	0.19	0.20	0.27	0.18	0.42	0.46	0.45	0.24	0.26	0.29	0.24	0.20	0.28	0.08	0.14
NL3	0.69	0.62	0.60	0.68	0.73	0.65	0.41	0.42	0.47	0.76	0.47	0.93	0.71	0.75	0.82	0.57	0.61	0.75	0.54	0.54	0.61	0.51
NL4	0.75	0.88	0.68	0.65	0.61	0.67	0.50	0.56	0.53	0.64	0.44	0.42	0.48	0.47	0.22	0.67	0.57	0.48	0.49	0.53	0.31	0.54
NL5	0.57	0.61	0.58	0.53	0.71	0.54	0.41	0.46	0.43	0.68	0.46	0.92	0.85	0.87	0.86	0.68	0.58	0.71	0.68	0.53	0.65	0.60
NL6	0.86	0.68	0.63	0.87	0.76	0.78	0.53	0.49	0.57	0.79	0.58	1.07	0.80	0.83	0.95	0.59	0.78	0.81	0.56	0.74	0.66	0.54

	F3	F4	L11	L1	L3	L4	L5	L6	L7	L8	NL1	NL2	NL3	NL4	NL5	NL6
V3	0.81	0.74	0.63	0.87	0.78	0.62	0.74	0.71	0.74	0.84	0.51	0.74	0.68	0.48	0.65	0.57
I28	0.81	0.73	0.64	0.92	0.80	0.64	0.80	0.79	0.76	0.88	0.59	0.77	0.68	0.51	0.64	0.58
V14	0.81	0.77	0.68	0.97	0.77	0.67	0.78	0.79	0.74	0.78	0.67	0.82	0.66	0.61	0.67	0.55
I1	0.78	0.73	0.65	0.73	0.70	0.62	0.70	0.54	0.67	0.77	0.54	0.72	0.53	0.50	0.58	0.45
I5	0.82	0.71	0.66	0.89	0.77	0.68	0.78	0.70	0.76	0.79	0.58	0.78	0.68	0.52	0.69	0.56
I17	0.80	0.72	0.66	0.87	0.79	0.65	0.80	0.74	0.76	0.84	0.61	0.79	0.64	0.55	0.63	0.56
V7	0.79	0.77	0.61	0.82	0.72	0.56	0.70	0.64	0.66	0.87	0.53	0.71	0.55	0.48	0.55	0.45
I13	0.79	0.68	0.65	0.76	0.81	0.66	0.72	0.68	0.80	0.73	0.48	0.76	0.72	0.51	0.65	0.64
I7	0.79	0.67	0.67	0.81	0.76	0.67	0.81	0.68	0.76	0.80	0.61	0.78	0.63	0.53	0.63	0.56
V4	0.80	0.77	0.66	0.86	0.76	0.62	0.69	0.65	0.71	0.79	0.53	0.74	0.65	0.53	0.66	0.52
I6	0.79	0.71	0.66	0.77	0.76	0.65	0.76	0.65	0.74	0.82	0.57	0.76	0.61	0.52	0.60	0.54
I10	0.79	0.72	0.67	0.79	0.76	0.64	0.78	0.67	0.74	0.83	0.57	0.75	0.59	0.52	0.59	0.52
V15	0.80	0.70	0.67	0.76	0.76	0.67	0.76	0.63	0.76	0.77	0.55	0.78	0.61	0.53	0.62	0.54
I21	0.78	0.68	0.62	0.80	0.78	0.61	0.77	0.72	0.75	0.87	0.53	0.73	0.64	0.48	0.59	0.57
I30	0.80	0.71	0.65	0.77	0.79	0.63	0.76	0.69	0.76	0.82	0.55	0.77	0.62	0.52	0.59	0.56
I23	0.80	0.66	0.66	0.80	0.78	0.69	0.78	0.68	0.79	0.76	0.58	0.79	0.72	0.52	0.70	0.63
I19	0.76	0.63	0.61	0.85	0.75	0.63	0.76	0.67	0.73	0.79	0.57	0.75	0.68	0.48	0.69	0.56
V8	0.80	0.73	0.65	0.77	0.77	0.63	0.68	0.60	0.74	0.76	0.46	0.74	0.66	0.49	0.65	0.55
I16	0.78	0.65	0.63	0.85	0.79	0.66	0.79	0.72	0.78	0.81	0.56	0.77	0.70	0.49	0.68	0.60
I12	0.79	0.66	0.62	0.82	0.79	0.64	0.74	0.69	0.76	0.78	0.54	0.76	0.72	0.48	0.68	0.62
V11	0.76	0.62	0.61	0.81	0.80	0.63	0.75	0.73	0.78	0.78	0.50	0.74	0.73	0.47	0.66	0.64
I20	0.74	0.66	0.61	0.93	0.77	0.61	0.75	0.80	0.74	0.77	0.58	0.76	0.68	0.54	0.65	0.56
I11	0.84	0.82	0.63	0.85	0.73	0.62	0.68	0.63	0.68	0.79	0.54	0.75	0.60	0.50	0.63	0.49

	F3	F4	L11	L1	L3	L4	L5	L6	L7	L8	NL1	NL2	NL3	NL4	NL5	NL6
I14	0.77	0.68	0.71	0.85	0.76	0.70	0.82	0.72	0.77	0.75	0.69	0.82	0.61	0.63	0.64	0.52
V10	0.77	0.69	0.64	0.91	0.80	0.63	0.81	0.78	0.76	0.87	0.60	0.76	0.67	0.53	0.64	0.57
I4	0.77	0.69	0.69	0.95	0.80	0.68	0.82	0.82	0.78	0.77	0.71	0.83	0.68	0.64	0.69	0.57
I2	0.73	0.64	0.66	0.89	0.76	0.66	0.81	0.77	0.74	0.76	0.70	0.80	0.61	0.61	0.63	0.53
I3	0.70	0.63	0.54	0.73	0.66	0.52	0.68	0.57	0.61	0.84	0.49	0.65	0.50	0.40	0.51	0.42
V6	0.80	0.81	0.65	0.80	0.73	0.58	0.64	0.59	0.66	0.78	0.51	0.72	0.56	0.52	0.59	0.45
I22	0.78	0.79	0.66	0.85	0.76	0.61	0.65	0.67	0.70	0.71	0.55	0.77	0.62	0.59	0.64	0.51
V9	0.76	0.75	0.66	0.90	0.78	0.62	0.69	0.75	0.73	0.70	0.60	0.80	0.67	0.62	0.67	0.54
I15	0.80	0.74	0.65	0.80	0.77	0.63	0.68	0.62	0.74	0.75	0.48	0.75	0.66	0.51	0.66	0.55
I27	0.79	0.75	0.61	0.86	0.77	0.58	0.70	0.69	0.70	0.84	0.51	0.73	0.64	0.48	0.61	0.52
V12	0.76	0.77	0.69	0.90	0.76	0.64	0.67	0.71	0.71	0.66	0.64	0.82	0.62	0.67	0.67	0.49
I24	0.80	0.74	0.61	0.90	0.79	0.60	0.77	0.80	0.74	0.89	0.57	0.74	0.66	0.50	0.61	0.58
■15	0.79	0.70	0.67	0.84	0.78	0.67	0.82	0.72	0.76	0.83	0.63	0.79	0.63	0.55	0.61	0.56
I18	0.73	0.79	0.60	0.87	0.71	0.53	0.68	0.75	0.63	0.81	0.65	0.73	0.50	0.59	0.51	0.44
V13	0.70	0.77	0.60	0.96	0.71	0.53	0.64	0.77	0.61	0.72	0.66	0.75	0.56	0.64	0.59	0.43
■18	0.79	0.72	0.62	0.82	0.76	0.60	0.77	0.71	0.73	0.88	0.56	0.73	0.62	0.48	0.59	0.55
I25	0.80	0.74	0.64	0.81	0.73	0.63	0.75	0.63	0.70	0.83	0.57	0.75	0.56	0.50	0.59	0.48
V16	0.84	0.81	0.56	0.69	0.66	0.58	0.57	0.50	0.64	0.72	0.35	0.67	0.53	0.38	0.53	0.46
I9	0.90	0.82	0.61	0.71	0.74	0.66	0.65	0.59	0.72	0.72	0.41	0.73	0.59	0.43	0.55	0.55
■20	0.87	0.78	0.65	0.72	0.73	0.70	0.72	0.60	0.75	0.74	0.50	0.77	0.59	0.49	0.58	0.55
■16	0.85	0.77	0.55	0.69	0.66	0.60	0.59	0.50	0.66	0.70	0.34	0.67	0.54	0.37	0.54	0.47
V2	0.85	0.83	0.54	0.66	0.61	0.60	0.53	0.46	0.60	0.65	0.35	0.67	0.48	0.37	0.49	0.43
●32	0.87	0.80	0.60	0.70	0.68	0.68	0.63	0.58	0.70	0.63	0.46	0.76	0.55	0.48	0.54	0.52
V5	0.81	0.73	0.70	0.80	0.78	0.69	0.77	0.70	0.78	0.76	0.60	0.81	0.63	0.59	0.63	0.57
△5	0.88	0.87	0.60	0.69	0.68	0.62	0.60	0.54	0.67	0.70	0.41	0.71	0.50	0.45	0.50	0.47
■19	0.82	0.77	0.72	0.89	0.79	0.71	0.78	0.75	0.77	0.74	0.69	0.85	0.63	0.66	0.66	0.55
I26	0.77	0.77	0.71	0.94	0.80	0.65	0.76	0.81	0.75	0.73	0.71	0.84	0.63	0.71	0.66	0.52
I8	0.83	0.72	0.72	0.83	0.80	0.72	0.78	0.67	0.80	0.77	0.59	0.82	0.68	0.57	0.69	0.58
V1	0.79	0.79	0.66	0.85	0.79	0.61	0.70	0.76	0.74	0.76	0.58	0.79	0.62	0.62	0.60	0.55
I29	0.82	0.77	0.69	0.88	0.81	0.66	0.78	0.77	0.77	0.84	0.62	0.80	0.66	0.59	0.64	0.58
▲12	0.84	0.82	0.60	0.79	0.69	0.60	0.62	0.56	0.65	0.77	0.43	0.69	0.56	0.42	0.58	0.46
■21	0.81	0.68	0.62	0.73	0.70	0.70	0.65	0.59	0.74	0.58	0.43	0.74	0.66	0.47	0.65	0.58

	F3	F4	L11	L1	L3	L4	L5	L6	L7	L8	NL1	NL2	NL3	NL4	NL5	NL6
●28	0.86	0.75	0.58	0.69	0.66	0.69	0.61	0.53	0.70	0.60	0.40	0.73	0.58	0.42	0.58	0.53
▲14	0.86	0.77	0.61	0.75	0.71	0.66	0.63	0.57	0.71	0.69	0.41	0.72	0.63	0.43	0.62	0.55
△7	0.87	0.74	0.68	0.77	0.77	0.73	0.75	0.66	0.80	0.71	0.50	0.80	0.66	0.52	0.64	0.60
●29	0.83	0.69	0.62	0.73	0.70	0.72	0.66	0.59	0.75	0.59	0.44	0.76	0.65	0.47	0.64	0.58
●30	0.83	0.72	0.60	0.57	0.61	0.69	0.60	0.44	0.67	0.51	0.42	0.73	0.51	0.45	0.49	0.53
▲11	0.81	0.67	0.60	0.61	0.61	0.71	0.61	0.45	0.69	0.47	0.42	0.74	0.50	0.47	0.55	0.46
△2	0.82	0.70	0.59	0.64	0.60	0.70	0.60	0.45	0.66	0.51	0.42	0.74	0.50	0.44	0.56	0.45
△6	0.81	0.69	0.57	0.55	0.58	0.68	0.58	0.41	0.65	0.49	0.40	0.72	0.45	0.43	0.49	0.44
▲13	0.84	0.73	0.61	0.58	0.61	0.79	0.60	0.43	0.70	0.57	0.43	0.74	0.48	0.46	0.51	0.50
■17	0.69	0.63	0.45	0.39	0.38	0.59	0.38	0.20	0.44	0.43	0.38	0.52	0.49	0.28	0.54	0.51
●31	0.74	0.70	0.54	0.78	0.59	0.60	0.58	0.56	0.59	0.54	0.51	0.72	0.47	0.49	0.54	0.38
❍23	0.80	0.73	0.57	0.69	0.59	0.65	0.56	0.48	0.63	0.48	0.44	0.72	0.50	0.47	0.57	0.42
□8	0.83	0.72	0.55	0.70	0.68	0.67	0.63	0.55	0.67	0.59	0.45	0.73	0.54	0.42	0.54	0.51
△4	0.85	0.77	0.61	0.69	0.68	0.68	0.66	0.58	0.71	0.63	0.52	0.78	0.55	0.51	0.56	0.53
■22	0.82	0.78	0.61	0.77	0.63	0.66	0.60	0.53	0.64	0.56	0.51	0.76	0.51	0.52	0.59	0.42
L9	0.84	0.82	0.61	0.70	0.65	0.67	0.61	0.66	0.67	0.60	0.52	0.78	0.48	0.54	0.49	0.47
❍27	0.86	0.80	0.64	0.70	0.66	0.69	0.62	0.49	0.68	0.60	0.49	0.78	0.52	0.51	0.58	0.46
△1	0.83	0.73	0.70	0.78	0.78	0.73	0.72	0.67	0.80	0.63	0.57	0.84	0.66	0.61	0.66	0.59
❍26	0.85	0.73	0.68	0.74	0.77	0.74	0.73	0.65	0.80	0.64	0.54	0.83	0.66	0.57	0.63	0.61
□9	0.82	0.70	0.69	0.70	0.72	0.75	0.69	0.57	0.76	0.57	0.55	0.82	0.62	0.59	0.65	0.57
□10	0.80	0.72	0.60	0.62	0.60	0.70	0.59	0.49	0.66	0.45	0.49	0.76	0.47	0.53	0.51	0.46
❍24	0.77	0.68	0.70	0.77	0.77	0.70	0.74	0.67	0.78	0.63	0.62	0.84	0.63	0.64	0.63	0.56
L10	0.59	0.67	0.62	0.77	0.81	0.43	0.68	0.78	0.64	0.63	0.60	0.66	0.39	0.66	0.40	0.34
△3	0.55	0.56	0.57	0.77	0.74	0.41	0.70	0.87	0.68	0.64	0.56	0.63	0.49	0.62	0.43	0.45
❍25	0.57	0.59	0.59	0.77	0.73	0.42	0.71	0.86	0.68	0.65	0.58	0.64	0.47	0.63	0.42	0.43
L2	0.67	0.69	0.74	0.76	0.74	0.46	0.70	0.81	0.69	0.59	0.70	0.79	0.44	0.80	0.42	0.39
F1	0.85	0.81	0.60	0.77	0.71	0.65	0.68	0.71	0.71	0.71	0.55	0.77	0.56	0.51	0.51	0.55
F5	0.80	0.84	0.58	0.91	0.67	0.58	0.61	0.73	0.62	0.68	0.59	0.75	0.54	0.56	0.56	0.46
F6	0.82	0.89	0.63	0.81	0.67	0.60	0.62	0.68	0.63	0.70	0.64	0.79	0.47	0.62	0.49	0.44
F7	0.87	0.89	0.65	0.80	0.72	0.63	0.65	0.71	0.69	0.71	0.61	0.82	0.58	0.61	0.51	0.57
F8	0.78	0.83	0.60	0.95	0.71	0.57	0.65	0.78	0.64	0.74	0.62	0.76	0.58	0.59	0.59	0.48
F2	0.94	0.93	0.75	0.81	0.72	0.65	0.68	0.72	0.70	0.68	0.74	0.92	0.54	0.73	0.52	0.51

	F3	F4	L11	L1	L3	L4	L5	L6	L7	L8	NL1	NL2	NL3	NL4	NL5	NL6
F9	0.99	0.87	0.75	0.73	0.74	0.70	0.73	0.64	0.75	0.78	0.61	0.87	0.60	0.58	0.55	0.58
F3	N/A	0.92	0.73	0.70	0.68	0.68	0.67	0.59	0.70	0.71	0.60	0.87	0.53	0.59	0.50	0.52
F4	0.09	N/A	0.64	0.72	0.60	0.52	0.52	0.58	0.54	0.68	0.60	0.79	0.38	0.60	0.38	0.37
L11	0.31	0.45	N/A	0.63	0.76	0.72	0.72	0.61	0.78	0.51	0.70	0.85	0.58	0.91	0.61	0.50
L1	0.36	0.33	0.46	N/A	0.73	0.58	0.73	0.84	0.67	0.74	0.73	0.77	0.61	0.65	0.63	0.49
L3	0.38	0.51	0.28	0.31	N/A	0.59	0.81	0.78	0.88	0.71	0.46	0.76	0.60	0.65	0.55	0.53
L4	0.39	0.65	0.33	0.55	0.53	N/A	0.61	0.44	0.71	0.54	0.55	0.74	0.62	0.59	0.63	0.62
L5	0.40	0.65	0.33	0.31	0.22	0.50	N/A	0.77	0.83	0.69	0.56	0.71	0.56	0.60	0.54	0.50
L6	0.54	0.54	0.50	0.17	0.25	0.81	0.26	N/A	0.72	0.69	0.59	0.66	0.52	0.64	0.45	0.46
L7	0.36	0.62	0.25	0.40	0.13	0.34	0.18	0.33	N/A	0.67	0.45	0.77	0.62	0.63	0.57	0.58
L8	0.34	0.38	0.67	0.30	0.34	0.62	0.37	0.37	0.41	N/A	0.42	0.58	0.53	0.36	0.43	0.52
NL1	0.50	0.50	0.36	0.32	0.77	0.59	0.58	0.52	0.80	0.86	N/A	0.77	0.53	0.80	0.61	0.48
NL2	0.14	0.24	0.17	0.26	0.28	0.30	0.34	0.42	0.26	0.55	0.26	N/A	0.60	0.82	0.62	0.54
NL3	0.63	0.96	0.55	0.49	0.50	0.48	0.58	0.65	0.48	0.63	0.63	0.52	N/A	0.45	0.94	0.95
NL4	0.53	0.52	0.09	0.43	0.43	0.53	0.51	0.45	0.47	1.03	0.22	0.19	0.79	N/A	0.52	0.38
NL5	0.69	0.97	0.50	0.46	0.60	0.47	0.62	0.80	0.56	0.85	0.49	0.47	0.06	0.66	N/A	0.85
NL6	0.66	0.99	0.70	0.72	0.63	0.47	0.69	0.77	0.54	0.66	0.74	0.62	0.05	0.98	0.16	N/A

8.4 Geographic distance between populations in kilometres. (Accession codes are given in Appendix 8.1.)

	I28	I1	I5	I17	I13	I7	I6	I10	I21	I30	I23	I19	I16	I12	I20	I11	I14	I4	I2	I3	I22	I15
I28	0																					
I1	61	0																				
I5	171	192	0																			
I17	273	229	247	0																		
I13	231	223	109	161	0																	
I7	212	230	42	250	96	0																
I6	126	85	165	147	154	192	0															
I10	163	120	180	110	145	201	37	0														
I21	245	201	228	28	151	235	119	82	0													
I30	83	24	212	224	234	249	87	118	196	0												
I23	112	106	99	184	120	130	67	90	159	121	0											
I19	124	66	212	174	208	243	55	74	146	52	113	0										
I16	133	116	111	158	106	136	56	70	133	128	26	109	0									
I12	200	155	204	75	148	219	74	37	46	150	122	101	99	0								
I20	125	78	177	152	168	205	14	42	123	77	79	41	70	77	0							
I11	162	124	166	112	130	185	39	16	84	125	79	85	57	43	48	0						
I14	92	39	184	190	195	217	48	81	162	40	87	36	90	116	39	86	0					
I4	204	217	40	230	78	20	175	182	215	235	115	226	118	199	188	166	141	0				
I2	145	113	146	131	123	168	32	35	105	118	57	86	36	65	46	22	65	150	0			
I3	208	222	43	233	79	17	179	186	218	240	120	231	123	203	193	171	145	5	154	0		
I22	102	71	146	172	154	177	28	63	144	80	48	66	48	100	35	61	108	161	44	166	0	
I15	122	101	122	161	122	150	43	63	134	112	27	94	16	96	56	53	85	133	31	137	32	0
I27	89	105	88	224	145	126	98	127	198	126	40	134	65	161	107	118	140	116	95	121	72	65
I24	113	88	133	164	136	162	33	60	137	98	35	81	30	95	44	53	94	145	33	150	18	14
I18	271	228	241	7	154	243	145	109	28	224	180	174	154	74	150	109	86	224	128	227	170	157
I25	119	110	100	176	114	129	65	85	151	125	8	113	18	116	77	73	93	113	51	118	48	23
I9	171	192	0	247	109	42	165	180	228	212	99	212	111	204	177	166	155	40	146	43	146	122
Δ5	741	683	837	632	785	858	673	657	635	660	741	634	726	640	662	672	703	838	691	842	696	715
I26	121	111	100	176	113	129	65	85	151	126	9	114	17	115	78	73	92	112	51	117	49	23
I8	141	136	83	174	90	107	85	97	151	150	31	136	28	122	98	83	85	90	63	95	73	43
I29	18	46	166	256	218	207	108	145	227	69	99	107	118	182	107	145	190	197	128	202	84	106
▲12	3365	3394	3498	3618	3590	3535	3479	3513	3591	3395	3476	3445	3498	3545	3471	3518	3569	3538	3505	3541	3461	3486

	I28	I1	I5	I17	I13	I7	I6	I10	I21	I30	I23	I19	I16	I12	I20	I11	I14	I4	I2	I3	I22	I15
■21	2462	2491	2597	2716	2688	2634	2576	2610	2688	2493	2574	2542	2595	2643	2569	2615	2667	2637	2603	2640	2559	2583
●28	2001	2007	2167	2198	2229	2208	2086	2111	2174	1999	2109	2038	2123	2135	2074	2122	2180	2203	2117	2207	2077	2108
▲14	5757	5760	5923	5942	5983	5964	5837	5861	5920	5751	5863	5788	5877	5883	5825	5872	5931	5959	5869	5963	5831	5861
Δ7	248	274	404	502	478	446	359	394	474	278	360	329	380	428	352	398	450	442	386	446	342	367
●29	1870	1872	2038	2055	2095	2080	1949	1973	2033	1863	1975	1899	1989	1995	1936	1984	2042	2074	1980	2078	1942	1973
▲11	3179	3200	3326	3416	3409	3365	3285	3316	3389	3198	3291	3245	3311	3346	3275	3323	3378	3366	3313	3369	3270	3297
Δ2	926	929	1093	1122	1151	1135	1008	1034	1098	921	1031	960	1046	1058	996	1044	1102	1129	1039	1133	1000	1030
Δ6	347	343	518	540	566	559	421	447	515	334	447	374	460	473	409	457	515	550	452	555	413	444
▲13	4839	4852	4996	5053	5069	5037	4933	4961	5028	4846	4949	4888	4966	4987	4922	4970	5028	5035	4964	5039	4922	4952
■17	1774	1823	1852	2047	1959	1879	1900	1937	2019	1834	1870	1887	1895	1974	1898	1935	1971	1890	1916	1891	1875	1889
○23	953	994	1064	1223	1164	1098	1076	1113	1195	1002	1059	1054	1083	1149	1072	1114	1158	1104	1098	1106	1054	1074
□8	2113	2166	2172	2383	2281	2195	2239	2275	2356	2180	2202	2231	2227	2312	2238	2272	2302	2207	2252	2208	2212	2223
Δ4	1494	1474	1663	1604	1688	1703	1534	1548	1587	1458	1579	1480	1585	1559	1520	1562	1619	1691	1566	1696	1537	1568
■22	2735	2772	2852	3001	2951	2886	2856	2892	2973	2777	2844	2828	2867	2926	2850	2894	2942	2892	2879	2894	2835	2857
L9	1862	1922	1809	2054	1900	1806	1953	1980	2036	1944	1890	1979	1911	2009	1960	1968	1964	1825	1946	1822	1926	1917
○27	1301	1331	1436	1557	1526	1474	1416	1450	1529	1333	1412	1383	1434	1483	1408	1455	1506	1477	1442	1480	1398	1422
Δ1	1221	1219	1391	1397	1442	1432	1294	1317	1375	1209	1323	1243	1335	1338	1281	1328	1387	1425	1325	1430	1288	1320
○26	1306	1336	1442	1562	1532	1480	1421	1456	1534	1338	1418	1388	1440	1489	1414	1460	1511	1483	1447	1485	1403	1428
□9	2326	2383	2241	2477	2317	2229	2399	2420	2464	2407	2332	2433	2350	2445	2408	2406	2390	2249	2386	2245	2374	2359
○24	706	739	841	967	931	879	823	858	939	743	817	794	839	892	817	862	912	881	848	884	804	828
L10	2888	2884	3058	3050	3106	3100	2956	2977	3030	2872	2989	2904	3000	2995	2943	2989	3048	3092	2988	3097	2952	2984
Δ3	1123	1114	1294	1279	1336	1335	1185	1205	1258	1102	1220	1133	1230	1223	1172	1217	1276	1326	1217	1331	1182	1214
○25	1271	1322	1343	1543	1450	1369	1397	1434	1515	1335	1364	1387	1389	1471	1395	1431	1465	1380	1412	1381	1371	1384
L2	1264	1322	1194	1438	1281	1188	1345	1370	1421	1346	1280	1376	1300	1397	1354	1357	1348	1208	1336	1205	1319	1307
L1	6460	6401	6537	6301	6461	6548	6380	6358	6314	6378	6446	6348	6428	6333	6371	6371	6387	6528	6392	6532	6406	6420
NL3	6216	6199	6386	6325	6414	6426	6260	6273	6310	6183	6304	6205	6310	6284	6246	6287	6344	6416	6291	6420	6262	6294

	I27	I24	I18	I25	I9	Δ5	I26	I8	I29	▲12	■21	●28	▲14	Δ7	●29	▲11	Δ2	Δ6	▲13	■17	○23	□8
I27	0																					
I24	67	0																				
I18	219	161	0																			
I25	47	33	172	0																		
I9	88	133	241	100	0																	
Δ5	768	706	639	738	837	0																
I26	48	34	171	1	100	738	0															
I8	59	56	169	26	83	754	24	0														
I29	81	97	253	106	166	729	107	128	0													
▲12	3446	3476	3619	3484	3498	3573	3485	3504	3380	0												
■21	2544	2573	2716	2581	2597	2694	2583	2601	2478	903	0											
●28	2091	2095	2200	2115	2167	1967	2116	2139	2012	1719	1003	0										
▲14	5846	5848	5946	5869	5923	5607	5870	5894	5767	2980	3634	3756	0									
Δ7	335	357	501	367	404	790	369	389	262	3120	2217	1763	5519	0								
●29	1960	1960	2058	1981	2038	1798	1982	2006	1880	1899	1160	181	3889	1638	0							
▲11	3265	3286	3417	3298	3326	3290	3300	3320	3193	491	810	1354	2829	2931	1534	0						
Δ2	1015	1017	1125	1037	1093	1012	1039	1062	935	2570	1683	1078	4832	694	945	2319	0					
Δ6	435	431	542	453	518	669	454	478	354	3092	2191	1665	5417	171	1529	2878	587	0				
▲13	4927	4939	5055	4956	4996	4812	4957	4979	4851	1828	2550	2864	1165	4593	3022	1740	3931	4514	0			
■17	1832	1884	2044	1878	1852	2289	1879	1890	1792	1904	1142	1568	4774	1574	1611	1945	1406	1638	3673	0		
○23	1024	1066	1222	1067	1064	1427	1068	1083	970	2447	1553	1369	5039	729	1308	2319	646	776	4036	863	0	
□8	2162	2219	2380	2210	2172	2673	2210	2218	2131	1910	1303	1914	4863	1933	1978	2053	1814	2012	3730	411	1248	0
Δ4	1576	1554	1608	1583	1663	1197	1584	1608	1497	2595	1816	879	4410	1312	699	2230	714	1157	3637	1955	1332	2360
■22	2810	2848	3000	2852	2852	3056	2853	2869	2752	762	472	1473	3714	2499	1625	941	2046	2501	2577	1152	1788	1153
L9	1855	1922	2048	1895	1809	2597	1895	1888	1876	3222	2531	2824	6153	1833	2810	3326	2182	1991	5033	1392	1562	1314
○27	1382	1412	1556	1420	1436	1612	1421	1440	1316	2064	1162	964	4605	1056	934	1901	631	1042	3607	811	434	1221
Δ1	1310	1306	1401	1329	1391	1178	1330	1354	1229	2407	1551	809	4545	998	658	2111	309	876	3673	1503	870	1913
○26	1388	1418	1562	1426	1442	1616	1427	1445	1322	2058	1156	960	4599	1062	930	1895	634	1048	3601	811	440	1220
□9	2302	2366	2470	2336	2241	3067	2336	2324	2338	3748	3110	3435	6708	2342	3420	3890	2761	2509	5573	1981	2159	1845
○24	786	818	966	825	841	1132	826	844	722	2659	1757	1401	5137	465	1305	2488	460	485	4175	1158	295	1542
L10	2977	2971	3054	2994	3058	2692	2995	3019	2896	1773	1523	962	2915	2664	1040	1284	1970	2542	2200	2441	2330	2731
Δ3	1210	1200	1283	1224	1294	1026	1226	1250	1129	2563	1706	949	4666	914	789	2264	276	776	3811	1606	907	2016

	I27	I24	I18	I25	I9	Δ5	I26	I8	I29	▲12	■21	●28	▲14	Δ7	●29	▲11	Δ2	Δ6	▲13	■17	○23	□8
○25	1325	1379	1540	1372	1343	1834	1373	1382	1289	2326	1481	1584	5086	1085	1568	2287	1067	1168	4027	511	432	848
L2	1247	1313	1432	1285	1194	2005	1285	1275	1277	3315	2512	2567	6132	1274	2515	3323	1744	1442	5062	1412	1207	1515
L1	6478	6413	6307	6442	6537	5719	6442	6454	6447	7679	7266	6293	6938	6483	6170	7189	6285	6329	7285	7646	6923	8053
NL3	6300	6280	6330	6308	6386	5829	6309	6334	6220	4283	4666	4379	1778	6015	4446	3964	5338	5873	2718	5763	5747	5969

	Δ4	■22	L9	○27	Δ1	○26	□9	○24	L10	Δ3	○25	L2	L1	NL3
Δ4	0													
■22	2263	0												
L9	2889	2461	0											
○27	1147	1460	1877	0										
Δ1	469	1959	2432	692	0									
○26	1147	1455	1880	6	693	0								
□9	3474	2996	612	2487	3023	2490	0							
○24	1174	2035	1731	596	747	602	2304	0						
L10	1495	1940	3762	1924	1668	1919	4373	2344	0					
Δ3	438	2110	2455	799	156	800	3037	735	1772	0				
○25	1722	1602	1243	636	1253	640	1854	713	2532	1315	0			
L2	2457	2562	626	1606	2029	1611	1069	1285	3529	2019	1047	0		
L1	5693	7735	8313	6835	6143	6835	8777	6670	5907	6051	7351	7723	0	
NL3	4726	4905	7138	5339	5028	5334	7741	5745	3417	5102	5932	6945	5523	0

8.5 – Chloroplast sequences. Full population names given at the end of the document.

***rps*16** – GenBank accession number DQ131606

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L. m. N                 -CTATCCATCATTTTCCATAGTAATT-TTAAATGCTCTTGGCTCGACATAGTCTGTTCTA
F. A. Dovey             GCTATCCTC--TTTTCCATA-TAATA--TAAATGCTCTTGGCTCGACATAGTCTGTTCTA
L. p. Magician          ---NNCCAC--ATTTCCATA-TAATA-TAAAATGCTCTTGGCTCGCAATAGTCTGTTCTA
L. p. 2419Roscommon     -CTATCCATC-ATTTCCATATTACGA--CTAATGCTCTTGGCTC-TCATAGTCTGTTCTA
Saccharum               GCCATCCATC-TTTTCCATAGTAATG--AAAATGCTCTTGGCTCGACATAGTCTGTTCTA
L. m. M                 ------TATTGGGATCCG--GTAGATAAATAACGCCCCCCCCCAATAAACGTATAGGAGG
F. p. W                 ------TATTGGGATCCG--GTAGATAAATAACGCCCCCCCCCAATAAACGTATAGGAGG
                                     ***     **        ** ** *    *      *  ** *

L. m. N                 TTCCTCCCGAACCAAATTTGCGCTGGGTTGTTTGTTTTTAA-GTAATTATAGTACACGAT
F. A. Dovey             TTCCTCCCGAACCAAATTTGCGCTGGGTTGTTTGTTTTTAA-GTAATTATAGTACACGAT
L. p. Magician          TTCCTCCCGAACCAAATTTGCGCTGGGTTGTTTGTTTTTAA-GTAATTATAGTACACGAT
L. p. 2419Roscommon     TTCCTCCCGAACCAAATTTGCGCTGGGTTGTTTGTTTTTAA-GTAATTATAGTACACGAT
Saccharum               TTCGTCCCGAACCGAATTTGCGCTGGGTTGTTTGTAAGTAAAGTAA--ATAGTACACGAT
L. m. M                 TTTTCTCCTCATACGGCTCGAGA-AAATGATTTGAATTTCTGTCTATAGTCTATAGTAAT
F. p. W                 TTTTCTCCTCATACGGCTCGAGA-AAATGATTTGAATTTCTGTCTATAGTCTATAGTAAT
                        **    **  *      * * *     *  ****    *      *   *         **

L. m. N                 GGAGCTCGAGA------GGATAGAATTTATTTTTTATCAAGGGAAAGAATCT---AGGGT
F. A. Dovey             GGAGCTCGAGCTCGAGAGGATAGAATTGATTTTTTATCAAGGGAAAGAATTT---ATGGT
L. p. Magician          GGAGCTCGAGCTC----GAATAGAATTTATTTTTTATCA-GGGAAAGAATTT---ATTGG
L. p. 2419Roscommon     GGAGCTCGAGCTC----GAATAGAATTTATTTTTTATCAAGGGAAAGAATTTT--ACGGT
Saccharum               GGAGCTCGAGA------GGACAGAATTTCTTTTTGATCAAGGGAAAGAATCT---AGGGT
L. m. M                 AGAAATTAGACTAT---GACGTGCATTAATTTCCTTACAGAAAAAACAAATTT---CATT
F. p. W                 AGAAATTAGACTAT---GACGTGCATTAATTTCCTTACAGAAAAAACAAATTT---CATT
                         **  *           *    * ***  ***     **    *** **  *

L. m. N                 TAGTGAAAACTAATAAA-TTAGGCCAACTTTGTCAGTCTATCCTTAATATAAAAATAGAA
F. A. Dovey             TAGTGAAAACTAATAAAGTTAGGCCAACTTTGTCAGTCTATCCTTAATATAAAAATAGAA
L. p. Magician          TAGTGAAAACTAATAAAAAT-GGCCAACTTTGTCAGTCTATCCTTAATATAAAAATAGAA
L. p. 2419Roscommon     TAGCGAAAACTAATAAA-TTATGCCAACTTTGTCAGTCTATCCTTAATATAAAAATAGAA
Saccharum               TATTGAAAACTAATAAA-TTAGGCCAACTTTGTCAGTCTATCCTTAATATAGAAATCAAA
L. m. M                 TATACTCATGTATTTAAAGTTGGCTAATTTTGACTGACAGACTTCAA-----AGACTAAA
F. p. W                 TATACTCATGTATTTAAAGTTGGCTAATTTTGACTGACAGACTTCAA-----AGACTAAA
                        **     *  ** * **  *  ** ** **** * * *   * * **     * *   **

L. m. N                 AGGTTAAAATAAGAAGAAAGTCC---------TCTTTTTGAAGATAGGGAAAACTCTTTC
F. A. Dovey             AGGTTAAAATAAGAAGAAAGTCCCCCTNCCCCTCCTTTTGAAGATAGGGAAAACTCTTTC
L. p. Magician          AGGTTAAAATAAGAAGAAAGCCC---------TCTTTTTGAAGATAGGGAAAACTCTTTC
L. p. 2419Roscommon     AGGTTAAAATAAGAAGAACGTCC---------TCTTTTTGAAGATAGGGAAAACTCTTTC
Saccharum               AGGTTAAAATAAGAA-AAAGTCT---------AATTTTGGAAGATTGGAAAAACTTTTTT
L. m. M                 TCCTTCCAA-AATTTTTGAGTCG----------TCTCTAAACTCTT----TTCTTTGTC
F. p. W                 TCCTTCCAA-AATTTTTGAGTCG----------TCTCTAAACTCTT----TTCTTTGTC
                           **  ** **       * *              *   **          ** * *

L. m. N                 AA-TTAAAAGTATATCAGAATTAATCCGGCTTATTTGATTTCTATATAAGAGGGATATGC
F. A. Dovey             AA-TTAAAAGTATATCAGAATTAATCCGGCTTATTTGATTTCTATATAAGAGGGATATGC
L. p. Magician          AAATTAAAAGTATATCAGAATTAATCCGGCTTATTTGATTTCTATATAAGAGGGATATGC
L. p. 2419Roscommon     AA-TTAAAAGTATATCAGAATTAATCCGGCTTATTTGATTTCTATATAAGAGGGATATGC
Saccharum               GA-TTAAAAGTCTATCTGAATCAATTGTTCATATTTGATTTCTATAGAAGAGTGAAATGC
L. m. M                 TCATTTCGAGCGAATTTACTTTTATCCCTTAT-TCTGATCCAATTCTGTTGTTGAGACAA
F. p. W                 TCATTTCGAGCGAATTTACTTTTATCCCTTAT-TCTGATCCAATTCTGTTGTTGAGACAA
                           **   **   **     *  **      * * ****     *        ** *
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L. m. N                 TTTATCGAAGGAA--ATAAGAAAAAGAGGGTATGTTGCTACTCTTTTGAAAGAAAG-AAA
F. A. Dovey             TTTATCGAAGGAA--ATAAGAAAAAGAGGGTATGTTGCTACTCTTTTGAAAGAAA--AAA
L. p. Magician          TTTATCGAAGGAA--ATAAGAAAAAGAGGGTATGTTGCTACTCTTTTGAAAGAAA---AA
L. p. 2419Roscommon     TTTATCGAAGGAA--ATAAGAAAAAGAGGGTATGTTGCTACTCTTTTGAAAGAAA--AAA
Saccharum               TTTATCGAGGAAATAAGAAAAAAGAAAGGGTATGTTGCTACTCTTTTGAAAGAAA--AAA
L. m. M                 TTGAAAATTGTGT--TTACTTGTTCTGGAATCCTTTATCTTTGATTTGTGAAATCCTTGG
F. p. W                 TTGAAAATTGTGT--TTACTTGTTCTGGAATCCTTTATCTTTGATTTGTGAAATCCTTGG
                        ** *    *       *         *  *   **     *  **** * *

L. m. N                 GAATAGAAATTCCCGAAGTAATGTCTAAACCCAAGGATTT-CACAAATCAAAGATAAAGG
F. A. Dovey             GAATAGAAATTCCCGAAGTAATGTCTAAACCCAAGGATTT-CACAAATCAAAGATAAAGG
L. p. Magician          GAATAGAAATTCCCGAAGTAATGTCTAAACCCAAGGATTT-CACAAATCAAAGATAAAGG
L. p. 2419Roscommon     GAATAGAATTCCCCGAAGTAATGTCTAAACCCAAGGATTTTCACAAATCAAAGATAAAGA
Saccharum               GAATAGGAGTTCCCGAAGTAATGTCTAAACCCAAGGATTT-CACAAATCAAAGATAAAGG
L. m. M                 GTTTAGACATTACTTCGGGAATTTCTATTCTTTTTTCTTTCAAAAGAGTAGCAACATACC
F. p. W                 GTTTAGACATTACTTCGGGAATTTCTATTCTTTTTTCTTTCAAAAGAGTAGCAACATACC
                        *  ***   *  *    * *** **** *       ***  * * *  *   * * *

L. m. N                 ATTCCAGA--------------------------------------------------------
F. A. Dovey             ATTCCAGA--------------------------------------------------------
L. p. Magician          ATTCCAGA--------------------------------------------------------
L. p. 2419Roscommon     TTTCCGGG--------------------------------------------------------
Saccharum               ATTCCGGA--------------------------------------------------------
L. m. M                 CTCTTTTTC-------------------------------------------------------
F. p. W                 CTCTTTTTC-------------------------------------------------------
                         *

L. m. N                 ----------------------------------ACAAGTAAACACAATTTTCAATTGTC
F. A. Dovey             ----------------------------------ACAAGTAAACACAATTTTCAATTGTC
L. p. Magician          ----------------------------------ACAAGTAAACACAATTTTCAATTGTC
L. p. 2419Roscommon     ----------------------------------ACAAGTAAACACAATTTTCAATTGTC
Saccharum               ----------------------------------ACAAGTAAACACGATTTTCAACCGTC
L. m. M                 -------------------------TTATTTCCTTCGATAAAGCATATCCCTC--TTATA
F. p. W                 -------------------------TTATTTCCTTCGATAAAGCATATCCCTC--TTATA
                                                  * *  ** **      **     *

L. m. N                 TCAACAACAGAATTGGATCAGAATAAGGGATAAAAGTAAATTCGCTCGAAATGA-GACAA
F. A. Dovey             TCAACAACAGAATTGGATCAGAATAAGGGAAAAAAGTAAATTCGCTCGAAATGA-GACAA
L. p. Magician          TCAACAACAGAATTGGATCAGAATAAGGGATAAAAGTAAATTCGCTCGAAATGA-GACAA
L. p. 2419Roscommon     TCAACAACAGAATTGG-TCAGA-TAAGGGATAAAAGTAAATTCGCTCGAAATGA-GACAA
Saccharum               TCAACAATAGAATTAGATCAGAATAAGGAATAAAAGTCAATTTGTTCGAGATGA-GATAA
L. m. M                 TAGAAAATCAAATAAGCCCGGATTAAT--TCTGATATACTTTTAATTGAAAGAG---TTT
F. p. W                 TAGAAAATCAAATAAGCCCGGATTAAT--TCTGATATACTTTTAATTGAAAGAG---TTT
                        *  * **   ***  *  * ** ***       *  *   **   * ** *

L. m. N                 AGAA-AAGAGTTTAGAGACGACTCAAA-AATTTTGGAAGGATTTAGTCTTTGAAGTC---
F. A. Dovey             AGAA-AAGAGTTTAGAGACGACTCAAA-AATTTTGGAAGGATTTAGTCTTTGAAGTC---
L. p. Magician          AGAA-AAGAGTTTAGAGACGACTCAAA-AATTTTGGAAGGATTTAGTCTTTGAAGTCCTC
L. p. 2419Roscommon     AGAA-AAGAGTTTAGAGACGACTCAAA-AATTTTGGAAGGATTTAGTCTTTGAATTCCTC
Saccharum               AGAA-AAGAGTTTAGAGACGACTCAAAAAATTTCGAA----TTT--CTTTTGAAGTT---
L. m. M                 TCCCTATCTTCAAAAAGAGGACTTTCTTCTTATTTTAACCTTTCTATTTTTATATTAAGG
F. p. W                 TCCCTATCTTCAAAAAGAGGACTTTCTTCTTATTTTAACCTTTCTATTTTTATATTAAGG
                             *       * *** ****       * *   *    **     ***  * *
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L. m. N                 -TCTC-AGTCAAAATTAG--CCAATCTT--ATTCATGAGTATAAATGAAATTTGTTTTT-
F. A. Dovey             -TGTC-AGTCAAAATTAG--CCAACATA--AATCATGAGTATAAATGAAATTTGTTTTT-
L. p. Magician          -TCCA-CCTCAAAATTAG--CCAACTTA--AATCATGAGTATAAATGAAATTTGTTTTT-
L. p. 2419Roscommon     -TCCAGTCCCAAAATTAG--CCAATTAA--ATTCATGAGTATAAATGAAATTTGTTTTT-
Saccharum               -TGTCCAGTCAAAATTAG--CCAACTTG--AGTCATGAGTATAAATGAAATTGGTTTTTG
L. m. M                 ATAGACTGACAAAGTTGGACCTAATTTATTAGTTTTCAACTTTCCCTAGATT--CTTTC-
F. p. W                 ATAGACTGACAAAGTTGGACCTAATTTATTAGTTTTCAACTTTCCCTAGATT--CTTTC-
                         *       **** ** *  * **     * *  * *   *     * ***   ***

L. m. N                 ----TCTGTAAGGAAATTAATGC-ACG----TCATAGTCTAATTTCTATTACTATAGACT
F. A. Dovey             ----TCTGTAAGGAAATTAATGC-ACG----TCATAGTCTAATTTCTATTACTATAGACT
L. p. Magician          ----TCTGTAAGGAAATTAATGC-ACG----TCATAGTCTAATTTCTATTACTATAGACT
L. p. 2419Roscommon     ----TCTGTAAGGAAATTAATGC-ACGC---TCATAGTCTAATTTCTATTACTATAGACT
Saccharum               ATTTTCTTTAAGGAAATTAATGC-AAG----TCATAATCGAATTTCTATCTATTTGCATT
L. m. M                 ----CCTGATAAAAAATAAATTCTATCCTC-TCGAGCTCCATCGTGTACTATAATTACTT
F. p. W                 ----CCTGATAAAAAATAAATTCTATCCTC-TCGAGCTCCATCGTGTACTATAATTACTT
                             **   *  **** *** * *      **    ** *   * **      *    *

L. m. N                 ATAGAC-AGAAATTCAAATCATTTTCTCGAGCC--GTATGAGG----AGAAAACCTCCTA
F. A. Dovey             ATAGAC-AGAAATTCAAATCATTTTCTCGATGCC-GTATGAGGA---GAAAGACCTCCTA
L. p. Magician          ATAGAC-AGAAATTCAAATCATTTTCTCGAGCC--GTATGAGGA---CCAAAACCTCCTA
L. p. 2419RoscommonATAGAC-ACAAATTCAAATCATTTTCTCGATTCC-GTATCAGGA---CCAAGACCTCCTA
Saccharum               ATAGACCAGAAATTCGAATCATTTTCTCGAGCC--GTATGAGGA----GGAAACCTCCTA
L. m. M                 AAAAACAAACAACCCAGCGCAAATTT--GGTTC----GGGAGGA---ATAGAACAGACTA
F. p. W                 AAAAACAAACAACCCAGCGCAAATTT--GGTTC----GGGAGGA---ATAGAACAGACTA
                        * * ** *  **  *    **  **   *   *       ***         **   ***

L. m. N                 TACGTTTATATG-GGGGGGCGTTATTTATCTACCT------------------
F. A. Dovey             TACGTTTATATG-GGGGGGCGTTATTTATCTACCT------------------
L. p. Magician          TACGTTTATAATGGGGGGGCGTTATTTATCTACCTCGGTCCCACT--------
L. p. 2419Roscommon     TACGTTTAAATA--GGGGGCGTTTTTTT--CTACCTCATTCCCAT---------
Saccharum               TACGTTCTAGGG--GGGGTTGTTTTTTGCGT----------------------
L. m. M                 TG---TCGAGCC--AAGAGCATTTATTAATTACTATGGAAAATGATGGTGTGC
F. p. W                 TG---TCGAGCC--AAGAGCATTTATTAATTACTATGGAAAATGATGGTGTGC
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***trn*L-F intergenic spacer** GenBank accession number DQ123585

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F. A. Dovey      ---AGCCAACTGATCTATCCTGACCTTTTCTTGTGCAT-ATCCT-AGTAGAGTATTTCGT
F. A. F          ---AACCAACTGCACTATCCTGACCTTTTCTTGTGCATTATCCT-AGTAGAGTATTTCGT
L. p. 2419Rosc   ---------------TATCCTGACCTTTTCTTGTGCATAATCCT-AGTAGAGTATTTCGT
L. p. 2483Wex    TTACTCAGACTGTCCTATCCTGACCTTTTCTTGTGCAACATCCTCAGTAGAGTATTT-GT
F. p. W          ----ACCAACTGATCT-TCCTGACCTTTA-TTGTGCACCTTCCT-AATAGAGTATTT-GT
L. m. N          -----ACCAACGCACT-TCCTGACCTTTTCTTGTGCATATACCT-AGTAGAGTATTTCGT
L. m. M          ---------------T-TCCTGACCTTTTCTTGTGCTATCTCCT-AGTAGAGTATTT-GT
Saccharum        ----------------ATCCTGACCCTTTCTTGTGC-TNNTCTT-AGTAAAGTATTT-CC
                                 ******** **  ******     * * * ** *******

F. A. Dovey      ATGCTATGT-CAATTAAAGGGACTAAAAAATAAATTAAATAAAGTATTTCAAATTCGAAA
F. A. F          AT-CTATGT-CAATTAAAGGGACTAAAAAATAAATTAAATAAAGTATTTCAAATTCGAAA
L. p. 2419Rosc   ATGCTATGTGCAATTAAAGGGACTAAAAAATAAATTAAATAAAGGATTTCAAATTCGAAA
L. p. 2483Wex    AT-CTATGT-CAATTAAAGGGACTAAAAAATAAATTAAATAAAGGATTTCAAATTCGAAA
F. p. W          AT-CTATGT-CAATTAAAGGGACTAAAAAATAAATTAAATAAAGGATTTCAAATTCCAAA
L. m. N          ATGCTATGT-CAATTAAAGGGACTAAAAAATAAATTAAATAAAGGATTTCAAATTCGAAA
L. m. M          AT-CTATGT-CAATTAAAGGGACTAAAAAATAAATTAAATAAAGGATTTCAAATTCGAAA
Saccharum        AT-CTATGT-CAATTAAAGGGACTAAAAAATCAAT-----AAAGTATTCC--ATTCAAAA
                 ** ****** ********************** ***     **** *** *  **** ***

F. A. Dovey      TTGTAAAATGGGGGGGG-----TAGTCCTATGC-ATTG-TACATGGCTTACTTAATAATA
F. A. F          TTGTAAAATGGGGGGGG-----TAGTCCTATGC-ATTG-TACATGGCTTACTTAATAATA
L. p. 2419Rosc   TTGTAAAATGGGGGGGGGGGG-TAGTCCAATGCTATTGTCACAGGGTTTACTTAAAAATA
L. p. 2483Wex    TTGTAAAATGGGGGGGGGGGGGTATCCCTATGCTATTG-CACAGGGCTTCATTTAATATT
F. p. W          TTGTAAAATGGGGGGGGGG---TAGTCCTATGC-ATTGTCACATGGCTTACTTAATAATA
L. m. N          TTGTAAAATGGGGGGGGG----TAGTCCTATGC-ATTG-TACATGGCTTACTTAATAATA
L. m. M          TTGTAAAATGGGGGGGGG----TAGTCCTATGC-ATTG-TACATGGCTTACTTAATAATA
Saccharum        TTAGGAAATGGGGAGGGG----TAGTCCTATGC-ATTG-TGGATGGCTTACTTAATAATA
                 **   ******** ***     **  ** **** ****    * ** **  ** *  **

F. A. Dovey      CTGAAAAATAGAGC-TGAATAACCGGGATTCTT-TCCCG----ATACTCTAATAAAAAAA
F. A. F          CTGAAAAATAGAGC-GTAATAACCGGGATTCTT-TCCCG----ATACTCTAATAAAAAAA
L. p. 2419Rosc   TTGGAAAATATAAACCGAAAAACCGGGTTTTTTGTCCGG----ATCCTTTGGTAAAAAAC
L. p. 2483Wex    TCGGAAAAAA-ACCGCGAATAACGGGGTTTTTT-TCCCA----ATCCTTTAATAAAAAAC
F. p. W          CTGAAAAATATAA--CGAATAACCGGGATTCGTTTCCCG----ATCCTCTAATAAAAACA
L. m. N          CTGAAAAATATAGT-CGAACAACCGGGATTCTT-TCCCG----ATACTCTAATAAAAAAC
L. m. M          CTGAAAAATATTAT-CTAAAACCCGGATTCTTT-GCCCG----ATGCTCTAATCGAAAAC
Saccharum        CTTAAAAAAATCGAATTATTAATCGAGATTCCTTGCCGGTGGTGTACTCTAATAAATAAA
                     **** *       *  *   *   *   *  **       * ** *  *  * *

F. A. Dovey      AAA--------------------TATATTCTA------GGATAAGATCCATTGAGTTCTC
F. A. F          AAA--------------------TATATTCTA------GGATAAGATCCATTGAGTTCTC
L. p. 2419Rosc   AAA--------------------TTTTTTTTG------GGGTAAAATCCTTTGATTTCTT
L. p. 2483Wex    AAA--------------------TTTTTTCTG------GGATAAAACCCTTTGGTTTTTT
F. p. W          AAT--------------------ATTATTCTA------GGATAAGATCCTTGGGTT-CTC
L. m. N          AAA--------------------TATATTATATTCT-AGGATAAGATCCATTGAGTTCTC
L. m. M          AAA--------------------TATATTATATTCTCAGGATAAGATCCATTGAGTTCTC
Saccharum        AAAAAGAAATAAAAAAGAAATCATCTATTTAATGAATAGCATAAGATTCATTGAGTTCTT
                 **                        * **         *  *** *  * * *  *  *
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F. A. Dovey      TTCGCACTCCTTTGTGAAAGAGTAGAATGAGAAAGCTAATGAATCTTAAACCC-GTGATA
F. A. F          TTCGCACTCCTTTGTGAAAGAGTAGAATGAGAAAGCTAATGAATCTTAAACCCCGTGATA
L. p. 2419Rosc   TTGGAATTCCTTTGGGAAAGAGTAAAATGAAAAAGCTAATGAATTTTAACCCCGGGGTTA
L. p. 2483Wex    TTCCCCCTCTTTTGTGAAAAATTAAATTGAAAAACCTAATGATTTTTAACCCTGGTGATA
F. p. W          TTCCCCCTCCTTTGTGAAAGAGTAGAATGAGAAAGCTAATGAATCTTAAACCCTGTGATA
L. m. N          TTCGCACTCCTTTGTGAAAGAGTAGAATGAGAAAGCTAATGAATCTTAAACCCTGTGATA
L. m. M          TTCGCACTCCTTTGTGAAAGAGTAGAATGAGAAAGCTAATGAATCTTAAACCCTGTGATA
Saccharum        GTCGCACTCCTTTGTGAAAGAGTAGAATGAGAAAGCTAGTGAATCTTAAACCCATTGATA
                  *     ** **** **** * ** * *** *** *** *** * **** **    * **

F. A. Dovey      AAAAGAAAAAAGAGGATAAATACTATAAGTTAGGG-AATAAAGGAGGG-TTTGGGGATAG
F. A. F          AAAAGAAAAAAGAGGATAAATACTATAAGTTAGGG-AATAAAGGAGGG-TTTGGGGATAG
L. p. 2419Rosc   AAAAGAAAAAAGAGGATAAATCCTATAATTTAGGG-AATAAAGGGGGG-TTTGGGGATAA
L. p. 2483Wex    AAAAAAAAAAAGGGGATAAATCCTTTATTTTAGGG-AATAAGGGGGGG-TTGGGGGTTAA
F. p. W          AAAAGAAAAAAGAGGATAAATACTATAATTTAGGG-AATAAAGGAGGG-TTTGGGGATAG
L. m. N          AAAAGAAAAAAGAGGATAAATACTATAAGTTAGGGGAATAAAGGAGGGGTTTGGGGATAG
L. m. M          AAAAGAAAAAAGAGGATAAATACTATAAGTTAGGG-AATAAAGGAGGG-TTTGGGGATAG
Saccharum        AAAGAAAAAAA--GGATAACAACTATG-GTTAGGG-AATAAAAGAGGG-TTTGGGGATAG
                 ***  ******  ******   ** *   ****** *****  * *** ** **** **

F. A. Dovey      AGGGACTTGAACCCTC-CAACTTAATAAAGTC-GACGGA----TTTTTCCTTTTAACTAG
F. A. F          AGGGACTTGAACCCTC-ACACTTA-TAAAGTC-GACGGGGAGGTTTTTCCTTTTAACTAG
L. p. 2419Rosc   AGGGACTTGAACCCTCACAACTTTATAAAGTC-GACGGA------TTTTCCTTTTACTA-
L. p. 2483Wex    AGGGACT-GACCCTTCACAACTT-ATAAAGT--GACGGA------TTTCC--TTTACTA-
F. p. W          AGGGACTTTAACCCTCCCAACTT-ATAAAGTC-CACGGA------TTTTCCTTTTCCTAC
L. m. N          AGGGACTTGAACCCTCACAACTT-ATAAAGTC-GACGGA------TTTTCCTTTTACTAG
L. m. M          AGGGACTTGAACCCTCACAACTT-ATAAAGTC-G-CGGA------TTTTCCTTTTACTAG
Saccharum        AGGGACTTGAACCCTCACGACTT-ATAAAGTCGGACGGA------TTTTCCTTTTACTAG
                 *******  * ** **   ****  ******    ***       *** *  **  ***

F. A. Dovey      AAATTTCATTGTTGGTCAG-TATT-GACATGTAAGAA--TGGGACT-CTCTCTTTGGTCC
F. A. F          AAATTTCCTTGGTGGTCAGGTATTTGACATGTAAAAA--TGGGACTTCTCTCTTTG--TC
L. p. 2419Rosc   GAAATTCATTGGTT-GCAG-TATT-GACATGTAGAAT---GGGACT-CTCTCTTTG-TCC
L. p. 2483Wex    GAATTTCATGTGT---CAG-TATT-GACATGTAGAAT---GGGACT-CTCTCTTTG-TCC
F. p. W          AAAATTCATTGTTG-TCAG-TATT-GACATGTAGAATATGGGGACT-CTCTCTTTG-TCC
L. m. N          AAATTTCATTGTTG-TCAG--GTT-GACCTGTAGAAT--GGGGACT-CTCTCTTTG-TCC
L. m. M          AAATTTCATTGTTG-CCAG-TATT-GACATGTAGAAT--G--GACT-CTCTCTTTG-TCC
Saccharum        AAATTTCATTGTTG-TCAG-TATTTGACATGTAGAAT---GGGACT-CTCTCTTTTATCC
                  ** *** *   *   ***   ** *** ****  *      **** ********    *

F. A. Dovey      CTCGTCCGGATTAATCCACTTTTTTAAAG-ACCTCAAAAACTTTGAATTGGAAG-GATTT
F. A. F          CTCGTCCGATTTAATCCACTTTTTTAAAG-ACCTCAAAA-CTTTGAATTG-AAG-GATTT
L. p. 2419Rosc   -TCGTC-GATTAATCC-ACTTTTTTAAAG-ACCTCAAAAC-TTTGAATTGAAG--GATTT
L. p. 2483Wex    -TCGTCCGATTAATCCCACTTTTTTAAAG-ACCTCAAAAC-TTTGAATTGAAG--GATTT
F. p. W          -TCGTCCGATTAAATCCACTTTTTTAAAGGACCTCAAAACCTTTGAATTGAAG--GATTT
L. m. N          CTCGTCCGATTAATCC-CCTTTTTTAAAGACCCTCCAAAACTTTGAATTGAAGGGGATTT
L. m. M          -TCGTCCGAT-AATCC-ACTTTTTTAAAGACCT--CAAAACTTTGAATTGAAG--GATTT
Saccharum        -TCGTCCGGATTAACCCACTTTTTTAAAAG-ATCTCGAAAACAATGAATTGAAG--GATTT
                  ***** *    *  *  ****** ****       ***    ******* *   *****
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F. A. Dovey      -GATTAC-AAAATATTCAATTGGAATGGATTCACAATAAAATAATTC--CAAAAAAAAAT
F. A. F          -GATTAC-AAAATATTCAATTGGAATGGATTCACAATAAAATAATTC--CAAAAAAAAAT
L. p. 2419Rosc   -GATTAC-AAAATATTCAATTGGAATGGATTCACAATAAAATAATTC--AAAAAAAAA-T
L. p. 2483Wex    -GATTAC-AAAATATTCAATTGGAATGGATTCACAATAAAATAATTC--AAAAAAAAA-T
F. p. W          TGATTAC-AAAATATTCAATTGGAATGGATTCACAATAAAATAATTC--AAAAAAAAAAT
L. m. N          -GATTCCAAAAATATTCAATTGGAATGGCTTCCCAATAAAATAATTCCAAAAAAAAAAAT
L. m. M          -GATTC--AAAATATTCAATTGGAATGGATTCCCAATAAAATAATTCAAAAAAAAAAAT
Saccharum        -GATTAC-TCAATATTCGATTGGAATAGATTCACAA-----TAATTC----TAAAAAAT
                  ****     ******* ******** * *** ***     ******     ****** *

F. A. Dovey      TCTGAAT-TTTTGATTTCATAATCATTTCTGTTTAAAATTGCATTCTAAAAA-AGAATAA
F. A. F          TCTGAAT-TTTTGATTTCATAATCATTTCTGTTTAAAATTGCATTCTAAAAA-AGAATAA
L. p. 2419Rosc   TCTGAAT-TTTTGATTTCATAATCATTTCTGTTTAAAATTGCATTCTAAAAA-AGAATAA
L. p. 2483Wex    TCTGAAT-TTTTGATTTCATAATCATTTCTGTTTAAAATTGCATTCTAAAAA-AGAATAA
F. p. W          TCTGAAT-TTTTGATTTCATAATCATTTCTGTTTAAAATTGCATTCTAAAAA-AGAATAA
L. m. N          TCCGAATCTTTTGATTTCATAATCATTTCTGTTTAAAATTGCATTCTAAAAA-AGAATAA
L. m. M          TCTGAAT-TTTTGATTTCATAATCATTTCTGTTTAAAATTGCATTCTAAAAA-AGAATAA
Saccharum        TATGAAT-TTTCTATTTCATAATCATTCCT------AATTTCATTCTAAAAATAAAATAA
                 *  **** ***  ************** **      **** *********** * *****

F. A. Dovey      AGAACCTATATTATAATATGGGTTCCATGATTAATCGTTTGCTATGCC-AGTATCTATAC
F. A. F          AGAACCTATATTATAATATGGGTTCCATGATTAATCGTTTGCTATGCC-AGTATCTATAC
L. p. 2419Rosc   AGAACCTATATTATAATATGGGTTCCATGATTAATCGTTTGCTATGCC-AGTATCTATAC
L. p. 2483Wex    AGAACCTATATTATAATATGGGTTCCATGATTAATCGTTTGCTATGCC-AGTATCTATAC
F. p. W          AGAACCTATATTATAATATGGGTTCCATGATTAATCGTTTGCTATGCC-AGTATCTATAC
L. m. N          AGAACCTATATTATAATATGGGTTCCATGATTAATCGTTTGCTATGCCGAGTATCTATAC
L. m. M          AGAACCTATATTATAATATGGGTTCCATGATTAATCGTTTGCTATGCC-AGTATCTATAC
Saccharum        AGAACCTATATT----------------------CCCCCCCCCCCCCC----------T
                 ************                        *    *    **

F. A. Dovey      GTGTTTATTAGATGTATAAAGCCCTTCTTTCTCAAATTTAGAA-GGATATACCACTAACA
F. A. F          GTGTTTATTAGATGTATAAAGCCCTTCTTTCTCAAATTTAGAACGGACATTCCACTAACA
L. p. 2419Rosc   GTGTTTATTAGATGTATAAAGCCCTTCTTTCTCAAATTTAGAAGGCATATTCCACTAACA
L. p. 2483Wex    GTGTTTATTAGATGTATAAAGCCCTTCTTTCTCAAATTTAGAAGGAA--TTCCACTAACA
F. p. W          GTGTTTATTAGATGTATAAAGCCCTTCTTTCTCAAATTTAGAAGGAA--TTCCACTAACA
L. m. N          GCGTTTATGAGATGTATAAAGCCCTTCTTTCTCA--TTTAGACCATA--TTCCACTAACG
L. m. M          GTGTTTATTAGATGTATAAAGCCCTTCTTTCTCAAATTTAGAAGGAA--TTCCACTAACA
Saccharum        GTGATTAATAGTTAT----------TATTTC------------------------------
                 * * ***  ** * *          * ****

F. A. Dovey      ACGCAAAATA-ATTATCCGATTCGTTAGAACAGCTTCCATCGAGTCTCTGCACCTATCCT
F. A. F          ACACAAAGTT-ATTATCCGATTCGTTAGAACAGCTTCCATCGAGTCTCTGCACCTATCCT
L. p. 2419Rosc   ACACAATGTATATTACCCGATTCGTTAGAACAGCTTCCATCGAGTCTCTGCACCTATCCT
L. p. 2483Wex    ACACAATCGTAATTACCCGATTCGTTAGAACAGCTTCCATCGAGTCTCTGCACCTATCCT
F. p. W          ACACAA-CGTAATTACCCGATTCGTTAGAACAGCTTCCATCGAGTCTCTGCACCTATCCT
L. m. N          GCGCGGGGTA-ATTACCCGATTCGTTAG--CAGCTTCCATCGAGTCTCTGCACCTATCCT
L. m. M          ACGCAAGGTATATTACCCGATTCGTTAGAACAGCTTCCATCGAGTCTCTGCACCTATCCT
Saccharum        -----------------GATTCGTTAGAACAGCTTCCATTGAGTCTCTGCACCTATCCT
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F. A. Dovey      TTTCCTTTGTATTCTAGTTCGAGAA--CCTCCTTGTTTTC-TCAAAAC-ACGGATTTGGC
F. A. F          TTTCCTTTGTATTCTAGTTCGAGAA--CCTCCTTGTTTTC-TCAAAAC-ACGGATTTGGC
L. p. 2419Rosc   TTTCCTTTGTATTCTAGTTCGAGATATCCTCCTTGTTTTCCTCAAAACCACGGATTTGGC
L. p. 2483Wex    TTTCCTTTGTATTCTAGTTCGAGAA--CCTCCTTGTTTTC-TCAAAAC-ACGGATTTGGC
F. p. W          TTTCCTTTGTATTCTAGTTCGAGAA--CCTCCTTGTTTTCCTCCAAAACACGGATTTGGC
L. m. N          TTTCCTTTGTATTCTAGTTCGAGAA--CCTCCTTGTTTTC-TCAAAAC-ACGGATTTGGC
L. m. M          TTTCCTTTGTATTCTAGTTCGAGAA--CCTCCTTGTTTTC-TCAAAAC-ACGGATTTGGC
Saccharum        TTTCCTTTGGGTTCTAGTTTGAGAA---CCACTTGTTTTT-TCAAAAAAGGGGATTTGGC
                 *********  ******** ****    *  ********  ** ***    *********

F. A. Dovey      TCAGGATTGCCCTTTTTT--AAGTCCAGGGTTTCTC-ATTTTTGGAAGTTACC-ACTTAG
F. A. F          TCAGGATTGCCCTTTTTT--AGTTCCAGGGTTTCTCTGAATTTGGAAGTTACC-ACTTAG
L. p. 2419Rosc   TCAGGATTGCCCTTTTTTTTAGATTCCAGGGTTTCTCTGAATTTGGAAGTTACC-ACTTAG
L. p. 2483Wex    TCAGGATTGCCCTTTTTT--AATTCCAGGGTTTCTCTGAATTTGGAAGTTACC-ACTTAG
F. p. W          TCAGGATTGCCCTTTTTTAAGTTGCCAGGGTTTCTCTAGTATTGGAAGTTACCCACTTAG
L. m. N          TCAGGATTGCCCTTTCTTTAGTTCCAGGCGTTTCTCAAGTTATAAAAGTT-CC-ACTTAG
L. m. M          TCAGGATTGCCCTTT-TTTAATTCCAGG-GTTTCTCTAGTTATGGAAGTTACC-ACTTAG
Saccharum        TCAGGATTGCCCATTCCT--CGTTCCAGGGTTTCTCAAAATTTGGAAGTTACC-ACTTAG
                 ************ **  *      *  * *******      *  ***** ** ******

F. A. Dovey      C------------------
F. A. F          CAGG---------------
L. p. 2419Rosc   CAGGTTTGCCATCACCA--
L. p. 2483Wex    CAGGTTTGCCAT-ACCA--
F. p. W          CAGGTTTACCATCACCAGA
L. m. N          CAGG---------------
L. m. M          CAGG---------------
Saccharum        CAGGGGCCCCCCCC-----
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***atp*B-*rbc*L intergenic spacer** GenBank accession number DQ123586

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L. p. 2419Rosc      ---NNCNAAANGACCTCCCNCGAACTTT-CTTTTTTTCTTG-TTGANTAATGCC----AA
L. m. N             --------------TCCCCCGAATTTTTCTTTTTTTCTTGGTTACCTAATGCC----AA
F. A. F             --------NGAGGATTCNC-CGAATTTTTCTTTTTTTCTTG-TTNAATAATGCC----AA
F. A. Dovey         ------AAAAAGGATTCCCGCGAATTTTTCTTTTTTTCTTG-TTGAATAATGCC----AA
L. p. Magician      ------------GANTCCC-CGAATTTTTCTTTTTTTCTTG-TTGAATAATGCC----AA
L. m. M             --------AGGGGGGACCGTCGAATTTTTCTTTTTTTCTTG-TTGAATAATGCC----AA
L. p. 2483Wex       ------TAAAAAGANACGTCGCAATTTTTCTTTTTTTCTTG-TTGAATAATGCCTGTCAA
F. p. W             -------AAAAGGGACCCGTCGAATTTTGCGTTTTTTCTTG-TTGAATAATGCC----AA
Saccharum           CTAAAAAAAAGGCAATTTGTCGAATTTT--TTTTTTTCTTG-TTGAATAATGCC----AA
                                        ** ***   ********** **   *******    **

L. p. 2419Rosc      ATCAAA-TCAAAAAAATATCCAAAAATACAAAAGNTAAAGAGAAAATGAATTAG-TTAAT
L. m. N             ATCAAA-TCAAAAAAATATCCAAAAATACAAAAGATAAAGAGAAAATGAATTAG-TTAAT
F. A. F             ATCAAA-TCAAAAAAATATCCAAAAATACAAAAG-TAAAGAGAAAATGAATTAGATTAAT
F. A. Dovey         ATCAAA-TCAAAAAAATATCCAAAAATACAAAAG-TAAAGAGAAAATGAATTAG-TTAAT
L. p. Magician      ATCAAA-TCAAAAAA-TATCCAAAAATACAAAAG-TAAAGAGAAAATGAATTAG-TTAAT
L. m. M             ATCAAACTCAAAAAAATATCCAAAAATACAAAAG-TAAAGAGAAAATGAATTAG-TTAAT
L. p. 2483Wex       ATCCAATGCAAAAAAATATCCAAAAATACAAAAG-TAAAGAGAAAATGAATTAG-TTAAT
F. p. W             ATCAAA-TCAAAAAAATATCCAAAAATACAAAAG-TAAAGAGAAAATGAATTAG-TTAAT
Saccharum           ATCAAA-----AAAAATATCCAAAAATCCAAAAGTCAAAAGGGAAATGAATTAT-TTAAT
                    *** **     **** *********** ******  ***  * **********  *****

L. p. 2419Rosc      TCAATAAGAGAGAAAAGGGGGCCAGGACTTTATT-TCGTTGCCCAAGCGAATCCCATTCA
L. m. N             TCAATAAGAGAGAAAAGGGGGCCAGGACTTTATT-TCGTTGCCCAAGCGAATCCCATTCA
F. A. F             TCAATAAGAGAGAAAAGGGGGCCAGGACTTTATT-TCGTTGCCCAAGCGAATCCCATTCA
F. A. Dovey         TCAATAAGAGAGAAAAGGGGGCCAGGACTTTATT-TCGTTGCCCAAGCGAATCCCATTCA
L. p. Magician      TCAATAAGAGAGAAAAGGGGGCCAGGACTTTATTATCGTTGCCCAAGCGAATCCCATTCA
L. m. M             TCAATAAGAGAGAAAAGGGGGCCAGGACTTTATT-TCGTTGCCCAAGCGAATCCCATTCA
L. p. 2483Wex       TCAATAAGAGAGAAAAGGGGGCCAGGACTTTATT-TCGTTGCCCAAGCGAATCCCATTCA
F. p. W             TCAATAAGAGAGAAAAGGGGGCCAGGACTTTATT-TCGTTGCCCAAGCGAATCCCATTCA
Saccharum           TCAATAAAAAAGAAAAGGGGACTCGCACTTGATT-TCGTTGCCCAAGCGAATCCCATTCA
                    ******* * ********** *  * **** *** *************************

L. p. 2419Rosc      ATCGTTTACTCATGGAATGAGTCCGTTGGAAA--ATTCAAT---CAATGTTTTTTTCCTA
L. m. N             ATCGTTTACTCATGGAATGAGTCCGTTGGAAA--ATTCAAT---CAATGTTTTTTTCCTA
F. A. F             ATCGTTTACTCATGGAATGAGTCCGTTGGAAATCATTCAAT---CG-TGTTTTTTTCCTA
F. A. Dovey         ATCGTTTACTCATGGAATGAGTCCGTTGGAAATCATTCAAT---CG-TGTTTTTTTCCTA
L. p. Magician      ATCGTTTACTCATGGAATGAGTCCGTTGGAAA--ATTCAAT---CA-AGTTTTTTTCCTA
L. m. M             ATCGTTTACTCATGGAATGAGTCCGTTGGAAA--ATTCAGGGGGCAATGTTTTTTTCCTA
L. p. 2483Wex       ATCGTTTACTCATGGAATGAGTCCGTTGGAAA--AGTTGGGGG-CAATGTTTTTTTCCTA
F. p. W             ATCGTTTACTCATGGAATGAGTCCGTTGGAAA--ATTCAAT---CAATGTTTTTTTCCTA
Saccharum           ATTGTTTACTTATGGAATGAGTCCGTTGGAAA--GTTCAAT---CAAT-TTTTTTTCATA
                    ** ******* ********************    *       *    ******** **

L. p. 2419Rosc      TACTATA-AATTTTGC---CTTTTG--TGGAAGATCTGTGCCTACTCTACTTTCCTATCT
L. m. N             TACTATACAATTTTGCACACTTTTGCCTGGAAGATCTGTGCCTACTCTACTTTCCTATCT
F. A. F             TACTATA-CATTTTGC---CTTTTG--TGGAAGATCTGTGCCTACTCTACTTTCCTATCT
F. A. Dovey         TACTATA-CATTTTGC---CTTTTG--TGGAAGATCTGTGCCTACTCTACTTTCCTATCT
L. p. Magician      TACTATA-AATTTTGC---CTTTTG--TGGAAGATCTGTGCCTACTCTACTTTCCTATCT
L. m. M             TACTATA-CATTTTGC---CTTTTG--TGGAAGATCTGTGCCTACTCTACTTTCCTATCT
L. p. 2483Wex       TACTATA-AATTTTGC---CTTTTG--TGGAAGATCTGTGCCTACTCTACTTTCCTATCT
F. p. W             TACTATA-CATTTTGC---CTTTTG--TGGAAGATCTGTGCCTACTCTACTTTCCTATCT
Saccharum           TA------AATTTCGC---CTTTTG--TGAAGGATCTGTGCCTACTCTACTTTCCTATCT
                    **       **** **   ******  ** * ****************************

L. p. 2419Rosc      AGGACTTCGATATACAAAATATATACTACTGTGAAGCATAGATTGCTGTCAACAGAGAAT
L. m. N             AGGACTTCGATATACAAAATATATACTACTGTGAAGCATAGATTGCTGTCAACAGAGAAT
F. A. F             AGGACTTCGATATACAAAATATATACTACTGTGAAGCATAGATTGCTGTCAACAGAGAAT
F. A. Dovey         AGGACTTCGATATACAAAATATATACTACTGTGAAGCATAGATTGCTGTCAACAGAGAAT
L. p. Magician      AGGACTTCGATATACAAAATATATACTACTGTGAAGCATAGATTGCTGTCAACAGAGAAT
L. m. M             AGGACTTCGATATACAAAATATATACTACTGTGAAGCATAGATTGCTGTCAACAGAGAAT
L. p. 2483Wex       AGGACTTCGATATACAAAATATATACTACTGTGAAGCATAGATTGCTGTCAACAGAGAAT
F. p. W             AGGACTTCGATATACAAAATATATACTACTGTGAAGCATAGATTGCTGTCAACAGAGAAT
Saccharum           AGGACTTCGATATACAAAATATATACTACTGTGAAGCATA-ATTGCTGTCAACAGAGAAT
                    ************************************** ******************
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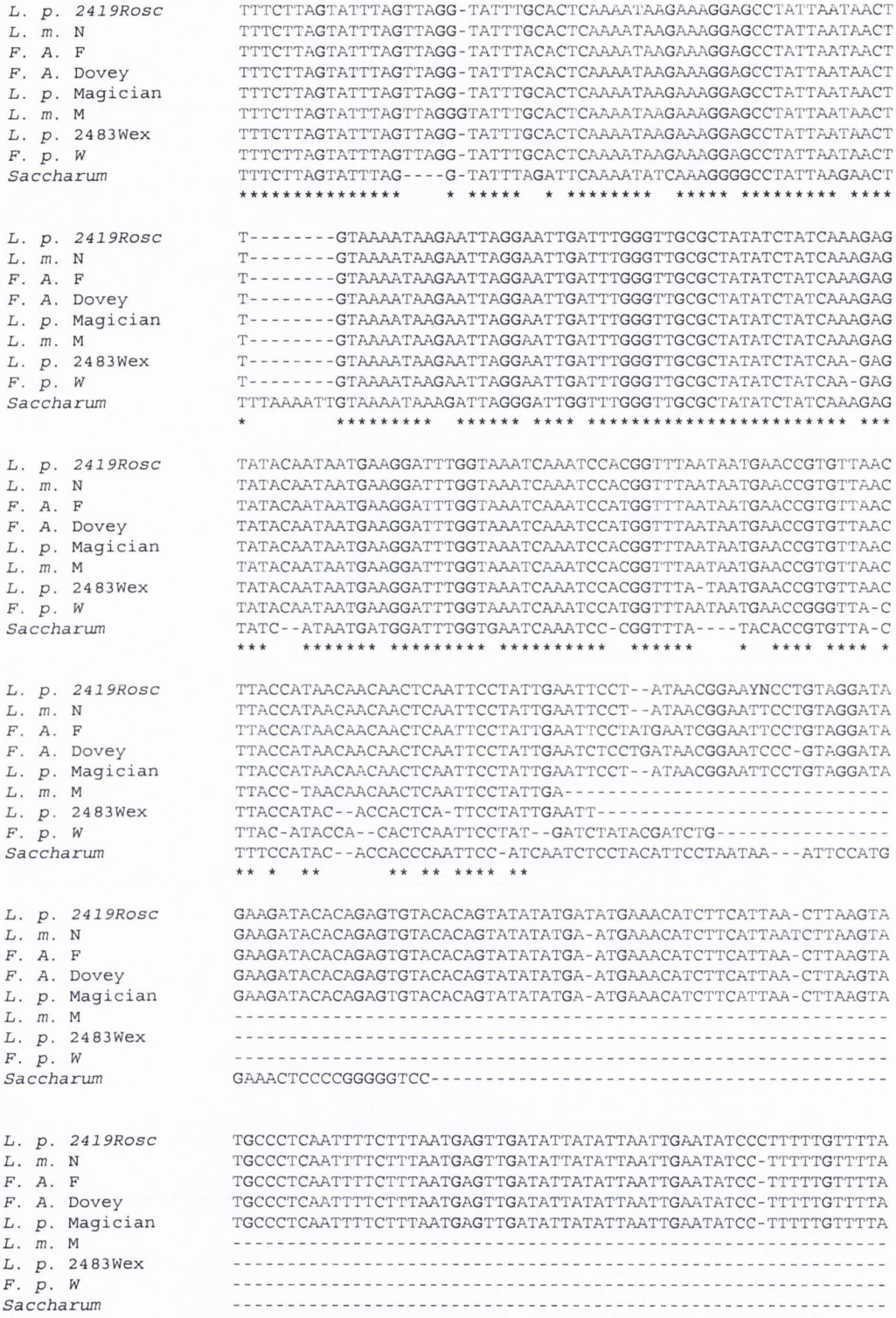
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L. m. N TTTCTTAGTATTTAGTTAGG-TATTTGCACTCAAAATAAGAAAGGAGCCTATTAATAACT
F. A. F TTTCTTAGTATTTAGTTAGG-TATTTACACTCAAAATAAGAAAGGAGCCTATTAATAACT
F. A. Dovey TTTCTTAGTATTTAGTTAGG-TATTTACACTCAAAATAAGAAAGGAGCCTATTAATAACT
L. p. Magician TTTCTTAGTATTTAGTTAGG-TATTTGCACTCAAAATAAGAAAGGAGCCTATTAATAACT
L. m. M TTTCTTAGTATTTAGTTAGGGTATTTGCACTCAAAATAAGAAAGGAGCCTATTAATAACT
L. p. 2483Wex TTTCTTAGTATTTAGTTAGG-TATTTGCACTCAAAATAAGAAAGGAGCCTATTAATAACT
F. p. W TTTCTTAGTATTTAGTTAGG-TATTTGCACTCAAAATAAGAAAGGAGCCTATTAATAACT
Saccharum TTTCTTAGTATTTAG----G-TATTTAGATTCAAAATATCAAAGGGGCCTATTAAGAACT
T--------GTAAAATAAGAATTAGGAATTGATTTGGGTTGCGCTATATCTATCAAAGAG
T--------GTAAAATAAGAATTAGGAATTGATTTGGGTTGCGCTATATCTATCAAAGAG
T--------GTAAAATAAGAATTAGGAATTGATTTGGGTTGCGCTATATCTATCAAAGAG
T--------GTAAAATAAGAATTAGGAATTGATTTGGGTTGCGCTATATCTATCAAAGAG
T--------GTAAAATAAGAATTAGGAATTGATTTGGGTTGCGCTATATCTATCAAAGAG
T--------GTAAAATAAGAATTAGGAATTGATTTGGGTTGCGCTATATCTATCAA-GAG
T--------GTAAAATAAGAATTAGGAATTGATTTGGGTTGCGCTATATCTATCAA-GAG
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TATACAATAATGAAGGATTTGGTAAATCAAATCCACGGTTTAATAATGAACCGTGTTAAC
TATACAATAATGAAGGATTTGGTAAATCAAATCCACGGTTTA-TAATGAACCGTGTTAAC
TATACAATAATGAAGGATTTGGTAAATCAAATCCATGGTTTAATAATGAACCGGGTTA-C
TATC--ATAATGATGGATTTGGTGAATCAAATCC-CGGTTTA----TACACCGTGTTA-C
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TTACC-TAACAACAACTCAATTCCTATTGA----------
TTACCATAC--ACCACTCA-TTCCTATTGAATT----------
TTAC-ATACCA--CACTCAATTCCTAT--GATCTATACGATCTG----------
TTTCCATAC--ACCACCCAATTCC-ATCAATCTCCTACATTCCTAATAA---ATTCCATG
GAAGATACACAGAGTGTACACAGTATATATGATATGAAACATCTTCATTAA-CTTAAGTA
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GAAGATACACAGAGTGTACACAGTATATATGA-ATGAAACATCTTCATTAA-CTTAAGTA
GAAGATACACAGAGTGTACACAGTATATATGA-ATGAAACATCTTCATTAA-CTTAAGTA
GAAACTCCCCGGGGGTCC----------
TGCCCTCAATTTTCTTTAATGAGTTGATATTATATTAATTGAATATCCCTTTTTGTTTTA
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TGCCCTCAATTTTCTTTAATGAGTTGATATTATATTAATTGAATATCC-TTTTTGTTTTA
TGCCCTCAATTTTCTTTAATGAGTTGATATTATATTAATTGAATATCC-TTTTTGTTTTA
TGCCCTCAATTTTCTTTAATGAGTTGATATTATATTAATTGAATATCC-TTTTTGTTTTA

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L. p. 2419Rosc      CG-AGATTTTTGCTAAAGTTGGA--TTTACGCCTAATTCACATCGAGTAGCACCCTGTTA
L. m. N             CG-AGATTTTTGCTAAAGTTGGG--TTTACGCCTAATTCA-ANNAAGTAG-ACCCTGTTA
F. A. F             CGGAGATTTTTGCTAAAGTTGGA--TTTACTCCTAATTCACANNGAGTAG-ACCCTGTTA
F. A. Dovey         CG-AGATTTTTGCTAAAGTTGGA--TTTACTCCTAATTCACATCGAGTAG-ACCCTGTTA
L. p. Magician      CG-AGATTTTTGCTAAAGTTGGGATTTTACGCCTAATTCACATCGAGTAG-ACCCTGTTA
L. m. M             ------------------------------------------------------------
L. p. 2483Wex       ------------------------------------------------------------
F. p. W             ------------------------------------------------------------
Saccharum           ------------------------------------------------------------

L. p. 2419Rosc      TTGTG-AGAGGCTTANTNCAAGNNTNGNGGGGGC--
L. m. N             TTGTG-AGAGG-TTAATCCA----------------
F. A. F             TTGGG-AGT--GTT----------------------
F. A. Dovey         TTGTG-AGAAGGTTANTCAAGGTTNGNGGGGGC---
L. p. Magician      TTGTGGAGANTGTTNCNTCCAGNTTTTAGGGGGGNC
L. m. M             -----------------------------------
L. p. 2483Wex       -----------------------------------
F. p. W             -----------------------------------
Saccharum           -----------------------------------
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L. p. 2419Rosc: *Lolium perenne* IRL-OP-02419 Roscommon
L. m. N: *Lolium multiflorum* cv 'Nivak'
F. A. F: *Festuca arundinacea* cv 'Festorina'
F. A. Dovey *Festuca arundinacea cv* 'Dovey'
L. p. Magician: *Lolium perenne cv* 'Magician'
L. m. M: *Lolium multiflorum* cv 'Multimo'
L. p. 2483Wex: *Lolium perenne* IRL-OP-02483 Wexford
F. p. W: *Festuca pratensis cv* 'Wendelmold'
F. p. B: *Festuca pratensis cv* 'Barpresto'
Saccharum

8.6 Euclidean distances between populations for morphological data. Accession codes given in Appendix 8.1

	V3	I28	V14	I1	I5	I17	V7	I13	I7	V4	I6	I10	V15	I21	I30	I23	I19	V8	I12	V11	I20	I11	I14
V3	0																						
I28	5.67	0.00																					
V14	4.38	5.98	0.00																				
I1	6.06	3.24	5.84	0.00																			
I5	5.62	2.76	5.73	3.46	0.00																		
I17	6.88	4.14	5.60	3.73	3.60	0.00																	
V7	5.68	3.96	6.44	5.68	5.15	7.06	0.00																
I13	6.15	4.72	6.40	4.97	3.67	3.66	7.17	0.00															
I7	5.98	4.01	4.86	3.87	3.51	2.76	6.41	3.84	0.00														
V4	5.27	2.92	6.12	4.50	3.48	6.06	2.89	5.53	4.94	0.00													
I6	5.12	4.45	3.73	4.32	3.89	3.53	5.88	4.83	2.19	5.06	0.00												
I10	4.94	4.32	5.18	4.47	4.10	4.92	6.56	5.36	3.17	4.97	3.39	0.00											
V15	3.64	3.70	4.31	4.09	3.21	5.23	4.08	5.13	4.56	3.22	3.74	4.16	0.00										
I21	6.75	3.40	5.30	2.79	3.34	2.55	6.24	4.63	2.15	4.87	3.06	3.86	4.64	0.00									
I30	5.16	4.56	4.70	3.50	3.50	3.85	6.76	3.04	3.43	4.94	4.09	4.36	3.95	3.71	0.00								
I23	5.99	3.39	6.69	2.88	3.98	5.43	5.56	5.09	4.51	3.52	5.38	4.47	4.66	4.02	3.89	0.00							
I19	6.18	1.83	6.37	2.94	3.45	4.82	3.63	5.14	4.64	2.71	5.06	5.31	4.00	3.79	4.62	2.88	0.00						
V8	3.50	4.06	4.62	5.29	5.32	6.42	3.93	6.36	6.00	4.31	5.50	5.53	3.91	6.00	5.47	5.18	4.32	0.00					
I12	5.75	4.77	5.93	4.02	3.32	3.47	7.67	3.13	3.73	5.88	4.57	4.25	5.02	4.07	2.66	4.55	5.44	6.30	0.00				
V11	2.99	4.66	3.08	4.63	4.32	5.09	5.60	4.43	4.24	4.45	3.91	4.49	3.28	4.83	2.84	4.63	4.90	3.62	4.21	0.00			
I20	6.59	3.47	5.60	4.11	4.58	3.39	6.01	3.96	3.74	5.16	4.64	5.54	5.38	3.44	4.08	4.54	3.61	4.99	4.92	4.55	0.00		
I11	7.18	4.55	5.65	4.79	4.67	2.42	7.74	4.54	3.25	6.72	4.02	4.54	5.92	3.05	4.64	5.93	5.54	6.44	4.31	5.55	3.45	0.00	
I14	6.19	3.23	5.84	4.38	4.26	3.76	5.59	3.56	4.02	4.63	4.88	5.71	5.21	3.99	4.11	4.39	3.47	4.73	4.72	4.24	1.45	4.23	0.00
V10	4.48	4.30	3.95	4.92	5.07	5.38	4.34	5.24	4.22	3.94	4.00	5.30	4.14	4.75	4.43	4.76	4.15	3.62	5.92	2.88	3.76	5.83	3.51
I4	6.16	3.61	5.38	3.89	3.80	3.08	5.79	3.22	2.91	4.62	3.73	5.17	4.92	3.06	3.48	4.15	3.65	5.32	4.16	3.97	2.07	3.93	1.85
I2	9.02	6.92	7.47	6.52	6.97	6.47	9.39	8.44	6.36	7.98	6.77	6.64	7.83	5.47	7.12	7.17	7.66	8.66	6.70	7.42	7.35	6.53	7.42
I3	6.09	4.66	4.78	3.09	3.85	3.00	7.18	3.90	2.88	5.71	3.40	4.14	4.62	2.58	2.20	4.25	4.76	6.00	3.06	3.98	3.89	3.51	4.45
V6	5.58	5.09	3.28	5.80	5.28	4.29	6.68	5.05	4.18	6.20	3.69	5.12	4.83	4.52	4.63	6.62	5.82	4.94	5.55	4.01	3.83	3.53	4.34
I22	7.25	4.45	6.25	3.79	3.70	1.96	7.72	4.08	3.60	6.50	4.23	4.73	5.38	2.90	4.01	5.46	5.19	6.86	3.34	5.68	4.26	2.41	4.78
V9	4.58	3.76	3.67	3.50	3.86	4.58	4.42	5.22	4.14	4.15	3.67	4.56	3.00	4.00	3.73	4.46	3.58	3.47	5.05	3.36	4.20	5.07	4.52
I15	6.43	2.75	6.15	2.73	2.98	2.93	5.77	3.14	3.33	4.33	4.41	4.92	4.70	2.74	3.21	3.14	2.70	5.35	3.55	4.50	2.38	3.87	2.36
I27	5.33	4.50	4.49	3.24	4.26	4.20	6.03	5.58	3.32	5.33	2.97	3.33	3.97	3.43	4.06	4.68	4.70	5.28	4.68	4.49	5.03	4.57	5.59

	V3	I28	V14	I1	I5	I17	V7	I13	I7	V4	I6	I10	V15	I21	I30	I23	I19	V8	I12	V11	I20	I11	I14
V12	5.02	4.50	5.39	4.16	3.26	4.41	6.53	3.03	3.12	4.34	4.10	3.89	4.21	4.08	2.12	3.66	4.79	5.76	2.72	3.16	4.71	5.27	4.31
I24	6.82	4.30	5.75	3.10	3.60	2.33	6.77	3.93	2.60	5.39	3.31	4.89	4.91	2.19	3.43	4.53	4.44	6.68	3.72	4.81	3.87	3.98	4.03
■15	5.95	4.21	5.67	4.45	2.91	3.93	6.09	2.75	3.52	4.16	4.25	5.18	4.05	3.97	2.87	4.62	4.44	6.12	3.78	3.88	4.28	5.22	3.74
I18	6.48	4.98	5.48	3.06	4.01	3.20	7.41	3.66	2.99	5.79	3.90	4.61	4.95	2.97	2.05	4.18	4.88	6.59	3.12	4.34	4.17	4.24	4.60
V13	5.50	3.75	6.06	5.01	3.72	5.49	4.20	4.01	4.58	2.53	4.99	5.72	4.02	5.00	4.25	4.15	3.46	4.92	5.42	3.97	4.31	6.40	3.47
■18	4.66	4.40	4.64	3.79	3.80	4.75	5.66	4.66	2.63	4.03	2.77	2.81	3.50	3.62	3.28	3.74	4.53	5.35	4.31	3.50	5.02	5.32	5.08
I25	5.96	4.47	5.28	3.15	3.65	2.99	6.84	3.43	2.00	5.28	3.03	3.81	4.67	2.65	2.52	3.93	4.58	6.16	3.05	4.10	3.93	3.90	4.23
V16	3.33	2.84	4.12	3.97	3.59	4.86	4.00	5.02	4.12	3.46	3.53	3.47	2.47	4.24	4.20	4.14	3.54	2.42	4.79	3.06	4.29	4.88	4.20
■20	6.88	4.55	5.73	3.13	4.36	3.45	7.03	5.07	2.69	5.81	3.47	3.85	5.37	2.30	4.01	4.21	4.64	6.42	4.28	5.28	4.27	3.65	5.00
■16	7.44	6.28	7.38	7.28	5.19	5.73	9.00	4.45	5.25	7.14	5.92	5.13	6.52	5.95	5.46	7.01	7.38	7.86	4.86	6.36	6.36	4.90	6.33
V2	4.11	3.06	4.32	3.76	3.39	3.88	4.89	3.91	2.64	3.90	2.77	2.96	3.37	3.48	3.50	3.92	3.71	3.78	4.06	3.14	3.50	3.99	3.55
•32	5.23	3.50	5.95	4.20	4.32	5.73	4.85	5.95	3.92	3.48	4.37	2.98	4.44	4.27	5.08	3.22	3.89	4.70	5.40	4.77	5.21	5.78	5.17
V5	4.78	3.60	4.22	4.95	4.58	4.68	4.62	4.98	3.40	4.18	3.17	4.18	4.27	4.05	4.77	4.86	4.15	3.75	5.55	3.66	3.40	4.46	3.42
Δ5	6.07	2.86	6.85	4.11	3.85	5.82	4.10	5.20	4.70	2.39	5.38	4.75	4.31	4.58	4.86	2.71	2.35	4.79	5.72	5.14	4.57	6.16	4.41
■19	5.14	5.45	4.58	5.05	4.55	5.30	6.35	5.09	3.08	4.76	3.33	3.95	4.58	4.45	3.86	4.83	5.62	6.07	4.73	3.65	5.65	6.02	5.51
I26	5.60	3.37	4.57	4.22	3.22	3.17	5.33	4.27	3.28	4.67	2.57	4.33	3.55	3.05	4.23	5.25	4.12	4.95	4.63	4.26	3.69	3.39	3.90
I8	5.36	3.64	4.87	2.82	3.21	3.31	5.55	3.74	2.54	4.02	3.20	4.28	3.68	2.83	2.64	3.66	3.69	5.38	3.71	3.40	3.70	4.72	3.55
V1	5.32	4.04	6.44	5.64	3.60	5.45	4.55	5.68	4.87	4.16	4.47	4.99	4.22	5.47	6.11	5.92	5.06	5.53	5.58	5.43	6.28	6.41	5.62

	V10	I4	I2	I3	V6	I22	V9	I15	I27	V12	I24	■15	I18	V13	■18	I25	V16	■20	■16	V2	●32	V5
V10	0.00																					
I4	3.33	0.00																				
I2	7.93	7.09	0.00																			
I3	4.94	3.36	6.69	0.00																		
V6	4.21	4.24	7.60	4.36	0.00																	
I22	6.46	4.21	6.66	2.87	4.81	0.00																
V9	3.52	4.16	8.10	3.69	4.18	4.98	0.00															
I15	4.44	2.00	7.07	3.09	5.04	3.38	3.99	0.00														
I27	4.85	4.77	7.60	3.18	4.94	4.31	2.69	4.45	0.00													
V12	4.40	3.59	7.15	3.47	5.47	4.87	4.52	3.57	4.55	0.00												
I24	4.91	2.72	6.02	2.72	5.15	3.18	4.73	2.82	4.25	3.76	0.00											
■15	4.44	3.18	7.11	4.05	5.12	4.66	4.83	3.35	5.44	2.56	3.22	0.00										
I18	5.15	3.46	7.06	1.36	5.20	3.35	4.18	3.09	3.56	3.13	2.48	3.71	0.00									
V13	3.31	3.37	8.51	5.34	5.66	6.29	4.52	3.73	5.88	3.61	4.82	2.87	5.21	0.00								
■18	3.83	4.07	7.40	3.45	5.20	5.16	3.53	4.21	2.81	2.71	3.90	3.99	3.41	4.22	0.00							
I25	4.58	2.90	7.08	1.74	4.91	3.42	3.96	2.87	3.07	2.74	2.32	3.64	1.45	4.73	2.57	0.00						
V16	3.42	4.19	7.60	4.38	4.02	5.07	2.61	4.09	3.57	4.33	5.01	4.79	5.03	4.19	3.54	4.39	0.00					
■20	5.33	3.86	6.98	2.34	5.28	3.46	4.02	3.47	2.54	4.42	3.25	5.23	2.70	5.92	3.40	2.12	4.64	0.00				
■16	7.54	6.06	8.66	5.62	5.61	5.10	7.08	5.90	6.92	5.25	6.39	5.47	6.09	6.60	6.18	5.69	6.14	6.40	0.00			
V2	3.16	3.16	7.64	3.52	3.78	4.40	2.75	3.31	2.93	3.35	4.11	4.13	3.95	3.87	2.56	2.99	1.99	3.49	5.48	0.00		
●32	4.38	4.83	7.46	5.09	6.13	6.04	4.19	4.55	3.83	4.26	5.35	5.49	5.31	4.71	2.97	4.37	3.31	4.19	6.88	2.90	0.00	
V5	2.34	3.17	7.66	4.64	3.38	5.53	3.59	4.14	4.30	4.59	4.78	4.81	5.20	3.96	3.73	4.25	2.67	4.52	6.24	2.10	3.63	0.00
Δ5	4.50	4.41	8.65	5.26	6.37	6.01	4.10	3.64	4.85	4.44	5.41	4.81	5.28	3.35	3.97	4.72	3.75	4.85	6.81	3.51	2.92	4.21
■19	3.96	4.39	7.42	4.29	5.61	6.12	4.45	5.06	4.11	2.79	4.45	4.13	4.14	4.46	1.98	3.36	4.66	4.44	6.65	3.59	3.96	4.22
I26	4.31	3.26	7.06	3.61	3.11	3.44	3.65	3.54	4.07	4.70	3.50	4.00	4.37	4.51	4.22	3.82	3.12	4.11	5.25	3.04	5.01	3.18
I8	3.51	2.71	6.35	3.15	4.89	4.28	3.77	2.88	3.82	2.61	2.17	2.42	2.75	3.53	2.77	2.49	4.00	3.80	6.55	3.20	4.28	4.02
V1	5.42	5.33	8.07	6.42	6.42	6.16	5.35	5.39	5.57	5.11	5.52	4.96	6.60	4.67	4.91	5.67	4.17	6.29	6.91	4.24	4.65	4.73

	Δ5	■19	I26	I8	V1
Δ5	0.00				
■19	5.12	0.00			
I26	4.92	5.11	0.00		
I8	4.37	3.30	3.77	0.00	
V1	5.21	5.19	4.54	4.81	0.00

8.7 Transformation of characters in Chapter 3

Square root transformation:

Data from the remaining non-normally distributed characters (spikelets per spike, date of ear emergence, spring growth, and summer growth) were transformed using a square root transformation. Histograms with fitted normal distributions were constructed (Figure 1). None of the characters had a typically normal distribution, and were either skewed to the left (square root transformed spikelets per spike and square root transformed date of ear emergence) or had too few values in the tails of the distribution (square root transformed spring growth and square root transformed summer growth). Probability plots were constructed for each character using the Kolmogorov-Smirnov test statistic (Figure 2). None of the plots followed a straight line. The non-normality of the transformed characters was confirmed by the Kolmogorov-Smirnov test statistic (Table 1).

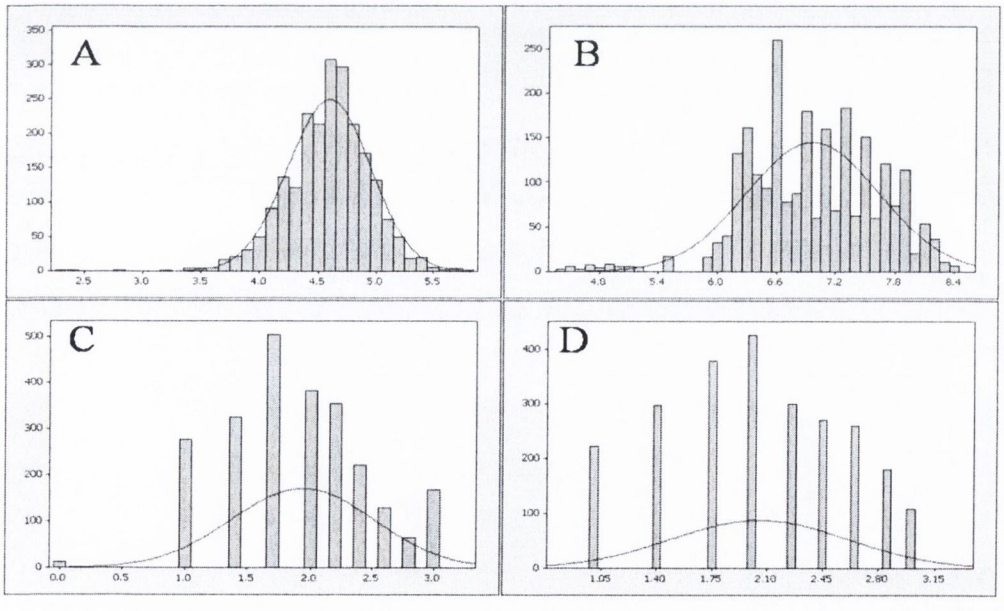

Figure 1 Histograms with fitted normal distribution curves for the data of square root transformed characters: A: spikelets per spike, B: date of ear emergence, C: spring growth, D: summer growth. Y-axis: frequency. X-axis: log transformed character of interest.

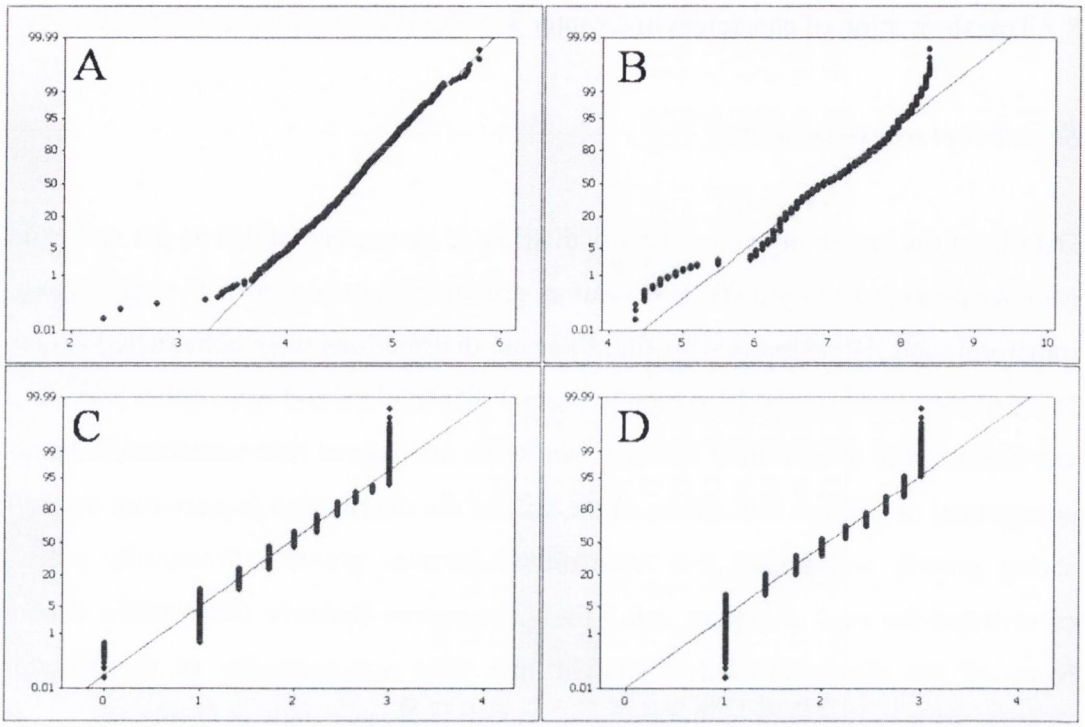

Figure 2 Probability plots using the Kolmogorov-Smirnov test for the following square root transformed data of characters: A: spikelets per spike, B: date of ear emergence, C: spring growth, D: summer growth. Y-axis: percentage. X-axis: character of interest.

Table 1 Kolmogorov-Smirnov statistics and p-values for each square root transformed character

Character	**Kolmogorov-Smirnov statistic**	**p-value**
Spikelets per spike*	0.035	<0.010
Date of ear emergence*	0.048	<0.010
Spring growth*	0.022	<0.010
Summer growth*	0.044	<0.010

*Non-normal characters

Reciprocal transformation:

Data from the remaining non-normal characters (spikelets per spike, date of ear emergence, spring growth, and summer growth) was transformed using a reciprocal transformation. Histograms with fitted normal distributions were constructed (Figure 3).

None of the histograms followed a typical normal distribution, and were either skewed to the right (reciprocal transformed spikelets per spike and reciprocal transformed date of ear emergence) or had too few values in the tails of the distribution (reciprocal transformed spring growth and reciprocal transformed summer growth). Probability plots were constructed for each character using the Kolmogorov-Smirnov test statistic (Figure 4). None of the normality plots followed a straight line. The non-normality of the reciprocal transformed characters was confirmed by the Kolmorgorov-Smirnov test statistic (Table 2).

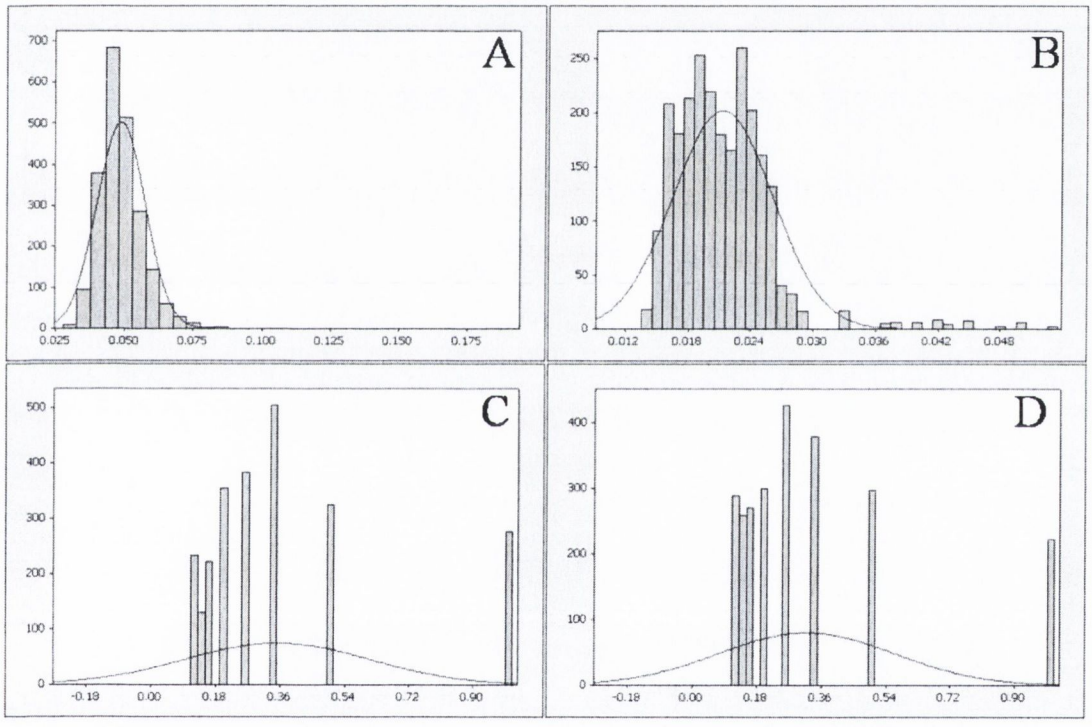

Figure 3 Histograms with fitted normal distribution curves for the data of reciprocal transformed characters: A: spikelets per spike, B: date of ear emergence, C: spring growth, D: summer growth. Y-axis: frequency. X-axis: log transformed character of interest.

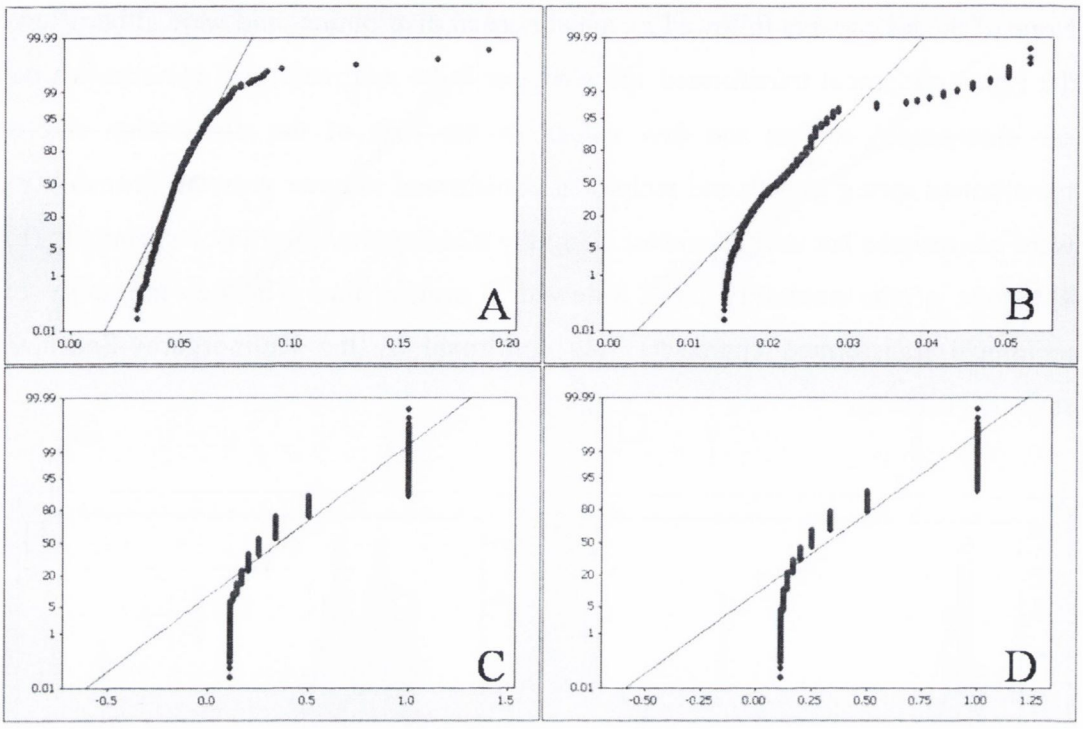

Figure 4 Probability plots using the Kolmogorov-Smirnov test for the following characters: A: Reciprocal transformed spikelets per spike, B: Reciprocal transformed date of ear emergence, C: Reciprocal transformed spring growth, D: Reciprocal transformed summer growth. Y-axis: Percentage. X-axis: character of interest.

Table 2 Kolmogorov-Smirnov statistics and p-values for each reciprocal transformed character

Character	**Kolmogorov-Smirnov statistic**	**p-value**
Spikelets per spike*	0.096	<0.010
Date of ear emergence*	0.086	<0.010
Spring growth*	0.178	<0.010
Summer growth*	0.188	<0.010

*Non-normal characters

Natural log transformation:

Data from the remaining non-normally distributed characters (spikelets per spike, date of ear emergence, spring growth, and summer growth) was transformed using a natural log

transformation. Histograms with fitted normal distributions were constructed (Figure 5). None of the histograms followed a typical normal distribution, and were either skewed to the left (natural log transformed spikelets per spike and natural log transformed date of ear emergence) or had too few values in the tails of the distribution (natural log transformed spring growth and natural log transformed summer growth). Probability plots were constructed for each character using the Kolmogorov-Smirnov test statistic (Figure 6). None of the normality plots followed a straight line. The non-normality of the natural log transformed characters was confirmed by the Kolmorgorov-Smirnov test statistic (Table 3).

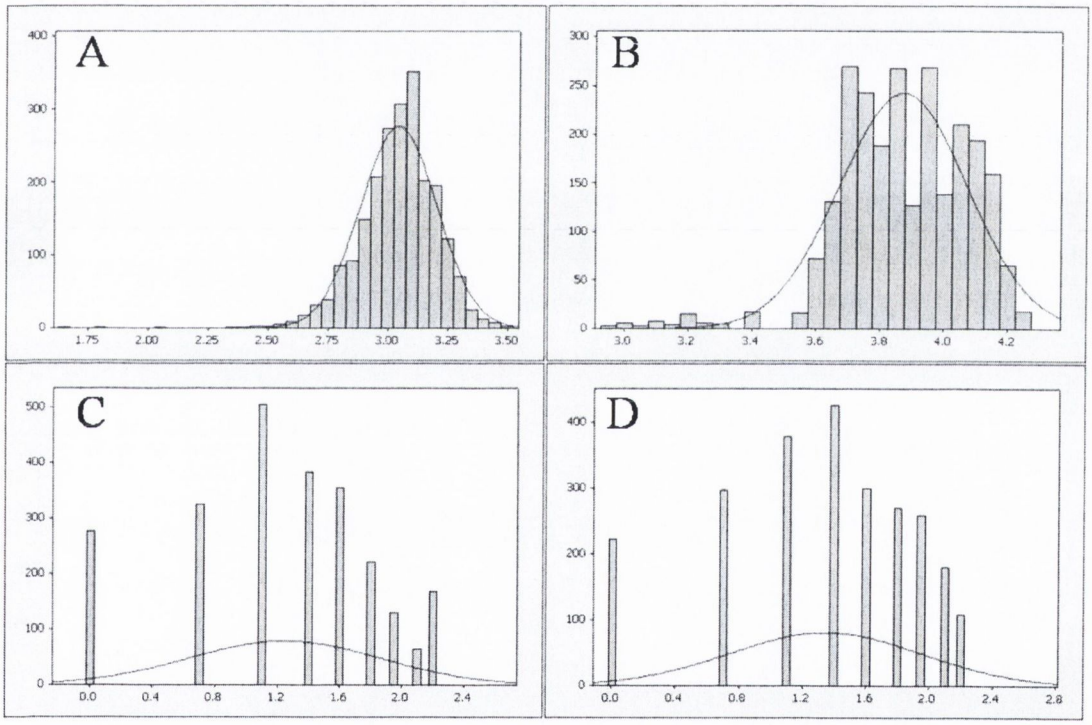

Figure 5 Histograms with fitted normal distribution curves for the data of natural log transformed characters: A: spikelets per spike, B: date of ear emergence, C: spring growth, D: summer growth. Y-axis: frequency. X-axis: log transformed character of interest.

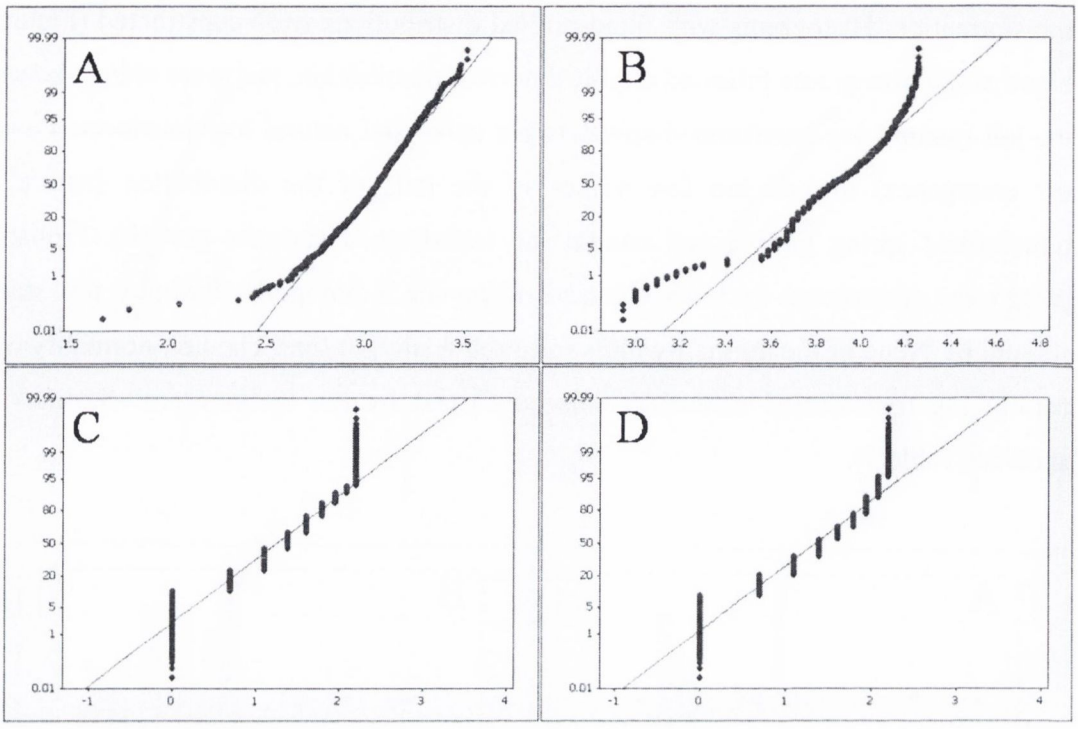

Figure 6 Probability plots using the Kolmogorov-Smirnov test for the data of natural log transformed characters: A: spikelets per spike, B: date of ear emergence, C: spring growth, D: summer growth. Y-axis: percentage. X-axis: character of interest.

Table 3 Kolmogorov-Smirnov statistics and p-values for each natural log transformed character

Character	**Kolmogorov-Smirnov statistic**	**p-value**
Spikelets per spike*	0.051	<0.010
Date of ear emergence*	0.053	<0.010
Spring growth*	0.053	<0.010
Summer growth*	0.069	<0.010

*Non-normal characters

8.8 Raw data, chapter 4. Values for dry matter, fructose, glucose, WSC, and crude protein. *missing data

	Dry Matter					Fructose					Glucose				
Cut	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
V3	18.7	21.2	25.7	20.8	17.6	6.67	6.69	5.84	7.11	5.22	2.98	4.83	2.56	4.08	1.85
	18.8	21.4	25.7	19.4	22.2	8.14	4.59	6.58	4.28	7.98	3.53	3.27	2.98	2.66	4.29
	19.1	21.7	26.4	20.6	17.9	7.66	7.83	5.13	4.58	8.11	3.01	4.99	2.66	2.88	3.39
	19.5	21.4	26.6	22.8	18.1	8.28	9.25	13.50	5.32	8.58	3.54	5.02	6.56	3.68	4.53
I28	20.3	25.1	25.6	21.7	16.3	3.79	8.18	10.85	2.61	4.73	1.57	5.43	6.00	2.46	2.46
	19.7	24.7	26.9	22.6	16.9	3.48	7.47	9.14	5.23	4.96	1.68	4.99	3.66	4.32	1.81
	19.7	23.3	26.2	21.6	17.1	6.44	6.86	9.95	3.21	4.82	2.50	4.89	5.51	2.11	2.41
	20.8	23.5	26.4	21	15.4	5.47	8.16	4.99	4.99	5.28	2.33	5.42	2.43	3.38	2.37
V14	17	22.4	22	17.3	14.8	7.38	3.91	6.07	5.04	4.90	3.03	2.72	3.47	3.89	2.61
	17.4	21	22.9	17.8	15	7.04	3.60	10.95	5.18	6.41	3.19	2.54	5.16	2.98	2.77
	16.9	20.4	22.8	17.2	15.3	7.63	6.72	4.80	3.93	6.47	3.89	5.36	3.00	3.31	2.98
	18.3	21.2	22.3	17.3	16.2	9.66	3.11	10.81	6.53	8.42	4.28	2.21	7.14	3.83	3.57
I1	20	24.7	26.4	24.9	17.3	7.04	4.37	6.10	6.25	7.09	3.55	3.54	4.00	3.43	3.61
	20.4	24.8	27.4	23.4	16.5	6.40	7.45	13.27	5.46	7.46	3.72	4.88	5.04	3.50	2.25
	21.1	25.1	27.9	24.8	15.9	7.02	7.54	6.78	5.45	6.59	2.83	4.77	3.57	3.49	1.86
	19.3	22.5	24.2	20.5	16.7	6.32	3.53	9.24	2.90	8.62	3.02	2.10	5.60	1.67	2.46
I5	21	27.1	29.1	22.8	16.3	7.96	2.82	4.16	5.01	5.81	2.48	2.21	1.87	3.34	3.50
	19.1	26	29.7	23.7	16.4	7.75	9.97	11.52	7.75	5.38	3.02	6.68	4.92	5.80	3.26
	20.8	27.6	28.5	22.3	15.4	12.37	10.76	3.79	5.48	5.02	3.53	6.50	4.91	3.84	1.86
	20.2	28.9	28.6	23.4	15.9	8.27	2.47	7.36	4.53	5.96	2.92	2.07	3.39	3.16	2.46
I17	22.3	28.4	28.4	25.5	16.8	14.86	3.34	13.05	5.22	6.04	4.70	2.92	6.09	3.33	2.20
	22.4	29.9	29.9	26.8	17	11.45	6.54	4.24	5.33	5.17	3.91	5.89	2.16	4.32	2.21
	21.7	26.5	28.4	26.6	18	9.62	4.51	5.26	2.94	7.09	3.76	3.09	3.33	2.48	3.21
	21.1	26.7	29.5	26.8	17.4	6.93	3.98	11.75	4.58	8.02	3.27	2.43	4.86	3.74	3.19
V7	17.8	22.4	24	18.9	15.7	10.15	7.98	11.13	4.44	5.17	4.27	5.97	3.65	2.93	2.59
	17.2	22.7	25.2	18.8	16.2	9.76	3.38	13.56	4.87	4.19	3.77	2.61	5.75	3.49	1.99
	17	21.9	24.6	18.2	15.2	6.66	6.68	13.52	3.64	5.66	3.13	5.26	5.73	3.01	3.78
	16.1	22.1	25	18.6	16.6	5.99	6.28	10.00	4.48	7.40	3.92	5.03	5.04	3.36	4.18
I13	22.8	27.5	29.1	26.5	19	10.81	7.76	11.76	3.54	6.28	3.86	5.74	4.87	2.03	2.81
	21.4	26.6	27.7	23.4	17	10.53	8.12	9.26	2.64	6.41	3.56	6.11	4.51	2.00	3.59
	21.5	27	30.2	22.7	18.1	9.96	7.80	16.82	3.39	7.92	3.22	6.04	6.25	2.91	4.58
	21.4	25.6	26.1	20.7	18.1	12.90	4.02	14.58	4.85	8.99	4.50	3.11	5.56	2.83	4.20
I7	21.7	26.6	27.7	26.7	18.3	8.76	7.46	8.11	3.52	7.96	3.90	4.72	2.95	2.66	4.98
	21.1	25.7	29.1	24.6	17.5	8.41	7.61	12.63	5.91	8.96	3.54	5.34	5.18	3.88	4.98
	19.2	23.6	25.3	23.9	18.2	4.86	7.82	9.19	4.11	7.52	2.79	6.01	6.46	3.28	4.21
	19.7	23.7	25.9	25.5	16.8	6.27	6.52	9.47	5.12	7.06	3.52	4.49	4.95	3.53	2.86
V4	20.7	24.7	27.1	21.2	17.9	4.13	7.04	10.98	3.14	7.03	2.20	5.01	4.92	2.83	4.51
	19.8	23.9	24.8	20.6	17.4	6.40	5.17	7.74	2.37	6.59	3.84	4.46	4.56	2.11	4.49
	19.9	23.5	26.7	19.9	17.4	3.15	3.45	6.72	4.13	6.83	2.00	3.31	3.24	3.30	4.84
	19.5	24	27.2	19.6	17.3	7.47	8.08	11.17	4.36	6.71	3.81	4.18	4.94	3.72	4.63
I6	19.7	27.1	28.1	22.2	18.9	7.90	7.07	14.45	5.42	6.06	3.98	6.23	5.71	3.15	3.77
	20.2	25.5	25.6	22.8	15.4	7.50	3.63	6.68	5.30	5.63	4.28	2.91	2.66	3.65	2.96
	21	27.6	28.3	24	18.2	10.19	6.95	5.80	5.17	5.00	4.45	6.20	2.60	3.16	3.37
	19.3	24.8	24.9	21.8	17.9	6.82	6.37	10.33	4.91	6.36	3.71	5.24	5.10	3.40	3.89
I10	20.8	25	26.9	26.2	17.3	5.57	5.31	14.08	5.95	10.57	3.20	4.48	5.57	3.89	6.58
	20.9	25	25.9	21.8	16.4	5.65	6.56	6.27	3.63	7.55	3.38	4.85	2.43	2.50	3.91
	19.9	25.2	25.8	23.8	15.8	7.13	3.50	4.99	5.91	7.29	3.97	2.50	2.43	4.11	3.98
	20.8	24.4	26.2	24.1	17.9	5.83	7.20	4.62	4.64	7.32	4.08	6.17	2.25	4.12	3.80
V15	20.7	25.5	29	22.5	18.6	5.62	9.59	7.06	5.65	8.04	3.19	5.67	3.29	3.10	4.18
	19.8	23.9	28.1	21.9	18.3	7.89	5.94	12.98	3.78	7.50	4.81	3.96	5.55	2.88	4.78

	20.2	26.2	28.8	22.5	18.6	3.33	9.09	12.78	4.93	7.14	1.97	6.16	5.22	3.14	4.81
	19.4	23.5	29.4	21.3	18.1	6.05	10.70	13.52	6.76	8.63	3.02	6.22	5.18	4.17	5.38
I21	22.8	27.8	29.9	24.9	19.2	7.33	7.44	16.85	2.20	6.08	3.06	5.17	5.73	1.84	4.42
	22.5	28.1	28.8	25.6	19.3	10.71	4.05	14.02	5.43	6.12	4.48	3.13	5.53	3.39	4.58
	22.3	27.5	28.7	25.7	21.1	11.55	11.29	13.81	3.30	8.70	5.10	6.06	5.55	1.99	4.98
	21.1	26.5	27.1	24.2	17.7	8.74	6.89	6.98	4.24	5.96	4.31	4.46	3.32	2.37	4.18
I30	19.7	25.5	27	23.3	18.7	3.68	7.93	10.97	3.80	8.00	2.18	5.77	6.32	3.54	4.99
	22.7	27.8	29.6	24.4	19.7	10.22	7.91	15.65	4.25	9.35	5.08	5.82	5.56	2.87	5.00
	21.5	25.7	28	24.9	18.9	9.13	7.52	17.55	3.68	8.22	4.43	5.50	6.94	3.13	4.61
	23.2	26.4	29.2	25.7	18.5	12.38	10.57	19.00	3.48	9.71	6.06	6.66	6.77	2.00	5.18
I23	21.6	24.4	26.2	22.1	19.4	6.83	4.71	12.45	5.23	6.53	4.58	2.99	5.70	4.25	4.59
	21.2	24.8	26.9	23.5	18.4	2.36	6.77	5.26	3.46	6.35	1.75	4.90	2.94	2.94	3.49
	20.8	24.4	27.9	24.4	21.3	5.06	4.51	6.18	5.26	7.78	2.56	3.36	2.42	3.74	4.29
	21.4	24.8	27.2	24	19.7	7.12	9.47	13.67	7.66	7.87	4.62	6.45	5.56	6.11	4.93
I19	23.5	25.8	26	24	18.6	5.22	8.11	4.44	5.26	7.10	2.91	5.48	2.24	4.91	4.70
	20.1	24	26.5	24.5	18.3	6.82	7.25	12.26	5.38	6.32	4.09	4.61	5.89	5.16	4.70
	19.7	24.7	26.2	24.5	18.4	5.70	8.51	7.93	2.82	7.97	2.88	4.83	3.48	2.32	4.70
	19.9	24.2	25.9	23.7	17.9	7.93	7.88	5.13	5.62	5.40	4.45	6.00	2.42	4.48	3.98
V8	21	21.4	24.7	18.2	17.5	13.11	3.77	4.74	4.51	9.56	5.10	2.64	2.49	2.47	5.98
	19.9	21.8	24	18.8	17.7	13.27	2.90	10.40	5.16	10.15	5.98	2.60	5.01	4.39	5.78
	19.4	21.4	24.1	19.3	17.2	11.00	6.36	10.80	3.13	10.82	6.17	4.09	4.86	2.66	5.85
	18.9	20.5	23.8	18.9	16.8	8.21	6.06	8.38	3.42	9.67	4.64	4.51	1.90	3.07	5.44
I16	21.2	27.6	26.7	22.7	18.6	12.37	2.87	11.58	4.17	7.15	5.43	2.21	5.36	3.87	4.86
	20.5	25.7	26.5	22.6	19	9.39	6.51	11.84	4.81	7.15	4.67	3.99	5.03	3.63	4.47
	21.9	27.1	27.1	22.3	19.1	10.37	6.64	5.37	4.09	7.02	4.80	3.54	3.17	3.65	4.55
	20.2	24.3	25.8	21.2	18.5	9.43	2.89	11.76	4.75	6.14	4.74	2.41	6.28	3.89	4.59
I12	21.6	25.6	28.8	22.6	19.2	9.63	8.44	9.80	5.79	7.74	4.81	5.20	4.68	4.59	4.75
	22.1	26.8	28	22.5	18.9	10.77	5.42	7.80	4.68	6.96	4.88	3.69	3.47	4.01	4.10
	22.1	26.5	27	22.7	18.6	10.29	5.32	10.81	4.28	6.57	5.32	3.85	5.90	3.75	3.89
	22.2	27.4	29.6	23	19.3	8.10	3.46	4.58	5.48	7.63	4.00	2.29	2.28	4.24	4.83
V11	20	21.5	26.3	22	18.4	10.29	6.86	11.88	3.13	8.91	4.45	4.83	5.18	2.86	5.38
	20.1	24.6	26	21.5	18.5	12.23	8.06	12.66	3.15	10.21	5.46	3.52	6.07	2.87	5.75
	20.1	23.4	24.6	22	18.1	9.10	5.46	11.59	3.29	8.97	3.89	4.26	6.08	2.67	5.60
	19.7	22.3	24.8	21.8	17.9	9.05	8.01	13.88	4.28	10.60	4.79	5.18	5.88	3.89	5.64
I20	24	24.8	27.5	23.2	20	12.39	7.90	4.92	4.83	7.11	6.19	5.99	3.50	3.99	4.17
	22.7	28.6	29.4	24.8	22	12.59	7.66	13.07	5.35	7.35	4.89	5.13	6.05	4.01	4.72
	21.3	25.8	26	22.6	20.1	7.35	5.36	11.74	2.26	7.35	3.03	3.71	6.08	1.88	4.61
	21.5	26.6	26.5	21.8	19.1	7.77	5.84	12.65	6.34	7.36	3.28	4.38	6.45	4.46	4.26
I11	21.3	28.9	29.2	25.2	19.5	10.05	6.70	4.71	4.88	5.74	4.28	3.72	2.64	3.89	3.38
	22.3	28.7	27.7	24	18.8	6.01	5.34	5.49	5.37	6.32	2.53	4.07	3.16	4.62	3.92
	21	28	27.9	23.5	18.8	11.88	7.06	9.57	6.50	6.68	4.85	4.65	5.04	5.34	3.74
	22.6	29.3	29.5	24	20	13.52	6.80	12.67	6.32	6.07	5.04	4.57	5.04	5.00	3.73
I14	10.4	25.3	29	23.9	21.4	10.35	8.81	11.84	2.69	7.47	4.47	5.45	5.56	2.50	5.15
	21.8	28.3	29.1	24.1	21.1	11.76	8.81	4.74	6.90	9.51	5.14	5.36	2.66	4.98	5.86
	20.9	26.1	29.4	22.9	20	9.52	2.98	6.55	4.22	7.43	3.64	2.40	3.84	3.24	5.35
	22.3	28.3	30.9	23.2	20	15.77	4.79	5.24	3.68	7.39	5.73	2.61	2.04	2.74	5.32
V10	21	25.6	27.8	21.6	20.7	4.39	4.56	4.59	4.83	8.58	2.28	3.14	2.64	3.00	6.04
	20	24.4	26.6	21.7	19.4	7.43	5.88	9.05	4.81	8.84	3.98	3.60	5.62	4.50	6.23
	20.5	25.3	27.7	22.7	20.9	12.21	4.46	8.37	4.55	7.54	4.80	3.15	4.34	4.11	5.52
	19.4	26.2	25.8	22	20.6	9.74	5.18	5.26	4.69	5.95	4.30	3.42	2.31	4.13	5.32
I4	20.1	27.1	28.2	22.6	19.6	8.78	7.25	5.11	3.52	7.04	4.25	4.26	3.23	2.42	4.81
	20.4	24.8	26	21	18.7	10.87	6.48	5.69	4.02	6.40	5.19	4.25	3.39	3.39	5.16
	19.3	26	27.5	22.5	18.9	8.55	5.99	12.44	4.41	7.33	4.69	4.41	3.91	3.16	4.98
	19.5	24.7	24.6	20.5	18.2	8.66	6.29	6.20	3.64	4.74	3.91	4.45	4.26	3.14	4.26
I2	21.7	26.4	27.1	23.3	22.4	11.21	6.13	5.13	5.11	8.53	4.95	4.17	3.37	3.99	5.85

	21.4	24.7	26.2	22.1	20.6	4.79	4.59	4.79	4.38	6.91	2.35	3.26	1.07	3.54	4.96
	21.7	26.8	27.8	24	21	7.36	2.77	5.38	3.75	8.97	2.56	2.22	1.95	2.51	6.72
	21.8	26.6	28	23.7	20.4	8.29	6.44	6.35	6.56	7.54	3.65	3.98	1.25	5.01	6.06
I3	21.1	25.2	27.8	23	20.5	11.45	7.52	12.45	5.23	9.25	5.15	4.24	3.38	2.75	6.22
	20.3	24.6	26	21.8	19.3	9.70	8.23	4.78	2.73	8.86	3.98	5.69	2.13	1.83	6.72
	19.9	25.6	27.3	23.7	22.2	3.97	7.18	14.84	4.73	9.27	1.81	4.55	12.34	3.66	6.77
	20.6	27.1	28.5	23.5	20.6	9.85	6.07	5.91	4.23	8.66	3.98	4.13	5.19	3.20	5.86
V6	18.5	22.3	25.4	19.3	18.3	9.96	6.20	*	2.35	11.60	4.74	4.54	*	3.72	6.35
	18.4	22.1	25.1	19.3	17.6	12.03	5.71	6.26	6.93	11.09	5.31	4.57	4.65	9.43	5.86
	19.7	23.4	24.7	20	18.7	11.55	3.87	5.23	5.36	11.50	4.57	2.77	3.83	7.72	7.06
	19.2	23.2	26.3	20.5	18.5	12.69	6.40	5.19	4.18	11.80	5.42	4.36	1.73	5.99	6.95
I22	21.4	26	28.8	23.4	22	11.71	2.88	3.43	3.13	7.82	4.74	2.04	1.56	5.42	5.60
	20.5	25.7	29.3	24.7	19.8	11.10	6.69	5.90	2.55	7.94	3.91	3.80	7.18	2.24	5.59
	22.9	27.7	29.7	25.7	21.8	13.77	5.99	12.37	5.26	7.99	5.16	4.10	11.95	4.25	4.39
	23.3	29.2	29.9	26.4	22.3	12.40	6.91	4.25	2.73	7.11	5.21	4.50	4.78	2.51	4.67
V9	19.5	21.9	23.8	20.1	19.7	10.94	6.52	6.12	2.86	10.86	4.64	4.51	7.96	2.29	6.79
	20	22.1	24.2	20.3	19.4	14.36	7.85	8.72	6.41	9.39	6.89	6.57	4.67	4.50	5.77
	20.1	22.4	23.7	19.7	17.9	6.86	4.37	9.07	5.44	6.60	2.71	3.69	5.33	3.87	4.91
	20.4	22.5	23.9	19.9	17.9	13.46	3.96	6.25	4.09	8.86	5.39	2.93	3.98	2.99	5.26
I15	20.4	24.6	26	22	20.6	10.35	7.22	4.14	5.75	11.11	4.10	4.33	1.91	4.24	6.94
	20.9	25.3	28.5	22.1	20	6.37	3.52	14.19	3.36	7.87	2.16	2.73	5.39	2.29	4.43
	21.5	26.5	29.6	23.9	20.2	10.56	7.19	5.46	3.52	7.92	4.15	5.21	3.16	2.50	4.76
	21.3	25.7	27.1	22	19	13.65	7.33	1.65	5.36	6.84	4.62	5.74	2.53	4.00	4.24
I27	21.3	24.6	27	23.4	21.9	9.23	6.94	5.08	6.61	8.41	4.84	5.06	5.59	5.48	5.96
	21.2	25	27.5	21.2	19.8	10.20	9.64	6.53	6.24	7.67	5.04	6.26	3.68	4.75	5.10
	21.2	25.6	28.8	23.9	20.6	11.34	7.25	*	7.91	8.95	5.18	5.25	*	4.85	5.76
	20.6	24	28	23.1	20.8	8.55	7.20	5.38	6.47	7.54	3.91	4.84	2.50	4.86	5.28

	WSC					Crude protein				
Cut	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
V3	9.65	11.52	8.40	11.19	7.06	22.68	23.26	19.35	23.29	26.63
	11.67	7.86	9.56	6.94	12.27	24.96	23.12	17.62	24.51	26.58
	10.67	12.83	7.79	7.46	11.51	25.22	22.83	18.41	21.01	27.16
	11.83	14.27	20.07	8.99	13.11	23.61	24.91	19.09	19.57	25.52
I28	5.36	13.62	16.85	5.07	7.20	24.62	21.23	19.4	22.29	28.6
	5.16	12.45	12.80	9.55	6.76	25.95	25.22	19.52	26.62	29.07
	8.94	11.75	15.46	5.32	7.23	26.99	25.11	19.52	22.51	28.3
	7.80	13.58	7.42	8.37	7.65	*	22.72	19.32	21.38	27.42
V14	10.41	6.62	9.54	8.93	7.50	25.17	22.68	19.93	22.85	27.14
	10.24	6.14	16.11	8.17	9.18	23.45	25.11	19.05	20.53	26.78
	11.53	12.07	7.79	7.24	9.45	25.25	24.48	19.5	21.07	26.92
	13.94	5.32	17.94	10.36	11.99	23.43	23.93	21.54	20.97	25.86
I1	10.59	7.91	10.10	9.68	10.71	26.23	20.75	19.88	20.51	27.26
	10.11	12.33	18.31	8.96	9.71	26.08	22.01	19.32	23.52	26.18
	9.85	12.30	10.35	8.94	8.44	26.61	21.94	19.37	24.71	25.48
	9.34	5.63	14.84	4.57	11.08	27.67	24.78	20.96	20.83	26.51
I5	10.44	5.03	6.03	8.34	9.31	20.89	22.56	19.93	24.21	27.7
	10.77	16.65	16.44	13.55	8.64	21.4	22.8	18.92	24.48	28.19
	15.89	17.26	8.70	9.32	6.88	21.2	21.93	20.12	25.25	27.89
	11.19	4.54	10.76	7.70	8.42	22.46	21.86	20.22	24.7	27.05
I17	19.56	6.26	19.14	8.55	8.24	21.9	19.51	18.69	21.95	25.69
	15.36	12.42	6.41	9.65	7.38	24.08	17.64	18.38	26.23	26.96
	13.38	7.60	8.60	5.42	10.29	23.67	19.31	19.37	24.69	26.31
	10.20	6.41	16.61	8.32	11.20	26.47	19.07	18.58	24.38	25.51

V7	14.42	13.96	14.78	7.38	7.76	22.93	22.66	18.12	20.22	27.4
	13.53	5.99	19.30	8.36	6.18	22.74	21.03	18.9	24.2	26.76
	9.79	11.94	19.25	6.65	9.43	31.32	21.74	22.42	25.08	28.66
	9.91	11.31	15.04	7.84	11.58	23.06	21.16	18.03	24.8	26.8
I13	14.67	13.49	16.63	5.57	9.08	23.87	20.66	17.81	25.15	25.91
	14.09	14.22	13.77	4.64	10.01	24.57	23.23	20.07	23.28	26.9
	13.19	13.84	23.07	6.30	12.51	24.06	21.79	18.09	23.04	25.64
	17.40	7.13	20.15	7.68	13.19	23.56	22.47	18.44	25.82	24.76
I7	12.65	12.18	11.06	6.18	12.94	25.4	19.78	19.43	22.33	23.3
	11.95	12.95	17.81	9.79	13.94	25.33	19.52	18.12	23.02	26.2
	7.65	13.82	15.66	7.40	11.73	27.45	20.3	18.82	23.94	27.9
	9.79	11.01	14.42	8.65	9.93	27.06	21.85	19.03	23.36	26.74
V4	6.33	12.05	15.91	5.97	11.54	25.7	21.98	19.3	26.7	26.55
	10.24	9.63	12.30	4.49	11.07	25.85	22.62	19.84	27.36	27.81
	5.15	6.76	9.96	7.42	11.67	26.52	24.16	20.13	26.24	26.99
	11.29	12.27	16.11	8.09	11.34	26.4	22.61	17.43	22.24	26.84
I6	11.89	13.30	20.16	8.57	9.83	27.39	20.63	18.71	24.26	27.27
	11.78	6.54	9.34	8.95	8.59	25.03	22.44	19.89	25.47	27.44
	14.64	13.15	8.40	8.33	8.36	25.07	19.48	19.02	22.34	23.3
	10.53	11.60	15.43	8.31	10.25	26.61	21.83	20.94	26.68	28.5
I10	8.77	9.79	19.64	9.84	17.15	25.58	23.64	23.27	24.81	21.31
	9.03	11.41	8.71	6.13	11.46	20.51	22.56	19.26	22.77	28.04
	11.10	6.00	7.42	10.02	11.26	26.47	22.75	20.43	24.78	27.16
	9.91	13.37	6.87	8.76	11.12	27.54	22.12	20.43	25.65	23.14
V15	8.81	15.27	10.36	8.75	12.22	23.08	22.95	19.21	26.15	27.84
	12.70	9.90	18.53	6.66	12.29	23.59	22.53	19.11	23.21	27.16
	5.30	15.25	18.00	8.07	11.95	23.21	22.8	18.97	25.16	26.51
	9.07	16.92	18.70	10.93	14.02	23.1	21.52	18.24	22.91	26.7
I21	10.39	12.60	22.58	4.04	10.50	24.98	20.47	18.91	21.95	27.4
	15.19	7.18	19.55	8.82	10.71	24.45	21.19	19.44	23.08	27.19
	16.66	17.35	19.36	5.28	13.68	26.34	22.02	19.23	26.03	23.57
	13.05	11.34	10.30	6.61	10.15	25.42	20.49	19.88	21.91	28.49
I30	5.86	13.69	17.29	7.34	12.98	25.57	21.24	18.7	23.36	27.72
	15.30	13.73	21.21	7.12	14.35	25.76	20.46	17.79	23.04	20.7
	13.56	13.02	24.49	6.81	12.83	24.78	22.3	19.93	21.32	23.9
	18.44	17.23	25.77	5.48	14.89	23.8	19.26	19.41	24.59	25.43
I23	11.41	7.69	18.15	9.49	11.12	26.93	23.06	19.45	25.64	27.66
	4.11	11.67	8.20	6.40	9.84	28.55	24.01	19.37	25.66	27.91
	7.62	7.87	8.60	9.00	12.07	26.31	21.88	17.4	22.21	26.72
	11.74	15.92	19.24	13.78	12.80	26.36	24.32	18.29	21.77	26.34
I19	8.13	13.58	6.68	10.16	11.80	24.26	22.43	19.64	26.05	27.67
	10.91	11.87	18.15	10.54	11.02	28.03	23.17	19.63	25.15	27.47
	8.59	13.34	11.41	5.14	12.67	26.45	20.22	18.9	21.8	26.18
	12.38	13.88	7.55	10.11	9.38	26.47	24.81	20.21	26.24	28.56
V8	18.20	6.41	7.23	6.97	15.54	21.16	21.12	20.14	25.04	26
	19.25	5.50	15.41	9.55	15.93	21.94	21.11	18.54	20.82	24.56
	17.17	10.45	15.66	5.79	16.67	22.98	20.14	19.09	20.34	23.45
	12.85	10.58	10.29	6.49	15.11	21.89	20.48	18.72	20.59	23.96
I16	17.80	5.09	16.95	8.04	12.02	23.23	19.14	19.51	23.26	26.81
	14.06	10.49	16.87	8.44	11.62	24.81	20.23	20.32	25.76	25.97
	15.17	10.18	8.54	7.74	11.57	22.35	21.59	21.79	23.43	26.58
	14.16	5.30	18.04	8.64	10.72	23.52	22.13	20.44	22.08	27.52
I12	14.44	13.65	14.48	10.38	12.49	23.5	20.76	18.02	25.45	26.66
	15.65	9.11	11.27	8.69	11.06	22.66	19.46	18.25	25.82	26.1
	15.62	9.17	16.71	8.03	10.46	22.5	19.15	20.09	25.99	26.58

	12.10	5.75	6.86	9.72	12.45	23.95	19.49	18.09	22.61	26.13
V11	14.74	11.69	17.06	5.99	14.29	22.97	23.42	18.84	22.07	26.31
	17.70	11.58	18.73	6.02	15.96	21.92	20.89	19.38	24.5	26.56
	13.00	9.71	17.67	5.95	14.57	22.73	21.95	19.94	24.22	26.19
	13.84	13.19	19.76	8.17	16.25	23.1	22.07	19.61	23.59	25.06
I20	18.58	13.89	8.42	8.82	11.28	22.13	23.19	19.43	22.95	27.59
	17.47	12.79	19.12	9.36	12.07	23.44	17.1	19.91	25.32	25.74
	10.38	9.07	17.82	4.14	11.96	24.1	20.21	20.06	23.95	26.45
	11.04	10.22	19.10	10.80	11.62	22.96	20.24	19.55	24.97	26.57
I11	14.32	10.42	7.35	8.76	9.12	23.09	21.34	19.59	24.39	26.96
	8.54	9.41	8.65	9.99	10.24	21.42	21.38	20.48	25.57	26.53
	16.73	11.70	14.60	11.83	10.42	21.89	21.04	19.84	23.63	26.75
	18.55	11.36	17.71	11.32	9.80	21.53	17.32	19.23	24.58	26.05
I14	14.82	14.26	17.40	5.19	12.62	23.32	20.53	19.07	23.33	26.76
	16.90	14.17	7.40	11.88	15.37	22.36	18.09	18.17	24.95	25.73
	13.16	5.38	10.39	7.46	12.78	22.37	20.81	18.31	24.31	26.71
	21.49	7.40	7.28	6.42	12.71	20.72	18.14	18.19	22.33	26.29
V10	6.67	7.70	7.23	7.83	14.62	24.74	25.1	20.36	26.9	28.42
	11.41	9.48	14.67	9.30	15.07	23.38	22.21	20.22	24.84	26.72
	17.01	7.62	12.71	8.66	13.05	21.85	20.38	19.85	26.84	27.36
	14.04	8.60	7.57	8.82	11.27	22.1	19.55	20.95	22.19	30.18
I4	13.03	11.51	8.34	5.94	11.85	24.95	20.1	19.31	25.93	25.76
	16.07	10.73	9.09	7.40	11.56	23.65	26.11	19.84	23.14	24.97
	13.23	10.40	16.34	7.57	12.32	25.18	20.08	19.11	25.36	26.97
	12.57	10.74	10.45	6.78	9.00	24.54	20.78	20.52	25.66	28.06
I2	16.16	10.30	8.50	9.11	14.38	23.12	20.03	16.82	25.37	26.03
	7.14	7.85	5.86	7.92	11.86	26.16	19.31	19.84	22.76	29.79
	9.91	4.99	7.34	6.26	15.68	21.24	19.63	18.24	25.22	25.8
	11.94	10.42	7.60	11.58	13.60	25.59	19.89	20.31	25.39	24.52
I3	16.60	11.75	15.82	7.98	15.47	22.99	20.3	18.06	24.01	25.98
	13.68	13.92	6.91	4.56	15.58	24.05	19.96	20.39	25.45	26.53
	5.79	11.73	27.19	8.39	16.05	24.64	19.4	18.96	25.32	25.51
	13.83	10.20	11.09	7.43	14.53	25.11	20.41	17.35	24.53	25.88
V6	14.70	10.73	*	6.07	17.95	24.19	20.04	19.95	21.95	26.59
	17.34	10.28	10.92	16.36	16.94	22.14	21.23	18.95	23.46	24.07
	16.12	6.64	9.07	13.08	18.56	23.44	20.56	19.65	21.86	25.11
	18.11	10.76	6.92	10.17	18.75	23.56	20.98	19	20.41	24.75
I22	16.45	4.92	4.99	8.55	13.42	24.22	18.76	17.76	25.15	26
	15.01	10.49	13.08	4.80	13.53	23.16	19.83	18.5	25.64	26.6
	18.93	10.09	24.32	9.51	12.38	22.65	18.78	18.74	25.2	25.5
	17.61	11.41	9.03	5.24	11.78	23.67	18.31	18.9	25.32	25.14
V9	15.58	11.03	14.08	5.15	17.64	24.54	18.91	19.19	21.68	23.93
	21.25	14.42	13.39	10.91	15.16	24.41	20.32	18.84	2.68	25.68
	9.56	8.06	14.40	9.31	11.51	22.86	20.98	19.33	21.42	25.33
	18.85	6.89	10.23	7.09	14.13	22.61	20.78	19.82	20.88	26.52
I15	14.45	11.55	6.05	9.99	18.05	23.49	19.49	19.18	26.31	26.54
	8.53	6.25	19.57	5.64	12.29	23.29	20.17	18.35	21.2	25.66
	14.71	12.39	8.62	6.02	12.68	23.75	18.49	19.09	25.55	26.4
	18.27	13.07	4.18	9.37	11.08	20.91	19.14	19.28	22.38	24.48
I27	14.08	12.00	10.68	12.09	14.36	26.92	21.53	18.76	26.49	26.07
	15.24	15.90	10.21	10.98	12.76	25.15	21.66	18.87	22.58	27.68
	16.52	12.50	*	12.76	14.71	24.06	20.1	18.04	25.04	26.71
	12.46	12.04	7.88	11.33	12.82	24.69	23.47	18.88	24.22	27.73

8.9 Nei's (1973) nuclear genetic identity (above diagonal) and genetic distance (below diagonal) between all populations

	V3	I28	I1	I5	I17	I13	I7	V4	I6	I10	V15	I21	I30	I23	I19	I12	V11	I20	I11	I14	V10	I4
V3	0	0.75	0.76	0.8	0.81	0.58	0.57	0.78	0.61	0.63	0.77	0.7	0.79	0.69	0.67	0.43	0.76	0.43	0.59	0.5	0.23	0.51
I28	0.29	0	0.8	0.85	0.86	0.74	0.66	0.85	0.77	0.62	0.79	0.81	0.78	0.77	0.83	0.7	0.82	0.58	0.78	0.6	0.38	0.65
I1	0.28	0.23	0	0.81	0.87	0.73	0.76	0.76	0.66	0.56	0.74	0.77	0.78	0.78	0.79	0.55	0.81	0.62	0.62	0.65	0.36	0.63
I5	0.22	0.16	0.21	0	0.84	0.8	0.7	0.81	0.71	0.63	0.77	0.78	0.81	0.83	0.82	0.62	0.84	0.61	0.74	0.63	0.44	0.57
I17	0.21	0.15	0.14	0.18	0	0.78	0.75	0.79	0.69	0.57	0.81	0.79	0.76	0.78	0.8	0.58	0.85	0.57	0.68	0.57	0.33	0.55
I13	0.55	0.3	0.31	0.23	0.25	0	0.74	0.62	0.64	0.53	0.63	0.8	0.69	0.83	0.76	0.61	0.81	0.63	0.74	0.56	0.58	0.5
I7	0.55	0.41	0.27	0.35	0.29	0.3	0	0.7	0.81	0.7	0.66	0.84	0.58	0.68	0.75	0.43	0.76	0.52	0.58	0.49	0.39	0.48
V4	0.25	0.16	0.27	0.21	0.24	0.47	0.35	0	0.8	0.63	0.85	0.77	0.82	0.76	0.84	0.55	0.85	0.52	0.6	0.54	0.28	0.6
I6	0.49	0.26	0.42	0.34	0.37	0.45	0.22	0.22	0	0.74	0.75	0.81	0.67	0.71	0.81	0.55	0.76	0.54	0.69	0.51	0.39	0.52
I10	0.47	0.48	0.58	0.46	0.56	0.64	0.35	0.47	0.3	0	0.57	0.7	0.55	0.62	0.59	0.46	0.6	0.48	0.63	0.47	0.42	0.44
V15	0.26	0.23	0.3	0.26	0.22	0.47	0.42	0.16	0.28	0.56	0	0.73	0.79	0.7	0.8	0.53	0.84	0.5	0.62	0.5	0.32	0.51
I21	0.36	0.22	0.26	0.24	0.24	0.22	0.18	0.27	0.21	0.36	0.31	0	0.73	0.76	0.76	0.59	0.85	0.58	0.73	0.57	0.48	0.59
I30	0.23	0.25	0.25	0.22	0.28	0.36	0.55	0.2	0.39	0.6	0.23	0.32	0	0.86	0.79	0.6	0.83	0.63	0.63	0.61	0.39	0.62
I23	0.37	0.26	0.25	0.19	0.25	0.19	0.39	0.27	0.34	0.49	0.35	0.27	0.15	0	0.82	0.66	0.85	0.75	0.78	0.63	0.51	0.6
I19	0.4	0.19	0.24	0.2	0.23	0.28	0.29	0.18	0.21	0.52	0.23	0.28	0.24	0.2	0	0.62	0.82	0.65	0.68	0.62	0.44	0.56
I12	0.85	0.36	0.6	0.48	0.54	0.5	0.84	0.59	0.6	0.77	0.64	0.52	0.52	0.41	0.48	0	0.62	0.82	0.65	0.79	0.6	0.8
V11	0.28	0.2	0.21	0.18	0.16	0.22	0.28	0.16	0.27	0.51	0.17	0.17	0.18	0.16	0.2	0.48	0	0.63	0.74	0.56	0.39	0.58
I20	0.84	0.55	0.48	0.5	0.56	0.46	0.65	0.65	0.62	0.73	0.69	0.55	0.46	0.29	0.43	0.19	0.46	0	0.57	0.85	0.64	0.73
I11	0.54	0.25	0.48	0.3	0.39	0.3	0.55	0.51	0.38	0.47	0.48	0.31	0.46	0.24	0.38	0.43	0.31	0.55	0	0.49	0.52	0.48
I14	0.69	0.5	0.44	0.46	0.57	0.58	0.71	0.62	0.68	0.76	0.7	0.56	0.5	0.46	0.48	0.23	0.59	0.16	0.71	0	0.58	0.78
V10	1.47	0.96	1.03	0.82	1.11	0.55	0.95	1.28	0.95	0.87	1.13	0.74	0.94	0.67	0.83	0.51	0.93	0.44	0.66	0.55	0	0.46
I4	0.68	0.43	0.47	0.56	0.6	0.69	0.74	0.51	0.65	0.81	0.68	0.53	0.48	0.52	0.58	0.22	0.55	0.32	0.74	0.24	0.77	0
I2	0.74	0.47	0.46	0.49	0.5	0.65	0.63	0.5	0.6	0.85	0.54	0.54	0.51	0.5	0.44	0.19	0.47	0.2	0.8	0.22	0.75	0.22
I3	0.92	0.64	0.45	0.61	0.62	0.59	0.54	0.7	0.62	0.7	0.75	0.57	0.72	0.54	0.47	0.33	0.62	0.22	0.88	0.23	0.53	0.35
I22	0.68	0.68	0.58	0.61	0.58	0.66	0.37	0.61	0.39	0.37	0.63	0.43	0.76	0.62	0.59	0.78	0.48	0.62	0.65	0.66	1.15	0.77
I15	0.63	0.32	0.36	0.33	0.35	0.24	0.41	0.47	0.41	0.5	0.44	0.35	0.44	0.22	0.29	0.4	0.26	0.41	0.21	0.51	0.48	0.59
I27	0.35	0.23	0.29	0.23	0.24	0.21	0.4	0.33	0.41	0.47	0.36	0.35	0.29	0.18	0.32	0.56	0.22	0.54	0.28	0.69	0.72	0.59
V12	1.19	0.64	0.75	0.65	0.76	0.5	0.76	0.92	0.7	0.9	0.84	0.69	0.77	0.51	0.53	0.31	0.71	0.29	0.56	0.45	0.26	0.62
■15	0.57	0.39	0.58	0.38	0.46	0.29	0.56	0.47	0.51	0.51	0.45	0.46	0.39	0.28	0.51	0.69	0.36	0.66	0.35	0.92	0.61	0.92
V13	0.83	0.48	0.63	0.47	0.63	0.5	0.7	0.6	0.56	0.59	0.61	0.57	0.49	0.33	0.45	0.29	0.45	0.21	0.46	0.4	0.45	0.57
■18	0.75	0.44	0.64	0.51	0.56	0.42	0.66	0.6	0.51	0.58	0.47	0.55	0.53	0.37	0.54	0.54	0.49	0.64	0.32	0.76	0.52	0.8
V16	0.9	0.64	0.61	0.5	0.65	0.35	0.7	0.9	0.7	0.81	0.79	0.62	0.6	0.37	0.52	0.34	0.6	0.28	0.47	0.39	0.23	0.5

	I2	I3	I22	I15	I27	V12	■15	V13	■18	V16	■20	■16	V2	•32	V5	Δ5	■19	V1
V3	0.47	0.4	0.51	0.53	0.7	0.31	0.56	0.44	0.47	0.41	0.49	0.69	0.52	0.43	0.46	0.54	0.38	0.41
I28	0.63	0.53	0.51	0.73	0.8	0.53	0.67	0.62	0.65	0.53	0.69	0.75	0.62	0.49	0.59	0.63	0.55	0.52
I1	0.63	0.64	0.56	0.7	0.75	0.47	0.56	0.53	0.53	0.54	0.59	0.74	0.65	0.56	0.57	0.68	0.48	0.51
I5	0.61	0.54	0.54	0.72	0.8	0.52	0.68	0.62	0.6	0.61	0.68	0.82	0.65	0.54	0.58	0.66	0.54	0.59
I17	0.61	0.54	0.56	0.7	0.79	0.47	0.63	0.53	0.57	0.52	0.59	0.78	0.63	0.55	0.57	0.64	0.51	0.55
I13	0.52	0.56	0.52	0.79	0.81	0.61	0.75	0.61	0.65	0.7	0.7	0.82	0.6	0.53	0.6	0.66	0.55	0.62
I7	0.53	0.58	0.69	0.66	0.67	0.47	0.57	0.5	0.52	0.5	0.58	0.72	0.56	0.45	0.48	0.53	0.39	0.45
V4	0.61	0.5	0.55	0.63	0.72	0.4	0.63	0.55	0.55	0.4	0.58	0.7	0.64	0.41	0.52	0.59	0.45	0.45
I6	0.55	0.54	0.68	0.66	0.66	0.49	0.6	0.57	0.6	0.5	0.63	0.65	0.63	0.4	0.54	0.56	0.51	0.46
I10	0.43	0.5	0.69	0.61	0.63	0.41	0.6	0.56	0.56	0.45	0.64	0.63	0.49	0.4	0.49	0.51	0.47	0.51
V15	0.58	0.47	0.53	0.65	0.69	0.43	0.64	0.54	0.63	0.46	0.57	0.66	0.61	0.46	0.55	0.62	0.57	0.47
I21	0.58	0.57	0.65	0.71	0.71	0.5	0.63	0.56	0.58	0.54	0.61	0.72	0.63	0.5	0.59	0.61	0.48	0.5
I30	0.6	0.49	0.47	0.65	0.75	0.46	0.68	0.62	0.59	0.55	0.66	0.72	0.67	0.52	0.61	0.75	0.57	0.54
I23	0.61	0.58	0.54	0.81	0.84	0.6	0.75	0.72	0.69	0.69	0.76	0.79	0.74	0.59	0.67	0.78	0.63	0.72
I19	0.65	0.62	0.55	0.75	0.73	0.59	0.6	0.64	0.58	0.59	0.66	0.75	0.7	0.46	0.56	0.67	0.51	0.56
I12	0.83	0.72	0.46	0.67	0.57	0.74	0.5	0.75	0.58	0.71	0.6	0.54	0.82	0.72	0.82	0.82	0.75	0.75
V11	0.62	0.54	0.62	0.77	0.8	0.49	0.7	0.64	0.61	0.55	0.63	0.76	0.72	0.47	0.6	0.7	0.5	0.58
I20	0.82	0.8	0.54	0.66	0.58	0.75	0.51	0.81	0.53	0.76	0.58	0.58	0.87	0.75	0.8	0.86	0.72	0.86
I11	0.45	0.41	0.52	0.81	0.76	0.57	0.71	0.63	0.72	0.62	0.76	0.65	0.56	0.46	0.57	0.62	0.54	0.62
I14	0.81	0.79	0.52	0.6	0.5	0.64	0.4	0.67	0.47	0.68	0.52	0.56	0.8	0.75	0.77	0.79	0.6	0.68
V10	0.47	0.59	0.32	0.62	0.49	0.77	0.55	0.64	0.59	0.79	0.64	0.52	0.49	0.61	0.61	0.58	0.57	0.63
I4	0.8	0.71	0.46	0.55	0.55	0.54	0.4	0.57	0.45	0.6	0.51	0.51	0.77	0.66	0.74	0.73	0.62	0.58
I2	0	0.82	0.5	0.55	0.48	0.65	0.38	0.65	0.42	0.63	0.46	0.55	0.83	0.7	0.76	0.77	0.62	0.68
I3	0.2	0	0.54	0.59	0.49	0.74	0.34	0.63	0.43	0.67	0.5	0.58	0.75	0.68	0.71	0.74	0.59	0.65
I22	0.69	0.62	0	0.53	0.48	0.37	0.43	0.47	0.41	0.42	0.39	0.45	0.59	0.43	0.51	0.55	0.4	0.48
I15	0.6	0.53	0.63	0	0.82	0.68	0.73	0.67	0.83	0.72	0.85	0.74	0.69	0.56	0.63	0.7	0.6	0.66
I27	0.74	0.72	0.74	0.2	0	0.54	0.8	0.62	0.75	0.63	0.81	0.84	0.57	0.5	0.58	0.65	0.59	0.59
V12	0.44	0.31	0.99	0.38	0.62	0	0.5	0.72	0.61	0.79	0.64	0.56	0.63	0.65	0.63	0.69	0.63	0.7
■15	0.98	1.09	0.84	0.31	0.22	0.7	0	0.61	0.82	0.6	0.8	0.74	0.45	0.53	0.51	0.6	0.59	0.61
V13	0.44	0.46	0.76	0.39	0.48	0.33	0.49	0	0.56	0.7	0.64	0.62	0.73	0.58	0.63	0.76	0.63	0.79
■18	0.88	0.84	0.89	0.19	0.29	0.49	0.2	0.58	0	0.62	0.86	0.67	0.51	0.56	0.62	0.63	0.66	0.56
V16	0.47	0.41	0.88	0.33	0.46	0.24	0.51	0.35	0.47	0	0.68	0.66	0.69	0.72	0.66	0.75	0.71	0.77

		V3	I28	I1	I5	I17	I13	I7	V4	I6	I10	V15	I21	I30	I23	I19	I12	V11	I20	I11	I14	V10
■20	0.71	0.38	0.53	0.39	0.53	0.35	0.54	0.54	0.46	0.45	0.57	0.49	0.42	0.28	0.42	0.5	0.46	0.54	0.28	0.65	0.45	0.67
■16	0.38	0.29	0.3	0.2	0.25	0.2	0.33	0.35	0.43	0.47	0.42	0.33	0.33	0.23	0.29	0.63	0.28	0.54	0.43	0.57	0.65	0.67
V2	0.66	0.47	0.43	0.44	0.46	0.52	0.58	0.45	0.47	0.71	0.49	0.46	0.41	0.3	0.35	0.2	0.33	0.14	0.57	0.22	0.72	0.27
•32	0.85	0.72	0.58	0.62	0.6	0.63	0.81	0.9	0.91	0.91	0.78	0.7	0.65	0.53	0.78	0.33	0.76	0.29	0.78	0.29	0.49	0.42
V5	0.77	0.53	0.56	0.54	0.56	0.5	0.74	0.65	0.61	0.72	0.59	0.52	0.49	0.4	0.57	0.19	0.51	0.23	0.56	0.26	0.49	0.29
Δ5	0.61	0.46	0.39	0.42	0.45	0.41	0.64	0.53	0.58	0.67	0.48	0.5	0.29	0.24	0.4	0.2	0.36	0.16	0.48	0.24	0.55	0.32
■19	0.97	0.59	0.73	0.62	0.66	0.6	0.95	0.8	0.67	0.77	0.56	0.73	0.57	0.47	0.67	0.28	0.7	0.33	0.62	0.5	0.57	0.48
V1	0.89	0.65	0.67	0.53	0.6	0.47	0.8	0.81	0.77	0.67	0.76	0.69	0.62	0.33	0.58	0.28	0.55	0.16	0.48	0.38	0.45	0.55

	I2	I3	I22	I15	I27	V12	■15	V13	■18	V16	■20	■16	V2	•32	V5	Δ5	■19	V1
■20	0.78	0.7	0.94	0.16	0.22	0.44	0.22	0.44	0.15	0.39	0	0.79	0.53	0.56	0.57	0.65	0.62	0.6
■16	0.6	0.54	0.8	0.3	0.17	0.58	0.31	0.49	0.4	0.42	0.23	0	0.54	0.55	0.56	0.63	0.52	0.57
V2	0.19	0.29	0.53	0.37	0.56	0.47	0.8	0.32	0.66	0.38	0.63	0.61	0	0.68	0.78	0.83	0.68	0.73
•32	0.35	0.39	0.85	0.59	0.7	0.43	0.64	0.54	0.58	0.33	0.59	0.59	0.38	0	0.77	0.74	0.78	0.67
V5	0.28	0.34	0.68	0.46	0.54	0.46	0.66	0.46	0.48	0.42	0.57	0.58	0.25	0.27	0	0.78	0.78	0.69
Δ5	0.26	0.3	0.6	0.35	0.42	0.38	0.51	0.28	0.47	0.29	0.44	0.46	0.18	0.31	0.25	0	0.74	0.78
■19	0.47	0.53	0.91	0.52	0.53	0.46	0.53	0.46	0.42	0.35	0.48	0.65	0.38	0.25	0.25	0.31	0	0.73
V1	0.39	0.44	0.74	0.42	0.53	0.36	0.49	0.23	0.57	0.27	0.52	0.56	0.31	0.4	0.37	0.25	0.32	0

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PRIMER NOTE

Development and testing of novel chloroplast microsatellite markers for *Lolium perenne* and other grasses (Poaceae) from *de novo* sequencing and *in silico* sequences

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Abstract

Twelve primers to amplify microsatellite markers from the chloroplast genome of *Lolium perenne* were designed and optimized using *de novo* sequencing and *in silico* sequences. With one exception, each locus was polymorphic with a range from two to nine alleles in *L. perenne*. The newly developed primer pairs cross-amplified in different species of *Lolium* and in 50 other grass species representing nine grass subfamilies.

Keywords: chloroplast microsatellites, *Lolium perenne*, plastid, Poaceae, SSRs, transferability

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Lolium perenne is an important forage and turf grass in temperate regions. As a diploid outbreeder, it can easily hybridize with other allogamous *Lolium* and *Festuca* species. Because of the economic importance of *L. perenne*, many studies have developed nuclear DNA markers (e.g. Kubik *et al.* 2001). However, there is a lack of chloroplast (plastid) DNA markers capable of detecting high levels of polymorphism in *Lolium* and closely related genera.

Although chloroplast DNA (cpDNA) generally has lower variability than nuclear DNA, chloroplast simple sequence repeat (cpSSR) loci have been shown to be polymorphic particularly at mononucleotide repeats (Provan *et al.* 2001). Chloroplastic markers have been used in *Lolium* to study phylogenetic relationships using restriction site (Balfourier *et al.* 2000) and DNA sequence variation (Catalan *et al.* 2004). Some cpSSR primers were reported for grasses (Provan *et al.* 2004). However, there is a need for highly polymorphic cpDNA markers for *Lolium* and other members of the *Festuca–Lolium* complex.

We report the identification of polymorphic chloroplast microsatellite regions within *Lolium* and on the development of primers for their amplification. Primers for cpSSR markers were designed and optimized using *de novo* sequencing and from sequences obtained from GenBank (Table 1). We tested these novel primers on *L. perenne* and a range of grasses from nine out of 10 subfamilies of Poaceae (Grass Phylogeny Working Group 2001; Table 2).

Freeze-dried leaves were ground with a Retsch bead mill. Total genomic DNA for the sequencing was extracted from *L. perenne*, *Lolium multiflorum*, *Festuca pratensis* and *Festuca arundinacea* using a modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle 1987). All other DNA was extracted using the magnetic bead-based QIAGEN MagAttract plant DNA core kit with a modification for use on a Hamilton Microlab Star robotic system.

From these DNA samples, polymerase chain reaction (PCR) products were amplified and sequenced (Table 1: Accession nos. DQ123585, DQ123586, DQ131606) using the primers of Taberlet *et al.* (1991) for the *trnL* and *trnL-F*, Oxelman *et al.* (1997) for the *rps16* and Samuel *et al.* (1997) for the *atpB-rbcL* regions. For PCR amplifications, a PTC-200 Peltier Thermal Cycler (MJ Research) was used as follows: 30 cycles each with 1 min denaturation at 97 °C, 1 min annealing at 52 °C, and 3 min extension at 72 °C, with a final extension step at 72 °C for 7 min. The PCR mixture (50 μL) contained 10× PCR buffer (Promega), 0.2 mM dNTPs (Invitrogen), 2 mM $MgCl_2$, 100 ng/μL of each primer, 0.25 U *Taq* DNA polymerase (Promega) and ~100 ng of template DNA. The amplified products were purified using the JETQUICK PCR purification kit (Genomed) and sequenced using a BigDye Terminator version 1.1 cycle sequencing kit and an ABI 310 automated DNA sequencer (Applied Biosystems).

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Table 1 Names, GenBank Accession nos, chloroplast genome region of SSR markers and their size ranges and allele numbers in *Lolium perenne* and other grass species

cpSSR marker	GenBank Accession no.	Chloroplast genome region	Primer sequences F (5′–3′) and R (5′–3′)	T_a (°C)	Fluorescent dye	Dilution ratio (pooling ratio)	Repeat motif	Size range (bp) *L. perenne* (other species)	Allele no. *L. perenne* (other species)‡
TeaSSR1*	DQ123586	*atpB-rbcL* intergenic spacer region	ATTGATTTGGGTTGCGCTAT TCATTAAAGAAAATTGAGGGCATA	60	VIC	1/20 (4/11)	$(AAC)_3$	217–230 (227–230)	6 (4)
TeaSSR2*	DQ123585	*trn-L* intron and *trn-F* intergenic spacer region	GGTTTGGGGATAAAGGGACT TCCATTCCAATTGAATATTTTGT	45	NED	1/40 (4/11)	$(CT)_4$	196–200 (196–202)	5 (3)
TeaSSR3*	DQ123585	*trn-L* intron and *trn-F* intergenic spacer region	AGGGACTTGAACCCTCACAA GCAAACGATTAATCATGGAACC	60	FAM	1/30 (2/11)	A_9	305–318 (305–318)	9 (10)
TeaSSR4†	L41587	*23S-5S* internal transcribed spacer	ACGAACGAACGATTTGAACC TGAAGCCCCAATTCTTGACT	60	NED	1/20 (4/11)	A_8	193–200 (185–200)	4 (9)
TeaSSR5†	AF363674	Herbicide binding protein D1 (*psbA*)	GCTATGCATGGTTCCTTGGT TTCCTACTACAGGCCAAGCAG	60	VIC	1/10 (1/11)	$(CTT)_3$	209–212 (209–212)	3 (2)
TeaSSR6†	AY694130	*trnV* gene for Valine transfer RNA (GAC)	CGGATTCTAACCGTAGACCTTC TCAAAGCCAGGAAGCAATCT	45	PET	1/10 (1/11)	T_{12}	194–203 (188–201)	6 (7)
TeaSSR7†	X53066	*rpl2* gene for ribosomal protein L2 and trnH gene for Histine transfer RNA (CAC)	GGAATTTGCAATAATGCGATG TCGATCGAGGTATGGAGGTC	60	NED	1/40 (4/11)	$(ATT)_5$	172–173 (171–177)	2 (5)
TeaSSR8†	Z77754	*rpoA* gene for RNA polymerase α subunit	TTGACAGTTTTCGTATGGAAGA GATTGTGCCAAAGATGCAAA	45	PET	1/1 (4/11)	T_{11}	179–182 (178–184)	3 (5)
TeaSSR9*	DQ123585	*trn-L* intron and *trn-F* intergenic spacer region	TCCTGACCTTTTCTTGTGCAT AACCCGGTTTTTCGGTTTAT	45	FAM	1/10 (2/11)	G_{12}	175–194 (176–194)	8 (5)
TeaSSR10*	DQ131606	*rps16* gene for 30S ribosomal protein CS16	TTCGCTCGAAATGAGACAAA CCTCATACGGCTCGAGAAAA	60	PET	1/10 (1/11)	T_6	219 (215–219)	1 (3)
TeaSSR11†	L25382	*rpoC2* gene for RNA polymerase β subunit	GCTTATTTTGACGATCCACGA TGGGCTGCCATACTCTTCTT	60	FAM	1/30 (2/11)	A_{12}	194–196 (194–195)	3 (2)
TeaSSR12†	U71014	NADH dehydrogenase subunit F	TGGAGCAATGGATAATGGAA CCGCGATTATATGACCAACTG	60	VIC	1/20 (4/11)	A_9	301–307 (304–305)	5 (2)

*, primers developed from *de novo* sequencing; †, primers developed from *in silico* sequences; ‡, the allele number given for other species represents the total allele number over the other species.

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Table 2 Allele sizes (in bp) of the 12 *de novo* developed cpSSR markers in 51 grass species representing nine subfamilies of Poaceae

		Chloroplast marker amplification											
Subfamily	Species	TeaSSR1	TeaSSR2	TeaSSR3	TeaSSR4	TeaSSR5	TeaSSR6	TeaSSR7	TeaSSR8	TeaSSR9	TeaSSR10	TeaSSR11	TeaSSR12
Anomochlooideae	*Streptochaeta spicata*	229	—	—	194	209	196	—	—	—	—	—	—
Arundinoideae	*Arundo donax*	229	—	—	195	209	195	—	—	194	—	—	—
Bambusoideae	*Phyllostachys flexuosa*	229	197	312	200	—	195	—	—	189	—	—	—
"	*Phyllostachys nuda*	229	—	312	194	—	—	172	181	189	—	—	—
Centothecoideae	*Chasmanthium latifolium*	—	—	—	—	—	195	—	—	—	—	—	—
"	*Orthoclada laxa*	229	—	—	195	212	196	—	—	190	—	—	—
Chloridoideae	*Chloris* sp.	—	—	—	195	—	194	—	—	—	—	—	—
"	*Eleusine coracana*	—	196	312	200	209	195	—	—	—	—	—	—
"	*Eragrostis chloromatus*	—	196	302	194	209	195	171	—	—	—	—	—
Danthonioideae	*Danthonia decumbens*	229	196	—	194	212	196	—	181	194	—	—	—
Ehrhartoideae	*Oryza sativa*	—	—	312	194	209	195	172	—	—	—	—	—
Panicoideae	*Miscanthus sinensis*	229	196	311	199	209	199	—	—	—	—	—	—
"	*Saccharum arundinaceum*	—	196	—	200	209	—	—	—	—	—	—	—
"	*Saccharum spontaneum*	229	196	311	199	212	201	—	—	—	—	—	—
"	*Zea diploperennis*	—	196	—	200	209	—	171	—	—	—	—	—
"	*Zea mays*	229	196	312	199	209	199	171	181	194	—	—	—
"	*Pharus latifolius*	229	196	—	195	209	195	—	—	189	—	—	—
Pooideae	*Aegilops speltoides*	229	—	312	194	—	195	—	—	194	—	—	—
"	*Agrostis canina*	229	196	312, 313, 316	195, 196	209, 212	188	172	179	—	—	—	—
"	*Agrostis capillaris*	229	196	313	194, 195	212	195, 196	172	184	—	—	195	—
"	*Agrostis stolonifera*	—	196, 197	311, 312	195, 196	209, 212	188	172	179	—	—	—	—
"	*Alopecurus pratensis*	228, 229	202	311, 312	194, 195	209, 212	195	171, 172	179	—	—	—	—
"	*Avena sativa*	—	196	312	200	—	—	172	181	—	—	—	—
"	*Briza media*	229	—	—	195, 196	209, 212	188	172, 173	—	—	—	—	—
"	*Bromus erectus*	229	196	311, 312	185, 186, 188	209	188	—	178	—	—	—	—
"	*Cynosurus cristatus*	229	197	305	193, 194, 195	209, 212	197	172	179	194	—	—	—
"	*Dactylis glomerata*	227, 229, 230	196	312, 314, 315	194, 195	209, 212	195	172	179, 180, 181	194	219	—	—
	Festuca arundinacea	229	196	313	194	209	195	172	181	—	—	—	—
"	*Festuca gigantea*	—	196	313	194	209, 212	195	—	181	—	—	—	—
"	*Festuca ovina*	229	196	312, 314, 317	194, 195	209, 212	195	172, 173	179, 180, 181	—	—	—	—
	Festuca pratense	229	196	313	194	209	195	172	181	194	—	—	—
"	*Festuca vivipara*	229, 230	196	314, 317	194, 195	209, 212	195	172, 173	180	—	—	—	—
"	*Festuca rubra*	229	196	311, 312	194, 195	209, 212	195	172	179, 180	189, 194	—	—	—
"	*Holcus lanatus*	—	196, 197	312	194, 195	212	195	174	180	194	—	—	—
"	*Hordeum vulgare*	—	196	—	—	209	—	172	181	194	219	194	—
"	*Koeleria macrantha*	—	196, 197	312, 313	195, 196	212	188	—	179	194	—	—	—
"	*Lolium canariense*	227, 229	196	308, 312, 313, 314	194, 195	209, 212	195	171, 172, 173	181	—	—	—	—
"	*Lolium hybridum*	227, 229	196	312	194, 195	209, 212	195	172	181	189, 194	219	194, 195	305
"	*Lolium multiflorum*	227, 229	196, 197	312, 314, 315	194, 195	209, 212	195	172	181	178	218, 219	—	—
"	*Lolium perenne*	217–230	196–200	305–318	193–200	209–212	194–203	172–173	179–182	175–194	219	194–196	301–307
"	*Lolium persicum*	228, 229	196	313, 317	194, 195	209, 212	195	—	181	—	—	194, 195	—
"	*Lolium remotum*	229	196	316	194, 195	212	195	172	181	176	219	—	304, 305
"	*Lolium rigidum*	229	196	312, 318	194, 195	209	195	172	181	176	215, 219	—	304
"	*Lolium subulatum*	228, 229	196	314, 317	194, 195	212	195	174	181	—	219	–	–
"	*Lolium temulentum*	227	196, 197	311, 312, 317	195	209	195	172	181	176	219	194, 195	305
"	*Phleum pratense*	229	196	311, 312, 317	194	—	201	172	—	189	—	—	—
"	*Poa pratensis*	229	197	—	194, 195	—	195	172, 177	179	194	219	–	–
"	*Poa palustris*	229	196	—	194, 195	209, 212	195	172, 177	—	—	—	—	—
"	*Secale cereale*	—	196	—	200	209	—	172	—	—	—	194	—
"	*Triticum aestivum*	—	—	312	—	209	—	—	181	—	—	194	—
"	× *Triticosecale*	—	196	—	—	209	—	172	181	194	218, 219	194	—

—, no amplification.

Further plastid DNA sequences from *L. perenne* and related species were obtained from GenBank (Table 1). All sequences were analysed for microsatellite motifs using a modified version of the MISA perl script (www2.unil.ch/software/).

Twelve primer pairs were designed using the Web-based PRIMER 3 software (Rozen & Skaletsky 2003) and were optimized for amplification and subsequent detection. DNA was then amplified from various populations of *L. perenne* and a range of other Poaceae (Table 2). All amplifications used a Px2 Thermal Cycler (Thermo Electron Corporation) with an initial 5 min denaturation at 95 °C, followed by 35 cycles each with a 1 min denaturation at 95 °C, 1 min at a primer-specific annealing temperature (Table 1) and a 1 min extension at 72 °C, followed by a final extension at 72° for 10 min. The 10 μL reaction mixture contained 10× reaction buffer (New England Biolabs) containing 2 mM $MgCl_2$, 0.625 mM dNTPs, 0.25 μM of each primer (one 5′ fluorescently labelled), 0.75 U *Taq* DNA polymerase (New England Biolabs) and approximately 10 ng template DNA. A poly A treatment at 65 °C for 30 min was done prior to dilution and pooling of PCR products. PCR products were sized using the LIZ 500 internal sizing standard on an ABI 3100 automated DNA sequencer and GENEMAPPER version 3.0 software (Applied Biosystems).

Size ranges and allele numbers were determined for each of the loci tested (Table 1). A single locus (TeaSSR10) was monomorphic in *L. perenne* but polymorphic across all grasses (Table 1). The 11 remaining loci were polymorphic within both *L. perenne* and all the other grasses. In *L. perenne*, the allele numbers ranged from locus TeaSSR10 with one allele to locus TeaSSR3 with nine alleles. All 12 loci also amplified in *Lolium temulentum* and *Lolium hybridum*. In the other grasses, allele numbers ranged from loci with two alleles (TeaSSR5, TeaSSR11 and TeaSSR12) to locus TeaSSR3 with 10 alleles. Particularly, cpSSRs from the regions *trnL-F*, *23S-5S* and *trnV* were highly polymorphic (Table 1). Successful cross-species amplification of the markers was found at all 12 loci (Table 2), with less successful cross-species amplification in more distantly related grasses.

The developed primers will be useful for a range of population genetic applications including the study of phylogeographic structure, for testing maternal inheritance and detecting introgression (especially for *Lolium*, *Festuca* and *Festulolium* hybrids), for species differentiation, gene flow studies and the identification of haplotype structures.

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ORIGINAL ARTICLE

Extremely high cytoplasmic diversity in natural and breeding populations of *Lolium* (Poaceae)

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Ten chloroplast microsatellite markers were used to characterise chloroplast genetic diversity at allelic and haplotypic level in 104 accessions of *Lolium perenne*, other *Lolium* species, *Festuca* species and × *Festulolium* cultivars. Furthermore, genetic relationships between the accessions and biogeographic distribution of haplotypes were investigated using a range of Nei's population genetic diversity measures and analysis of molecular variance (AMOVA). An extremely high number (511) of haplotypes was detected in 1575 individuals. Nei's gene diversity values among *L. perenne* accessions ranged between 0 and 0.333. Much of the *L. perenne* European ecotype diversity (61%), as calculated using AMOVA, could be attributed to within-population variance and this is likely caused by, and maintained by, high levels of natural and anthropogenic seed dispersal. Plastid gene pools and maternal lineages for *L. perenne* could be clearly identified. Evidence was found, using AMOVA, to show a likely migration route of *L. perenne* from Southern regions of Europe northwards.
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Keywords: chloroplast; cytoplasm; *Lolium*; microsatellites; plastid; Poaceae

Introduction

Lolium species are highly important forage and turf grasses in Europe and other temperate zones of the world. Perennial ryegrass, *Lolium perenne*, is the most economically important species within *Lolium* and is the main constituent of productive pastures (Humphreys *et al.*, 2006). Genetic studies are required for *L. perenne* to better understand the extent of infraspecific variation, how this diversity is partitioned within and among populations, and to help infer phylogeographic patterns. Chloroplast microsatellite (cpSSR) markers have been previously used successfully to assess variation and chloroplast DNA (cpDNA) diversity in a range of other plant species (Provan *et al.*, 2001; Flannery *et al.*, 2006). cpDNA restriction fragment length polymorphism (RFLP) markers and DNA sequencing have been used to assess the phylogenetic relationships of *L. perenne* to other *Lolium* and *Festuca* species (Darbyshire and Warwick, 1992; Charmet *et al.*, 1997; Catalan *et al.*, 1997, 2004; Torrecilla *et al.*, 2004). These studies showed the separation of narrow-leaved fescues (for example, *Festuca alpine, Festuca ovina*) from the broad-leaved fescues (for example, *Festuca arundinacea, Festuca pratensis*), with *Lolium* species grouping either close to the broad-leaved fescue group (Catalan *et al.*, 1997) or within this group (Darbyshire and Warwick, 1992). Within *Lolium*, self-pollinating species tend to separate phylogenetically from the open pollinating species. In the study undertaken by Catalan *et al.* (2004), two autogamous species, *Lolium canariense* and *Lolium rigidum*, grouped together while the allogamous species *L. perenne* grouped with a second allogamous species, *Lolium multiflorum*.

Genetic characterization of natural and breeding populations of *L. perenne* has so far largely utilized nuclear molecular DNA markers (Cresswell *et al.*, 2001; Bolaric *et al.*, 2005). Few studies have assessed chloroplast or mitochondrial organelle diversity partly because easily applicable organelle markers have until recently not been easily produced (Huang *et al.*, 2002; McGrath *et al.*, 2006). Chloroplast genomes of plants have distinct features. They are generally uniparentally inherited, haploid, non-recombinant and have a conserved gene order (Provan *et al.*, 2001), making cpDNA a useful tool for studying inter-relationships of plants at many taxonomic levels (Catalan *et al.*, 1997; Hodkinson *et al.*, 2002). Although cpDNA generally has lower variability than nuclear DNA (Wolfe *et al.*, 1987), chloroplast simple sequence repeat (cpSSR) loci have been shown to be polymorphic particularly at mononucleotide repeat loci (Powell *et al.*, 1995) and can be used to investigate plant population structure, diversity and differentiation (reviewed by Provan *et al.*, 2001). They can be used to monitor the transmission of chloroplast genomes during hybridization and introgression in wild or breeding populations or to characterize plastid genome type for breeding purposes (Flannery *et al.*, 2006).

cpSSRs can be used in breeding programmes as cultivar identifiers as an addition to nuclear DNA markers (Joshi *et al.*, 1999). Furthermore, detailed

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characterization of plastid type is essential in studies investigating nucleo-cytoplasmic effects (Hallden *et al.*, 1993), since plastid signals controlling nuclear gene expression can have both positive and negative effects on gene expression (Gray, 2005). Several chloroplast genes may have importance for genetic engineering such as those involved in synthesis of fatty acids, amino acids and vitamins (Saski *et al.*, 2005) or for the directed manipulation of plant lines in breeding programmes (Daniell *et al.*, 2005). Worldwide breeding efforts for the improvement of *L. perenne* and its allies are ongoing and depend upon well-characterized genetic resource collections. Therefore, it is necessary to learn more about the diversity found within it and within closely related species and genera as these form part of the readily accessible gene pool of *L. perenne* for breeding purposes. cpSSR markers have not been used before to study plastid types for plant breeding purposes in *Lolium* species.

L. perenne is thought to have originated in the Near East, with Europe as a secondary centre of origin (Balfourier *et al.*, 2000). It has subsequently been introduced to almost all of the rest of the temperate world (Charmet *et al.*, 1996). cpDNA RFLP polymorphisms have been used to assess phylogeographic structure in wild-*Lolium* populations and to infer methods and pathways of geographic migration of *Lolium* populations (Balfourier *et al.*, 2000). Balfourier *et al.* (2000) recognized three major clusters of haplotypes in their European sample of *Lolium*. Their results suggest a single origin for *Lolium* as well as a geographical structure following an east/west cline, matching known historical processes such as the emergence of agriculture and cereal crops from the Fertile Crescent 10 000 years ago and the spread of these crops towards Europe. As yet, cpDNA SSRs have not been used to study plastid diversity in populations of *L. perenne* or other *Lolium* species; neither have they been used to study the phylogeography of these species or *Festuca* species. Given the agronomic importance of *L. perenne* for European agriculture, migration routes from its centres of origin require investigation.

The aims of this study are to (1) describe cpDNA allelic and haplotypic diversity in natural and breeding populations of *Lolium* including Irish and other European *L. perenne* ecotypes and bred *L. perenne* and × *Festulolium* cultivars; (2) assess the potential of a set of previously developed cpSSR markers (McGrath *et al.*, 2006) for plastid genome identification and to assess their value for the study of hybridization and introgression and for the definition of cytoplasmic pools in plant breeding; and (3) determine the level of biogeographic population genetic structure in Irish and European *L. perenne* populations and to gain insights into their phylogeography.

Materials and methods

In total, 104 grass accessions including 78 *L. perenne* accessions were studied (Table 1). These 78 accessions consisted of 30 Irish ecotypes, 32 European ecotypes and 16 cultivars. In addition, 11 other *Lolium* species, 6 *Festuca* species and 9 × *Festulolium* cultivars were used. The term ecotype is used broadly in this article to define locally adapted populations. The Irish ecotypes were selected from the Teagasc Oak Park collection holding 419 *L. perenne* accessions collected from old Irish pasture ecosystems (Connolly, 2000). This collection was made between the years 1980 and 1982 as part of the *Lolium* Core Collection Project that was coordinated by the European Co-operative Programme for Genetic Resources (ECPGR). The populations originated from collection sites where, according to the farmer, no reseeding had been carried out for 50 years or more. For this study, accessions were selected from the Teagasc Oak Park collection to cover a widespread of diverse geographic regions from the Republic of Ireland. The other European accessions were selected to provide a wide European geographic coverage to allow genetic diversity comparisons to be made with the rest of Europe and to help address possible geographic migration routes of *L. perenne*. The included × *Festulolium* cultivars had a defined maternal lineage. The definition of geographic groups was based on the United Nations definition of geographical subregions (United Nations Publication, 1999).

Seeds were germinated on seed testing paper, seedlings transferred to pots and the plants raised in the greenhouse. The ecotype accessions and cultivars were subsequently planted as spaced plants in the field at Oak Park using 50 individual plants per population. Leaf material from generally 16 individuals of each accession was harvested, freeze-dried and ground with a Retsch bead mill. DNA was extracted using a magnetic bead-based method (MagAttract Plant DNA Core kit, Qiagen, Hilden, Germany) as described previously in McGrath *et al.* (2006). PCR reactions were carried out using the primers developed by, and the thermal cycling parameters used in, McGrath *et al.* (2006). Ten of the 12 described primers were used: TeaCpSSR1, TeaCpSSR2, TeaCpSSR3, TeaCpSSR4, TeaCpSSR5, TeaCpSSR7, TeaCpSSR8, TeaCpSSR10, TeaCpSSR11 and TeaCpSSR12. PCR products were analysed using an ABI 3100 automated DNA sequencer and alleles were sized using GeneMapper V3.7 software (Applied Biosystems, Warrington, UK).

A genetic distance matrix was calculated based on the Nei's genetic distance measure (Nei, 1972), using allele data (characters) without size information. Distances based on a stepwise mutation model, such as the $\delta\mu^2$-distance (Goldstein *et al.*, 1995; Flannery *et al.*, 2006), were not used because the size variation of alleles could be attributed to both SSR length variation and other types of substitutions (non-SSR indels). From this matrix, a dendrogram showing the similarities between populations was constructed using the unweighted pair group method with arithmetic means (UPGMA) method (Sneath and Sokal, 1973) as implemented in POPGENE (Yeh and Boyle, 1997). Bootstrapping analysis was performed on the UPGMA data with 1000 replicates as implemented in NTSYSpc V2.2 software (Rohlf, 2005).

A geographic distance matrix was constructed, using the 56 accessions where an exact geographical origin was known (Table 1). A Mantel test was used to correlate the pairwise comparisons in the geographic distance matrix and the genetic distance matrix using NTSYSpc V2.2 (Rohlf, 2005). A total of 10 000 permutations were employed to test for significance.

Since polymorphisms in the chloroplast genome are considered to be linked, each haplotype can be con-

Table 1 Name, source, original location and genetic diversity information on accessions of *Lolium*, × *Festulolium* and *Festuca* used in this study

Species	*Accession number*	*Group*[a]	*Country of origin*	*Location*	*Latitude*	*Longitude*	*Seed source*	N	H	N *Haplotypes/ population*	N *Unique haplotypes*
L. perenne	IRL-OP-02337	I 1	Ireland	Kellistown Farm, Carlow	N 52.47.60	W 06.49.73	Teagasc Oak Park	15	0.180	9	2
L. perenne	IRL-OP-02059	I 2	Ireland	Moyneroe, Scarrif, Clare	N 52.54.35	W 08.30.32	Teagasc Oak Park	16	0.202	12	1
L. perenne	IRL-OP-02007	I 3	Ireland	Bromcloc, Bantry, Cork	N 51.39.95	W 09.31.07	Teagasc Oak Park	16	0.177	11	2
L. perenne	IRL-OP-02011	I 4	Ireland	Crowleys Pub, The Square, Bantry, Cork	N 51.42.15	W 09.27.67	Teagasc Oak Park	14	0.207	11	0
L. perenne	IRL-OP-02015	I 5	Ireland	South Ring, Clonakilty, Cork	N 51.37.10	W 08.53.71	Teagasc Oak Park	16	0.268	15	6
L. perenne	IRL-OP-02048	I 6	Ireland	Carrigeen, Conna, Old Kents, Fermoy, Cork	N 52.05.88	W 08.03.50	Teagasc Oak Park	16	0.202	10	2
L. perenne	IRL-OP-02192	I 7	Ireland	Horse Island, Roaring Water Bay, West Cork	N 51.30.80	W 09.29.03	Teagasc Oak Park	16	0.197	11	1
L. perenne	IRL-OP-02312	I 8	Ireland	Fortlands House, Charleville, Cork	N 52.20.86	W 08.42.27	Teagasc Oak Park	16	0.219	12	3
L. perenne	IRL-OP-02320	I 9	Ireland	Clonakilty, Co. Cork	N 51.37.10	W 08.53.71	Teagasc Oak Park	16	0.202	11	3
L. perenne	IRL-OP-02064	I 10	Ireland	Kilreekill, Loughrea, Galway	N 53.13.23	W 08.28.75	Teagasc Oak Park	16	0.190	10	3
L. perenne	IRL-OP-02078	I 11	Ireland	Ballycahalan, Peterswell, Galway	N 53.05.64	W 08.36.72	Teagasc Oak Park	18	0.324	15	5
L. perenne	IRL-OP-02230	I 12	Ireland	Clough, Cummer, Tuam, Galway	N 53.27.11	W 08.53.29	Teagasc Oak Park	16	0.333	15	8
L. perenne	IRL-OP-02128	I 13	Ireland	Mahera Beg, Commonage North, Castlegregory, Kerry	N 52.17.79	W 10.01.38	Teagasc Oak Park	16	0.122	5	3
L. perenne	IRL-OP-02538	I 14	Ireland	Colt, Ballyroan, Laois	N 52.58.08	W 07.20.70	Teagasc Oak Park	16	0.198	8	2
L. perenne	IRL-OP-02274	I 15	Ireland	Buffanoka, Cappamore, Limerick	N 52.39.29	W 08.18.62	Teagasc Oak Park	16	0.174	9	1
L. perenne	IRL-OP-02480	I 16	Ireland	Inch St, Lawrence, Caherconlish, Limerick	N 52.35.47	W 08.30.99	Teagasc Oak Park	16	0.276	16	5
L. perenne	IRL-OP-02442	I 17	Ireland	Doughmakean, Roonagh, Westport, Mayo	N 53.44.74	W 09.53.69	Teagasc Oak Park	16	0.270	15	3
L. perenne	IRL-OP-02444	I 18	Ireland	Barnabawn, Killadoon, Westport, Mayo	N 53.41.48	W 09.54.91	Teagasc Oak Park	16	0.191	10	1
L. perenne	IRL-OP-02068	I 19	Ireland	Ballycommon, Tullamore, Offaly	N 53.17.50	W 07.23.11	Teagasc Oak Park	15	0.287	14	7
L. perenne	IRL-OP-02241	I 20	Ireland	Clonohill, Birr, Offaly	N 53.05.20	W 07.53.73	Teagasc Oak Park	16	0.253	13	6
L. perenne	IRL-OP-02419	I 21	Ireland	Johnstown,Cornafulla, Athlone, Roscommon	N 53.38.05	W 09.30.71	Teagasc Oak Park	16	0.253	14	4
L. perenne	IRL-OP-02258	I 22	Ireland	The Lawn, Drum, Tipperary	N 52.46.32	W 07.52.89	Teagasc Oak Park	16	0.191	11	3
L. perenne	IRL-OP-02272	I 23	Ireland	Ballycrana, Kilross, Tipperary	N 52.25.28	W 08.15.88	Teagasc Oak Park	16	0.301	16	6
L. perenne	IRL-OP-02250	I 24	Ireland	Glown, Upperchurch, Tipperary	N 52.42.55	W 08.07.99	Teagasc Oak Park	17	0.260	13	3
L. perenne	IRL-OP-02267	I 25	Ireland	Ballyhoulihan, Emly, Tipperary	N 52.26.88	W 08.22.06	Teagasc Oak Park	16	0.292	15	4
L. perenne	IRL-OP-02269	I 26	Ireland	Ballycurrane, Emly, Tipperary	N 52.27.42	W 08.22.95	Teagasc Oak Park	17	0.248	15	2
L. perenne	IRL-OP-02173	I 27	Ireland	Deerpark, Lismore, Waterford	N 52.08.04	W 07.55.62	Teagasc Oak Park	16	0.273	12	3
L. perenne	IRL-OP-02483	I 28	Ireland	Edwardstown, Cleriestown, Wexford	N 52.16.20	W 06.38.25	Teagasc Oak Park	16	0.262	16	1

Table 1 Continued

Species	*Accession number*	*Group*[a]	*Country of origin*	*Location*	*Latitude*	*Longitude*	*Seed source*	N	H	N *Haplotypes/ population*	N *Unique haplotypes*
L. perenne	IRL-OP-02491	I 29	Ireland	Heath Park, Newbawn, Wexford	N 52.23.32	W 06.48.61	Teagasc Oak Park	16	0.248	14	2
L. perenne	IRL-OP-02018	I 30	Ireland	Ballynure Demesne, Grangecon, Wicklow	N 52.59.95	W 06.44.92	Teagasc Oak Park	16	0.286	14	6
L. perenne	GR 5092	Δ1	Germany	Malchow/Poel	N 54.00.00	E 11.28.00	IPK Gatersleben	16	0.199	12	2
L. perenne	PI 598445	Δ2	Netherlands	Unknown	N 53.07.00	E 07.02.00	GRIN	12	0.146	8	0
L. perenne	ABY-Ba 12896	Δ3	Denmark	Unknown	N 55.00.00	E 09.46.59	IGER	16	0.128	8	5
L. perenne	NGB14250	Δ4	Sweden	Unknown	N 57.45.50	E 14.51.25	Nordic Gene Bank	12	0.285	11	3
L. perenne	16-7-62-2 Nordic	Δ5	Norway	Sola	N 58.54.00	W 05.34.99	Teagasc Oak Park	16	0.268	13	6
L. perenne	PI 619024	Δ6	England	Unknown	N 53.17.00	W 01.46.00	GRIN	12	0.106	6	2
L. perenne	W6 9339	Δ7	Wales	Unknown	N 51.57.00	W 03.03.00	GRIN	16	0.247	14	2
L. perenne	PI 610958	□8	Tunisia	Unknown	N 36.53.42	E 09.11.13	GRIN	16	0.260	14	7
L. perenne	ABY-Ba 11315	□9	Morocco	Unknown	N 31.30.00	W 09.48.00	IGER	16	0.206	12	5
L. perenne	E1	□10	Egypt	Unknown	Unknown	Unknown	PGG-Wrightson	16	0.066	4	3
L. perenne	W6 11325	▲11	Turkey	Karabuk, Ankara	N 41.07.12	E 32.22.12	GRIN	16	0.114	6	1
L. perenne	PI 598512	▲12	Turkey	Antalya	N 36.54.45	E 30.41.23	GRIN	16	0.188	9	2
L. perenne	PI 547390	▲13	Iran	Karaj	N 35.28.48	E 51.00.00	GRIN	12	0.118	5	1
L. perenne	PI 317452	▲14	Afghanistan	North of Hari Rud River, 4 miles West of Besha	N 34.46.00	E 63.46.00	GRIN	16	0.165	9	1
L. perenne	No 10 Spain	■15	Spain	Unknown	Unknown	Unknown	Teagasc Oak Park	16	0.250	13	6
L. perenne	3408 Italy	■16	Italy	Unknown	Unknown	Unknown	Teagasc Oak Park	16	0.264	15	4
L. perenne	W6 16127	■17	Italy	Sardinia	N 40.34.37	E 09.12.18	GRIN	15	0.191	9	8
L. perenne	3013 Romania	■18	Romania	Unknown	Unknown	Unknown	Teagasc Oak Park	17	0.235	15	3
L. perenne	3199 Romania Podoloni	■19	Romania	Unknown	Unknown	Unknown	Teagasc Oak Park	16	0.235	14	0
L. perenne	920 Bulgaria	■20	Bulgaria	Unknown	Unknown	Unknown	Teagasc Oak Park	16	0.206	11	0
L. perenne	PI 418701	■21	Yugoslavia	Prizren	N 42.13.00	E 22.44.00	GRIN	16	0.120	6	1
L. perenne	ABY-Ba 11478	■22	Greece	Unknown	N 38.00.00	E 22.10.00	IGER	16	0.132	14	3
L. perenne	W6 9286	○23	France	Unknown	N 47.33.00	E 04.28.00	GRIN	16	0.155	9	3
L. perenne	ABY-Ba 11514	○24	France	Unknown	N 49.57.00	E 02.46.00	IGER	16	0.182	10	3
L. perenne	CPI 44924	○25	France	Arles	N 43.40.01	E 04.37.58	PGG-Wrightson	16	0.205	11	7
L. perenne	GR 5095	○26	Germany	Kempten	N 47.49.00	E 10.19.59	IPK Gatersleben	16	0.205	12	3
L. perenne	GR 5105	○27	Germany	Kempten	N 47.49.59	E 10.15.00	IPK Gatersleben	16	0.238	12	5
L. perenne	PI 274637	•28	Poland	Lublin	N 51.13.48	E 22.33.00	GRIN	16	0.158	10	1
L. perenne	PI 267058	•29	Poland	Warszawa	N 52.35.00	E 21.05.00	GRIN	16	0.099	5	1
L. perenne	PI 182857	•30	Czech Republic	Central Bohemia	Unknown	Unknown	GRIN	16	0.232	6	6
L. perenne	PI 321397	•31	Czech Republic	Central Bohemia	Unknown	Unknown	GRIN	16	0.232	12	5
L. perenne	IV-51-161 Hungary	•32	Hungary	Unknown	Unknown	Unknown	Teagasc Oak Park	16	0.245	14	6
L. perenne	cv. Aurora	V 1	NA	NA	NA	NA	IGER	17	0.249	12	1
L. perenne	cv. Barlenna	V 2	NA	NA	NA	NA	Barenbrug Holland BV	16	0.229	10	5
L. perenne	cv. Cancan	V 3	NA	NA	NA	NA	DLF-Trifolium	16	0.302	15	6
L. perenne	cv. Cashel	V 4	NA	NA	NA	NA	Teagasc	16	0.172	5	1
L. perenne	cv. Fennema	V 5	NA	NA	NA	NA	Norddeutsche Pflanzenzucht	16	0.207	11	3
L. perenne	cv. Greengold	V 6	NA	NA	NA	NA	Teagasc	17	0.161	9	0
L. perenne	cv. Magician	V 7	NA	NA	NA	NA	Teagasc	16	0.254	14	3

Table 1 Continued

Species	*Accession number*	*Group*[a]	*Country of origin*	*Location*	*Latitude*	*Longitude*	*Seed source*	N	H	N *Haplotypes/ population*	N *Unique haplotypes*
L. perenne	cv. Millenium	V 8	NA	NA	NA	NA	Teagasc	16	0.157	9	0
L. perenne	cv. Navan	V 9	NA	NA	NA	NA	DARDNI	16	0.272	11	4
L. perenne	cv. Odenwaelder	V 10	NA	NA	NA	NA	IPK Gatersleben	16	0.259	14	4
L. perenne	cv. Portstewart	V 11	NA	NA	NA	NA	DARDNI	16	0.193	9	3
L. perenne	cv. Premo	V 12	NA	NA	NA	NA	Mommersteeg International BV	16	0.167	9	0
L. perenne	cv. S24	V 13	NA	NA	NA	NA	IGER	17	0.199	12	5
L. perenne	cv. Sarsfield	V 14	NA	NA	NA	NA	Teagasc	16	0.238	12	2
L. perenne	cv. Shandon	V 15	NA	NA	NA	NA	Teagasc	16	0.213	11	2
L. perenne	cv. Talbot	V 16	NA	NA	NA	NA	Van der Have Grasses BV	16	0.235	13	3
L. canariense	PI 320544	L 1	Canary Islands	Iqueste de San Andres, Tenerife	N 110.36.00	E 12.00.29	GRIN	16	0.239	7	7
L. hybridum	ABY-Ba 13122	L 2	Portugal	Unknown	N 40.56.00	W. 07.33.00	IGER	16	0.157	9	9
L. hybridum	GR11849/94	L 3	NA	Unknown	Unknown	Unknown	IPK Gatersleben	8	0.109	5	3
L. multiflorum	GR11855/98	L 4	NA	Unknown	Unknown	Unknown	IPK Gatersleben	8	0.231	6	6
L. persicum	PI 229764	L 5	Iran	Unknown	Unknown	Unknown	GRIN	16	0.065	3	4
L. remotum	GR11839/99a	L 6	Germany	Unknown	Unknown	Unknown	IPK Gatersleben	8	0.052	3	3
L. rigidum	GR11848/91	L 7	Iran	Between Kalafabad and Ramhormos	Unknown	Unknown	IPK Gatersleben	8	0.066	4	4
L. subulatum	PI 197310	L 8	Argentina	Unknown	Unknown	Unknown	GRIN	16	0.156	6	7
L. temulentum	ABY-Ba 13643	L 9	Morocco	Unknown	N 35..34.00	W 05.22.00	IGER	16	0.227	13	11
L. temulentum	ABY-Ba 8917	L 10	Iran	Unknown	N 52.19.00	E 36.25.59	IGER	16	0.174	9	9
L. temulentum	GR11880/82	L 11	Italy	South of Monte Mutry, 1 km east of Santa Crocella	Unknown	Unknown	IPK Gatersleben	8	0.147	5	6
× *Festulolium braunii*	cv. Perun	F 1	NA	NA	NA	NA	Plant Breeding Station Hladke Zivotice	16	0.240	13	9
× *Festulolium braunii*	cv. HD 14 DK	F 2	NA	NA	NA	NA	Plant Breeding Station Hladke Zivotice	16	0.247	13	6
× *Festulolium braunii*	cv. Paulita	F 3	NA	NA	NA	NA	Plant Breeding Station Hladke Zivotice	16	0.207	11	4
× *Festulolium braunii*	cv. Achilles	F 4	NA	NA	NA	NA	Plant Breeding Station Hladke Zivotice	16	0.187	10	1
× *Festulolium Lolium multiflorum* × *Festuca arundinacea BC to F. arundinacea*	cv. Lesana	F 5	NA	NA	NA	NA	Plant Breeding Station Hladke Zivotice	16	0.213	13	0
× *Festulolium Lolium multiflorum* × *Festuca arundinacea BC to F. arundinacea*	cv. Korina	F 8	NA	NA	NA	NA	Plant Breeding Station Hladke Zivotice	16	0.215	11	0
× *Festulolium Lolium multiflorum* × *Festuca arundinacea BC to F. arundinacea*	cv. Felina	F 9	NA	NA	NA	NA	Plant Breeding Station Hladke Zivotice	12	0.214	8	1
× *Festulolium Lolium multiflorum* × *Festuca arundinacea BC to L. multiflorum*	cv. Becva	F 6	NA	NA	NA	NA	Plant Breeding Station Hladke Zivotice	16	0.251	13	1

Table 1 Continued

Species	*Accession number*	*Group*[a]	*Country of origin*	*Location*	*Latitude*	*Longitude*	*Seed source*	N	H	N *Haplotypes/ population*	N *Unique haplotypes*
× *Festulolium Lolium multiflorum* × *Festuca arundinacea BC to L. multiflorum*	cv. Lofa	F 7	NA	NA	NA	NA	Plant Breeding Station Hladke Zivotice	16	0.273	15	10
Festuca arundinacea	cv. Dovey	NL 1	NA	NA	NA	NA	Barenbrug Holland BV	8	0	1	1
Festuca gigantean	PI 440362	NL 2	Kazakhstan	Near river between Alma Ata and and Medeo	Unknown	Unknown	GRIN	8	0.122	5	5
Festuca ovina	PI 634304	NL 3	China	Xinjiang	N 43.28.05	E 81.06.39	GRIN	16	0.148	9	7
Festuca pratensis	cv. Northland	NL 4	NA	NA	NA	NA	PGG-Wrightson	8	0	1	1
Festuca rubra	IRL-OP-02174	NL 5	Ireland	Kilworth Range, Fermoy, Cork	N 52.12.88	W 08.15.80	Teagasc Oak Park	8	0.115	4	3
Festuca vivipara	PI 251118	NL 6	Yugoslavia	Unknown	Unknown	Unknown	GRIN	16	0.109	5	3

Abbreviations: BC, Backcross; *H*, Nei's (1973) gene diversity; L, *Lolium* species; *N*, number of individuals; NA, not applicable; NL, non-*Lolium* species; V, *Lolium perenne* variety.
[a]Group: I, Irish ecotype; Δ, Northern Europe group 1; □, North Africa group 2; ▲, Near East group 3; ■, Southern Europe group 4; ○, Western Europe group 5; λ, Eastern Europe group 6.

sidered as one allele at a single haploid locus. Allele size information was used to construct haplotypes. Haplotype frequencies were used to calculate Nei's (1973) gene diversity (H) and the total diversity (H_t), the mean within-population diversity (H_s) and the coefficient of variation (G_{st}) following the procedures of Nei (1987) using the program POPGENE (Yeh and Boyle, 1997).

An analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was carried out with ARLEQUIN 2.0 software (Schneider *et al.*, 2000) based on the number of different haplotypes, which is the equivalent of a weighted F_{st} over all loci when estimating genetic structure (Weir and Cockerham, 1984; Michalakis and Excoffier, 1996). The level of significance for variance component estimates was calculated by non-parametric permutation procedures using 1000 permutations. The data were partitioned in several combinations to display among- and within-population variation of Irish and European *L. perenne* accessions, to assess biogeographic differentiation and to address possible migration routes of *L. perenne*.

AMOVAs were calculated to test for evidence of geographic patterns of genetic structuring. In addition, we used AMOVAs to test if there was evidence of migration of *Lolium* (a) following a Mediterranean route, this involved comparisons of Near Eastern vs Southern European and Southern European vs Western European *L. perenne* accession groups; (b) following a Danubian migration route, this involved comparisons of Near Eastern vs Southern European, Southern European vs Eastern European and Eastern European vs Northern European *L. perenne* accession groups; (c) following a Northern African route, this involved comparisons of Near Eastern vs Northern African and Northern Africa vs Southern European *L. perenne* accession groups; (d) following a northerly retreat route of the glaciers after the last ice age, this involved comparison of all *L. perenne* ecotype groups south of the Alps (Near East, North Africa and Southern Europe) against all *L. perenne* ecotype groups North of the Alps (Northern Europe, Eastern Europe, Western Europe, Ireland); and (e) following routes into Ireland consistent with migration from neighbouring geographical regions, this involved comparisons of Irish *L. perenne* ecotypes with three European accession groups (Southern Europe, Western Europe and Northern Europe).

An Edward's Venn diagram was constructed to display shared haplotypes between six accession groups: Irish *L. perenne* ecotypes, European/Near Eastern *L. perenne* ecotypes, *L. perenne* cultivars, other *Lolium* species, × *Festulolium* cultivars and *Festuca* species. A second Edward's Venn diagram was constructed to display shared haplotypes between seven *L. perenne* accession groups: Irish ecotypes, Northern European ecotypes, Western European ecotypes, Southern European ecotypes and Near Eastern ecotypes, North African ecotypes and Near Eastern ecotypes.

From the genetic distance matrix, a dendrogram showing the similarities between six major groups of accessions was constructed using UPGMA (Sneath and Sokal, 1973) as implemented in POPGENE (Yeh and Boyle, 1997). Bootstrapping analysis was performed on the UPGMA data with 1000 replicates as implemented in NTSYSpc V2.2 software (Rohlf, 2005).

Results

Allelic variation

All ten cpSSR marker loci used in this analysis of 1575 individuals were found to be polymorphic, ranging from marker TeaCpSSR7 with 4 alleles to marker TeaCpSSR3 with 22 alleles (Table 2). The distribution of alleles in the populations varied between *L. perenne* ecotypes, *L. perenne* cultivars and the groups of other species. At locus TeaCpSSR8, there was only one allele present in the × *Festulolium* cultivars, but ten alleles present in Irish *L. perenne* ecotypes. Loci TeaCpSSR3 and TeaCpSSR8 had the largest number of alleles for *L. perenne* ecotypes. Loci TeaCpSSR2, TeaCpSSR3 and TeaCpSSR5 had the largest number of alleles for *L. perenne* cultivars. Locus TeaCpSSR3 was extremely rich in alleles, including 11 alleles for the other tested *Lolium* species.

Generally, there were more alleles unique to *L. perenne* ecotypes than in all the other species groups. Marker locus TeaCpSSR8 was the richest locus for unique alleles in *L. perenne* in general. There were five alleles unique to the Irish *L. perenne* ecotypes, five alleles unique to the European/Near Eastern ecotypes and one allele unique to the other *Lolium* species. None of the alleles at this locus was unique to the × *Festulolium* cultivars or to *Festuca* species. Locus TeaCpSSR7 was an exception with no allele being unique to *L. perenne* accessions. None of the alleles, across all loci, was diagnostic by itself for a single population, but some were for a defined group of populations. However, unique alleles were present only in groups containing more than one allele. Three alleles were unique for non-*L. perenne Lolium* species (at loci TeaCpSSR3 and TeaCpSSR8). Generally across all ten marker loci, no unique alleles for *Festuca* and × *Festulolium* accessions were found (Table 2).

An UPGMA dendrogram showing the similarities between all 104 populations was constructed and it produced groupings that were similar to the UPGMA analysis of 11 defined groups of accessions (Figure 1). For this reason only the latter dendrogram is shown. The dendrogram of all 104 accessions is provided as Supplementary Information (Supplementary Material A).

The UPGMA dendrogram showing the similarities between the 11 groups of accessions was constructed to support the AMOVA analysis (below) and to investigate the broad-scale geographical structuring (Figure 1). The group of *Festuca* species were outlying all other groups (Figure 1, group I). The rest of the dendrogram was split into two major groups (Figure 1, groups II and III). The first group (II) contained the Irish *L. perenne* ecotypes, the *L. perenne* cultivars, the *Lolium* species and the × *Festulolium* cultivars. The second group (III) contained all European/Near Eastern *L. perenne* ecotypes and could be split into two subgroups (IIIa and IIIb). Subgroup IIIa consisted of the Southern European, Western European and Northern European ecotypes, while subgroup IIIb consisted of the North African, Near Eastern and Eastern European ecotypes. There was moderate-to-good bootstrap support for many branches of the tree.

A Mantel test to check for a correlation between genetic distances and geographic distances among 56 accessions out of 62 ecotypes resulted only in a weak and nonsignificant correlation ($r = 0.338$).

Haplotypic variation

The 104 tested populations had a large amount of haplotypic variation (Table 1 and Figure 2). Of the 511 haplotypes present, 366 of these were unique to individual populations. Generally with a few exceptions, each of the 104 populations had a range of unique haplotypes (Table 1). Nine populations had no unique haplotypes, while four populations (*L. temulentum* L10, *F. arundinacea*, *F. gigantea* and *F. pratensis*) were composed of completely unique haplotypes (Table 1). No single haplotype was present in all groups of populations. One hundred and twelve of the haplotypes were only present in Irish *L. perenne* ecotypes (Figure 2) of which 98 were unique to single populations. Thus 15 haplotypes were shared only among Irish *L. perenne* ecotypes and were diagnostic for these accessions (Supplementary Material B). One hundred and twenty-four haplotypes were unique to European *L. perenne* ecotypes of which 105 were unique to single populations. Twenty haplotypes could be considered as diagnostic for European *L. perenne* ecotype accessions (Supplementary Material B), especially for ecotypes of the Northern European and Western European geographical regions. Forty-five haplotypes were found only in *L. perenne* cultivars of which 42 haplotypes were unique to single populations. Three haplotypes were shared among *L. perenne* cultivars (Supplementary material B), and were found in cultivars 'Aurora', 'Cancan', 'Magician' and 'Shandon'. Fifty-two unique haplotypes were detected in × *Festulolium* cultivars of which 32 were unique to single populations. Seventy-four haplotypes were specific to other *Lolium* species of which 69 were unique to single populations. Twenty-one haplotypes were found to be specific for *Festuca* species of which 20 were unique to single populations. In total, 29 haplotypes were shared between × *Festulolium* accessions and *Lolium* accessions, of which 26 haplotypes were shared with *L. perenne* ecotypes (Figure 2).

Nei's gene diversity values (H; Nei, 1972) among *L. perenne* accessions ranged between 0 (NL1 and NL4) and 0.333 with the Irish ecotype I 12 (Table 1). Only one haplotype was present in the *F. arundinacea* and *F. ovina* populations, thus their H values were 0. The lowest H value of 0.052 was found in a *L. remotum* population (L6, Table 1). H values were higher in the Irish and European/Near Eastern *L. perenne* ecotypes ranging between 0.211 and 0.219. In the other groups of accessions, values for cultivars were $H = 0.145$, other *Lolium* species $H = 0.187$, × *Festulolium* cultivars $H = 0.086$ and *Festuca* species $H = 0.148$. The total gene diversity (H_t; Table 3) ranged from Near Eastern *L. perenne* accessions ($H_t = 0.233$) to Southern European *L. perenne* accessions ($H_t = 0.359$). H_t values for other accession groups were within this range. The gene diversity within subdivided populations (H_s) ranged between 0.077 for *Festuca* species and 0.238 for Irish *L. perenne* ecotypes. The highest H_s value in the Irish *L. perenne* ecotypes was closely followed by the value for × *Festulolium* accessions ($H_s = 0.227$). The H_s value for Near Eastern *L. perenne* ecotypes was the lowest ($H_s = 0.146$) among *L. perenne* accessions. As G_{st} becomes closer to one, the populations within the groups are more markedly different from each other. G_{st} values ranged from 0.238 in the Irish *L. perenne* ecotypes to 0.716 in the *Festuca* species.

Table 2 Number of alleles (and unique alleles) per locus in each group of accessions

	N	*TeaCpSSR1*	*TeaCpSSR2*	*TeaCpSSR3*	*TeaCpSSR4*	*TeaCpSSR5*	*TeaCpSSR7*	*TeaCpSSR8*	*TeaCpSSR10*	*TeaCpSSR11*	*TeaCpSSR12*	*Total number of alleles/group*
Irish *Lolium perenne* ecotypes	480	6 (2)	6 (1)	11 (1)	6 (1)	7 (3)	3	10 (5)	4 (1)	3 (1)	4 (2)	60
European and other geographic regional *L. perenne* ecotypes (total)	496	3 (1)	8 (3)	16 (3)	5	3 (1)	2	9 (5)	4 (2)	4 (2)	4 (1)	58
Northern Europe Δ	100	2	2	11	3	2	2	3	1	2	3	31
North Africa □	48	2	3	5	3	2	2	3	2	1	2	25
Near East ▲	60	2	4	4	2	2	2	1	1	2	4	24
Southern Europe ■	128	3	4	8	4	2	2	3	3	2	2	33
Western Europe ○	80	2	5	6	2	2	2	4	2	3	3	31
Eastern Europe •	80	2	3	5	3	3	2	1	1	2	2	24
L. perenne varieties	259	5 (1)	5	8	4 (1)	5 (2)	2	2	3	3 (1)	4 (2)	41
× *Festulolium*	140	2	1	8	2	2	2	1	1	1	2	22
Other *Lolium* species	136	2	3	11 (2)	2	2	4	2 (1)	1	3	3	33
Festuca species	64	1	1	5	2	2	2	4	2	1	1	21
Total number of alleles/locus		8	10	22	7	10	4	17	6	7	8	99

Abbreviation: *N*, number of individuals.
The numbers of unique alleles are shown in parentheses.

AMOVA analysis

AMOVA analysis was carried out on 15 different subgroups of accessions, to test for differences in genetic structure within and between Irish and European ecotypes, to test possible geographic migration routes and to test for differences between and within *L. perenne* accessions and × *Festulolium* cultivars (Table 4). In general, the variation among populations accounted for less of the total variation than that found within populations, for example, for the Irish and European *L. perenne* ecotype comparison, the within-population variation accounted for 63% of total variation. Among-population variation accounted for 26% of total variation and among-group variation accounted for 11% of total variation. These results were comparable to the variation found within and among partitions in the *L. perenne* and × *Festulolium* comparison (64% within-population variation, 17% among-population variation and 19% among-group variation). However, there was more variation among groups and less variation at the among-population level in the *L. perenne* and × *Festulolium* comparison (Table 4).

Within-population variation of Irish *L. perenne* ecotypes accounted for 82% of the variation and among-population variation accounted for 18%. For the European *L. perenne* ecotypes, the situation was different. The within-population variation accounted for only 61% of the total variation, and the among-population variation accounted for 39% (Table 4). For all AMOVA calculations, all results were highly significant ($P \leqslant 0.001$). For analyses comparing the phylogeographic structure in the European/Near Eastern ecotypes, the percentages of variation accounted for by among- and within-population variation were similar for all the partitions tested (data not shown).

For all calculations comparing migration routes among-group and among-population variation was highly significant ($P \leqslant 0.001$). All within-population variations were not significant. In both tests for evidence of a Mediterranean migration route, among-group variation was $\leqslant 0$ (Table 4). For the two tests investigating phylogeographic structure on a possible Danubian migration route (Southern vs Western Europe and Southern vs Eastern Europe), among-group variations were low but significant, 3 and 4%, respectively (Table 4). Similarly, for both tests investigating phylogeographic structure on a possible North African migration route, among-group variations were low but significant, 2 and 4%, respectively (Table 4). A post-glacial migration route appeared to be possible since among-group variation for this partition was 0 ($P \leqslant 0.001$). For migration into Ireland from three neighbouring geographical groups, the lowest among-group variation was found in the partition between Southern European and Irish *L. perenne* ecotypes with only 5%. This result was confirmed by a second Edward's Venn diagram (Supplementary Material C), where the highest number of haplotypes was shared among Irish *L. perenne* ecotypes and Southern European *L. perenne* ecotypes (14, Supplementary Material C). The values for Western European and Northern European groups were higher, each 10%, respectively.

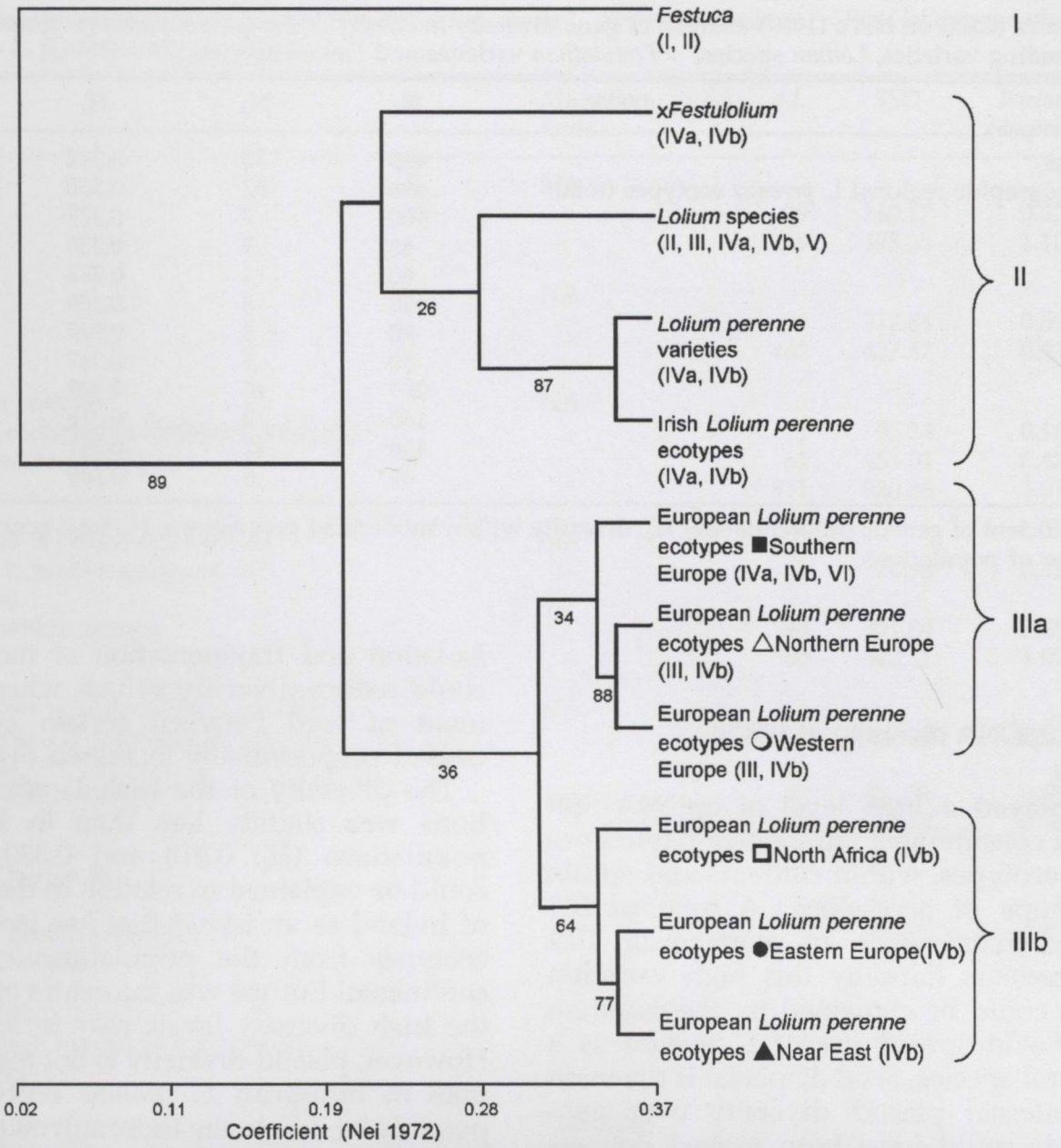

Figure 1 Unrooted dendrogram showing similarities between groups of accessions, constructed using the unweighted pair group method with arithmetic means (UPGMA) method (Sneath and Sokal, 1973) as implemented in NTSYSpc V2.2 (Rohlf, 2005), based on Nei's genetic distance measures (Nei, 1972). Numbers on the branches are percentage bootstrap values generated in NTSYSpc V2.2 (Rohlf, 2005). Symbols in parentheses are the groups where those species are present in Supplementary Material A.

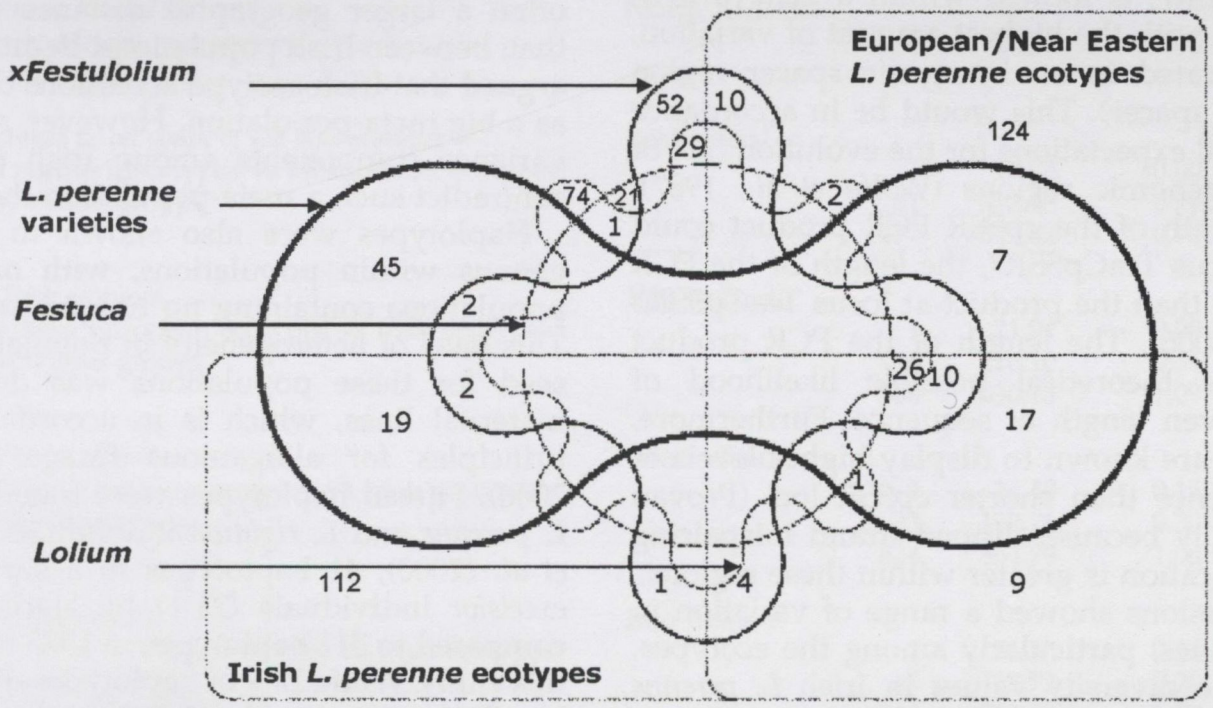

Figure 2 Edwards' Venn diagram demonstrating shared and unique haplotypes for the Irish *L. perenne* ecotypes, European/Near Eastern *L. perenne* ecotypes, *L. perenne* commercial varieties, other *Lolium* species, × *Festulolium* varieties and *Festuca* species. Shared haplotypes among groups are in intersects.

Table 3 Diversity statistics based on Nei's (1987) analysis of gene diversity in subdivided populations for geographical groups of *L. perenne* ecotypes, *L. perenne* breeding varieties, *Lolium* species, × *Festulolium* varieties and *Festuca* species

	N_i	N_p	H_t	H_s	G_{st}
Irish *L. perenne* ecotypes	480	30	0.312	0.238	0.238
European and other geographic regional *L. perenne* ecotypes (total)	496	32	0.330	0.188	0.431
Northern Europe △	100	7	0.327	0.197	0.397
North Africa □	48	3	0.254	0.177	0.301
Near East ▲	60	4	0.233	0.146	0.373
Southern Europe ■	128	8	0.359	0.217	0.395
Western Europe ○	80	5	0.347	0.191	0.449
Eastern Europe •	80	5	0.247	0.166	0.328
L. perenne varieties	259	16	0.302	0.216	0.285
× *Festulolium*	140	9	0.318	0.227	0.285
Other *Lolium* species	136	11	0.341	0.142	0.585
Festuca species	64	6	0.269	0.077	0.716

Abbreviations: G_{st}, coefficient of genetic differentiation; H_s, diversity within subdivided populations; H_t, total gene diversity; N_i, number of individuals; N_p, number of populations.

Discussion

Characterization of cpDNA diversity at allelic and haplotypic level

All accessions displayed a high level of cpDNA SSR allelic variation and considerable numbers of haplotypes were found within ecotypes, within cultivars and within closely related groups of accessions. A total of 511 haplotypes were detected with an average of 10.4 haplotypes per accession. Partially this high variation within populations could be explained by the common and geographically widespread use of *L. perenne* as a cultivated agricultural species. Seed dispersal is the main factor affecting maternal plastid diversity over geographic space. Seeds could have been moved deliberately and accidentally by grazers, birds and wind or by human involvement including seed trade. We have detected numerous chloroplast haplotypes within *Lolium* populations, which would suggest that seed dispersal is high between populations over large geographical areas.

Some of the marker loci tested in our study were more variable than others. There are several possible reasons for this. For example, the locus with the least amount of variation, TeaCpSSR7, is located within a gene (*trn*H), whereas the locus with the highest amount of variation, TeaCpSSR3, is located in an intergenic spacer region (*trn*L-F intergenic spacer). This would be in accordance with the theoretical expectations for the evolution rate of these particular genomic regions (Wolfe *et al.*, 1987). Moreover, the length of the cpSSR PCR product could play a role. At locus TeaCpSSR7, the length of the PCR product is shorter than the product at locus TeaCpSSR3 (McGrath *et al.*, 2006). The length of the PCR product could reduce the theoretical possible likelihood of variation for a given length of sequence. Furthermore, longer cpSSR loci are known to display higher levels of molecular divergence than shorter cpSSR loci (Provan *et al.*, 2001) partially because slipped-strand mispairing during DNA replication is greater within these regions.

Individual accessions showed a range of variation in gene diversity values, particularly among the ecotypes. For example, gene diversity values in Irish *L. perenne* ecotypes ranged from 0.122 to 0.333 (Table 1). Different factors acting on the individual populations may have affected the cytoplasmic diversity of the ecotypes. Isolation and fragmentation of individual populations could reduce diversity values, whereas increased movement of seed between certain ecotypes could have caused proportionally increased diversity.

The diversity of the Irish *L. perenne* ecotype populations was slightly less than in European *L. perenne* populations (H_t: 0.312 and 0.330, respectively). This could be explained in relation to the geographic position of Ireland as an island that has isolated Irish *L. perenne* ecotypes from the populations on the continent. If continental Europe was the centre of origin for *L. perenne*, the Irish diversity levels may be expected to be lower. However, plastid diversity is not markedly lower in Irish than in European *L. perenne* ecotypes and this could possibly be due to the thorough collection strategy of the Irish team in establishing the ECPGR collection that aimed to maximize the ecogeographical spread of Irish *L. perenne* populations. The G_{st} value of the Irish *L. perenne* ecotypes is almost half that of the European/Near Eastern *L. perenne* ecotypes (0.238 and 0.431, respectively). This indicates that the European/Near Eastern *L. perenne* ecotypes are more markedly different from each other than the Irish *L. perenne* ecotypes are from each other. This was not unexpected as there was often a larger geographic distance between European than between Irish populations. Because of this it may be argued that Irish ecotype accessions could be considered as a big meta-population. However, significant AMOVA variance components among Irish populations would contradict such a meta-population theory.

Haplotypes were also shown to be highly heterogenous within populations, with only 11 out of 104 populations containing no unique haplotypes (Table 1). This level of heterogeneity in populations indicated that seed for these populations was derived from many maternal lines, which is in accordance with breeding principles for allogamous forage species (Acquaah, 2006). Fifteen haplotypes were found in a study of 447 *L. perenne* and *L. rigidum* individuals (3%) by Balfourier *et al.* (2000), 41 haplotypes in a study of 168 *Fraxinus excelsior* individuals (24%) by Harbourne *et al.* (2005) compared to 511 haplotypes in 1575 individuals (32%) in this study. While 27% of haplotypes detected in the study by Balfourier *et al.* (2000) were unique to single populations, 71% of haplotypes detected in this study were unique to single populations. Our findings are in

Table 4 Analysis of molecular variance (AMOVA) for Irish and European *L. perenne* accessions, × *Festulolium* varieties and subgroups within European/Near Eastern *L. perenne* ecotype accessions

Source of variation	*Migration route*	*d.f.*	*SSD*	*Variance component*	*Variance (%)*	P[a]
Irish ecotypes	NA					
Among populations		29	140.17	0.23	18	***
Within populations		449	493.29	1.10	82	***
European ecotypes	NA					
Among populations		31	312.84	0.59	39	***
Within populations		462	427.57	0.93	61	***
Irish ecotypes vs European ecotypes	NA					
Among groups (Irish ecotypes vs European ecotypes)		1	95.24	0.18	11	***
Among populations/within groups		61	453.01	0.42	26	***
Within populations		911	920.86	1.01	63	***
Irish L. perenne ecotypes vs × Festulolium varieties	NA					
Among groups (Irish *L. perenne* ecotypes vs × *Festulolium* varieties)		1	74.98	0.32	19	***
Among populations/within groups		38	201.84	0.28	17	***
Within populations		580	622.31	1.07	64	***
European L. perenne ecotypes vs × Festulolium varieties	NA					
Among groups (European *L. perenne* ecotypes vs × *Festulolium* varieties)		1	47.00	0.17	10	***
Among populations/within groups		40	374.51	0.56	34	***
Within populations		593	556.59	0.94	56	***
Near East ▲ vs Southern Europe ■	Mediterranean, Danubian					
Among groups (Near Eastern ecotypes vs Southern European ecotypes)		1	8.95	0.00	−1	***
Southern Europe ■ vs Western Europe ○	Mediterranean					
Among groups (Southern European ecotypes vs Western European ecotypes)		1	11.76	0.00	0	***
Southern Europe ■ vs Eastern Europe •	Danubian					
Among groups (Southern European ecotypes vs Eastern European ecotypes)		1	18.48	0.06	4	***
Eastern Europe • vs Northern Europe Δ	Danubian					
Among groups (Eastern European ecotypes vs Northern European ecotypes)		1	12.92	0.04	3	***
Near East ▲ vs North Africa □	North African					
Among groups (Near Eastern ecotypes vs North African ecotypes)		1	10.01	0.03	2	***
North Africa □ vs Southern Europe ■	North African					
Among groups (North African ecotypes vs Southern European ecotypes)		1	15.65	0.08	4	***
All north of the alps ecotypes vs all south of the alps ecotypes	Post-glacial					
Among groups (All northern ecotypes vs all southern ecotypes)		1	10.63	0.00	0	***
Among populations/within groups		31	312.45	0.59	39	***
Within populations		477	444.82	0.93	61	NS
Southern Europe ■ vs Irish ecotypes	Into Ireland					
Among groups (Southern European ecotypes vs Irish ecotypes)		1	21.91	0.08	5	***
Among populations/within groups		36	216.10	0.31	21	***
Within populations		568	630.13	1.09	74	**
Western Europe ○ vs Irish ecotypes	Into Ireland					
Among groups (Western European ecotypes vs Irish ecotypes)		1	26.49	0.15	10	***
Among populations/within groups		33	192.76	0.30	19	***
Within populations		524	564.79	1.08	71	***
Northern Europe Δ vs Irish ecotypes	Into Ireland					
Among groups (Northern European ecotypes vs Irish ecotypes)		1	31.30	0.16	10	***
Among populations/within groups		35	198.72	0.29	19	***
Within populations		542	583.69	1.08	71	***

Abbreviations: d.f., degrees of freedom; NA, not applicable; NS, not significant; SSD, sum of squared differences.
[a]$^{**}P<0.01$ and $^{***}P<0.001$.

accordance with findings of Fjellheim *et al.* (2006) in *Festuca* study including *L. perenne* for comparative reasons. Their analysis demonstrated that *L. perenne* accessions had four times as many haplotypes as *F. pratensis*. These authors suggested that a lower variation in *F. pratensis* could be due to the greater age of the species *F. pratensis* and because the chloroplast variation in *F. pratensis* was largely decimated in the Eemian interglacial period, where most of Europe was densely forested, while *F. pratensis* is a shade intolerant species.

Plastid genome identification, cytoplasmic gene pool characterization, and the study of hybridization and introgression

While none of the alleles for each of the ten cpSSRs were diagnostic for individual populations, several of the alleles found were unique to specific population groups (Table 2). It is possible that germplasm from these collections could be identified by genotyping these cpSSR markers if sufficient numbers of individuals are tested. Particularly useful for this purpose could be marker TeaCpSSR8, where half of the alleles were unique to Irish or European *L. perenne* ecotype accessions, respectively (Table 2). At the haplotype level, the majority of haplotypes detected were unique to specific groups of populations (Figure 2). While the high level of heterogeneity of haplotypes within populations made it difficult to assign individuals to specific populations, it was possible to use these haplotypes to assign individuals to specific groups. For example, 22% of haplotypes were specific to the Irish *L. perenne* ecotypes, and 24% of haplotypes were specific to the other European *L. perenne* ecotypes. These haplotypes have potential to distinguish geographic *L. perenne* ecotypes and accessions (Supplementary Material B).

The high level of variation, both allelic and haplotypic, in the European and Irish *L. perenne* ecotype collection in comparison with *L. perenne* cultivars suggests that the full cytoplasmic diversity is still underexploited in breeding material (Table 2 and Figure 2). Ecotypes with unique plastid variation not present in breeding material could be useful to expand the cytoplasmic gene pool for breeding of the species. A wide variation in plastid type can be useful to enhance the possibility of yield gains and yield stability as demonstrated on a data set for potato (Provan *et al.*, 1999). For both UPGMA dendrograms (Supplementary Material A and Figure 1), the ten cpSSR markers were able to distinguish among Irish and European *L. perenne* ecotypes. This outcome indicated the usefulness of these ten cpSSR markers to identify cytoplasmic gene pools in ecotypes and breeding *L. perenne* germplasm.

Identification of plastid type is also useful for the study of introgression and hybridization (Hodkinson *et al.*, 2002; Johannessen *et al.*, 2005), as plastid marker information can identify the source of introgression and can be used in parentage analysis. For example, *L. temulentum* (L11) grouped with two *Festuca* species, NL1 and NL4 (Supplementary Material A, group II). This could be an indication of introgression of the plastid genome from *Festuca* species into *Lolium*. AMOVA analysis of Irish and European and Near Eastern *L. perenne* ecotypes vs the × *Festulolium* cultivars showed there was almost twice as much of the among-group variation between Irish *L. perenne* ecotypes and × *Festulolium* cultivars than between the European/Near Eastern *L. perenne* ecotypes and × *Festulolium* cultivars (Table 4). This could suggest more movement of cytoplasmic material between European ecotypes and × *Festulolium* cultivars than with Irish *L. perenne* ecotypes. Six out of nine × *Festulolium* cultivars grouped with the European *L. perenne* ecotypes (Supplementary Material A), which also could indicate introgression from European and Near Eastern *L. perenne* ecotypes into × *Festulolium*.

Plastid identification could also be used to verify that seed or seedlings derived from crosses in breeding programmes were assigned to the correct maternal parent (Gauthier *et al.*, 1997). This could be particularly helpful for *Lolium* breeding in which multiple maternal lines are used in plant breeding (top cross breeding).

Phylogenetic and phylogeographic genetic structure of *Lolium*

Studying plastid DNA variation can contribute to phylogenetic analysis. UPGMA data demonstrated that two broad-leaved *Festuca* species, *F. arundinacea* and *F. pratensis*, and three narrow-leaved *Festuca species*, *F. pratensis*, *Festuca rubra* and *Festuca vivipara*, grouped together, respectively (Supplementary Material A). The broad-leaved *Festuca* species grouped closer to *Lolium*. Both of these findings were in agreement with previous studies (Darbyshire and Warwick, 1992; Catalan *et al.*, 1997, 2004; Charmet *et al.*, 1997; Torrecilla *et al.*, 2004). However, some unusual groupings have occurred in the UPGMA dendrogram (Supplementary Material A). For example, one of the European *L. perenne* ecotypes (■17) was positioned outside all other *Lolium* accessions. This particular accession was from Sardinia where previously a high degree of diversity has been found for other species (Papa *et al.*, 1998). Moreover, unlike other studies (Catalan *et al.*, 1997, 2004; Charmet *et al.*, 1997; Torrecilla *et al.*, 2004), no separation of allogamous and autogamous *Lolium* species was found. These unusual groupings could be explained by high homoplasy in the data set caused by rapid molecular evolution at the loci studied. Parallel evolution at these loci would therefore be expected to be high and this would obscure phylogenetic signal of the markers (Flannery *et al.*, 2006). They are thus potentially more useful for assessing variation within species than among species.

A loose correlation of genetic and geographic distances was detected with a Mantel test for the ecotypes were an exact geographic position was available ($r = 0.33$). While studies have tested the correlation between nuclear and geographic distances such as Cresswell *et al.* (2001), this is the first study to test the correlation between plastid genetic and geographic distances in *L. perenne*. We believe the lack of correlation is due to both the high within population plastid diversity and the high degree of seed-mediated gene flow, natural and human related.

The AMOVA analyses indicated that most of the variation in populations used in this study was within-groups and individual populations, but that there was also significant population genetic structuring among groups (Table 4). Generally, higher among-population variance component values are comparable to AMOVA

analysis results of other studies of *L. perenne* populations using nuclear markers (Bolaric *et al*., 2005).

The results for the AMOVA analysis showing the proportion of variance within or among groups were also useful for assessing broad-scale biogeographical patterns. For comparisons among groups examining the Mediterranean migration route in relation to other possible migration routes, the variance components between partitions for the Danubian and North African routes were 0 or close to 0. When a variance component is close to 0, it can mean that there is no population genetic structure (Schneider *et al*., 2000). Close to 0 or 0 values can also be an indication that samples among groups are more closely related to each other than samples within groups. This would indicate that these population groups are closer to each other than to groups showing a higher among-group variance component. For this data set it could be an indication of a Mediterranean movement of *L. perenne* from the Near East across Southern Europe into North Western Europe, Ireland. This is in accordance with one movement theory of *L. perenne* across Europe as proposed by Balfourier *et al*. (2000). This finding was substantiated in our study by the result of an AMOVA for post-glacial partitioning of south of the Alps ecotypes against north of the Alps ecotypes. In this case, among-group variation was 0 as well (Table 4). The post-glacial movement hypothesis can be further supported by our UPGMA data (Figure 1). Southern European, Northern European and Western European *L. perenne* ecotypes were grouped together and were distinct from the other European/Near Eastern *L. perenne* ecotypes. This indicated that these population groups were closer to each other than to other geographic groups and that movement of seed between these groups has occurred. Finally, the hypothesis that *L. perenne* most likely moved from the southern Europe into Ireland can be supported by the lowest among-group variation value in the AMOVA analysis (Table 2: 5%) for Southern Europe/Irish ecotype partition.

Conclusion

Allelic and haplotypic variation was extremely high within and between Irish and European *L. perenne* ecotypes. Migration of seed material by natural or anthropogenic means, including plant breeding, could contribute to this high level of variability. High plastid diversity was clearly persisting in populations. The cpSSR markers were shown to be extremely useful for characterizing variation in our accessions and have enabled the identification of cytoplasmic genepools and maternal lineages. The plastid type of individual populations could not be unambiguously identified, but groups of populations could be successfully identified. This suggests that an increase in the number of cpSSR markers would increase the likelihood of identifying individuals within population groups. Our findings describe broad-scale biogeographical patterns of population genetic structure in this highly heterogenous species. Furthermore, some evidence was provided to support possible broad-scale prehistorical geographical migrations. A pathway of migration from Southern Europe to Northwest Europe including Ireland is most likely.

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