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The Origin and Evolutionary History of the Turlough form of *Ranunculus repens*

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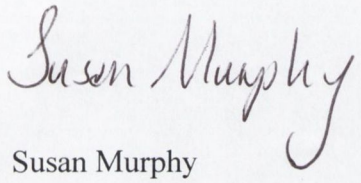

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Summary

Turloughs are temporary lakes that occur in karstic limestone areas predominantly in the West of Ireland. They generally flood with groundwater in Autumn and empty through swallow holes in late Spring. This strong ecological gradient allows scope for adaptation of the plant populations that inhabit this environment. Turloughs harbour a morphologically distinct from of the clonal species *Ranunculus repens* (Ranunculaceae); this form has more dissected and glabrous leaves than the typical broad-leaved form. The main aim of this thesis was to determine whether these apparent differences in leaf shape had an underlying genetic basis potentially induced by the turlough environment.

Molecular techniques such as DNA sequencing, Amplified fragment length polymorphism (AFLP), and Inter-simple sequence repeat (ISSR-PCR) were employed to investigate genetic variation within and among populations of *R. repens* from contrasting habitat types (turlough, wet pasture and dry pasture). This thesis had a three-tiered approach: Firstly, a broad scale, inter-population, nationwide study of *R. repens* from various habitat types was undertaken. Secondly, the effect of environmental change on *R. repens* individuals growing along an ecological gradient within a turlough basin was assessed. The final study focused on the clonal growth structure of a turlough population of *R. repens*.

Morphological analysis of leaf shape variation between populations of *R. repens* was carried out in conjunction with the molecular analyses. The morphological research revealed leaf dissection to be a distinguishing trait that separates turlough populations of *R. repens* from non-turlough populations of the species. Statistical analysis (ANOVA) showed that leaf dissection differed significantly among all pairwise combinations of the three habitat types (turlough, wet pasture, dry pasture).

Of all the molecular techniques employed throughout the three-tiered studies presented in this thesis AFLP proved to be the most suitable. From the broad scale of inter-population relationships, to the intermediate level of a population growing along an ecological gradient, and finally to the fine scale of the identification of *R. repens* genets within a turlough basin, this method proved successful in its yielding of comprehensive results. The use of AFLP at an inter-population level revealed ecotypic differences in the species with the dissected-leaf turlough form of *R. repens* being more genetically similar to each

other than to non-turlough broadleaved populations of the species regardless of geographic location. This may infer a disparate evolutionary history of the turlough form. Significant differences in AFLP marker frequency between individuals within a turlough population of *R. repens* growing along an ecological gradient were also revealed by the technique. Several AFLP markers showed heterogeneous distribution in individuals along the turlough gradient, which could be related to differences in leaf dissection among individuals. Finally, AFLP was found to be a suitable tool for the identification of *R. repens* genets spreading clonally within a turlough basin.

In conclusion, this study revealed that the turlough form of *R. repens* is both morphologically and genetically distinct from the more common weedy, broadleaved form of the species. These differences are likely to be related to ecological preferences of the different forms, the susceptibility to prolonged periods of inundation being the most obvious factor in the case of the turlough form. The hypothesis of different evolutionary histories of the two forms was put forward and supported by this population genetic study of the turlough form of *R. repens*. The story of the origin of the turlough buttercup is far from complete but hopefully the research presented here will lay down the foundations for future investigations on the turlough form of *R. repens*.

Abbreviations

AFLP	Amplified Fragment Length Polymorphism
Bp	base pair
BS	Bootstrap
BSA	Bovine Serum Albumin
CsCl	Caesium Chloride
CTAB	Cationic Haexadecyl Trimethyl Ammonium Bromide
EDTA	Ethylenediaminetetra-Acetic Acid
DNA	Deoxyribonucleic Acid
DNTP's	2'-deoxynucleotide 5'-triphosphates, i.e. dATP, dGTP, dCTP, dTTP
ISSR	Inter-simple Sequence Repeats
ITS	Internal transcribed Spacer
LDI	Leaf Dissection Index
$MgCl_2$	Magnesium Chloride
NJ	Neighbor Joining
NMDS	Non-parametric Multidimensional Scaling
PCO	Principal Co-ordinate analysis
PCR	Polymerase Chain Reaction
pers. comm.	Personal communication
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
SSR	Simple Sequence Repeat
TE	Tris HCl and Ethylenediaminetetra-Acetic Acid
UPGMA	Unweighted Pair Group Method with Arithmetic mean

Chapter 1

General Introduction

It is interesting to contemplate a tangled bank, clothed with many plants of many kinds, with birds singing on the bushes, with various insects flitting about, and with worms crawling through the damp earth, and to reflect that these elaborately constructed forms, so different from each other, and dependent on each other in so complex a manner have all been produced by laws acting around us. These laws, taken in the larger sense, being growth and reproduction; Inheritance which is almost implied by reproduction; Variability from the indirect and direct action of the conditions of life, and from use and disuse; a ration of increase so high as to lead to a Struggle for Life, and as a consequence to Natural Selection, entailing Divergence of Character and the extinction of the less-improved forms.

Charles Darwin 1859

1.1 Plant populations and their genetic make up

Plant populations typically consist of a mixture of genotypes, each of which can potentially exhibit a variety of phenotypes, depending on environmental conditions (Cheplick 1991). The expression of an individual genotype can be modified by its environment (Trewavas & Jennings 1986). The phenotype is the expression of all the morphological and physiological characteristics of the genotype that are in interaction with the environment during the course of the growth and development of the organism (Scheiner 1993). The structures that we can identify in populations of plants result from the action of biotic and abiotic forces to which their members, and in many cases their ancestors, have been exposed in the past. The forces experienced by the ancestors of a population can clearly affect its genetical structure (Hutchings 1997). Several authors have compared the genetic structure of plant populations of species with limited geographic range, or a small number of individuals per population, with widespread species with many individuals per population (Loveless & Hamrick 1984; West Eberhard 1989). In many of these cases, restricted species with fragmented populations are found to have lower levels of genetic diversity than those of widespread congeners. There are several documented causes for this, including founder effects, genetic drift, strong directional selection producing genetic uniformity in a small range of habitat types, and high levels of inbreeding (Franklin 1980; Templeton 1990; Young *et al.* 1996; White *et al.* 1999; Gustafsson 2000).

Each individual in a population may have a unique genotype in a variable gene pool. Alternatively, a local population of plants may consist of the genetically identical clonal offspring of a single ancestor, or the genetic variation of a population may have a more complex structure depending on mating system, gene flow, dispersal and selection (Bachmann 1994). The prediction of low genetic variation in populations of clonal species that rarely recruit from sexually produced seed is well documented (Richards 1973; Barnes 1975; Cook 1983; Smith, Bruhn & Anderson 1992). Even if these populations are founded by recruitment from many seeds, the fittest clone might be expected to gradually eliminate all others, resulting in genetically monotonous population structure. However, comprehensive reviews of genetic diversity in clonal plant populations, where seedling recruitment is a rare occurrence, have revealed that populations of most such species are multiclonal (Silander 1985; Ellstrand & Roose 1987; Brookfield 1992; Bachmann 1994; Gornall 1999).

To understand how a species population structure has evolved one needs to realise what processes and evolutionary forces have been paramount in moulding its genesis and maintaining its presence. Mutation, genetic drift, and natural selection are all involved in the creation of genetic differentiation of local populations. In contrast, gene flow opposes that differentiation. Because genetic structuring reflects the number of alleles exchanged between populations, it has major consequences on the genetic composition of individuals themselves. The exchange of genes between populations homogenises allele frequencies between populations and determines the relative effect of selection and genetic drift (Balloux & Lugon-Moulin 2002). High gene flow often precludes local adaptation (i.e. the fixation of alleles, which are favoured under local conditions), and will therefore also impede the process of speciation (Barton & Hewitt 1985). On the other hand, gene flow generates new polymorphism in the populations, and increases local effective population size (the ability to resist random changes in allele frequencies), thereby opposing random genetic drift, generating new gene combinations on which selection can potentially act (Balloux & Lugon-Moulin 2002).

Species that have a wide geographic range are likely to encounter a multitude of different environmental conditions, and hence a variety of selective forces. Clausen *et al.* (1940) demonstrated a correlation between the gross morphology of plants and features of their natural environment. Species that grow in a wide range of habitats are therefore likely to exhibit variation between populations in different habitats. The distinction between genetically (between-genotype) and environmentally (within-genotype) invoked phenotypic diversity is applicable at the population level for two reasons: (1) gene frequency change over time may allow a population to move beyond existing norms of reaction and accommodate an environmental shift outside the range to which existing genotypes are tolerant, and (2) genetic diversity may enhance the population's phenotypic and thus ecological breadth beyond that of single genotypes. The relation of the population to the environmental range it encompasses involves two dynamic levels: at one level are the gene frequencies of the entire population, which underlie its within- and between-individual phenotypic diversity and may change over generations; at another level is the phenotypic diversity due to interaction of existing genotypes with the current distribution of environments, which is contained within the individual life span (Sultan 1987). Thus at the population level there is an interplay of

genetic diversity and phenotypic plasticity. There are numerous processes acting on both the phenotype and genotype of plants within a population that mould the general population structure. Starting at the level of phenotype, plasticity may play an important role in a plants response to its environment.

1.2 Phenotypic plasticity in plants

Plant plasticity is perhaps the single most important factor determining the behavioural uniqueness of plant populations and individuals (Sarukhan 1976). Phenotypic plasticity is the ability of a single genotype to produce more than one alternative form of morphology, physiological state, and / or behaviour in response to environmental change (West-Eberhard 1989). The amount by which the expressions of individual characteristics of a genotype are changed by different environments is a measure of the plasticity of these characters. Plasticity of a character appears to be (a) specific for that character, (b) specific in relation to particular environmental influences, (c) specific in direction, (d) under genetic control not necessarily related to heterozygosity, and (e) able to be radically altered by selection. An individual genotype assumes particular characteristics in a given environment, and that in a second environment it may remain the same or it may be different (Bradshaw 1965). Sultan (1987) describes plasticity as variation in phenotypic expression of a genotype that occurs in response to particular environmental conditions and which enhances the capacity of the individual to survive and reproduce under those conditions. Plastic responses include both inevitable effects of environmental limits on growth and physiology, and adaptive adjustments that enhance the organism's success in the environment that elicits them (Sultan 1995). It is these adaptive plastic responses that allow individual organisms to maintain function and hence fitness across a range of diverse environments, and that therefore influence both ecological breadth and response to natural selection (Sultan 2000).

West-Eberhard (1989) emphasises the importance of plasticity as a diversifying factor in evolution – a factor contributing to the origin of novel traits and to altered directions of change. A common misconception regarding phenotypic plasticity is that it represents a non-genetic means of dealing with a change in the environment (Schlichting 1986). Bradshaw (1965) emphasised that the plasticity of a character is a character in and of itself, that it is under genetic control, and that it has evolved in

some cases in relation to specific environmental influences. Bradshaw (1965) and others (Breese & Hayward 1972; Jain 1979) have shown that: (1) phenotypic plasticity is under genetic control, (2) plasticity can be subject to selection, (3) levels of plasticity may vary within and among populations, and (4) each trait may vary in the level of plasticity within and among individuals. The multidimensional plasticity of a living organism means that the functional phenotype is complexly malleable, like a clay figure that can be variously reshaped without falling apart (West-Eberhard 1989).

1.3 Evolution of phenotypic plasticity

Forces responsible for evolutionary changes in phenotypic plasticity can be assigned to three general categories: selection, drift and disruption of the genetic system (Schlichting 1986). If genotypes with broad norms of phenotypic response are able to survive and reproduce more successfully over time than are genotypes with less plastic expression, selection will promote evolution of plasticity (Sultan 1987). This very much depends on the strength of the selection, that is, the capacity for flexibility. Selection will necessarily favour individual plasticity unless a set of narrowly adaptive genotypes tolerates a greater range of environmental conditions than will any single genotype in the population (Lewontin 1957). Plasticity provides a phenotypic alternative to micro-differentiation in a heterogeneous environment where variation is random or large relative to generation time or clone size (Silander 1985). In species that occupy a range of environments, a single genetic type with infinite plasticity is not found, but rather a myriad of genetically distinct individually adapted populations. Some species may be 'jack of all trades' but no single species can be 'master of all' (Crawley 1986). Although adaptation by plasticity plays an important role in many situations, permanent adaptation by genetic change is more common (Bradshaw 1965). Phenotypic plasticity is thus one solution to the problem of adaptation to heterogeneous environments (Via *et al.* 1995), but in general genetic adaptation plays a more important role in plant populations, allowing diversification of genotypes and thus evolutionary change.

1.4 Natural selection

Natural selection can be defined as a force that creates new systems of adaptations to the environment by eliminating all but the favourable gene combinations out of the

enormous diversity of random variants that could otherwise exist (Stebbins 1950). There are three main processes that are deemed responsible for natural selection which are described by Harper (1981). Directional selection occurs when the adaptive optimum for a character does not coincide with the modal frequency for that characteristic in the phenotype of the population, therefore selection acts more strongly against one tail of the frequency distribution of the phenotypes. Stabilising selection occurs when the adaptive optimum for a characteristic coincides with the modal frequency for that characteristic in the phenotypes of the population, therefore both tails of the frequency distribution are selected against. Disruptive selection occurs when two or more adaptive optima exist and selection acts against the norm. When selection is acting in a stabilising manner it can permit a population to assume a uniform phenotype, while retaining a considerable degree of genetic variation (Bradshaw 1965).

1.5 Adaptation

Adaptation, or the ability to change according to ones environment, is proposed as just one of several factors that may cause variation in amounts and patterns of plastic response between taxa (Schlichting 1986). Adaptation is under genetic control and is the "memory" that is passed on to the next generation (Antonovics 1972). Over many generations a tendency towards adaptation to trends in the physical and biotic environments develop, which are threatened by more transient environmental changes of a largely unpredictable nature. Genetic changes underlying adaptation involve genes that are segregating in natural populations as polymorphisms, this means that genetic changes will follow the favouring of phenotypic extremes by the reorganisation of segregating genes (Myers & Giller 1988). Sexuality even at low levels provides sufficient genetic variation through recombination to permit genetic variation, which is the building block of evolutionary change (Silander 1985).

Adaptation can be demonstrated at the phenotypic level in terms of ecological phenotypes having obvious relationships with habitat; the relationship with genotype seems to be more obscure (Myers & Giller 1988). Underlying the phenotype is a harmonious integration of the physiological effects of many genes incorporating interactions within and between loci; this means that the effects of selection may be difficult to predict and will vary among populations differing in their genetic

backgrounds (Mayr 1963). If a response is adaptive then the genotype in question may show fitness in one habitat but may be totally unsuitable for another. Many transplant experiments have been conducted (Bradshaw 1960; Lewis 1969; Lovett-Doust 1981b; Aarssen & Turkington 1985; Schmid 1985; Lynn 1998) in order to study the effect of varying habitats on particular phenotypes and to investigate whether the phenotypic traits exhibited by the particular plant in question are plastic responses or whether these phenotypic characters are genetic in origin and thus will be passed on to their offspring. Lovett-Doust (1981b) showed strong interaction effects between site of origin and site of transplanting in *Ranunculus repens*, which indicates that certain plant populations are specialised to their site of origin. Habitability is the degree to which the additive genetic effects of multiple factors determine the phenotype, as compared with parallel effects on the same phenotype caused by environmental modification (Stebbins 1950).

1.6 Patterns of adaptive variation

Turesson (1922a) used the term "ecotype" to describe an ecological subunit that arises as a result of a genotypical response to a particular habitat and Clausen *et al.* (1940) used the same term to define an intraspecific unit originating in one climatic region. Sultan (1987) describes them as genetically distinct and especially adaptive populations or sub-populations. Genetically based adaptive differences can evolve in a population if gene flow is low and if the existing range of phenotypic expression of individuals cannot accommodate the entire range of environments encountered (Sultan 1987). Ecotypes may fail to form for various reasons, such as the case when the physiological and morphological responses available to the populations can successfully accommodate the full environmental range encountered in the various habitats, or when gene flow between the different habitats prevents differentiation regardless of the local selection pressures (Sultan 1987). Variability of ecological phenotypes and their underlying genotypes tends to be high under severe environmental conditions, so providing circumstances conducive to rapid evolutionary change. While biological species are unique genetical and ecological systems, the development of geographic races within species shows that the environment across the distribution of species is not uniform (Myers & Giller 1988). Environmental stress caused by heavy metals such as copper in the vicinity of old mines can result in extremely localised clines for resistance to the metals; copper

tolerance has been shown to evolve in just one or two generations in the grass *Agrostis stolonifera* (Wu *et al.* 1975). Therefore among-habitat heterogeneity in terms of biotic factors may also lead to micro-geographic variation in a species. When ecotypes are distinguished on the basis of some character, and that character remains distinct when the plants are transplanted, it is generally concluded that the trait is adaptive, i.e. that it confers a selective advantage to the organism in its native habitat, and that it has a genetic component (Lovett-Doust 1981b). There are many ways in which a population can maximise its fitness in a heterogeneous environment such as maintaining genetic variability (Bradshaw 1959) and the formation of ecotypes (Clausen *et al.* 1940) as well as developing a variety of phenotypes (Cook & Johnson 1968).

1.7 Morphological variation in plant populations

The notion that plants respond morphologically to changes in their environment has a long tradition (Schlichting 1986). Many cases have been documented, classic examples of responses to herbivory (Lubchenco & Cubit 1980), mowing (Warwick & Briggs 1979), competition (Turkington 1983), and heterophylly (Cook & Johnson 1968) show that these responses are adaptive in relation to some environmental cue. Where morphological differentiation is found to be correlated with environmental factors, it can be taken to indicate the existence of adaptive divergence between populations without any knowledge of the physiological meaning of the differences observed, since the only reasonable explanation for their existence is the differential effect of selection in the various habitats (Heslop-Harrison 1964). The structural features of leaves are particularly important in determining rates of gaseous exchange and heat transfer between plants and environment. It is therefore not unexpected that a close correlation between leaf morphology and habitat is one of the most conspicuous trends in genecological differentiation (Lewis 1969). Plant population variation in leaf size and shape is a commonly observed phenomenon (Clausen *et al.* 1940; Erickson 1945; Briggs 1962; Cook & Johnson 1968; Andersson 1995). There are many approaches to studying the genetic basis of morphological variation in plant species some of which include common garden experiments (Lynn & Waldren 2001a), and quantitative genetic analysis in which the transmission of morphological traits from parents to offspring of selected crosses is scored (Briggs 1962). The work in this

thesis is based upon morphologically different populations of the species *Ranunculus repens*.

1.8 *Ranunculus repens*

1.8.1 Species description and classification

Ranunculus repens L. (Creeping Buttercup) is a polycarpic, perennial, semi-rosette hemicryptophyte with strong creeping stems rooting at the nodes, with erect flowering stems to 60cm; the basal leaves are triangular-ovate in outline, with three main segments, the middle one long-stalked and borne above the two laterals, usually pubescent (Harper 1957; Grime *et al.* 1988). *Ranunculus repens* belongs to the subgenus *Ranunculus* section *Ranunculus*. The 17 species in this section are characterised by having a nectary scale attached only at its base, compressed achenes with a distinct beak, and a receptacle that in fruit is not more than three times its length when in flower (Tutin 1964). All species in the subgenus have yellow petals except *Ranunculus aconitifolius* L. (white).

The majority of the European members of this section can be placed in two clearly definable groups; the first of these has a base chromosome number of x=7, and is characterised by having terete pedicels, globose flower buds and glabrous receptacles (e.g. *R. acris* L.) (Coles 1977). The second group, to which *Ranunculus repens* belongs, has a base chromosome number of x=8, 2n=32 and is characterised by having sulcate pedicels, ovoid flower buds and pubescent receptacles (Coles 1977). *Ranunculus repens* can be distinguished from all other members of its section in Europe in that it produces numerous creeping stolons which root at their nodes (Coles 1977).

Synonyms of *R. repens* L., Sp. Pl., 554 (1753) include *R. prostratus* Poir. In Lam. Encycl. Meth. Bot., 6:113 (804), *R. pubescens* Lag., Gen. Sp. Nov., 19 (1816), *R. intermedius* Eaton, Man. Bot., 3rd ed., 424 (1822), *R. lagascanus* DC., Prodr., 1:43 (1824), *R.. clintonii* Beck, Bot. N. & Mid. States, 9 (1833), *R. oenanthifolius* Ten. & Guss., in Atti Accad. Napoli, 6: 318 (1843), *R. reptabundus* Jordan, Diagn., 83 (1864) and *R. hortensis* Schur, Enum. Pl. Transs., 21 (1866).

1.8.2 Geographical distribution

Ranunculus repens is a widespread species which occurs in Ireland, Britain and most of Europe, as well as in Asia; it has also been introduced to the Americas and New Zealand. The species inhabits four main habitat types: arable land, grassland semi-aquatic communities such as fens and marshes but not bogs, and open patches and paths in woodlands and is most commonly found as a constituent of grassland communities. It occurs on a wide range of soils, but it is most abundant on soils that are heavy and wet, it is especially abundant where drainage is impeded (Harper 1957; Coles 1977). The species is variable, particularly in respect to stolon production, and it is apparently unaffected by severe frosts, moderate drought and water logging (Harper 1957).

1.8.3 Life form, demography, and population dynamics

Ranunculus repens is possibly one of the best-studied weeds, from a demographic perspective (Harper 1957; Sarukhan 1974; Lovett-Doust *et al.* 1990; Lynn & Waldren 2001a,b). Strategies of clonal spread may be described as either 'guerrilla', in which widely spaced ramets are produced and rapid spread is achieved, or "phalanx" in which lateral spread is less rapid but current position is consolidated (Lovett-Doust 1981a). *Ranunculus repens* exhibits plasticity in stolon production and internode length, and thus can express a range of strategies along the phalanx-guerrilla continuum depending upon local conditions (Lovett-Doust *et al.* 1990).

1.8.4 Reproduction

1.8.4.1 Floral biology and sexual reproduction

Ranunculus repens has hermaphroditic chasmogamous flowers that are insect pollinated (Lovett-Doust *et al.* 1990). The flowers are visited by *Apis mellifera* L., the honey bee and potentially by all Sryphidae flying at the time of flowering (Harper 1957), as well as by butterflies, moths, bugs and beetles (Harper 1957). It flowers mainly in May and June and sets seed primarily in June and July. Stolons give rise to newly formed ramets produced in early summer at the same time as the flowers (Grime *et al.* 1988). The parent plant does not persist for more than one flowering season, it is replaced by daughter rosettes that are formed at the nodes of the stolons (Coles 1977). Reproduction is by seed and stoloniferous daughter plants (Harris *et al.* 1998).

1.8.4.2 Seed production and dispersal

Although produced in smaller numbers, seeds of *R. repens* are more persistent in the soil than those of *R. acris* L. and *R. bulbosus* L. (Sarukhan 1974). Seeds are dispersed by wind, in the dung of birds, by farm animals that have ingested the seeds, by small rodents that store the seed for winter food, and by humans (Darlington & Brown 1975). Seed of *R. repens* can acquire enforced dormancy when buried (Sarukhan 1974). Large populations of viable seed of *R. repens* may occur buried deeper than 10cm and these are ready to germinate as soon as they are exposed (Chippindale && Milton 1934). Germination takes place during late spring and early summer, sporadic germination may occur later in the summer or autumn (Sarukhan 1974). Factors related to the soil atmosphere may play an important role in keeping the seeds under enforced dormancy for long periods but the quantity and quality of the light to which the seeds are exposed may also be important factors in the control of dormancy (Sarukhan 1974). *R. repens*, with its dependable vegetative reproduction, weak flowering and seed production but great seed longevity, contrasts strikingly with the other species (*R. acris* and *R. bulbosus*) with little or no vegetative reproduction, high seed output, rapid germination and a short life of the buried seed (Sarukhan 1974).

1.8.4.3 Vegetative reproduction

Clonal growth can be envisaged as the means of maintaining a successful genotype in a favourable environment (Soane & Watkinson 1979). The vegetative spread of a species in a community has many genealogical implications (Harberd 1958). A single genet (originally one seedling) may extend its ramets over a considerable area in which the species is then represented by a single genotype (Sarukhan 1974). Population structure can be broken down into two parts; one level can be described as the number of genetic individuals present (genets) each consisting of sub-units or modules (ramets). Population dualism is particularly conspicuous in clone forming plants such as *R. repens* when not only does the plant (genet) develop from a seedling as a subpopulation of parts, but some of these parts may root and eventually become severed from the original (Soane & Watkinson 1979). The ramet, which may often follow independent existence if severed from the parent plant, is the unit of clonal growth (Soane & Watkinson 1979).

In Spring, lateral stolons are produced in the axils of leaves of over-wintering rosettes, and stolon production continues until late summer when the stolon begin to wither, these stolons may produce daughter ramets at their rooting nodes (Lovett-Doust *et al.* 1990). The usual growth cycle in *R. repens* is for leaves on a parent rosette to die at about the same time as daughter ramets become established (Lovett-Doust 1981b). Immediate response to changed environmental conditions is through increased stolon development rather than seed production (Harper 1957). Rosette density of *R. repens* increases with increased grazing (Sarukhan 1974). The safety of the vegetative reproduction as against the risky sexual reproduction in plants is illustrated with the probabilities of survival of seedlings and daughter plants of *R. repens* to the year next to their 'birth'; these probabilities are 0.12 for a seedling and 0.77 for a daughter plant (Sarukhan 1976). Sarukhan and Gadgil (1974) found that *R. repens* depends more heavily on vegetative than sexual reproduction.

1.8.5 Variation in *Ranunculus repens*

Species within *Ranunculus* show extensive morphological adaptations for life in very different habitats (Usherwood *et al.* 1997). Variation in leaf morphology has been found in many species in the genus (Lovett-Doust 1981a,b; Cook 1983; Lynn & Waldren 2001a). Many studies have shown that *Ranunculus repens* varies greatly in morphological characters, which are found to vary greatly in plants from any one locality; those characters that were found to show regional variation most clearly were the degree of dissection of the leaf and the hairiness of the plants (Fisher 1954, 1960; Cook *et al.* 1968; Hintikki 1975; Coles 1977; Lynn & Waldren 2001a) as well as in its population dynamics in varying habitats, and growth of ramets (Sarukhan & Harper 1973; Sarukhan 1974; Lovett-Doust 1981a; Lynn & Waldren 2001b).This is not a new phenomenon as it has been found that different types of response to environmental conditions may be shown not only by closely related species, but also by different populations of a single species (Bradshaw 1965).

1.8.6 Measurement of morphological variation in *R. repens*

Quantitative measures have been used to analyse leaf shape in a number of Ranunculus species with varying degrees of success. Melville (1960) used leaf dimensions to distinguish *R. concinnus* L. and *R. decurvus* L. Menadue and Crowden (1988) concluded that leaf size appeared to be habitat-modified and therefore was not

a reliable taxonomic character. Briggs (1962) used quantitative leaf shape techniques originally developed by Anderson (1949, 1954) to show evidence of natural hybridisation in a number of Australian *Ranunculus* species. Coles (1977) measured the degree of dissection of the leaflets by the ratio W/L where L is the length of the terminal leaflet and W is the width of the main lateral lobe of the terminal leaflet at the position just above its main dissection. Leaf shape was found to be variable, even among plants from the same locality, and a full range of leaf-shapes were found in many geographical regions. This is probably due to the wide ecological tolerance and ecotypic variation shown by *R. repens* (Coles 1977). In species that occupy a range of environments, a single genetic type with infinite plasticity is not found, but rather a myriad of genetically distinct individually adapted populations, and although adaptation by plasticity plays an important role in many situations, permanent adaptation by genetic change is more common (Bradshaw 1965). *Ranunculus repens* is found in a broad range of ecosystems, the environmental factors in each affecting the species in various ways, and resulting in high levels of phenotypic diversity. One such environment where populations of *R. repens* are found to have a strikingly different morphological form to the more common broadleaved form is the temporary limestone lakes (turloughs) in the West of Ireland.

1.9 Turloughs

'As to those places called turloughs, quasi terrain lacus, or land-lakes, they answer the name very well, being lakes one part of the year of considerable depth, and level smooth fields the rest. There holes are in these, out of which the water rises in winter, and retires again in summer..' King (1685) in Coxon (1986).

1.9.1 Physiography

A turlough, simply defined, is a limestone basin, covered by glacial drift of varying thickness, that floods annually with groundwater but is without overground outflow. The 'typical' turlough has an impermeable floor of lake clay or marl with one or several connections to groundwater called swallow holes (Goodwillie 1992). It appears very difficult to attribute a general classification to turloughs, as they are so various in characteristics such as size, depth, and adjacent land use and drainage pattern. They have been described as ecotones – transition zones between aquatic and terrestrial environments (Reynolds 1996). The name is derived from the Irish word

'tuar loch' meaning dry lake (Praeger 1932). Turloughs have been called 'disappearing lakes', and 'vanishing fields' (Mac Gowran 1985).

1.9.2 Hydrology

Turloughs are the boundary zones ('lakeshores') of underground water bodies and each year they experience different hydrological conditions (Goodwillie *et al.* 1998). Turloughs usually flood in the autumn, form a lake for several months in the winter, and empty underground through swallow holes in late spring (Plate 1.1) (Coxon 1987a). The changing water levels are not always strongly seasonal, but often reflect relatively local weather patterns (Reynolds 1996). The permanent lacustrine deposits present in at least 50% of turloughs imply that the present day flooding regime was preceded in many turloughs by a period of all-year-round flooding (Coxon 1986). The cause of change from permanent to seasonal inundation remains in doubt, but it is suggested that factors involved may have included sedimentation causing shallowing of the depression, the development of better connections between the lake and groundwater, and the development of the zone of higher permeability associated with the turlough (Coxon 1986).

1.9.3 Distribution

Past methods of mapping turloughs were by no means consistent; many turloughs are depicted only as areas of flooding or 'liable to floods'. In several instances, the town land or village name provides a clue (e.g. Turloughmore, or Killaturly) (Coxon 1986). The majority are located in the counties of Galway, Mayo, Roscommon, and Clare, with a few outliers in Counties Limerick, Sligo, Tipperary, Longford, Kilkenny and Donegal, all of these being situated in carboniferous limestone regions of Ireland. A broad belt extends eastwards from Lough Corrib and Lough Mask linking with others in Roscommon west of Lough Ree. Another cluster occurs at the head of Galway Bay and southwestwards into Clare. A few isolated examples are found in Sligo, south Clare, Limerick, Tipperary and Kilkenny but there is no overall figure for the number of turloughs in the country (Goodwillie 2002).

1.9.4 Classification and origin

Despite the volume of data available it is often difficult to classify a turlough as being a turlough due to their ambiguous description and wide variability. Coxon (1986)

Plate 1.1: Cooloorta turlough basin; Flooded in the winter months (above) and dry during the summer months (below).

used two main criteria to characterise turloughs; (I) hydrological criteria such as the need for the geographical feature in question to flood seasonally and to empty to groundwater, and (II) occurrence on well bedded, pure, grey calcarenite limestone which is lithologically similar to the Burren limestone and is frequently in the same stratigraphic position. Both glacial and karstic processes appear to have been involved in the formation of turloughs and Coxon (1986) proposes three possibilities for the formation of turloughs; (I) Glacial hollows, with flow lines developing postglacially either independently of the hollows or controlled by the hollows, (II) Glacial hollows along the line of preglacial flow routes, and (III) Preglacial karstic hollows and flow routes, modified by glaciation, concluding that turloughs are possibly polygenetic features formed by more than one of the suggested methods, and that the variation in turlough characteristics does tend to suggest this.

1.9.5 Similar land forms

No exact equivalent of turloughs have been found outside Ireland. Turloughs have been compared to the Poljes of Yugoslavia, although theses land forms can remain dry for a number of years (Williams 1964), Uvalas in Slovenia, temporary lakes in low lying karst areas of Florida (Sweeting 1972), and Coxon (1986) suggests that intermittent lakes from the Canadian Northwest territories and the glaciokarstic depressions in Newfoundland provide the closest equivalents to turloughs. Campbell *et al.* (1992) concluded that the behaviour of a fluctuating water body in Pant-y-llyn situated on the northern perimeter of the South Wales Coalfield, near Llandtbie in Dyfed, UK (Blackstock *et al.* 1993) shares many of the characteristics of ephemeral lakes or turloughs. This small basin in South Wales, on carboniferous limestone, lacks surface drainage outlets and empties via swallow holes (Lynn 1998). Similar landforms, coined 'flooding meadows' can be found in the Saone Valley in France (Broyer & Lauranson-Broyer 1996). Turlough-like water bodies have also been discovered at Clot d' Espolla in Catalonia (Vila *et al.* 1989). The Breckland Meres, in Norfolk, England are also considered to be similar to the turloughs of Ireland. The Meres are glacially formed hollows with a thin cover of drift (Trist 1979) that have fluctuating water levels. The main difference is that because the bedrock is chalk as opposed to limestone there is a lag in the rise and fall of the water table. Thus there is a lack of a seasonal basis for flooding.

1.9.6 Priority habitat status

Turloughs are listed as a priority habitat in Annex 1 of the 1992 European Union Habitats Directive (92/43/EEC). Karst regions are rare in Europe as a whole and this has led to special attention being given to them by the EU Habitats Directive. Limestone pavement, calcareous fens and springs with tufa (calcium carbonate) formation are also listed as priority habitats in the Directive. A priority habitat is a natural habitat that is in danger of disappearing. The regulations outlined in the Directive were brought into effect in Ireland in 1997 and incorporated into the 1976 Wildlife Act. Article 11 in the Directive states that *Member states shall undertake surveillance of the conservation status of the natural habitats with particular reference to priority natural types*. Therefore Ireland has both a national and international obligation to survey the conservation status of these habitats and to protect their biodiversity. Several turloughs have been proposed as Special Areas of Conservation (SAC's) but one third of the turloughs over 10 hectares have been irreparably damaged by drainage (Coxon 1986).

1.9.7 Threats to the turlough habitat

The main threats to turloughs are eutrophication and drainage. Wetland drainage has been a feature of Ireland since the Famine (Baldock 1984). Numerous turloughs have been drained at various times, particularly in the 1840's as Famine relief work, and in more recent OPW drainage schemes from the 1950's onwards (Coxon 1986). Drainage in the nineteenth century eliminated many of the great turloughs of East Galway in the creation of the Clare river (Reynolds 1996). Many turloughs are interconnected by groundwater and are thus sensitive to any changes in hydrology or farming practices (Drew 1990). At sites geographically remote from a given turlough any decline or change in hydrology will have repercussions on the turlough habitat such as changes in the vegetation community due to a changed water regime. Several turloughs occur in a farmed landscape and thus they are not independent of the agricultural activities that prevail. Turloughs, which are fed by groundwater, are becoming enriched in many parts of the country due to fertiliser application, this enrichment can also occur indirectly from farm runoff. A recent quantitative study on the effect of fertiliser application on turlough vegetation found that it resulted in a change in the composition of dominant species (Lynn *et al.* in prep). Grazing animals can also affect the vegetation directly by removing some of the palatable species as

well as through physical damage and manuring (Goodwillie *et al.* 1998). It is crucial to understand the way a species or ecosystem functions before any attempt at management can be initiated.

1.9.8 Turlough vegetation

The most successful and consequently most abundant plants of the turloughs are those, which can best withstand submergence or grazing or most commonly both (Praeger 1932). Many detailed phytosociological surveys have been carried out on turlough vegetation (Ivimey-Cook & Proctor 1966; Louman 1984; O'Connell *et al.* 1984; Mac Gowran 1985). Coxon (1986) correlates vegetation communities to depth and duration of flooding, and finally Goodwillie (1992) classifies the vegetation communities of turloughs firstly on the basis of depth of submergence, and subsequently in terms of differing substrates or grazing patterns. Thirteen vegetation types were recorded, ranging from dry woodlands to sedge communities. The dry woodlands, dominated by species such as *Fraxinus excelsior* L., *Euonymus europaeus* L., *Quercus robur* L., *Glechoma hederacea* L., *Festuca gigantea* (L.) Villars, and *Moehringia trinervia* (L.) Clairville, are only occasionally flooded for short periods. In contrast, sedge communities on peaty substrates dominated by *Carex nigra* (L.) Reichard, *Eleocharis palustris* (L.) Roemer & Schultes, *Juncus articulatus* L., *Caltha palustris* L., and *Senecio aquaticus* Hill, are regularly submerged for several months each year.

It is evident from these surveys that the vegetation of turloughs is dependent on the depth and duration of flooding and that the variability of the turlough environment is reflected in the vegetation found. However, there are a few ubiquitous species occurring from the edge of the turlough almost to its base: *Agrostis stolonifera* L., *Potentilla anseria* L., *Carex panicea* L., *and Ranunculus repens;* the two species best adapted to turlough life being *Ranunculus repens* and *Agrostis stolonifera* as they survive in all types of sites (Goodwillie 1992). *Ranunculus repens* occurred in 43 % of the turloughs under observation (60 sites) in a survey conducted by Coxon (1987b).

1.9.9 The turlough form of *Ranunculus repens*

Ranunculus repens is one example of a terrestrial species that traverses the boundary between pastureland and turlough habitats (Lynn & Waldren 2003a). The populations

of *R. repens* individuals that inhabit turloughs are of a morphologically distinct form from those found in the surrounding pastureland (Plate 1.2). The turlough form has more finely dissected leaflets than the more typical form and the leaf surfaces are glabrous as opposed to densely hairy (Lynn & Waldren 2001b, 2003b). Leaf morphology in this turlough form correlates with susceptibility to inundation with groundwater (Lynn & Waldren 2001), with leaf dissection generally increasing in areas that are prone to longer periods of flooding. It is most likely to be a result of the hydrological pressure exerted by this habitat that has caused these differences to arise

1.9.10 Physiological variation in the turlough form of *R. repens*

Turlough populations of *Ranunculus repens* must endure prolonged submergence, often in several metres of water, where any attempt at amelioration, such as 'depth accommodation', would not be feasible (Lynn & Waldren 2002). Lynn & Waldren (2002) showed that turlough populations have a higher rate of photosynthesis and respiration than ruderal populations, and that the turlough population had a higher stomatal conductance than the ruderal population. In addition, the turlough populations had a relatively higher stomatal index on the upper leaf surface and a relatively lower index on the lower surface than the ruderal populations. Compared with ruderal populations, turlough populations of *Ranunculus repens* had higher concentrations of total non-structural carbohydrates in their roots (Lynn 1998; Lynn & Waldren 2002), thus enhancing their chances of survival following extensive bouts of submergence. Lynn and Waldren (2002) suggest that the differences in leaf morphology between turlough and more typical populations of *R. repens* have resulted in enhanced gas exchange, which may lead to increased concentrations of storage carbohydrates for use in times of inundation when rates of photosynthesis are low in turlough populations. Both leaf dissection and leaf size will significantly affect rates of CO_2 uptake, water loss and convective heat transfer of the leaf due to what is called the boundary layer effect (Raschke 1960). The boundary layer is that layer of air immediately adjacent to the leaf surface through which gaseous exchange and heat flux are limited by static diffusion or turbulence. The effective size of the boundary layer is proportional to the geometry and dimensions of the leaf as well as wind speed. The more dissected or smaller the leaf, the less is the resistance to heat transfer or gaseous exchange due to this effect (Lewis 1969). Lynn (1998) postulates reasons

Plate 1.2: The dissected leaf form of *Ranunculus repens* that is found in the turlough habitat (above and below).

why the turlough populations are able to survive the conditions that prevail in the turlough habitat:

(I). A more dissected leaf reduces the boundary layer and enhances photosynthesis in submerged conditions; a greater oxygen release may ameliorate anoxia in a stagnant media.

(II). A relatively smaller plant size with a relatively greater oxygen output again would reduce tissue damage.

(III). Enhanced aerial photosynthesis due to leaf shape and stomatal allocation to the upper leaf surface would increase the potential for starch accumulation for subsequent use in submerged conditions.

(IV). The observed conservation of carbohydrate resources in submerged conditions due to a reduction in respiration would increase the chances of survival during periods when photosynthetic capacity is severely reduced.

1.9.11 Theories on the evolution of the turlough form of *R. repens*

Lynn & Waldren (2003a) speculate on the origin of this unique turlough form of *R. repens*, stating that the land surrounding turlough would have been dominated by mixed deciduous woodland (Mitchell 1990), or pine and hazel scrubland in the open limestone areas (Watts 1984) before the impact of agriculture. *R. repens* is often present in the community at the edges of woodlands (Lovett-Doust 1981a) and may have initially invaded the fringes of the turlough basins. Following the clearance of woodland by man and the onset of domestic farming, large areas were likely to be open for exploitation by colonising species such as *R. repens*, particularly in the more freely drained turlough basins which would have been and still are the more heavily grazed. Thus, the authors state that the more disturbed fringes of the high water mark may have provided an ideal habitat for the colonisation of a weedy species such as *R. repens*. If disturbance of an area continues for a long period of time and a weedy species remains in the area, selection for closer local adaptation may occur (Baker 1972).

An alternative theory posed by Lynn & Waldren (2003a) is that the turlough populations are post-glacial relict populations. The vast expanses of moraine, outwash sand and gravel, and ground uncovered by melting ice following the retreat of the glaciers is considered to have offered ideal conditions for the rapid spread of weedy

species, including *R. repens* (Godwin 1959). This herb community would have occurred around the fringes of turloughs as many turloughs are believed to have been permanent water bodies in the late glacial due to the presence of lacustrine deposits in the sediment (Coxon 1986). Following the transition of these lakes to turloughs in the early Holocene (Mitchell 1990) the turloughs may have provided a refuge for some of the late glacial herbaceous species, providing they were tolerant of the fluctuating water levels. The clearance of the high forests by man approximately 5000 years ago (Mitchell 1990) may have resulted in contact being re-established between the turlough and the weedier forms. Every species has a history and the turlough form of *R. repens* is no exception. The resolution of its history may be aided with the use of molecular techniques.

1.10 Molecular markers

1.10.1 Molecular tools in plant population biology

Integration of ecological and genetic data has provided a clearer understanding of the impact of ecological processes on the evolution of plant populations. Genetic markers have contributed to the study of plant evolutionary ecology by providing methods for detecting genetic differences among individuals (Cruzan 1998). Techniques such as DNA fingerprinting enable both the genetic variation in populations and its spatial structure to be analysed. Molecular tools can overcome genotype – environment interaction effects and elucidate genetic differences between and within populations that would prevent the problems associated with the classical techniques, particularly the time factor involved in the propagation and establishment of material for morphometric comparison (Lynn 1998). Bachmann (1994) states that it is advisable to complement the general population genetic information with an approach that begins with the identification of physiological and morphological characters relevant for the ecological status of the plants under investigation, which in the case of the turlough form of *R. repens*, has been thoroughly explored by Lynn & Waldren (2001a,b).

With the genesis of the polymerase chain reaction (PCR; Mullis & Faloona 1987) came a monumental increase in molecular biology studies. PCR is a deceptively simple but remarkably versatile procedure that has applications in virtually all areas of molecular biology research (Brown *et al.* 1990). The isolation of thermally stable DNA polymerase from the hot springs bacteria *Thermus aquaticus* (*Taq* polymerase)

led to an efficient means of amplifying short fragments of DNA using automated thermal cyclers (Parker *et al.* 1998). The PCR involves replicating target regions of DNA, which are flanked by regions of known sequences (Ehrlich 1989). Synthetic oligonucleotide primers (usually 20-30 bases long) that are complementary to each of the flanking regions are needed; these are combined with a small sample (nanograms) of genomic DNA, plus free deoxynucleotides, a reaction buffer, and *Taq* DNA polymerase (Parker *et al.* 1998). During a series of heating and cooling cycles the DNA is repeatedly denatured into single stranded molecules. The single stranded DNA is used as a template for primers to anneal to their complementary sequences on either side of the target region and *Taq* polymerase replicates the region downstream from each primer using the free deoxynucleotides as building blocks. The growth of target DNA is exponential in each cycle thus resulting in numerous copies of the target sequence being produced (micrograms).

Adequate controls are particularly important in a PCR experiment because the extreme sensitivity of the technique means that small amounts of contaminating template DNA can give rise to a PCR product that is visible when the results are examined by agarose gel electrophoresis (Brown *et al.* 1990). Therefore when conducting standard PCR reactions both negative and positive controls are always included. Means of contamination can include aerosol droplets and indirect contamination via stock solutions. Brown *et al.* (1990) lists a number of precautionary steps that can help decrease the risk of such contamination:

- Minimise handling of the solutions
- Use sterile technique
- Autoclave reagents
- Prepare small aliquots of reagents
- Separate workspaces

PCR can significantly decrease the amount of time required to isolate a desired segment of the genome; however, for most uses of PCR, one must determine the sequences of regions flanking a given locus, and this can entail considerable effort when working with a new species (Parker *et al.* 1998). The technique of using arbitrary or random primers was developed to overcome this limitation. The use of "random chosen primers" does allow one to identify genetic markers relatively

quickly in species for which extensive sequence information is not available (Parker *et al.* 1998).

1.10.2 Classes of DNA markers

DNA markers provide a means for powerful, fine-grained analysis of individual genotypes (Parker *et al.* 1998). There are various categories of molecular techniques available, the key is to discover the one most suited to the problem posed and most likely to solve the questions asked. Therefore population biologists and taxonomists often require a different suite of techniques as they are working on different levels of classification. Time and money also play a major role when choosing a molecular technique, for example if one method has high enough resolving power to answer a particular question it could be argued that it would be futile to employ a much more expensive and time consuming technique. Among the many reviews published (Bachmann 1994; Karp *et al.* 1996; Parker *et al.* 1998; Ouborg *et al.* 1999) concerning the suitability of molecular markers, Sunnack (2000) gives a comprehensive summary of favourable attributes of genetic markers in population biology:

Assayable by PCR: PCR can use low quantities of even degraded DNA and can target specific DNA regions.

Comparability: PCR primers that amplify homologous regions over a wide taxonomic range generate directly comparable data thus facilitating profound insights by meta-analysis (Burke *et al.* 1998).

DNA rather than protein: unlike allozymes DNA can be extracted from old material, is more convenient for storage and collection of samples, is more variable, is PCR-assayable and can yield gene genealogies.

Gene genealogies and frequency data: molecular genealogies uniquely can untangle current and historical processes and can yield information on demographic trends (Templeton 1998).

Many separate loci available: the use of multiple markers has two main advantages: (1) overcoming stochastic biological sampling that can cause different patterns in loci with similar histories, and (2) detection of nonconcordant characters that are likely to be biologically interesting (e.g. affected by natural selection).

Rapid development and screening: ideal markers would be useable in new taxa with little further development and amenable to rapid screening.

Single-locus as opposed to multilocus markers: multilocus approaches (e.g. RAPD and AFLP) are technically convenient but imprecise and have many major technical and / or analytical drawbacks, such as dominance (Perez *et al.* 1998; Hurme & Saolainen 1999; Rabouam *et al.* 1999). Data are of limited comparability among studies. By contrast, single-locus, codominant markers supply robust data for input into precise analyses. Data are comparable among studies, thus contribute globally.

Unlike with codominant markers such as microsatellites and allozymes, with a dominant marker the heterozygote cannot be directly distinguished from the dominant homozygote phenotype at individual loci. Consequently, the estimation of allele frequencies from dominant markers presents some statistical difficulties (Krauss 2000). Krauss documents two possible solutions to this problem:

(I) The frequency of the null allele at a given locus can be estimated by taking the square root of the frequency of the null homozygote, or the absence of a band (Bernstein 1930).

(II) To avoid statistically biased estimates, Lynch & Milligan (1994) introduced a procedure and recommended that loci with three or less null homozygotes be excluded from null allele frequency estimations. However, this procedure leads to severely biased estimates of average heterozygosity and genetic distances (Isabel *et al.* 1995; Szmidt *et al.* 1996; Zhivotovsky 1999).

More recently, Zhivotovsky (1999) introduced a Bayesian approach to the estimation of null allele frequencies for dominant DNA markers. The Bayesian method gave good estimations of heterozygosity, genetic distances and F-statistics for simulations and RAPD data on two conifer species where macrogametophyte data were available to infer allele frequencies (Zhivotovsky 1999).

1.10.3 Choosing and using a molecular technique

In the last decade, diverse arrays of new molecular genetic tools have become available for high-resolution genetic studies of population-level processes (Parker *et al.* 1998). As a result many review papers and books have been published (Hoelzel 1992; Avise 1994; Bachmann 1994; Milligan 1994; Cruzan 1998; Parker *et al.* 1998)

to try to help ecologists in this transitionary period between true field ecology and the world of molecules. Highly variable regions of DNA can sometimes provide a unique "fingerprint" for each individual, and access to such fine scale genetic variation is one of the most compelling reasons for choosing to work with DNA markers (Parker *et al.* 1998). The application of methods dealing with the nucleotide sequence of DNA to ecology may either look like the surrender to an irrelevant fashion and likely to distract from the essential questions of the field, or it may be welcomed as the long-awaited simple and general tool to unravel ecological complexity from the inside out (Bachmann 1994).

DNA markers can behave like single-gene morphological markers or like allozymes, with the added advantage that their expression is not influenced by development or by environmental factors (Bachmann 1994). Different regions of the genome experience different selective pressures, depending on the genetic product and / or the tendency of the DNA to withstand changes in nucleotide sequences (Li *et al.* 1985). For example many coding regions of the genome have been found to be highly conserved and thus are useful for phylogentic studies but not of much use to population studies (Jarne & Lagoda 1996; Schaal *et al.* 1998). On the other hand, noncoding regions can accumulate mutational change in a neutral manner, such that only genetic drift influences the level of polymorphism in a population (Parker *et al.* 1998). There is normally a risk in using neutral genetic markers for making inferences about adaptive processes unless there is a very close linkage between the selected locus and the neutral marker (Palacios & Gonzales-Candelas 1999).

Hamilton (1997) states that the use of DNA fingerprinting techniques is more relevant than allozymes for population studies. Allozymes are less useful for estimating the balance of evolutionary forces acting on polygenic characters due to their limited coverage of the genome (Hamilton 1997). Conversely due to their random nature and wide coverage of the genome, multilocus techniques such as AFLP cover a high proportion of the genome, therefore there is a better chance of detecting differences between individuals.

1.11 General aims

The aim of this project was to use molecular techniques to elucidate the genetic relationships between turlough and non-turlough populations of *R. repens*. An interesting point to consider is that not all turlough populations of *R. repens* have corresponding broad-leaved populations growing in close proximity. The question arises as to how this species came to inhabit these ephemeral environments; do they share a unique recent common ancestor or have they evolved along separate evolutionary lineages? By combining molecular, morphological and ecological data, the holistic approach was anticipated to provide a better understanding of the turlough populations of *Ranunculus repens* and may add to the conservation value of turloughs. The approach taken for this project is three tiered:

I. The evolutionary history of the turlough form of *Ranunculus repens* - By employing appropriate molecular techniques it was hoped that an understanding of the genetic relationship between the turlough and non-turlough form would be elucidated. This study was undertaken on an inter-population, wide geographic scale, incorporating individuals from many locations and habitat types in order to compare the genetic relatedness of the turlough and non-turlough forms.

II. The genetic variation within a population of the turlough form of *R. repens* - This component of the project involved studying one population of the species along a strong ecological gradient within a turlough basin. This study was conducted to ascertain whether or not there was a genetic difference between those individuals subjected to different periods and depths of inundation.

III. The genetic structure of a turlough population of *Ranunculus repens* - This was undertaken by conducting an in-depth turlough basin study. Unlike tier two, no major ecological gradient in terms of period of inundation was present as all plants were selected from a similar microenvironment. This part of the project aimed to tackle such issues as population genetic structure and growth strategy of the turlough form of *R. repens*.

Therefore each one of the three separate tiers form a distinct chapter; Chapter two focuses on the origin and evolutionary history of the turlough form of *R. repens* by using a suite of molecular techniques on an inter population level, Chapter 3 focuses

on the effect of a strong ecological gradient on the genetic structure of one of these turlough populations of the creeping buttercup, and Chapter 4 tackles population genetic structure, growth strategy and genotypic fitness of this species within a turlough basin.

Chapter 2

Evolutionary history of the turlough buttercup

It is almost impossible with any brevity to exemplify the notion of adaptation. Just because adaptation consists, even in the simplest cases, in a multiplicity of correspondences between one sufficiently complicated system, the organism itself, and another equally complicated, the environment in which it finds itself. It is indeed, just this multiplicity that makes the thing recognisably adaptive.

Fisher 1934

2.1 Introduction

2.1.1 Plant population differentiation and the measurement of genetic diversity

Genetic structure in populations can arise from the localised sampling of genotypes that takes place at reproduction as well as from dispersal of individuals over an area less than that occupied by the total population (Hamilton 1997). Wright (1946) was the first to coin this phenomenon as isolation by distance: populations isolated by distance form a network of demes that have gene frequencies regulated by limited migration from other demes, finite sampling within the deme, and the local effects of selection (Hamilton 1997). Plant populations are particularly subject to the effects of isolation by distance because plants are sessile, often exhibit mixed mating systems, and have gametes that are dispersed indirectly via vectors that can be highly localised (Hamilton 1997).

The population genetic structure of a species is both influenced by its past history, by current gene flow and by genetic drift (Despres *et al.* 2002). Genetic drift in small populations at the edge of the species range, or in populations which are isolated, may lead to reduced genetic diversity and high levels of differentiation between populations. Conversely, populations that are in contact with others experience gene flow and thus have the potential for increased genetic diversity and often show lower levels of among population differentiation. Despite these hypotheses, Antlfinger (1981) states that local differentiation in plant populations does not require geographic discontinuity but can manifest as a result of strong selection pressures. Neutral markers such as AFLP will be subject to genetic drift and gene flow alone, unless the markers are linked to genes acted on by selection. Pakniyat *et al.* (1997) found this to be true in a study using AFLP to distinguish wheat genotypes from geographically distinct areas, discovering that some AFLP markers correlated closely with salt tolerance.

The 'niche variation' hypothesis of Van Valen (1965) proposes that genetic polymorphism can be maintained within local populations if different genotypes have different relative fitnesses in different microhabitats or niches. Mengoni *et al.* (2000) found that genetic diversity observed in copper mining deposit populations of *Silene paradoxa* L. was as high as that of the presumed primary neighbouring populations in

uncontaminated soil; they state this result is not theoretically expected for a recent colonisation event but that a high number of tolerant plants in the natural populations could have reversed the effect of the initial genetic bottleneck produced by selection. Pollen flow from the neighbouring populations could also be a possible source of variation. Similar results were reported by Wu *et al.* (1975) for copper tolerance in *Agrostis stolonifera*. Therefore, with an increasing number of genetic studies being published, it is becoming more apparent that genetic diversity can be relatively high in small populations, and that genetic differentiation between plant populations does not require some sort of gene flow barrier, sometimes an ecological factor such as metal levels in the soil, salt tolerance, or even water tolerance in these populations are enough to make them genetically different ecotypes. This suggests that although dominant marker techniques such as AFLP produce neutral markers it is not impossible to detect markers that are somehow linked to genes and are therefore under selection.

Understanding the genetic diversity both within and among natural plant populations is a fundamental goal of plant molecular ecology. Recent developments in molecular genetic techniques have provided several types of genetic markers that are suitable for the screening of genetic variability in plant populations. There have been numerous recent attempts to estimate levels of genetic diversity using dominant marker techniques such as RAPD, ISSR and AFLP (Lynch & Milligan 1994; Jorde *et al.* 1999; Zhivotovsky 1999; Mengoni *et al.* 2000; Deshpande *et al.* 2001; Arafeh *et al.* 2002). Genetic studies of complex traits, such as salt tolerance, have become easier with the development of informative molecular markers such as AFLPs (Pakniyat *et al.* 1997). Techniques such as AFLP and RAPD have been employed to assess the genetic relationships among and within plant populations growing on metal contaminated soil (Mengoni *et al.* 2000) and also along salinity gradients (Pakniyat *et al.* 1997).

By measuring genetic variation and by applying population genetic models, inferences can be made about the biology of organisms. By examining genetic markers with appropriate rates of change, and therefore, suitable signals, information can be gathered about almost any population and evolutionary process through the hierarchy of life. Selecting appropriate genetic analysis methods is vital to the success

of applying molecular genetics to population biology. There has been considerable debate about which method is most appropriate to analyse empirical data on the population structure of species (Sunnacks 2000).

2.1.2 The evolutionary history of the turlough buttercup

Turloughs harbour a morphologically distinct form of the common weedy species *Ranunculus repens.* A description of the turlough form, as well as hypotheses regarding its formation, was discussed in chapter one. Lynn and Waldren (2002) showed physiological differences to be present among turlough and non-turlough populations of the species. A morphological difference in terms of leaf shape between turlough and non-turlough populations was found to be a heritable trait (Lynn & Waldren 2001b). These findings indicate that the morphological differences are genetically based. However, it is still unclear how these turlough populations have evolved to be so different. Do these turlough populations represent a different evolutionary lineage to non-turlough forms? Or has the distinct turlough form repeatedly evolved from non-turlough populations?

Baker (1972) states that plasticity plays the greatest role in the colonising phase of a weedy species like *R. repens*. If it then manages to establish and persist for a long enough time period, the ability to survive in the habitat changes from being plastic or from having a 'general purpose genotype' to a locally adapted genotype. Could this be the case with the turlough buttercup? Has it survived in these conditions for a long period of time so that it has become an ecotype, genetically adapted to the turlough environment? The hypothesis is certainly plausible. Many studies in plant adaptation have been conducted on plant populations that inhabit environments that impose strong selection pressures such as metal contamination and salinity (Antonovics 1972; Wu *et al.* 1975; Antlfinger 1981). In the advent of the prolific growth in knowledge and the widespread availability of molecular techniques plant population genetic analysis has been incorporated into these examinations. Many studies have shown these phenotypic differences in plant populations to have a genetic basis (Prentice *et al.* 1995; Cordell *et al.* 1998; Creswell *et al.* 2001; Fujii *et al.* 2001). Considering the obvious morphological variation in populations of *R. repens*, and with the advantage of having prior knowledge on its genetic basis, a molecular study of numerous populations was undertaken. Background knowledge on the suite of techniques

available is critical in choosing the most suitable markers and relevant information is given below.

2.1.3 Molecular analysis

2.1.3.1 Choosing the appropriate techniques

DNA markers provide a means for powerful, fine-grained analysis of individual genotypes (Parker *et al.* 1998). There are various categories of molecular techniques available; the key is to discover the one most suited to the problem posed and most likely to solve the questions asked. Therefore population biologists and taxonomists often require a different suite of techniques as they are working on different levels of classification. Genetic markers are simply heritable characters with multiple states of each character. All genetic markers reflect differences in DNA sequences, usually with a trade-off between precision and convenience (Sunnacks 2000). Multi-locus techniques such as AFLP, RAPD and ISSR are technically convenient but often less precise, and have many technical and/or analytical drawbacks such as dominance (Perez *et al.* 1998; Hume & Saolainen 1999; Rabouam *et al.* 1999). Data from multilocus methods are often of limited comparability among studies. By contrast, single-locus, codominant markers supply robust data for input into precise analyses, are comparable among studies, and thus contribute globally (Sunnacks 2000).

A fundamental limitation to multilocus methods is dominant inheritance – DNA fragments can be scored only as present or absent in contrast to codominant inheritance where each of the alleles at a locus in an individual can be identified and thus analysed more precisely. As a consequence of simultaneous visualisation of many dominant markers, multilocus data typically are analysed as pairwise comparisons of complex patterns that only have meaning relative to others in the same study. Thus multilocus data can be compared only superficially among studies. By contrast, single-locus markers are far more flexible, informative and connectible because they can be analysed as genotypic arrays, as alleles with frequencies and as gene genealogies (Sunnacks 2000). Therefore, in this study both multilocus and single locus techniques were employed to assess whether or not the turlough populations of *R. repens* are in fact different, in terms of evolutionary history, to the populations residing in other habitat types.

2.1.3.2 Inter simple sequence repeat

Inter simple sequence repeat (ISSR) analysis involves the anchoring of designed primers to a subset of simple sequence repeats (SSRs) and results in the PCR amplification of regions between adjacent, inversely orientated microsatellites using a single SSR containing primer (Zietkiewicz *et al.* 1994; Kantety *et al.* 1995). ISSR markers are generated from PCR primers that consist of a core sequence of repeated di-, tri-, tetra-, or penta-nucleotide motifs. The addition of a different base or anchor at the 5'- or 3'-end renders their binding sites more specific and reproducible. The sequence between two binding sites in opposite orientation within suitable distance is amplified. Insertions and deletions (Indels) within this region and loss or gain of binding sites are detected as band polymorphisms (Yang *et al.* 1996). Primers are usually longer (16-20 bp) than those employed in RAPD (10 bp), and this allows increased stringency (Hodkinson *et al.* 2002). The process is not entirely random. The polymorphisms detected by ISSR-PCR are from the sequences between the two simple sequence primer sites and are not necessarily simple sequence repeats. The nature of the amplified fragments therefore depends on both the simple sequence primers used and on genome structure (Lu *et al.* 1996).

ISSR primers anneal directly to SSRs and because SSRs are common in higher plants, no prior knowledge of target sequences is required (Tsumura *et al.* 1996). ISSR analysis can be undertaken for any species that contains a sufficient number and distribution of SSR motifs (Zietkiewicz *et al.* 1994). For successful ISSR analysis, pairs of SSRs must occur within a short distance (in bps) that is amplifiable by a PCR reaction that produces a size fragment, revealed as a band that is resolvable on polyacrylamide gels, agarose gels or similar detection methods (Zietkiewicz *et al.* 1994). The potential supply of ISSR markers depends on the variety and frequency of microsatellites, which changes with the species and the SSR motifs that are targeted. If microsatellite distribution is completely random, the length of the intervening region between SSRs of the same motif is a function of their frequency (Blair *et al.* 1999).

2.1.3.3 Amplified Fragment Length Polymorphism

The Amplified fragment length polymorphism (AFLP) technique developed by Vos *et al.* (1995) generates a large number of markers, spanning the whole genome. AFLP

assays require no previous sequence knowledge and can regularly detect 20-100 loci per assay (Maughan *et al.* 1996; Powell *et al.* 1996). This relatively new molecular technique is considered a highly informative fingerprinting tool (Powell *et al.* 1996). The advent of AFLP has provided a new level of precision in genotyping and offers great potential in studies of genetic diversity (Pakniyat *et al.* 1997). AFLP analysis combines the ease of RAPD with the reliability of the laborious restriction fragment length polymorphism (RFLP) (Vos *et al.* 1995). Fluorescent fragment labelling applicable on automated sequencers can add an extra advantage by providing a radioactive-free system. Moreover, if a combination of fluorescent dyes with distinct emission spectra can be multiplexed in one lane, higher throughput per gel and the application of an internal size standard in each lane can be achieved (De Riek *et al.* 1999). AFLP technology is extremely robust and proficient at revealing intra-population diversity and estimating genetic distance between individuals and populations (Travis *et al.* 1996; Arens *et al.* 1998; Winfield *et al.* 1998). Time and money play a major role when choosing a molecular technique, for example if RAPD has high enough resolving power to answer a particular question it could be argued that it would be futile to employ a much more expensive and time consuming technique such as AFLP. The relatively new technique of AFLP (Vos *et al.* 1995) has many advantages over other marker systems in use such as ISSR and RAPD. It produces a high number of polymorphic informative markers per primer pair, is highly sensitive, requires small amounts of DNA, and has proved to be robust, reliable and reproducible. AFLP fingerprinting is based on the selective amplification of restriction fragments from a digest of total genomic DNA and has been adapted for use on an automated sequencer (Hodkinson *et al.* 2002).

2.1.3.4 Plastid DNA microsatellites

Microsatellites or simple sequence repeats (SSRs) are tandemly repeated sequences whose unit of repetition is between one and five base pairs. They are found in a wide variety of eukaryotes and also in the chloroplast genome of plants. They may be classified into three families – pure, compound and interrupted repeats (Jarne & Lagoda 1996). Alleles at a given locus are identified following specific PCR amplification by their relative electrophoretic migration, by comparison with a ladder of known size. Pedigree analyses have indicated that microsatellites are often codominant and inherited in a Mendelian fashion (Jarne & Lagoda 1996). In

population studies, allele size of microsatellites may be known with an accuracy of one base pair and alleles are subsequently characterised by their number of repeats at the locus analysed (Jarne & Lagoda 1996). Mean density of microsatellites within species varies widely for reasons that remain unclear, so that no *a priori* rule can be forged for predicting their occurrence and density. Microsatellites may be found within expressed regions of the genome (Jarne & Lagoda 1996). The fact that a size ceiling on allele size among microsatellites in exons has been detected in surveys of natural populations suggests that these loci are under selective pressure (Sutherlands & Richards 1995).

Microsatellite regions occur frequently within intron and spacer regions. Microsatellites are generally variable with high mutation rates due to slipped strand mispairing or 'stuttering'. The nonrecombinant, uniparentally inherited nature of organelle genomes makes them useful tools for evolutionary studies (Provan *et al.* 2001). Microsatellite markers are routinely used to investigate the genetic structuring of natural populations (Balloux & Lugon-Moulin 2002). Microsatellite analysis is more discriminative than AFLP as it involves the use of co-dominant markers; microsatellite loci have high levels of allelic diversity, making them valuable as genetic markers (Mc Couch *et al.* 1997). The technique is well documented by Powell *et al.* (1996).

One approach to finding these microsatellites is to sequence a particular gene region with forward and reverse primers; these sequences can then be scanned for microsatellites that are revealed as a repeat sequence of bases. The unique sequences bordering the SSR motifs provide templates for specific primers to amplify the SSR alleles via the polymerase chain reaction (PCR) (Mc Couch *et al.* 1997). In order to reveal these variable microsatellite regions, primers need to be designed to amplify the target microsatellite region. If length variation occurs in the microsatellite regions of different individuals, then DNA fragments of different length will be produced. For the chloroplast microsatellite analysis to be of any benefit for screening *R. repens* populations these repeat regions must vary between individuals. This method of analysis has not previously been used on *R. repens*, and was anticipated to be applicable on an inter-population or national scale but not for detecting local population differentiation.

When sequence data is limited for the taxa under observation, a less time consuming method of chloroplast microsatellite analysis can be achieved by using universal primers that amplify certain regions of the chloroplast genome in many different plant species. Several of these have been developed (Taberlet *et al.* 1991; Demesure *et al.* 1995; Dumolin-Lapegue *et al.* 1997; Weising & Gardner 1999). These primers work on the premise that short runs of mononucleotide repeats are scattered throughout the chloroplast genome of higher plants, and that such repeats are variable among closely related taxa. Consensus primers are designed and constructed to amplify chloroplast microsatellite regions across a wide range of taxa. According to Weising and Gardner (1999) these primer pairs must fit three criteria to be successfully applicable to many different plant species; Firstly, target sequences need to be sufficiently conserved to generate a fragment from unrelated species; secondly, the amplification products should be polymorphic; and thirdly the products should be relatively short in order that they can be resolved on sequencing gels. The one major drawback of this technique is that because of their inherent universal nature, the detection of intra specific variation is not always feasible.

2.1.4 Aims

This upper tier of the project was undertaken to investigate the origin of the turlough form of *R. repens* (see section 1.11). An among-population study of the species, taking samples from a broad range of habitats was conducted. A number of techniques were employed, with the belief that the solution lies in the use of more sophisticated methods of analysing morphological data and of complementary characters offered by molecular analyses.

Molecular screening at an interpopulation level was employed to compare turlough and non-turlough populations of *R. repens*. Genetic distances between geographically isolated turlough populations were estimated to help answer the evolutionary history of the form. Two opposing hypotheses were put forward for the origin of the turlough buttercup. The first, coined 'adaptation by isolation', or 'common ancestry' suggests that if turlough populations were discovered to be more similar to each other than to adjacently positioned non-turlough populations this would imply genetic separation of turlough populations, and somewhat imply a breeding barrier being in place between the turlough and non-turlough forms. The second theory, which will be called 'local

adaptation', would result from turlough populations originating from adjacently positioned non-turlough populations. These broadleaved populations of *R. repens* would be the 'genetic source' for the newly forming turlough populations. The selection pressures present in the turlough environment would then 'weed' out the genotypes 'unfit' for these conditions, resulting in a number of 'fit' genotypes suited to the turlough habitat remaining over generations.

In addition, other questions arise such as 'if turlough and adjacent non-turlough populations are genetically similar then why do they have such different leaf morphologies?' and 'where have the dissected leaf populations come from when there are no nearby broadleaved populations present?' Elucidating the genetic structure of the populations may also help to distinguish between the hypotheses of either glacial survival or postglacial immigration. Low levels of intra-population genetic variation and distinct geographical clustering of genetic variation would point towards long term *in situ* isolation, whereas high levels of intra-population genetic variation combined with the absence of genetic differentiation between different regions may indicate recent gene flow and establishment of the populations.

Occasionally, the dissected leaf form can be found to occur in non-turlough populations. Thus it may be present at low levels in all populations, and the reason for its low levels of expression is that it is selected against. If this scenario proves true, it may be difficult to find any population genetic structure or separation, other than a geographical one, in the molecular data obtained. One final issue to note is that leaf morphology is likely to be determined by a small number of genes. AFLP produces neutral markers so the chances are that the markers will bear no relation to the morphological and physiological differences between turlough and non-turlough forms.

Ranunculus repens individuals from numerous populations of varying habitat type were screened using the techniques of AFLP, universal chloroplast microsatellites, and sequencing. In addition, quantitative morphological measures were taken for each sample and habitat types were recorded for each population sampled. By taking a holistic approach and combining molecular, morphological and ecological data, it was hoped that a better understanding of the turlough form of *R. repens* would be reached.

2.2 Materials and Methods

2.2.1 Site details

Sampling sites of *R. repens* populations can be found in Table 2.1 and Figure 2.1. The habitat at each site was described and associated species for each site were recorded (see Appendix 1). The location of each collecting site was fixed with a Geographical Positioning System (GPS, Garmin 12 x 2). Each individual from each site was given a unique collection number.

Table 2.1: Sampling sites of *R. repens*, including unique population code, county of origin, grid reference and short description. Each population is classified as one of four habitat types; T-turlough, TA-turlough adjacent, WP-wet pasture, and DP-dry pasture.

Site No.	Pop Code	Location	County	Grid Reference	Description	Habitat type
1	RC	River Carey, Ballycastle	Antrim	D140408	Wooded river bank	TA
2	LN	Lough Neagh	Armagh	J034599	Flooded field	WP
3	CV	Lough Ramor, Virginia	Cavan	N603865	Wet lakeshore	WP
4	TG	Turloughnagullan	Clare	M280040	Turlough	T
5	TGA	Turloughnagullan A	Clare	M280040	Pasture	TA
6	GK	Gortlecka	Clare	R310940	Turlough	T
7	LBG	Lough Baile na gCea	Clare	R284914	Fen margins	T
8	CR	Carran	Clare	R290990	Turlough	T
9	CFS	Carran A	Clare	R288991	Roadside	TA
10	LG	Lough Gash	Clare	R390680	Turlough	T
11	CO	Cooloorta	Clare	R337979	Turlough	T
12	GG	Glengariff	Cork	V920568	Woodland verge	DP
13	MP	Magilligan Point	Derry	C675355	Damp field	WP
14	B2	Ballyshannon 2	Donegal	G853601	Turlough	T
15	B4	Ballyshannon 4	Donegal	G860603	Turlough	T
16	WN	Whinney Hill, Holywood	Down	J795425	Pasture	DP
17	DD	Dodder	Dublin	O150297	Dry river bank	DP
18	TBG	Trinity Botanic Gardens	Dublin	O160305	Garden	DP
19	FE	Knockninny Quay	Fermanagh		Lake shore	WP
20	GM	Glenamaddy	Galway	M640610	Turlough	T
21	KT	Kiltiernan	Galway	M430410	Turlough	T
22	CH	Caherglassaun	Galway	M410060	Turlough	T
23	CE	Coole	Galway	M430030	Turlough	T
24	KLT	Killtullagh	Galway	M370300	Turlough	T
25	TM	Turlough Monaghan	Galway	M330460	Turlough	T
26	KK	Kilkorcan	Galway	R350941	Occasional flooded	WP
27	HK	Hawkhill	Galway	M411025	Turlough	T
28	HKA	Hawkhill A	Galway	M413025	Wooded laneway	TA
29	MC	Muckross, Killarney	Kerry	V963864	Wet bog area	DP
30	URL	Urlingford	Kilkenny	S310640	Turlough	T
31	NR	Inistoige	Kilkenny	S639384	River callow	WP

Site No.	Pop Code	Location	County	Grid Reference	Description	Habitat type
32	LT	Leitrim	Leitrim		Wet pasture	WP
33	FT	Fortwilliam	Longford	N020630	Turlough	T
34	FTA	Fortwilliam A	Longford	N025630	Pasture	TA
35	WT	Whitestown	Louth	J233064	Roadside ditch	DP
36	ML	Millgrange	Louth	J208089	Garden	DP
37	KLY	Killaturly	Mayo	M410980	Turlough	T
38	SL	Slishmeen	Mayo	M220790	Turlough	T
39	BL	Balla	Mayo	M260850	Turlough	T
40	MY	Moylough	Mayo	G540080	Turlough	T
41	MYA	Moylough A	Mayo	G544080	Turlough	T
42	RM	Ramolin	Mayo	M260540	Pasture	WP
43	CHV	Cahervoostia	Mayo	M260650	Turlough	T
44	MN	Whitelough	Monaghan	H728206	River flood plain	WP
45	P1	Pingo 1, Thompson Common	Norfolk	TL936966	Wet grassland	WP
46	P2	Pingo 2, Thompson Common	Norfolk	TL937965	Wet hollow	WP
47	LM	Langmere	Norfolk	TL909885	Mere	WP
48	DPB	Devils Punchbowl	Norfolk	TL878892	Mere	WP
49	CL	Clonmacnoise	Offaly	N013312	River callow	WP
50	CS	Castleplunket	Roscommon	M780770	Turlough	T
51	CSA	Castleplunket A	Roscommon	M788776	Pasture	TA
52	BN	Ballinturly	Roscommon	M840600	Turlough	T
53	BNA	Ballinturly A	Roscommon	M841599	Pasture	TA
54	SW	Swallow hole	Sligo	G740174	Permanent pool	WP
55	GT	Gorteen	Sligo	G675055	Marsh	WP
56	LB	Lough Beg	Tyrone	J578927	Lake flood plain	WP
57	WX	Wexford	Wexford	T040201	Garden	DP

2.2.2 Plant collections

In the summer of year 2000 *R. repens* samples were collected from 53 sites throughout Ireland (Plate 2.1). This entailed collecting clean, undamaged leaf samples from four to five individuals from each population, and preserving them in silica gel (Chase & Hills 1991). The number of individuals collected per population was low as it was considered more beneficial to sample few individuals from a large number of populations as opposed to a large number of individuals from a small number of populations. This strategy was chosen in order to sample as many different turlough and non-turlough populations as possible, considering the economic limitations of the project. Due to the fact that this is a clonal species showing profuse vegetative spread, samples were collected at least 10m apart, to reduce the chance of collecting clones of the same genotype. Twenty-five of these sites were turlough habitats, the remainder consisted of varied habitats including river callows, pastures occasionally flooded, marshes, woodlands, river banks, agricultural meadows and roadsides. Turlough populations were collected in counties Clare, Donegal, Galway, Longford, Mayo, Roscommon, Sligo, and Tipperary. Non-turlough populations were collected from all

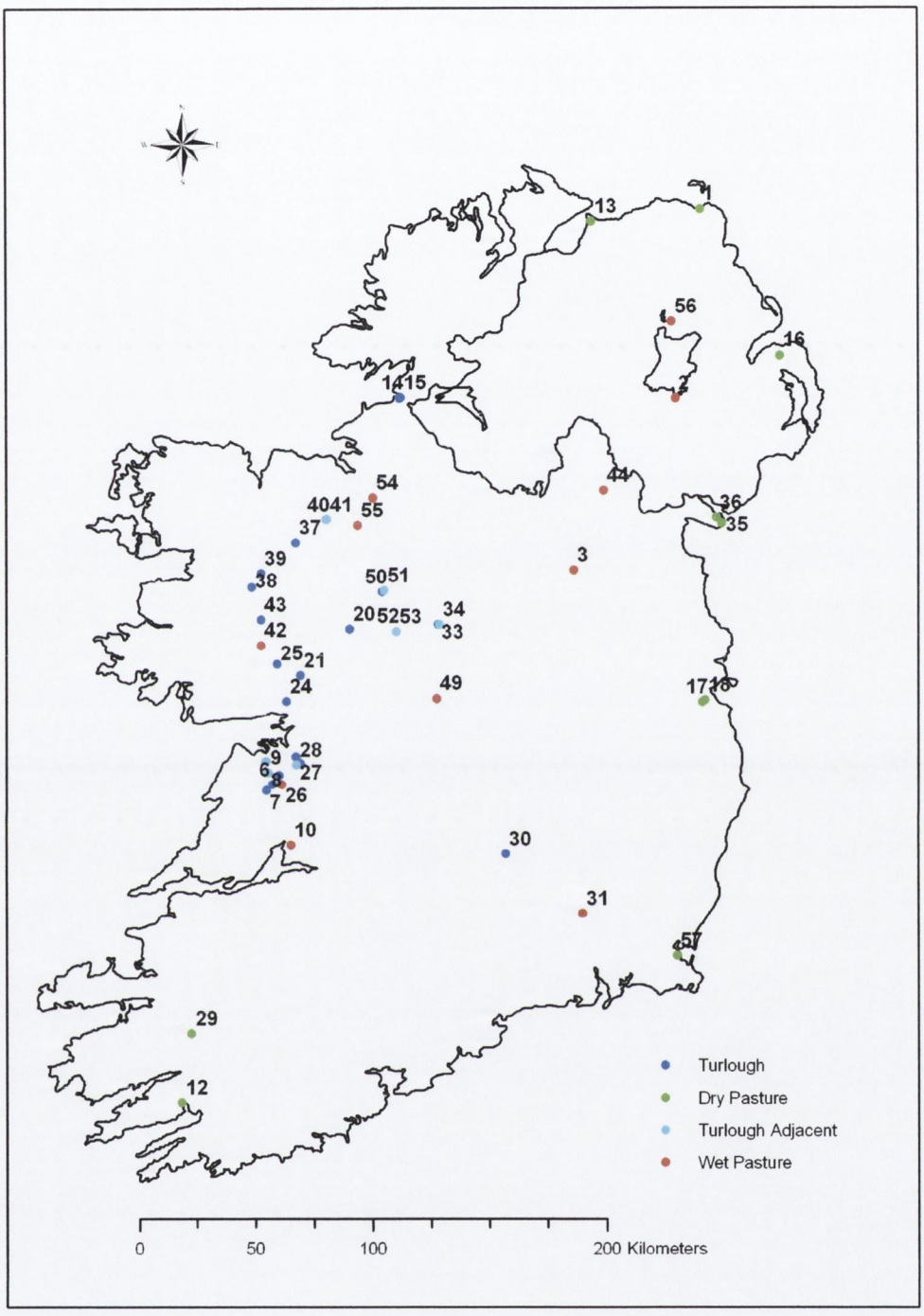

Figure 2.1: *Ranunculus repens* collection sites throughout Ireland. Populations are labelled according to four habitat types. Dark blue dots represent turlough populations, light blue dots represent populations adjacent to turloughs, red and green dots denote wet pasture and dry pasture populations respectively. Numbers represent population codes that are described in full in Table 2.1.

Plate 2.1: Turlough collection sites; Caherglassaun, Co. Clare (above) and Coole, Co. Galway (below).

of the above counties as well as from many others around Ireland, including Northern Ireland, in order to obtain a geographical spread of *R. repens* populations throughout the island.

In the Spring of 2002 *R. repens* samples were collected from 4 sites in Norfolk, England (Plate 2.2). Collection methods were identical to those already described in the preceding paragraph. Two of these sites were meres, natural lakes that are fed by rising groundwater. Like turloughs, water levels change over the year but unlike turloughs water levels are generally at their highest in the summer. The other two collection sites were from wet grassland communities surrounding pingos - permanent shallow ponds that were formed periglacially towards the end of the last Ice age, at Thompson Common in Norfolk. Full site details are given in Appendix 1.

2.2.3 Leaf collection and morphological measures

At the same time that leaf samples were collected, one clean, undamaged mature leaf was taken from each individual, labelled, and stored in a paper envelope. Leaf outline for each individual (5 per population) was traced within 24 hours of collection and the leaf outlines were later scanned using a flatbed Apple Scanner that gave a bitmap image of each leaf. Area and perimeter values for each individual image were obtained using the image software analysis package Scion Image on a PC following the procedure of Lynn and Waldren (2001b). These data were then used to obtain a dissection index by calculating the ratio of the perimeter of an outline to the square root of its area; this value is standardised so that a circle has a value of 1. The more deeply lobed or dissected the leaf the larger the value (Kincaid & Schneider 1983). Therefore a leaf dissection index was obtained for each individual:

$$\text{Dissection Index} = \frac{\text{Perimeter}}{2\sqrt{(\text{area} \times \pi)}} \quad \text{[Equation 2.1]}$$

For leaf dissection analysis each population was assigned to one of three habitat types: turlough, wet pasture or dry pasture. All turlough adjacent populations were assigned to the dry pasture category, as all of them were dry pasture populations. The criteria for allocation to habitat type are described in Appendix 1. The relationship between perimeter and $\sqrt{}$area of populations from different habitat types (turlough,

wet pasture, dry pasture) was analysed by comparing the regression slopes of the association between perimeter and √area using the interaction between the discrete variable (habitat type) and the continuous variable (√area). This method allows one to decipher whether the regression slopes are different between different habitat types. The relationships between perimeter and √area of the three groups were plotted in Microsoft Excel.

Differences in leaf dissection between populations from different habitat types were analysed using nested ANOVA with populations nested within habitat type (turlough, wet pasture, dry pasture). The data were analysed using Datadesk 6 (Data Description Inc., Ithaca, New York.)

2.2.4 DNA Extraction

DNA was extracted from silica gel dried leaf material (3 individuals per population) following a modified CTAB (Hexadecyltrimethylammonium bromide) method (Doyle & Doyle 1986). 10ml of CTAB extraction buffer (150mM Tris [hydroxymethyl] aminomethane hydrochloride (Tris-HCl) (pH 7.5), 1M NaCl, 15mM ethylenediaminetetra-acetic acid (EDTA) (pH 8.0) and 2% CTAB) was heated to 65°C with 40µl of mercaptoethanol in labelled 30ml chloroform resistant centrifuge tubes. All extractions were carried out in a fume hood. Approximately 0.2g of dried leaf material was used for each individual and placed in a clean, labelled weighing boat. Each leaf sample was transferred to a pre-heated pestle and mortar and subsequently ground to a pulp by adding hot CTAB and grinding with a pestle.

After grinding the solution was poured back into the labelled centrifuge tubes and incubated for a further 10 minutes at 65°C. 10ml of chloroform: isoamyl alcohol (24:1) was then added to each tube and the samples were placed on an automatic shaker for 30 minutes in a horizontal position. This step extracts constituents present in the organic solvent such as polysaccharides and chlorophyll. Tubes were then centrifuged for 10 minutes at approximately 3000 g [MSE Harrier 15/80, 4x200 swing out rotor (MSE 43124-134) 4500rpm max. speed (3351g), with 2-place 50ml trunnion (34136-109) and 50ml spherical end bucket (MSE 53156-602)]. The upper aqueous phase was then pipetted into a fresh 50ml storage tube and an equal volume of ice-

cold isopropanol was added before storing at –20°C for at least 24 hours to precipitate the DNA.

The samples were then removed from the freezer and centrifuged in a Harrier 15/80 centrifuge (see previous page for details) at approximately 1500g for 10 minutes to pellet the DNA. The supernatant was poured off carefully and 3ml of wash buffer was added (70% ethanol). This was mixed gently and centrifuged at approximately 1500g for 5 minutes to pellet the DNA once more. The supernatant was poured off and the tubes were inverted and placed on clean paper towel for 5 minutes to drain the excess wash buffer. The tubes were then turned the right way up and placed in a fume hood for 20 minutes to allow the evaporation of the remaining ethanol. Once the pellets were dry they were resuspended in 500µl of TE buffer [10mM Tris-HCl (pH 7.5), 1mM EDTA (pH 8.0)] and then transferred using a transfer pipette to labelled 1.5ml tubes, and stored in a freezer at –20°C.

2.2.5 DNA Clean Up

The Gibco BRL ® Concert ™ Rapid PCR purification system was used to clean all total DNA samples. They are designed for purification of PCR products less than 10kb in length but also function well for total DNA, although some large fragments of DNA are lost (T. Hodkinson, pers comm.). The manufacturers information on solutions contained in the kit are as follows: Binding solution (H1), which contained concentrated guanidine hydrochloride, EDTA (Ethylenediaminetetra-acetic acid), Tris-HCl and isopropanol. Wash buffer (H2), which consisted of NaCl, EDTA and Tris-HCl. TE buffer (TE) which was used to resuspend cleaned DNA contained 10mM Tris-HCl (pH 8.0) and 0.1mM EDTA.

The DNA cleaning protocol was as follows. An aliquot of TE (number of samples X 50µl) was preheated to 69°C. 100µl of each total uncleaned DNA sample was mixed with 400µl of H1 (binding solution). A spin cartridge was placed into a labelled 2ml tube and the DNA and H1 mixture were added to the cartridge and centrifuged at 11600g for 1 minute using an IEC Micromax centrifuge. The flow through was discarded and 700µl of H2 (wash buffer) was added to each sample. The tubes were again centrifuged for 1 minute at 11600g. The flow through was discarded and the

tubes were spun at 11600g for 1 minute to dry and eliminate any remaining wash buffer from the spin cartridge membrane. The spin cartridge was then placed in a labelled 1.5ml centrifuge tube and 50µl of the preheated TE was added directly to the centre of the spin cartridge membrane and left for 2 minutes at room temperature. The 1.5ml tube, containing the spin cartridge, was centrifuged at 11600g for 2 minutes to elute the DNA. Spin cartridges were removed from the tubes and the tubes were stored at -20°C until needed for use.

2.2.6 DNA Quantification

Two methods were employed to determine the concentration and purity of the extracted DNA. Firstly, gel electrophoresis was employed and involved running 5µl of the samples mixed with 1µl of loading dye [40% sucrose, 0.25% bromophenol blue] on a 1.5% agarose gel (7 x 14 cm) stained with Ethidium bromide in 1xTBE [45mM tris-HCL, 44mM boric acid, 1mM EDTA], at 110 volts for 30 minutes in a Horizon ® 11.14 gel rig. The DNA samples were run against a standard of known molecular weight (Gibco BRL 1kb ladder). The gels were illuminated under UV light and the concentration of the resulting DNA bands was estimated by comparing with the ladder of known DNA concentration using a digital camera and Kodak 1D 2.0.2 image analysis software.

Secondly, the concentration and quality of the extracted DNA was assessed using an Eppendorf Bio Photometer (spectrophotometer). The spectrophotometer was used for direct measurement of nucleic acids at 260nm, where 1 absorbance value is equal to 50ug/ml DNA. A blank consisting of 5µl TE and 95µl of distilled water was placed in a Eppendorf disposable single sealed 50-2000µl cuvette, which was then placed in the spectrophotometer. 5µl of each DNA sample and 95µl of distilled water was placed in a clean cuvette, and the solution was mixed thoroughly to insure accurate readings. By entering the dilution the machine automatically calculates the concentration of DNA based on the absorbance of light at 260nm. DNA concentration in ng/µl was recorded for each sample. Purity (DNA versus RNA) of each sample was assessed by the ratio of absorbance at 260nm and 280nm.

2.2.7 AFLP procedure

The restriction endonucleases *EcoR*1 (rare 6-base cutter), and *Mse*1 (frequent 4-base cutter) were used for DNA digestion. Adapter ligation, preselective and selective amplification were conducted according to the AFLP ™ Plant Mapping Kit protocol from Perkin Elmer with certain modifications. See below for full protocols and reaction conditions.

2.2.7.1 AFLP reactions

The total volume of this reaction was 5.5µl. Between 700 and 800ng of DNA in a total volume of 2.75µl was used in the restriction-ligation of *EcoR*1 and *Mse*1 adaptors.

AFLP Protocol:

AFLP reactions consisted of three stages.

1. Ligation and Preselective Amplification module containing *EcoR*1 and *Mse*1 adaptor pairs, preselective primers and preselective amplification mix.

2. Core Mix module which composed of a mix of buffer, nucleotides and AmpliTaq® DNA polymerase.

3. The Selective Amplification module which contained a set of primers complementary to the *EcoR*1 adaptor sequence and labelled at the 5' end with a fluorescent dye (either FAM (blue), JOE (green) or TAMRA (yellow)) and a set of primers complementary to the *Mse*1 adaptor sequence.

The AFLP protocol was a modified version of that suggested by the manufacturer as follows.

1. The adaptor pairs were annealed by heating to 95°C for five minutes and allowed to cool over a ten-minute period. The tube was then centrifuged at maximum speed for 10 sec.

2. An enzyme master mix was prepared to contain the following for each restriction ligation reaction:

10 x Promega T4 DNA Ligase buffer	0.05 µl
0.5M NaCl	0.05 µl
BSA (at 1mg/ml)	0.025 µl
*Mse*1	0.5 Units
*EcoR*1	2.5 Units
Promega T4 DNA Ligase	0.5 Units
Sterile distilled water	To make a volume of 0.5 µl

3. Restriction-ligation reactions were prepared to contain the following for each reaction:

Promega T4 Ligase buffer	0.5 µl
0.5M NaCl	0.5 µl
BSA (at 1mg/ml)	0.25 µl
*Mse*1 Adaptor	0.5 µl
*EcoR*1 Adaptor	0.5 µl
Enzyme master mix (from step 2 above)	0.5 µl
DNA template	700-800 ng
Sterile distilled water	To make up to a reaction volume of 5.5 µl

4. The restriction-ligation reactions were carried out at 37° C for 2.5 hr in a PTC 200 MJ Research Peltier Thermal Cycler (the heated lid option was used).

5. A total of 94.5µl of $TE_{0.1}$ were added to the reactions and the reactions were stored on ice or at -20°C until used.

6. A preselective amplification was carried out using the preselective primers. The reactions contained:

AFLP Preselective Primers	8.0µl
Core Mix	7.5µl
Diluted restriction-ligation product (from step 5 above)	2.0µl

The reaction specifications for the PCR amplification were as follows:

Denaturing	Annealing	Extension	Number of Cycles
-	-	72°C for 2 min	1
94°C for 1 sec	56°C for 30 sec	72°C for 2 min	20
-	-	60°C for 30 min	1

A 4°C soak ended the reaction. All ramp times were set at one degree per second.

7. A total of 5 µl of the PCR product were diluted in 95µl $TE_{0.1}$ and the reactions were stored on ice or at 4°C until used.

8. A selective amplification reaction was performed for each primer pair with each reaction containing:

*EcoR*1 labelled primer	0.5 µl
*Mse*1 primer	0.5 µl
Core Mix	7.5 µl
Diluted PCR product (from step 7 above)	1.5 µl

These primer pairs were chosen based on the criteria listed in Section 2.2.7.2. The primers used in the final analysis were:

- FAM (blue) labelled *EcoR*1-ACT and *Mse*1-CAG
- JOE (green) labelled *EcoR* 1-AGG and *Mse*1-CTC
- TAMRA (yellow) labelled *EcoR* 1-AAC and *Mse*1-CAG

The reaction specifications for the selective amplification PCR are much more stringent in the early cycles of the PCR than those for the preselective amplification. The conditions are set out below.

Denaturing	Annealing	Extension	Number of Cycles
94°C for 2 min	65°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	64°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	63°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	62°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	61°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	60°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	59°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	58°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	57°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	56°C for 30 sec	72°C for 2 min	23
–	–	60°C for 30 min	1

The three PCR products for each sample were combined as follows:

0.6µl of FAM product

0.8µl JOE product

1.3µl TAMRA product

0.25µl ROX 500S size standard

24µl of formamide.

Each of the four multiplexed dyes constituted a fingerprint for each individual. Three dyes (FAM, JOE, TAMRA) represented primers used in the PCR and the fourth dye (ROX) labelled the internal size standard. This was heated to 95°C for five minutes and then stored on ice until being placed in the sequencer. The samples were then run on an ABI PRISM® 310 Genetic Analyser following the manufacturers guidelines with 310 Collection Software (Applied Biosystems).

2.2.7.2 AFLP optimisation

A number of *Mse*1 and *EcoR*1 primers were tested with a number of *R. repens* individuals from various locations. Suitable primer pairs were chosen on the basis of clear, reproducible, polymorphic banding patterns, see Table 2.2 below for details.

Table 2.2 Primer trial combinations; codes (01 to 27) represent pairs of primers that were used. *EcoR*1 primers are listed in the first and fourth columns; *Mse*1 primers are given in the third and sixth columns. Primer combinations producing the most scorable band patterns, and which were used in all subsequent AFLP reactions, are in bold.

***Eco*R1 primer anchor**	**Fluorescent label**	***Mse*1 primer anchor**	***Eco*R1 primer anchor**	**Fluorescent label**	***Mse*1 primer anchor**
01 ACT	FAM	CAA	15 AAG	JOE	CAC
02 AGC	TAMRA	CTT	16 ACT	FAM	CAC
03 AAG	JOE	CAT	**17 AAC**	**TAMRA**	**CAG**
04 ACT	FAM	CTA	18 AGG	JOE	CTA
05 AAC	TAMRA	CAT	19 ACT	FAM	CAT
06 AGG	JOE	CAG	20 ACC	TAMRA	CTA
07 ACA	FAM	CTG	21 AAG	JOE	CAA
08 AAC	TAMRA	CTC	22 ACA	FAM	CTC
09 ACG	JOE	CTA	23 AAC	TAMRA	CAC
10 ACA	FAM	CAG	**24 AGG**	**JOE**	**CTC**
11 AGC	TAMRA	CTC	**25 ACT**	**FAM**	**CAG**
12 AGC	TAMRA	CTT	26 AGC	TAMRA	CAA
13 ACA	FAM	CTT	27 ACG	JOE	CTG
14 ACC	TAMRA	CTG			

Several concentrations of DNA from the same individuals were used in AFLP reactions to determine whether or not the amount of template DNA present made a difference to the resulting electropherograms, see Table 2.3 below for details. Generally between 700 and 900ng/µl template DNA gave optimal results.

Table 2.3: DNA concentration trials; each sample was analysed at three different concentrations. Weak banding patterns are given as *, moderate as ** and optimum DNA concentrations for AFLP reactions denoted by *.**

Sample ID	Stock [DNA] (ng/µl)	AFLP reaction DNA template volume (ng/µl)	Banding pattern strength / consistency
17	596	357	*
17	596	715	***
17	596	1072	*
57	1420	426	*
57	1420	852	***
57	1420	1278	*
77	1055	316	*
77	1055	738	***
77	1055	1055	*
80	937	281	*
80	937	562	**
80	937	843	***
184	259	259	*
184	259	518	**
184	259	712	***
193	808	485	*
193	808	727	***
193	808	969	**

2.2.7.3 Band scoring

Sizing of the fragments was performed by Applied Biosystems Genescan ® software Version 3.1. Only the fragments between 35 and 500bp were used for scoring. Resolution of the capillary gel system (i.e. the capacity to separate two adjacent bands in one lane) is 1bp. However, due to lane to lane variation between samples the scoring of the same band between different lanes varied within 1bp. All data were collected under a Genescan® baseline setting of 50 units (manufacturers recommendation) and attributed to a marker category (closest bp). Genscan® files were then processed using Genotyper® Software Version 3.7. Electropherograms were scored manually for the presence (1) or absence (0) of bands. Genotyper does this automatically, but it was found that by processing the results in this way high scoring error occurred due to the software placing peaks in the wrong size category. Three individuals per population were analysed with AFLP and the results for each individual for each primer were used to construct a 0 / 1 matrix.

2.2.8 Data Analysis

Several methods of analysis were employed to process the data from the resulting 0/1 matrix obtained from the AFLP data.

2.2.8.1 Cluster analysis

Neighbor – joining (NJ), and unweighted pair-group method with arithmetic mean (UPGMA) (Sneath & Sokal, 1973) analysis, two forms of tree building or cluster analysis, were applied to the distance matrix based on Nei and Li (1979) distances (equates to Dice distances) using PAUP 4.0; (Swofford 1998) for the AFLP data.

$$GSNL_{(ij)} = \frac{2a}{2a + 2b + 2c} \quad \text{[Equation 2.2]}$$

Where: GSNL (ij) is the Nei and Li (1979) measure of genetic similarity between individuals i and j,

a is the number of polymorphic fragments that are shared by i and j

b is the number of fragments present in i and absent in j

c is the number of fragments present in j but absent in i

With this coefficient twice the number of fragments in common is divided by the sum of the fragments in both phenotypes. The data were analysed using both methods of analysis to check for robustness of the data. The results for UPGMA were similar to those obtained by the NJ method. Internal support for grouping was assessed using 1000 bootstrap replicates (Felsenstein 1985) of NJ or UPGMA with the same parameters as described above (implemented in PAUP 4.0).

2.2.8.2 Distance measures for the ordination techniques

Jaccard's similarity coefficient (Jaccard 1908) was used to construct a pairwise similarity matrix between individuals. Compared to Euclidean distance this distance measure retains sensitivity in more heterogeneous data sets and gives less weight to outliers. This measure also allows more weight to shared presences than to shared absences. The similarity matrix was then converted (1-S, where S is similarity) to genetic distance matrices. The equation for the Jaccard similarity coefficient is given below.

$$GSJ_{(ij)} = \frac{a}{a + b + c} \quad \text{[Equation 2.3]}$$

Where: GSJ (ij) is the Jaccard measure of genetic similarity between individuals i and j, and a, b and c are as above.

The 0/1 matrix was used to construct genetic similarity estimates representing all possible pairwise comparisons of individuals within and among populations using Jaccard's similarity coefficient, the similarity matrix was generated using The R Package 4.0 d1 (Casgrain *et al.* 1999).

Ordination techniques are used to simplify a complex multivariate data set into a small number of dimensions that explain most of the variation. Generally, ordination is the ordering of objects along axes according to their similarities. The major objective is to achieve an effective data reduction, expressing many-dimensional relationships in a small number of dimensions. Objects close in the ordination space are generally more similar than objects distant in the ordination space.

Firstly, principal co-ordinate analysis (PCO) (Gower 1966) was employed to assess and describe the major patterns of variation within and between populations. PCO works on a distance or similarity matrix of all the samples and combines these values to give a representation of the data in n dimensional space (where n = number of taxa). Thus, the dimensions are not reduced but are condensed because generally the first few dimensions account for a greater proportion of the overall variation and dominant patterns in the data are reflected in these first few dimensions (Digby & Kempton 1987). PCO was conducted with The R Package (PCOORD) version 4.0d (Casgrain 1999) and the eigenvectors of the first three axes were graphed in Microsoft Excel.

Secondly, ordination of the data was undertaken using nonmetric multidimensional scaling (NMDS). NMDS is an ordination method that is well suited to data that are nonnormal or are on arbitrary, discontinuous, or otherwise questionable scales. This method can be used both as an ordination technique and as a method for assessing the dimensionality of a data set (plots of minimum stress against k, the number of dimensions in the ordination space). NMDS differs fundamentally in design and

interpretation from other ordination techniques (McCune & Mefford 1997). NMDS is an iterative search for a ranking and placement of n entities on k dimensions (axes) that minimizes the stress of the k-dimensional configuration. The calculations are based on an n x n distance matrix calculated from the n x p-dimensional main matrix, where n is the number of rows and p is the number of columns in the main matrix. "Stress" is a measure of departure from monotonicity in the relationship between the dissimilarity (distance) in the original p-dimensional space and distance in the reduced k-dimensional ordination space (Mc Cune & Mefford 1997).

Program NMDS in PC-ORD is largely based on Mather's program NMMDS (Mather 1976; includes listing of source code). The central computational algorithm (steepest descent minimization to find minimum stress) in NMMDS is based on Kruskal (1964b). NMDS was calculated using PC-ORD Version 3.04; all possible combinations of axes (eigenvectors) were plotted in Microsoft Excel.

Although NMDS is a very powerful and useful technique, it is important to understand how its use and interpretation differ from other ordination methods.

1. The first dimension in a 2-D solution is not the same as the first dimension in a 3-D solution or a 1-D solution. For a given number of dimensions, the solution for a particular axis is unique.
2. Axis numbers are arbitrary, so that the percent of variance on a given axis does not necessarily form a descending series with increasing axis number.
3. An appropriate number of dimensions (axes) should be determined by plotting or tabulating final stress vs. the number of dimensions. The number of axes beyond which reductions in stress are small are chosen. However, graphics in PC-ORD are limited to three axes (McCune & Mefford 1997).

The Jaccard similarity coefficient was used as a distance measure in NMDS; to test the robustness of the data a Monte Carlo (randomisation) test of 100 runs was employed. Each run consists of a separate randomisation or permutation of the data. A large number of runs is generally desirable, but is dependent upon computer power, and data set size, and the desired precision of the resulting p-value. Note that the p-value for a randomization test can be no smaller than 1/N where N is the total number of runs (McCune & Mefford 1997). After initial analysis trials using different

numbers of axes, rotated and unrotated it was decided that NMDS using 3 rotated axes gave the lowest stress levels for the population AFLP data.

2.2.8.3 Diversity statistics

In addition to the ordination analyses obtained from PC-ORD and The R Package, several other quantitative estimates of diversity were measured;

- Within population diversity values (H), (Nei's unbiased diversity statistic, 1987).
- Nei's (1973) genetic distance between populations.

These were calculated using POPGENE version 1.31 (Yeh *et al.* 1997). Within population diversity values were calculated using Nei's unbiased diversity statistic (Nei 1987), averaging over individual AFLP products. A UPGMA dendrogram showing the relationships between populations based on Nei's genetic distance (Nei 1973) was constructed using POPGENE version 1.31, itself modified from PHYLIP Version 3.5. POPGENE computes both comprehensive genetic statistics (*e.g.* allele frequency, gene diversity, genetic distance, *G*-statistics, *F*-statistics) and complex genetic statistics (*e.g.* gene flow, neutrality tests, linkage disequilibria, multi-locus structure).

AFLPs are predominantly dominant markers so that the difference between the 1/1 and the 1/0 AFLP phenotypes in diploid tissues cannot be distinguished. The procedure to estimate AFLP frequencies is straightforward if the study populations are in Hardy-Weinberg Equilibrium (HWE); however, most populations under investigation are not. POPGENE enables one to estimate population genetic parameters under HWE and Hardy-Weinberg Disequilibrium (HWD).

2.2.9 PCR Amplification

The polymerase chain reaction was used to amplify DNA with universal chloroplast microsatellite primers (Weising & Gardner 1999) from a number of *R. repens* individuals. See Table 2.4 below for chloroplast loci used (all from Weising & Gardner 1999).

Table 2.4: Universal chloroplast microsatellite primers used for the amplification of selected *R. repens* individuals showing primer code, location on the chloroplast genome and primer base sequence.

CODE	LOCATION	PRIMER BASE SEQUENCE
ccmp 1	*trn*K intron	Forward primer (5' – 3'): CAGGTAAACTTCTCAACGGA Reverse primer (3' – 5'): CCGAAGTCAAAAGAGCGATT
ccmp 2	5' to *trn*S	Forward primer (5' – 3'): GATCCGGACGTAATCCTG Reverse primer (3' – 5'): ATCGTACCGAGGGTTCGAAT
ccmp 6	ORF 77 – ORF 82 intergenic	Forward primer (5' – 3'): CGATGCATATGTAGAAAGCC Reverse primer (3' – 5'): CATTACGTGCGACTATCTCC
ccmp 8	*rpl* 20 – *rps* 12 intergenic	Forward primer (5' – 3'): TTGGCTACTCTAACCTTCCC Reverse primer (3' – 5'): TTCTTTCTTATTTCGCAGDGAA
ccmp 10	*rpl* 12 – *rps* 19 intergenic	Forward primer (5' – 3'): TTTTTTTTTAGTGAACGTGTCA Reverse primer (3' – 5'): TTCGTCGDCGTAGTAAATAG

The reverse primers for each primer pair were fluorescently labelled as listed below:

ccmp 1 FAM

ccmp 2 JOE

ccmp 6 TAMRA

ccmp 8 FAM

ccmp 10 JOE

PCR reactions were conducted at a volume of 50 µl, using 2.5µl primer at a concentration of 2µM, 1µl of template DNA (the concentration of which varied slightly depending on the sample), 2.5µl PCR buffer x 10 [10mM Tris-HCl (pH 9.0), 50mM KCL], 3mM $MgCl_2$, 250µM each of dATP, dCTP, dGTP, and dTTP, and 0.5 units *Taq* polymerase. Reactions were made up over ice and all samples were centrifuged at approximately 5000g in an MSE microcentrifuge and overlaid with 20

µl of mineral oil. Amplification was carried out on a PTC 200 MJ Research Peltier Thermal Cycler (the heated lid option was used). See Table 2.5 for cycling parameters.

Table 2.5: Cycling parameters for universal chloroplast microsatellite PCR.

Step	Temperature (°C)	Time (minutes)	Cycles
Denature	97	1	30
Annealing	50	1	30
Strand extension	72	3	30
Final strand extension	72	7	1
Incubation	4	infinite	

PCR products were diluted 1 in 30 and combined as follows:

0.6µl of FAM product

0.7µl JOE product

0.8µl TAMRA product

0.25µl ROX 500S size standard

24µl of formamide.

This mixture was heated to 95°C for five minutes and then stored on ice until ready. The samples were then run on an ABI PRISM® 310 Genetic Analyser. Electropherograms were read and fragments were sized using Genscan® Analysis Software Version 3.1 and then processed using Genotyper® Software Version 3.7.

2.2.10 Search for novel chloroplast DNA markers suitable for population genetic studies on *R. repens*

The PCR was also used to amplify target plastid DNA sequences in a number of *R. repens* individuals. See Table 2.6 for primer pair base sequence. See table 2.7 for individuals used in the analysis. Samples were chosen to include a range of *R. repens*

individuals from different habitat types. If variation was not detected in these samples it is unlikely that the regions would be useful at a local population level.

Table 2.6. Gene regions used to sequence samples of *R. repens*, showing genetic origin, and base sequence of primers.

GENETIC ORIGIN	TARGET REGION	PRIMER BASE SEQUENCE
*cp*DNA	*trn*L-*trn*F (Taberlet *et al.* 1991) *trn*L intron and *trn*F intergenic spacer	Forward primer c: CGAAATCGGTAGACGCTACG Reverse primer f: ATTGAACTGGTGACACGAG
*cp*DNA	*rps* 16 (Oxelman *et al.* 1997)	Forward primer 16f: GTGGTAGAAAGCAACGTGCGACTT Reverse primer 2R: TCGGGATCGAACATCAATTGCAAC
*cp*DNA	*atpB – rbcl* (Chiang *et al.* 1998) non coding spacer	Forward primer 2R: GAAGTAGTAGGATTGATTCTC Reverse primer 1R: GTTTCTGTTTGTGGTGACAT

PCR reactions were conducted at a volume of 50 µl, using 0.5µl primer at a concentration of 100ng/µl, 1µl of template DNA (the concentration of which varied slightly depending on the sample), 5µl PCR buffer x 10 [10mM Tris-HCl (pH 9.0), 50mM KCl], 4mM $MgCl_2$, 250µM each of dATP, dCTP, dGTP, and dTTP, and 0.5 units Taq polymerase. Reactions were made up over ice and all samples were centrifuged at approximately 5000g in an MSE microcentrifuge and overlaid with 20 µl of mineral oil. Amplification was carried out on a Perkin Elmer DNA thermal Cycler 480. See Table 2.5 for cycling parameters. All PCR products were cleaned using the Concert Rapid PCR Purification system as described in Section 2.2.5.

Table 2.7. *R. repens* individuals used for PCR amplification and subsequent cycle sequence reactions. All individuals were sequenced using the gene regions listed in Table 2.6.

Sample ID Number	Site	County
42	Urlingford	Kilkenny
72	Fortwilliam	Galway
74	Killaturly	Galway
113	Gortlecka	Clare
140	Ballyshannon 2	Donegal
152	Trinity College Botanic Gardens	Dublin
170	Wexford	Wexford
188	River Carey	Antrim
197	Glengariff	Cork
305	Pingo 1, Thompson Common	Norfolk
309	Pingo 2, Thompson Common	Norfolk
314	Langmere	Norfolk
319	Devils Punchbowl	Norfolk

2.2.11 Sequencing

Cycle sequencing reactions were carried out on the amplified and cleaned DNA using fluorescently labelled dye terminators (Big Dye Terminator sequence mix; Applied Biosystems™). Forward and reverse strands were sequenced in separate reactions. See Table 2.8 for cycling parameters used in the sequencing reaction. Total reaction volume was 10µl, which consisted of the following:

Sequence mix:	1.0µl
Sterile ultra pure water:	1.3µl
Sequence Buffer:	3.5µl
Primer:	0.7µl
PCR product:	3.5µl

Table 2.8: Cycling parameters for sequencing reactions carried out on *R. repens* individuals from several geographically separate populations.

Step	Temperature (°C)	Time	Cycles
Denature	96	10 seconds	25
Annealing	50	5 seconds	25
Strand extension	60	4 minutes	25

Prior to the automated sequence reaction purification was undertaken to remove excess dye terminators. This was achieved by adding each 10µl reaction mix (PCR product) to 52µl clean up mix consisting of 50µl 100% ethanol and 2µl sodium acetate, in a labelled 1.5ml tube. Samples were vortexed and then left at room temperature for 5 minutes, after which they were placed on ice for a further 5 minutes. Samples were then centrifuged for 25 minutes at 11600g and the supernatant was decanted. Further purification involved washing with 70% ethanol and the samples were once again centrifuged at 11600g for 5 minutes, this step was repeated. Finally all samples were left to air dry at room temperature overnight.

Subsequently, the cycle-sequenced samples were denatured (95°C for 5 minutes in a heat block (Grant), then placed directly on ice until ready for use), and run on the ABI PRISM® 310 Genetic Analyser, using the ABI Prism™ 310 module SEQ POP6 (1.0-mL) E or the module SEQ POP6 RAPID (1.0-mL) E run files. The sequences were then processed using ABI Prism™ DNA Sequencing Analysis Software, version 2.1.1.

2.2.12 Sequence Data analysis

Successfully sequenced DNA samples were edited and assembled using the SEQUENCHER software package (© Gene Codes Corporation, 1998) version 3.1. The sequences were then imported into PAUP version 4.0b (PPC) (Swofford 1999), and aligned by eye, and also in SeqAl alignment package (Rambaut 1999). The resulting matrix was then assessed by eye to look for regions that showed variation in terms of base composition.

2.3 Results

2.3.1 Quantitative leaf shape analysis

The association between perimeter and √area of populations within habitat types are presented in Figure 2.2. The interaction between the discrete variable (habitat type) and the continuous variable (√area) showed that the slopes for each habitat type were different at the less than 5% level ($F = 6.32$, $df = 2, 224$, $P < 0.0001$). Regression analysis was conducted on populations from each of the habitat types separately. The turlough populations had a significantly greater slope than the wet pasture and dry pasture populations which was apparent from the interaction between √area and habitat type (20.33, 15.03, and 16.445 respectively, $P<0.0001$) indicating that the perimeter was relatively greater for a given √area in the turlough populations than for either the wet pasture or dry pasture populations. Regression equations for the relationship between perimeter and √area for each of the three habitat types are given in Table 2.9. The turlough populations had a higher perimeter relative to √area than both the wet pasture populations and dry pasture populations; this is reflected in the higher slope (Table 2.9) in the pooled data for the turlough than the wet pasture and dry pasture populations.

Table 2.9 Regression equations for the relationship between perimeter and √area for each of the three habitat types.

Habitat type	Regression equation	F value	P value	df
Turlough	Y=20.33x – 19.57	453	<0.0001	1, 82
Wet pasture	Y=15.03x – 7.73	131	<0.0001	1, 71
Dry pasture	Y=16.45x – 20.52	277	<0.0001	1, 71

Table 2.10 shows mean leaf dissection index for each population sampled and populations are listed according to habitat type. Mean leaf dissection index ranged from 2.56 in the Leitrim population to 4.91 in the Turloughnagullan population. There was a significant difference in leaf dissection among habitat types ($F = 5.96$, $df = 2, 37$, $P<0.0001$) and between populations within habitat types ($F = 1.83$, $df = 2, 17$, $P<0.02$) using nested ANOVA, although no post hoc tests were carried out to

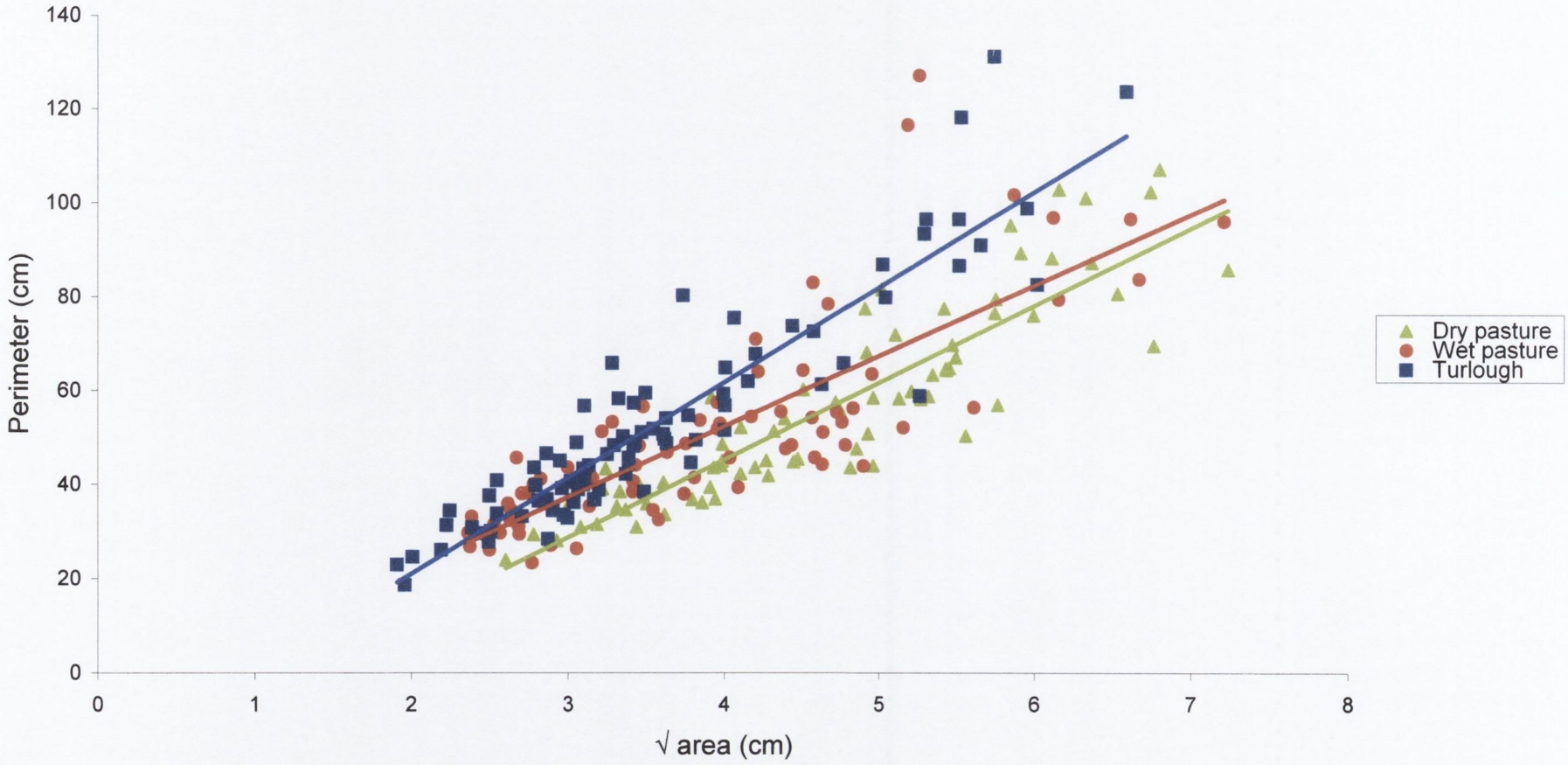

Figure 2.2: The association between perimeter and √ area in *R.repens* populations categorised into three habitat types. Regression equations are given in Table 2.9.

determine which populations differed significantly from each other. From Table 2.10 it is evident that turlough populations such as Gortlecka, Turloughnagullan and Turlough Monaghan (GK, TG, and TM respectively) have a much higher dissection index than dry pasture populations such as Hawkhill adjacent and Turloughnagullan adjacent (HKA and TGA respectively). Figure 2.3 shows mean leaf dissection index for each of the three habitat types.

Table 2.10: Mean leaf dissection index (LDI) for each *R. repens* population sampled throughout Ireland. Populations are grouped according to one of three habitat types; T – turlough, WP – wet pasture and DP – dry pasture.

Habitat	Site	Mean LDI	Habitat	Site	Mean LDI
T	Balla	3.83	WP	Inistoige / Nore	2.93
T	Ballinturly	4.3	WP	Kilkorcan	3.43
T	Bals 2 Donegal	4.58	WP	Langmere	3.95
T	Bals 4 Donegal	4.2	WP	Leitrim	2.56
T	Caherglassaun	3.62	WP	Lough Beg	3.51
T	Cahervoostia	3.28	WP	Lough Gash	3.52
T	Carron	3.94	WP	Lough Neagh	3.84
T	Castleplunket	4.49	WP	Magilligan	3.37
T	Coole	3.75	WP	Monaghan	3.14
T	Cooloorta	4.05	WP	Pingo 1	4.67
T	Fortwilliam	3.41	WP	Pingo 2	4.78
T	Glenamaddy	3.98	WP	Swallowhole	3.56
T	Gortlecka	4.83	DP	Ballinturly Adj	3
T	Killaturly	3.99	DP	Carron Field Station	3.16
T	Kiltiernan	3.66	DP	Castleplunket Adj	3.19
T	Lough Baile na gCea	2.82	DP	Fortwilliam Adj	3.01
T	Moylough	3.6	DP	Glengariff	3.14
T	Ramolin	3.74	DP	Hawkhill Adjacent	2.91
T	Slishmeen	3.61	DP	Millgrange	2.94
T	Turlough Monaghan	4.85	DP	Moylough Adj	3.17
T	Turloughnagullan	4.91	DP	Muckross	3.32
T	Urlingford	3.68	DP	R. Carey	3.53
WP	Cavan Virginia	3.76	DP	TBG	4.08
WP	Clonmacnoise	3.98	DP	TurloughnagullanAdj	2.89
WP	Devils Punchbowl	3.72	DP	Wexford	3.11
WP	Fermanagh	3.32	DP	Whinney Hill	3.88
WP	Gorteen / Aglish	3.69	DP	Whitestown	4.06

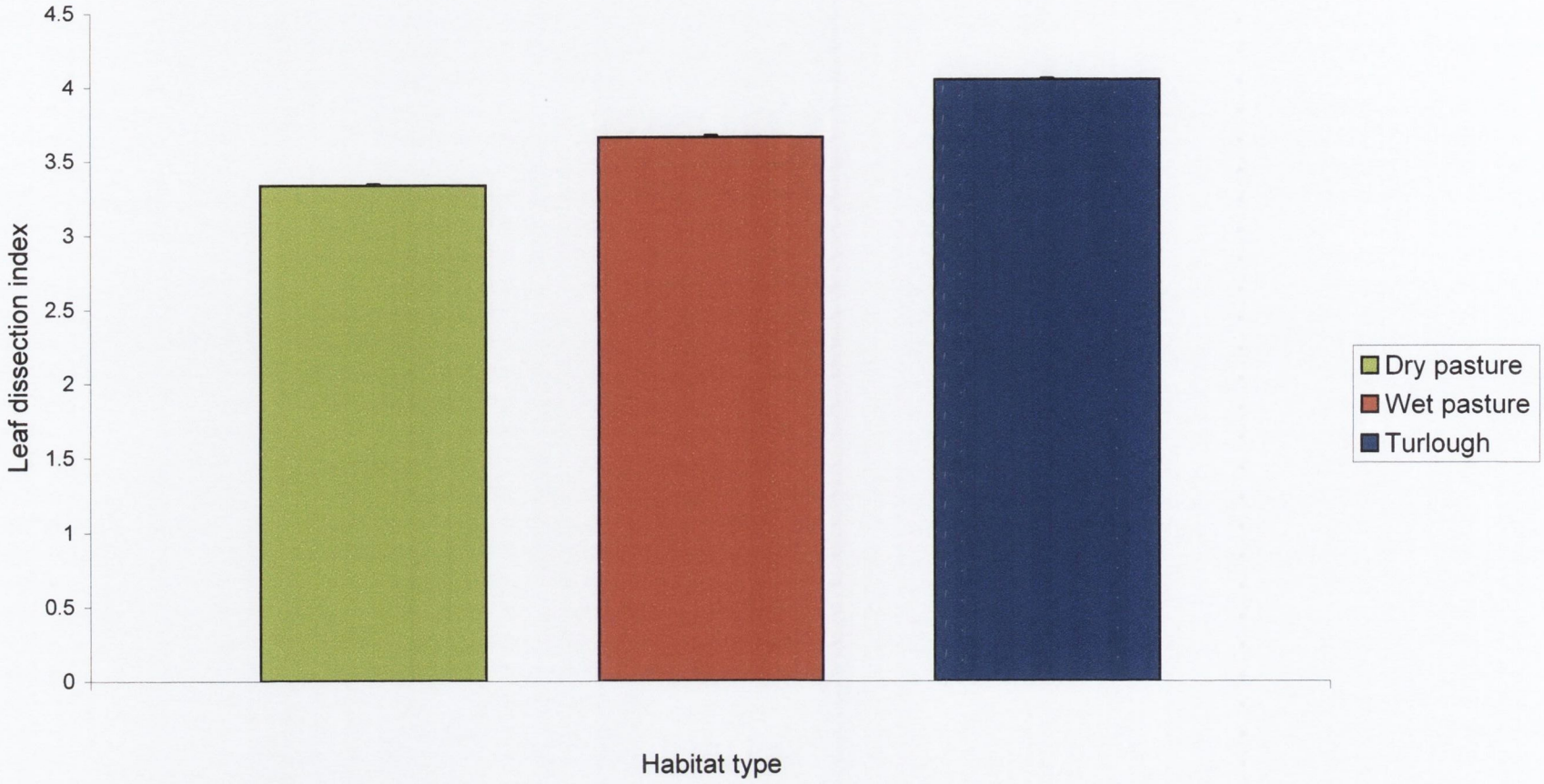

Figure 2.3 Pooled dissection index values for each of the three habitat types. Error bars represent standard errors of the means.

2.3.2 DNA extractions and AFLP band scoring

Extracted, cleaned DNA concentration varied greatly between samples, ranging from 188 ng/μl to 1470ng/μl. Data were first analysed for the Irish populations from three primer pairs and secondly analysed with the inclusion of the four Norfolk (UK) populations using two primer pairs. Therefore all subsequent analyses discussed that include the Norfolk populations are from data obtained from two primer pairs rather than three.

Initial scoring of electropherograms from the AFLP analysis of 132 individuals from 42 Irish populations using three pairs of AFLP primers revealed a total of 229 markers. The removal of uninformative, ambiguous bands was based on the following criteria:

(I) Bands that are present in all samples (monomorphic markers). These were removed from the PCO analysis but were included in the calculation of the diversity statistics.

(II) Bands which, from duplicate samples, and repeated reactions proved to be unreproducible (ambiguous markers).

Fifty-three ambiguous markers were removed from the analysis. The final analysis consisted of 176 markers, 29 of which were monomorphic, leaving a total of 147 polymorphic AFLP markers (83.52% polymorphism). When the Norfolk samples were included, analysis based on two sets of primer pairs revealed a total of 152 AFLP markers, 123 of which were polymorphic (80.92% polymorphism). Scoring error, based on duplicate samples, was calculated from the distance matrix and averaged at 7% over all individuals.

The primers differed in their ability to detect polymorphism within populations. The mean number of polymorphic bands, over all populations, amplified per primer pair combination varied from 63 ACT-CAG, to 37 AGG-CTC to a mid value of 47 AAC-CAG. The frequency of individual markers also varied between primers with ACT-CAG marker presence ranging from 6 to 121 individuals, AGG-CTC marker presence ranging from 4 to 116 individuals and, AAC-CAG marker presence ranging from 4 to 106 individuals. Markers generated from the primers ACT-CAG and AAC-CAG bands occurred in a mean of 32 individuals, and AGG-CTC bands occurred in a mean

of 37 individuals. The mean number of total polymorphic markers (all three primers) per individual ranged from 12 in an individual from the Kiltiernan population (Co. Galway), to 70 in a Ballinturly (Co. Roscommon) individual.

Each of the 132 individuals was assigned to one of three habitat types, as for the leaf dissection analysis: turlough, wet pasture or dry pasture. The frequency of several of the AFLP markers showed significant heterogeneous distribution among individuals from different habitat types (Chi sq. $P<0.05$). Markers with uneven distribution are denoted in bold in Table 2.11 below. None of the markers showed ubiquitous presence in one category while being completely absent in the remaining two categories (none were turlough diagnostic). Some of the markers did show higher occurrence in individuals sampled from the turlough habitat but these markers were never present in all turlough individuals.

Table 2.11: AFLP marker occurrence in *R. repens* samples in each of three habitat types; T-Turlough (n=60), W-wet pastures or flood plain (n=30), and D-dry pasture (n=42). Possible informative markers in terms of inundation tolerance are denoted in bold, possible dry pasture population markers are denoted in italics.

Marker	T	W	D	Marker	T	W	D	Marker	T	W	D
B40	38	16	36	B226	23	5	6	G223	12	7	11
B44	41	13	29	B228	11	4	6	G226	31	7	6
B46	16	10	6	B229	7	3	2	Y40.5	1	8	19
B50	6	3	8	B239	8	0	0	Y42.5	16	6	22
B53	35	6	7	B246	8	2	4	Y47	2	0	2
B52	8	1	8	**B257**	**6**	**0**	**0**	**Y48.5**	**7**	**0**	**0**
B54	40	8	28	B260	19	4	10	Y50	17	3	3
B56	11	5	8	B262	0	4	7	Y51	29	7	16
B55	24	6	21	B269	8	0	1	Y52	2	5	0
B61	6	0	1	B282	14	0	10	Y53	24	10	11
B62.5	3	0	3	B287	11	2	6	Y54	39	5	15
B64	23	12	20	B301	4	0	2	Y55	16	6	4
B65	13	0	1	B309	8	2	0	Y56	34	11	17
B67	29	9	15	B314	5	0	1	Y59.5	34	10	17
B71	38	10	21	G62.5	23	6	13	Y61	9	4	1
B74	15	5	10	G65	26	4	9	Y61.5	1	4	16
B79	49	21	51	G68	40	21	51	Y62	4	6	0
B85	46	21	50	G70	29	15	32	Y67	2	6	12
B87	11	3	2	G71	5	9	14	Y68.5	7	9	16
B103	19	8	15	G72	7	5	0	Y71	0	1	11
B105	33	12	23	G73	7	1	2	Y71.5	13	7	5
B107	30	10	25	G78	14	0	4	Y72	23	3	23
B110	19	4	4	G79	47	19	45	Y77	7	0	1
B116	6	6	5	G81	17	2	1	Y78	6	1	4
B119	7	0	5	**G86**	**9**	**1**	**0**	Y79	9	4	4
B120	6	1	3	G91	47	19	50	Y90	19	6	7
B124	26	5	6	G122	28	14	13	Y92	15	3	8
B126	10	4	17	G125	49	19	48	Y94	12	4	5
B135	24	11	26	**G126**	**4**	**0**	**0**	Y101	9	10	21
B136	48	16	18	G128	22	5	8	Y117	6	3	2
B138	15	5	3	G130	14	8	5	Y121	38	15	20
B140	15	2	4	G144	16	4	4	Y122	19	12	14
B144	13	0	12	G148	4	5	18	Y135	1	5	4
B146	17	0	4	**G149**	**10**	**0**	**0**	Y138	16	10	26
B153	38	14	13	G152	6	1	12	Y158	21	9	4
B160	10	2	9	G153	15	6	21	Y161	31	10	5
B161	19	2	1	G158	12	2	3	Y169	15	20	37
B163	39	9	30	G177	22	1	2	Y172	9	1	5
B164	13	0	1	G186	11	1	2	Y182	11	2	1
B169	**8**	**0**	**0**	G187	1	2	16	Y184	36	7	12
B170	*0*	*0*	*6*	G188	9	0	7	Y195	1	5	7
B172	2	1	8	G201	22	9	16	Y197	3	0	5
B174	2	3	4	G205	15	6	5	Y210	31	11	12
B184	*0*	*1*	*23*	G206	16	3	2	Y224	21	6	12
B184.5	30	6	14	G213	40	14	20	**Y217**	**4**	**0**	**0**
B188	*0*	*0*	*11*	G216	10	7	7	Y219	10	6	7
B199	**6**	**0**	**0**	G218	6	0	2	Y226	40	18	48
B213	8	0	2	G220	21	2	3	Y235	8	6	8
B227	22	1	3	G222	5	4	3	Y222	4	7	20

2.3.3. Cluster analysis

Figure 2.4a and 2.5a display the phylograms obtained from the UPGMA and NJ analyses of AFLP data from 42 populations of a total of 132 *R. repens* individuals. Figures 2.4b and 2.5b are the bootstrap consensus trees associated with each analysis. Due to the large number of samples in these analyses mid point rooting phylograms rather than unrooted phylograms were used in order to make the graphic representation of the results more readily interpretable.

Generally, the results from the UPGMA were similar to those obtained by the NJ method, although a few differences can be seen. In general, individuals were grouped within population units. This finding was common in both sets of analysis. Populations in which all individuals did not group together include Castleplunket, Cavan, Gorteen, Kilkorcan, Kiltiernan, River Carey and Turloughnagullan adjacent (CS, CV, GT, KK, KT, RC, and TGA respectively), again this result was apparent from both sets of analysis. Incongruence between the UPGMA and NJ results include the clustering of all three individuals from the Cooloorta (CO), and Muckross (MC) populations in the NJ analysis, only two individuals from each of these populations grouped together in the UPGMA analysis. Other positions on the trees which are not in agreement include the clustering of all three individuals from the Balla (BL), Ballyshannon 2 (B2), Glenamaddy (GM), Swallowhole (SW), and Lough Neagh (LN) in the UPGMA analysis, only two individuals from each of these populations cluster together in the NJ analysis. Apart from the above differences both modes of analysis resulted in the same large groupings, with non-turlough populations clustering together in one group.

Although there appears to be some grouping of turlough, wet pasture and dry pasture populations, many of these clusters are not supported by bootstrap values. The only groupings with high bootstrap support are for the clustering of individuals within their population units (Figure 2.4b). There does not appear to be any geographic explanation to the clusters, as turlough populations are clustering with other turlough populations which are not spatially close, in fact, in some instances populations are grouping with other populations which are found to be relatively distant in terms of geography (B2, B4 grouping with URL and CR, CH and CS). Groupings which tend to make some geographical sense include the branch grouping HK with GK as both

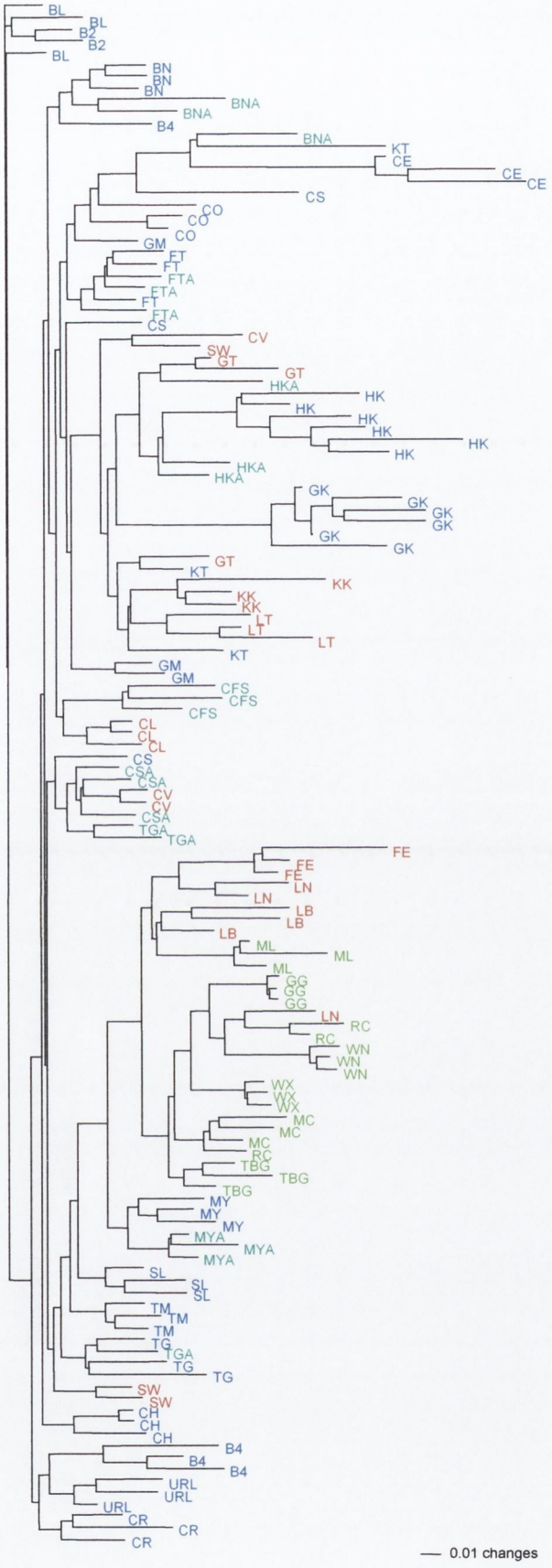

Figure 2.4a: A NJ tree based on Nei & Li (1979) distance measure of the AFLP data from 42 populations of *R. repens* sampled from different habitat types; Turlough populations are denoted in blue, turlough adjacent in light blue, wet pasture in red and dry pasture in green.

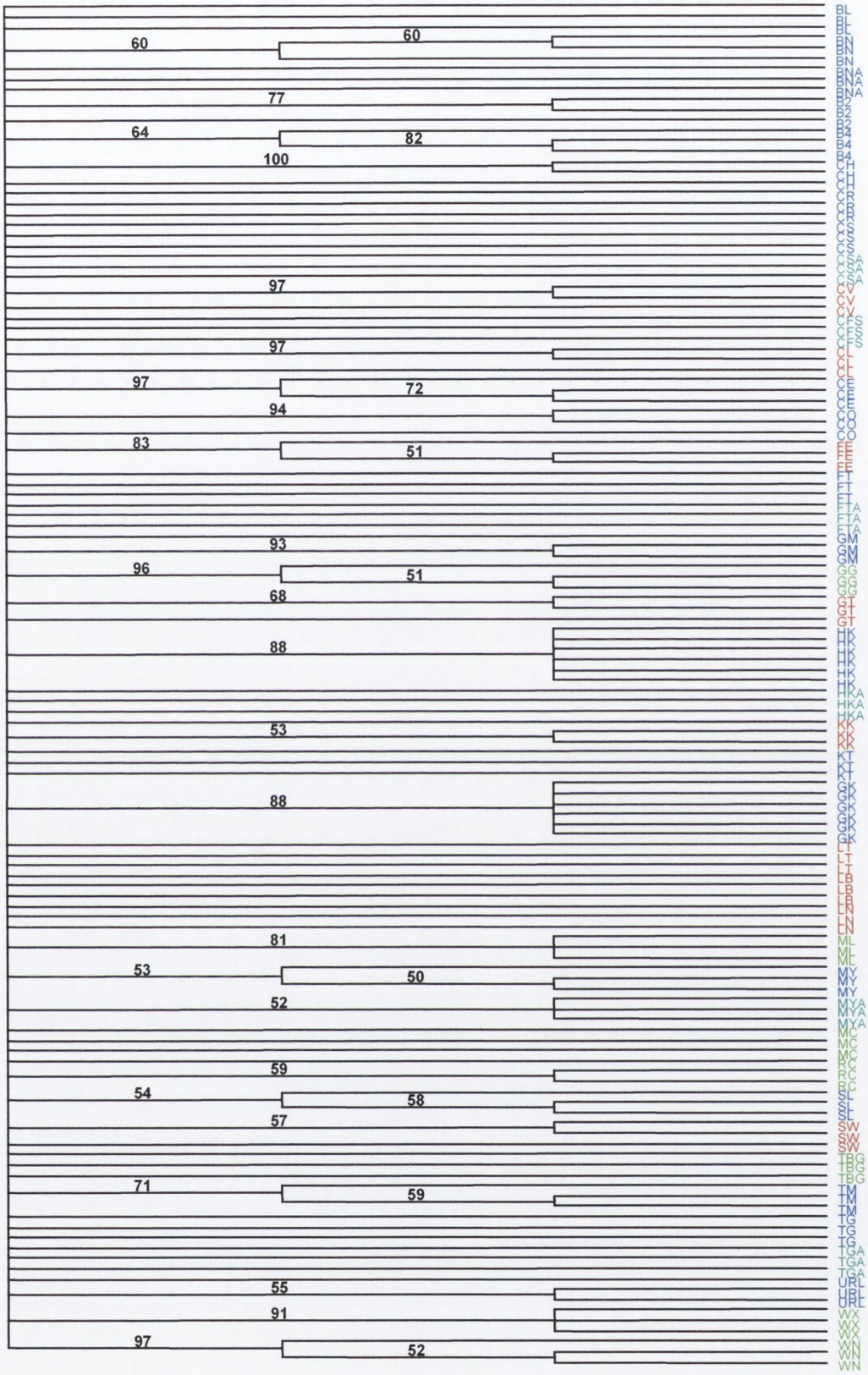

Figure 2.4b: A NJ BS tree based on Nei & Li (1979) distance measure of the AFLP data from 42 populations of *R. repens* sampled from different habitat types; Turlough populations are denoted in blue, turlough adjacent in light blue, wet pasture in red and dry pasture in green.

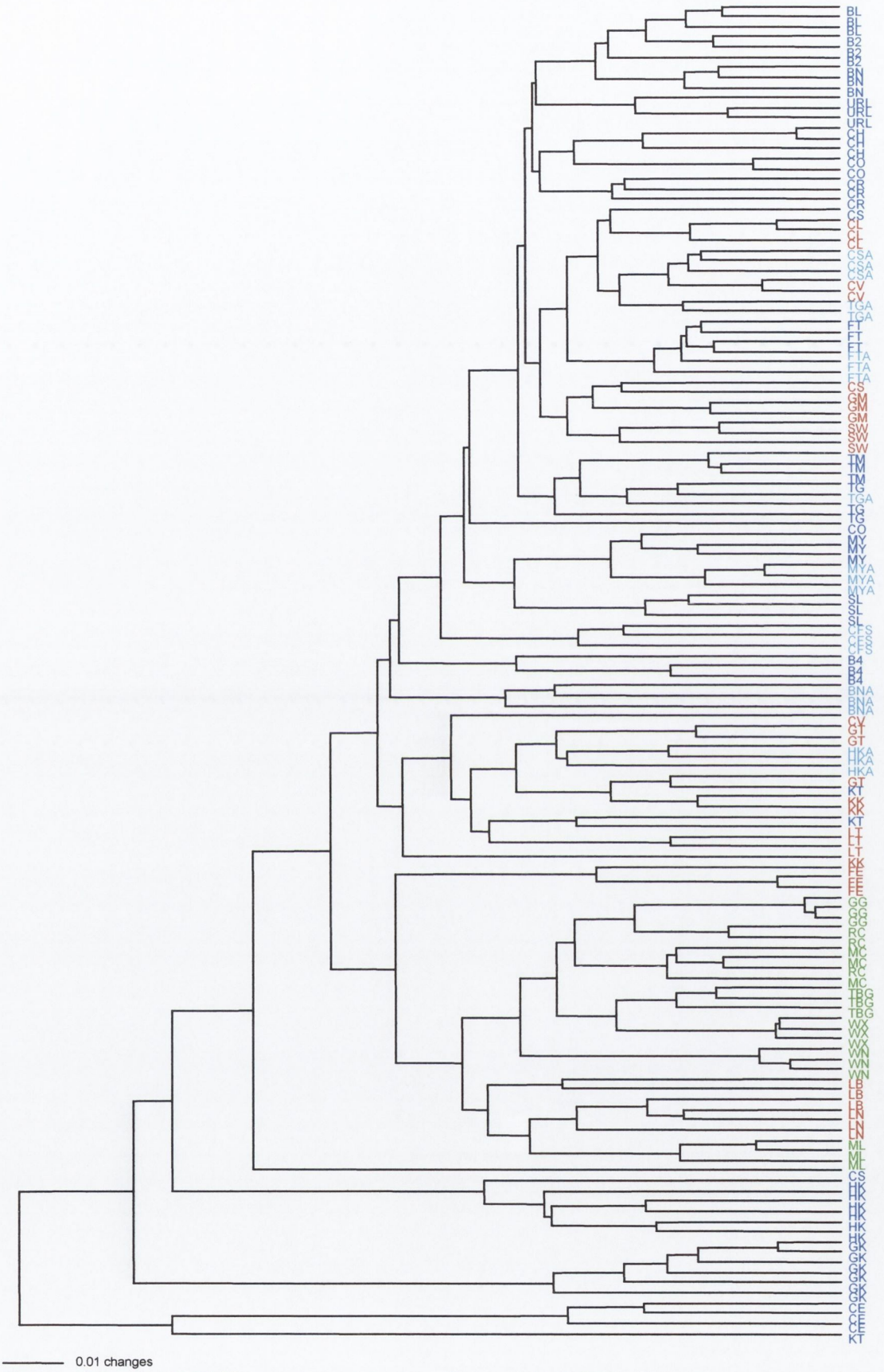

Figure 2.5a: A UPGMA tree based on the Nei and Li (1979) distance measure of the AFLP data from 42 populations of *R.repens* sampled from different habitat types; Turlough populations are denoted in blue, turlough adjacent in light blue, wet pasture in red and ruderal in green.

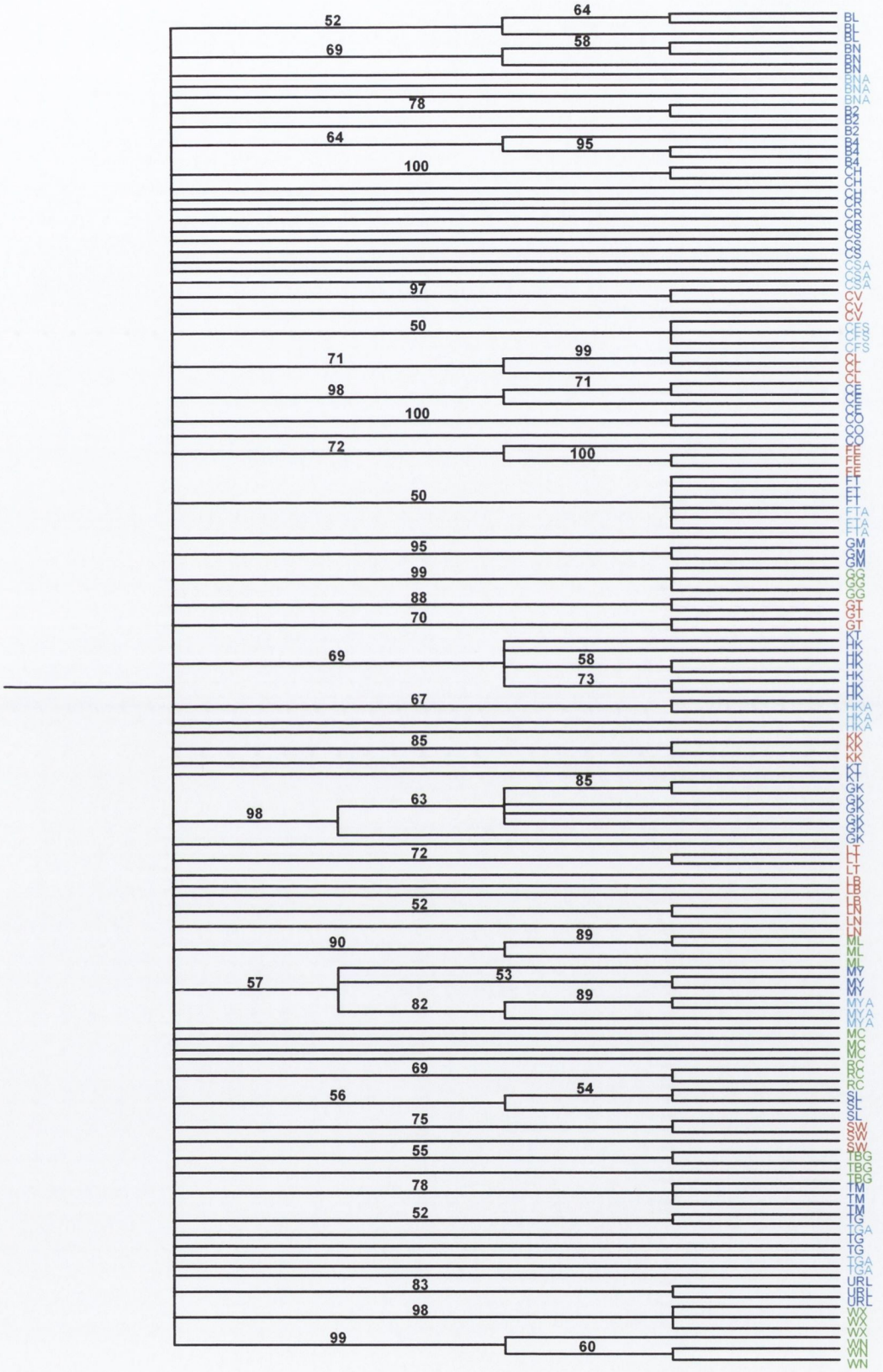

Figure 2.5b: A UPGMA BS tree based on the Nei and Li (1979) distance measure of the AFLP data from 42 populations of *R.repens* sampled from different habitat types; Turlough populations are denoted in blue, turlough adjacent in light blue, wet pasture in red and ruderal in green.

these turloughs are found relatively close to each other; and the populations sampled from the north of Ireland (WH, ML, CV, RC, LB, and LN) grouping together.

Figure 2.4b and 2.5b show the bootstrap consensus trees for the UPGMA and NJ analyses respectively. Few of the groupings were given bootstrap support (values less than 50), and some branches were given higher bootstrap support than others in both sets of analyses, such as those grouping individuals from the same populations together. All bootstrap support in both sets of analysis above 50% is for the grouping of individuals in their population units. There is no bootstrap support above 50% assigned to different groups such as turlough populations from wet pasture or dry pasture populations (Figures 2.4b, 2.5b).

An additional NJ analysis was conducted which included the individuals sampled from the four Norfolk populations. The data were not analysed using UPGMA as this method showed similar results to the NJ analysis for the 42 Irish populations. Figure 2.6a shows the NJ unrooted phylogram of the 42 Irish populations as well as the 4 Norfolk populations of *R. repens*, with the Norfolk populations included as an outgroup. Individuals from the same populations generally group together. High bootstrap support is given for the separation of the Norfolk mere populations from the rest of the 42 Irish populations (99%), (Figure 2.6b).

2.3.4 Principal co-ordinates analysis (PCO)

The Jaccard (1908) similarity coefficient was used in the PCO analysis of the AFLP data from 42 populations of *R. repens* comprising of 132 individuals in total. The first three principal coordinates accounted for 23% of variation in the AFLP data. The resulting ordinations were plotted in numerous ways, in order to try to make ecological sense of the results. Firstly, the scatterplots of the individuals are labelled according to population (Figures 2.7a, 2.7b, and 2.7c) for each combination of the first three axes. Secondly, ordinations for each individual were categorised in terms of one of four habitat types: turlough, wet pasture, dry pasture and the additional category of populations adjacent to turloughs (turlough adjacent) (Figures 2.8a, 2.8b, and 2.8c). This fourth category is important, as although the populations in this category are generally dry pasture, they were collected nearby their respective turlough populations.

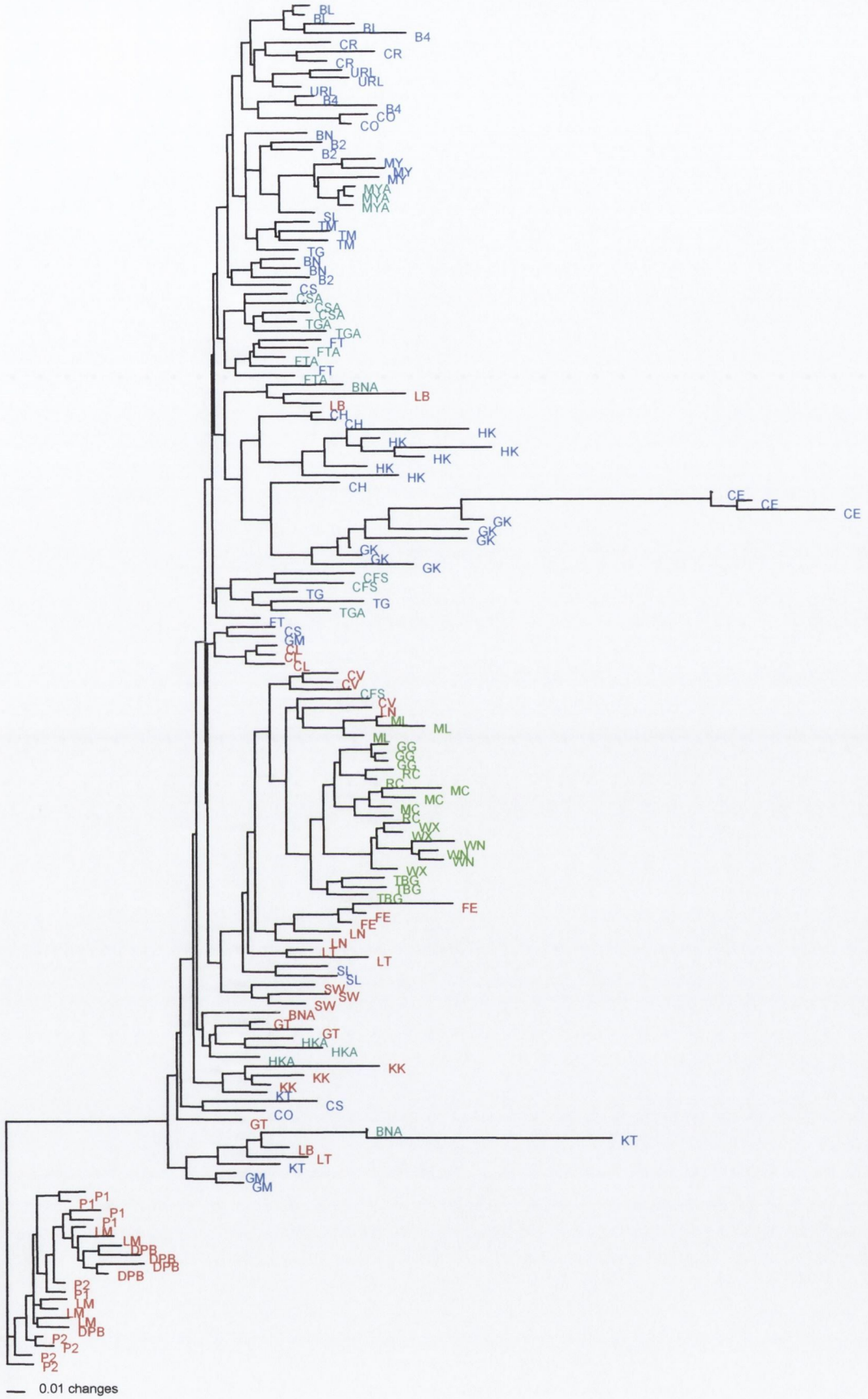

Figure 2.6a: A NJ tree based on the Nei & Li (1979) distance measure of the AFLP data from 42 Irish populations, and 4 Norfolk populations of *R. repens* sampled from different habitat types; turlough populations are denoted in blue, turlough adjacent in light blue, wet pasture and mere in red and dry pasture in green.

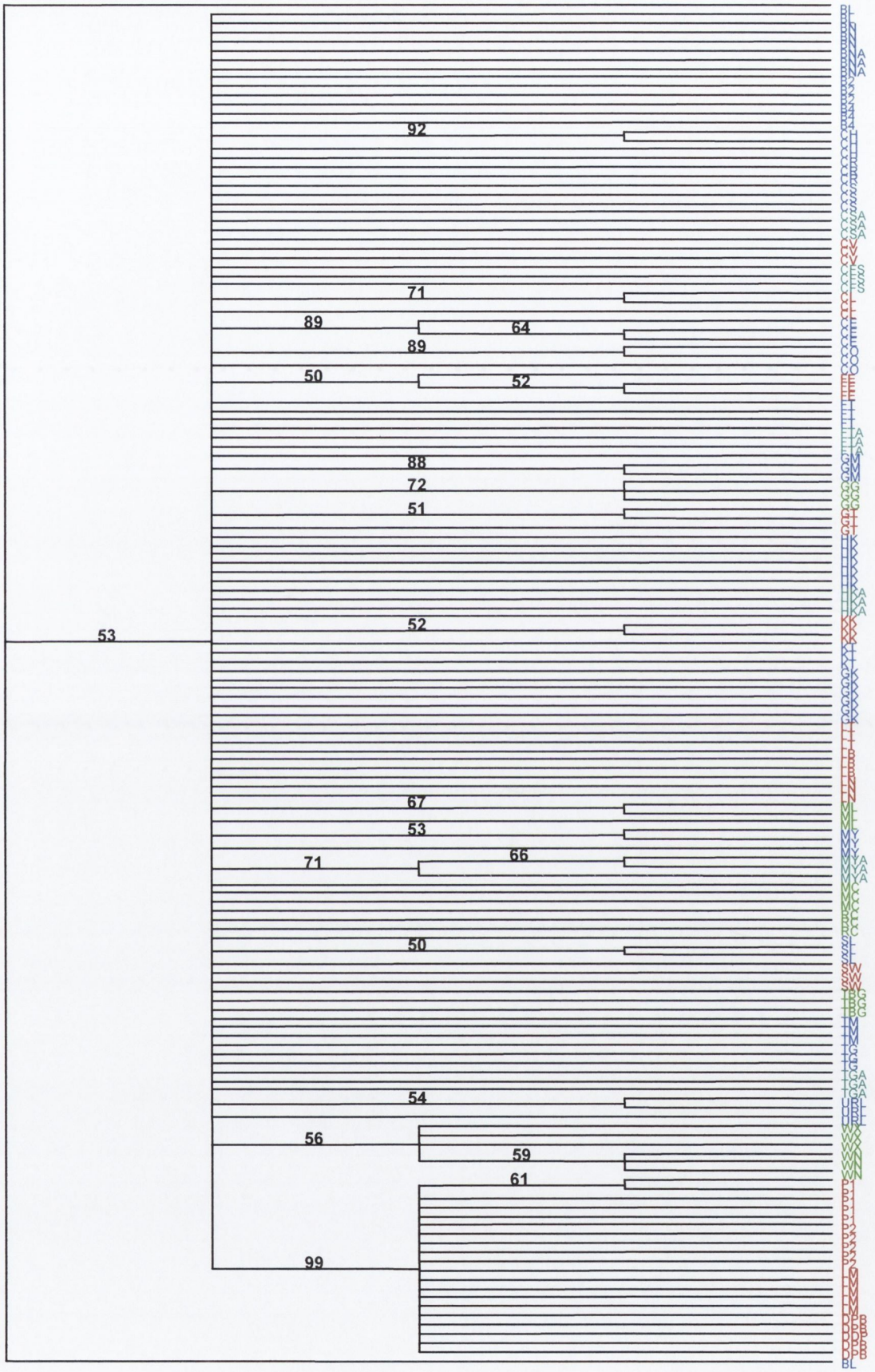

Figure 2.6b: A NJ BS tree based on the Nei & Li (1979) distance measure of the AFLP data from 42 Irish populations, and 4 Norfolk populations of *R. repens* sampled from different habitat types; turlough populations are denoted in blue, turlough adjacent in light blue, wet pasture and mere in red and dry pasture in green.

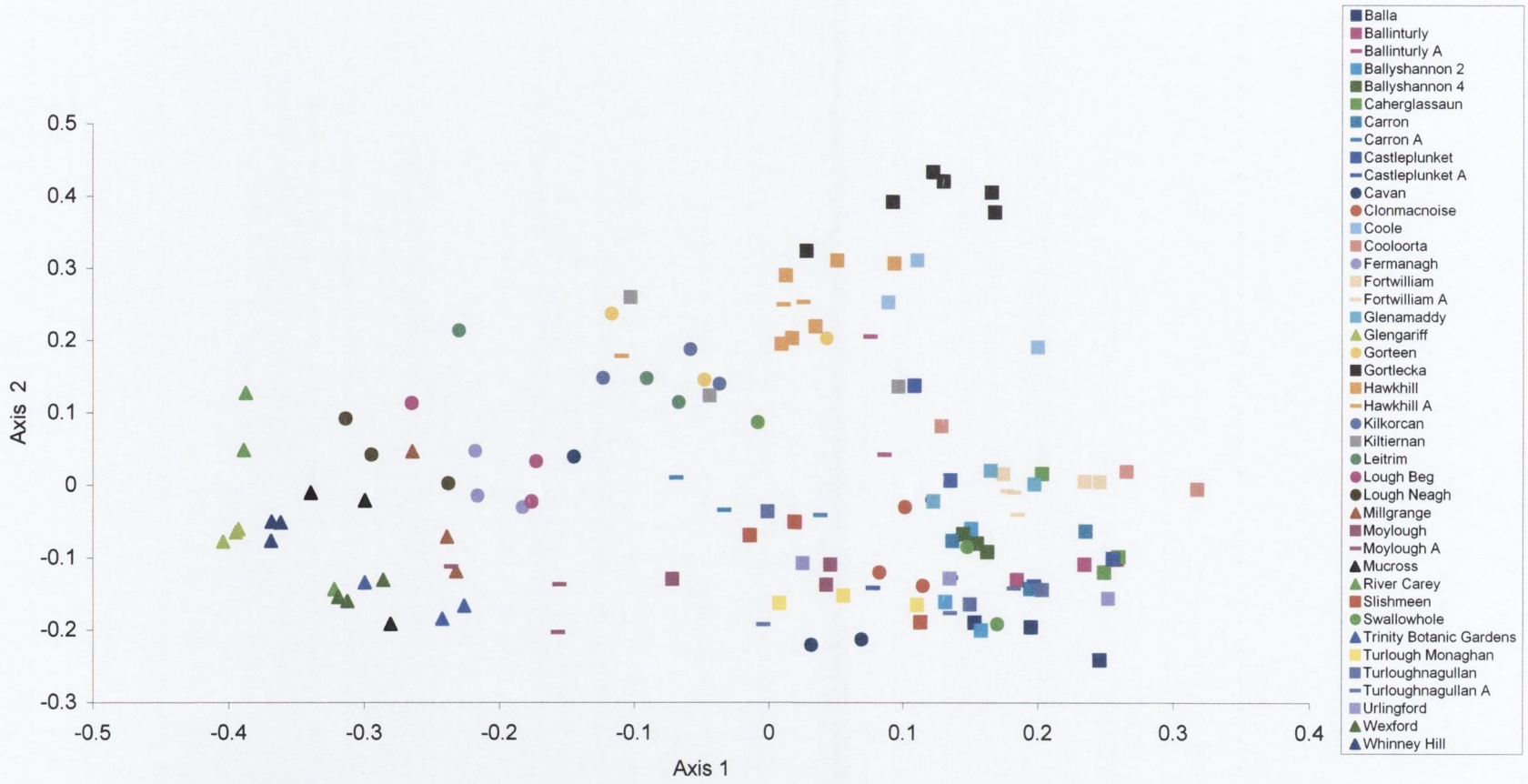

Figure 2.7a: Axis 1 and 2 of a PCO analysis of AFLP data from 42 populations of *R.repens* sampled from a number of habitat types. Each symbol corresponds to a different habitat type, and colours represent different populations within these habitat types.

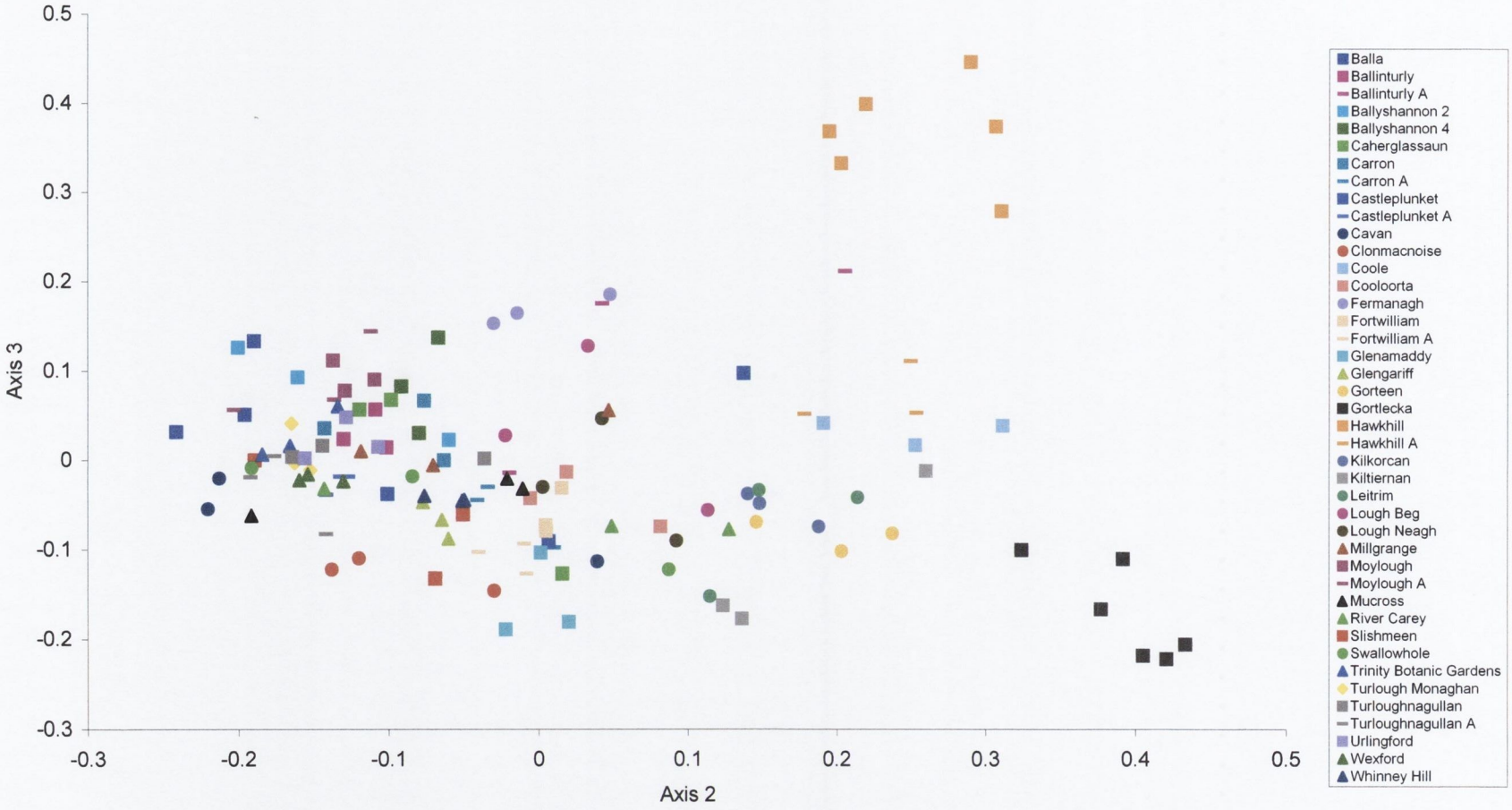

Figure 2.7b: Axis 2 and 3 of a PCO analysis of AFLP data from 42 populations of *R.repens* sampled from a number of habitat types. Each symbol corresponds to a different habitat type, and colours represent different populations within these habitat types.

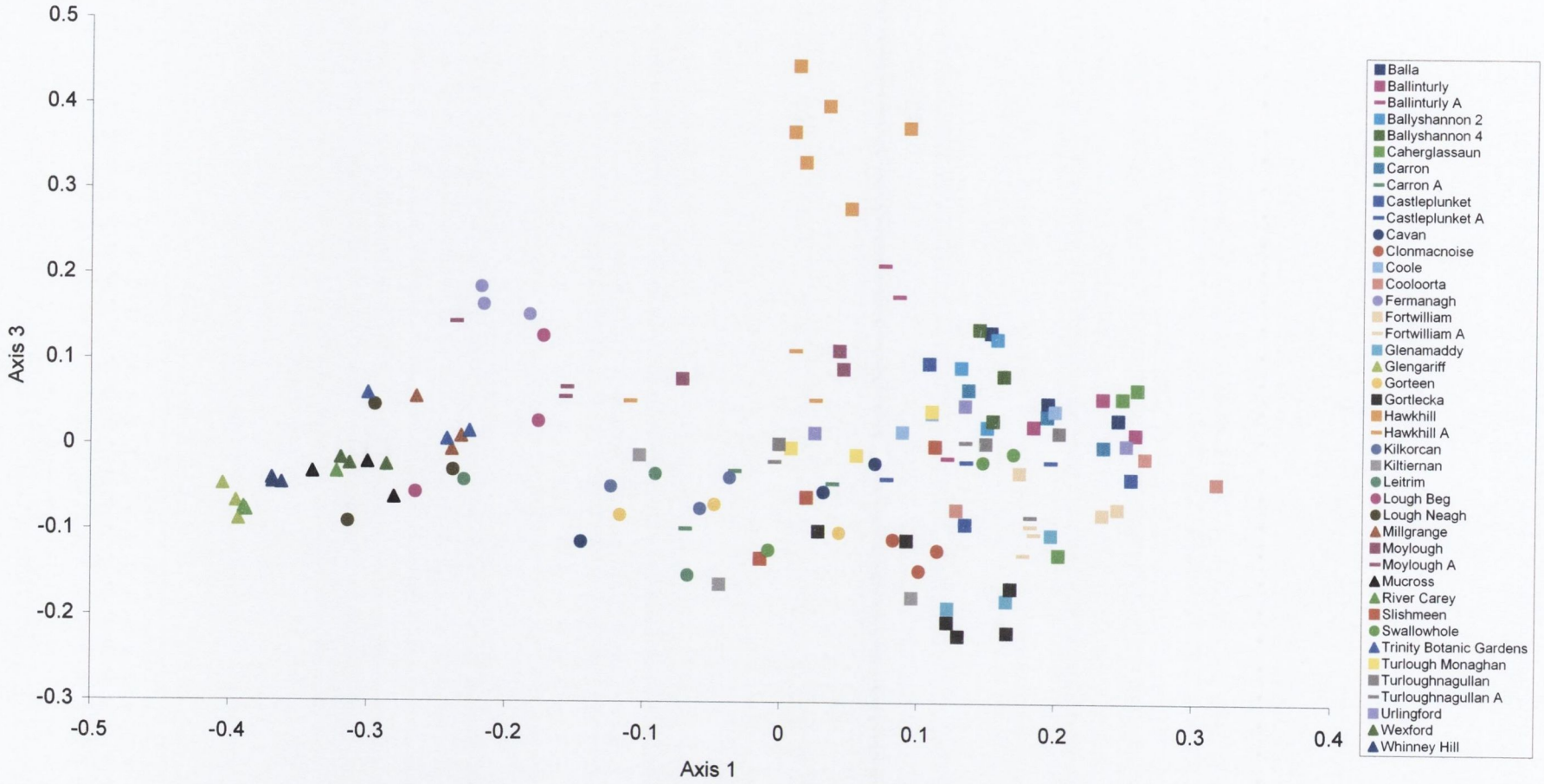

Figure 2.7c: Axis 1 and 3 of a PCO analysis of AFLP data from 42 populations of *R.repens* sampled from a number of habitat types. Each symbol corresponds to a different habitat type, and colours represent different populations within these habitat types.

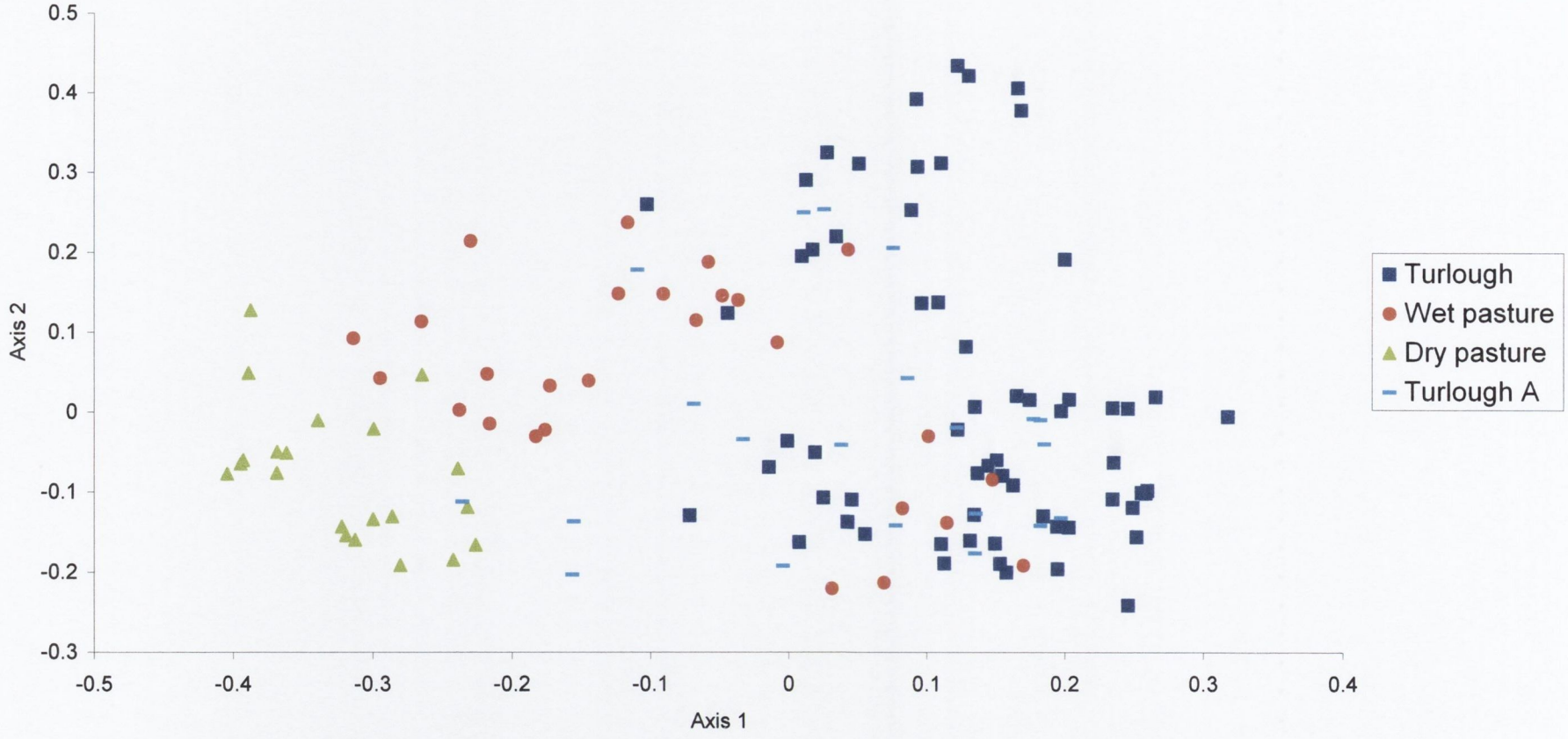

Figure 2.8a: Axes 1 and 2 of a PCO analysis obtained from AFLP data for all individuals from 42 populations of *R.repens*. Samples are coded according to one of four habitat types.

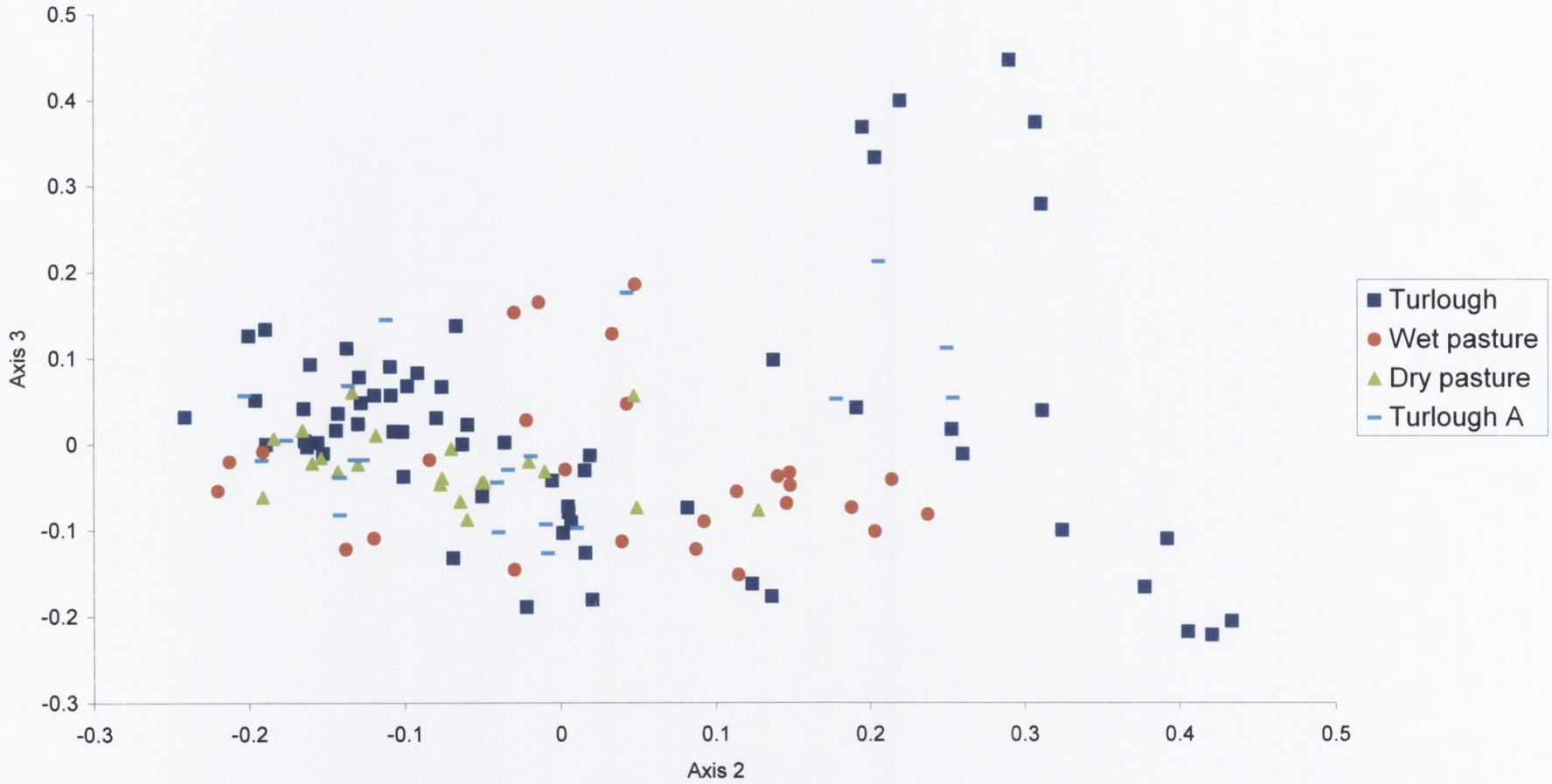

Figure 2.8b: Axis 2 and 3 of a PCO analysis obtained from AFLP data for all individuals from 42 populations of *R.repens*. Samples are coded according to one of four habitat types.

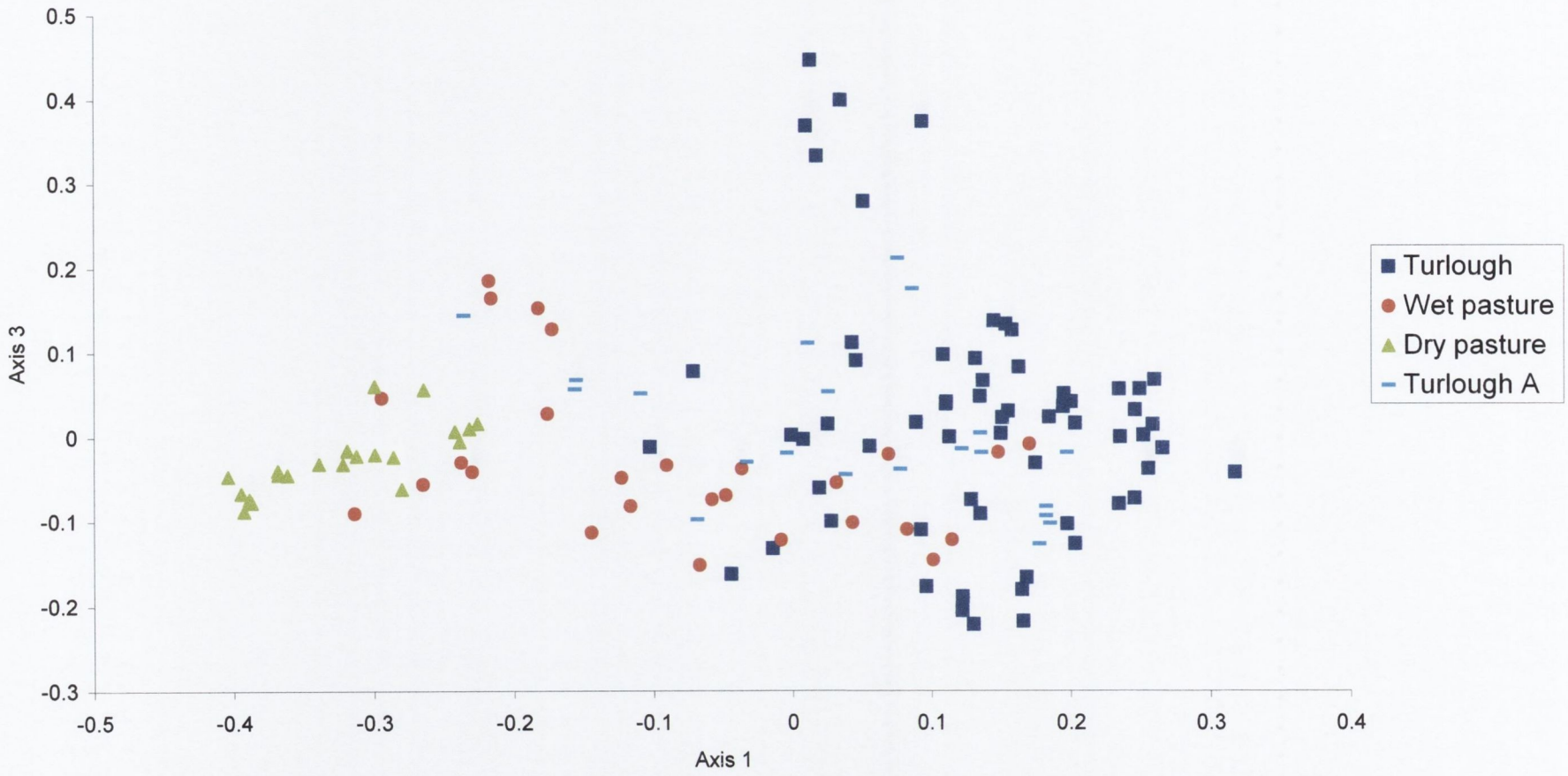

Figure 2.8c: Axis 1 and 3 of a PCO analysis obtained from AFLP data for all individuals from 42 populations of *R.repens*. Samples are coded according to one of four habitat types.

When individuals were plotted in terms of their population unit (Figures 2.7a, b and c) little sense could be made from the scatterplots. Due to the high levels of variation and low number of individuals sampled from each population there appears to be little clustering in the ordination space. However, it was evident from these figures that individuals clustered into their respective population unit. The use of different symbols for different habitat types shows that populations generally separate in terms of ecology. Generally, the first principal coordinate separates individuals from turlough populations from the rest. At the negative end of axis 1 are positioned individuals from the Glengariff, Whinney Hill, Wexford, and Muckross populations that are all dry pasture, and at the positive end of axis 1 are found turlough populations such as Turloughnagullan, Balla, Cooloorta, Fortwilliam and Caherglassaun. The remaining two principal component ordinations of individuals are more difficult to interpret. There is a tendency for the second principal coordinate to separate a sub group of turlough populations from the remaining populations; at the negative end of the spectrum are found turlough populations such as Balla and Moylough, whereas the positive end represents Hawkhill, Gortlecka and Kiltiernan. Axis three is difficult to interpret but it separates a turlough population subgroup, that population being Hawkhill. Axis two and three only account for approximately 8 and 4% of the variation respectively so it is not surprising that they appear to show relatively no pattern in the data. Not all turlough adjacent populations behave in the same manner with some being close to their respective turlough population in the ordination space (HK and HKA, TG and TGA, FT and FTA) while others form separate population units in the ordination space (MY and MYA, BT and BTA).

Figures 2.8a, 2.8b and 2.8c show scatterplots for individuals plotted according to one of four habitat types (turlough, wet pasture, dry pasture and turlough adjacent) with no discrimination between individual populations. These figures make much more ecological sense than do Figures 2.7a, b, and c. The first axis separates turlough individuals from dry pasture individuals. Turlough individuals are positioned to the right on the first ordination axis whereas dry pasture samples are found to the left of axis 1. Wet pasture individuals are found scattered between the two extreme habitat types of turlough and dry pasture. Generally turlough adjacent individuals are found nested among the turlough individuals but there are some exceptions such as MA and CFS individuals that are grouped together in their population units but are separate

from their turlough neighbours in the ordination space (Figures 2.8a, c). Axes two and three are more troublesome to interpret. Figure 2.8b reveals a subgroup of turlough individuals to the right of axis two, this loosely grouped cluster consists of GK, CH, and BL individuals. Figure 2.8c again separates turlough individuals from dry pasture individuals on the first axis, principal coordinate three separates GK turlough individuals from the rest.

A PCO analysis was also conducted with the inclusion of the four Norfolk mere populations (Figure 2.9a, b and c). Individuals were categorised in terms of five habitat types; turlough, turlough adjacent, dry pasture, wet pasture and mere. The first three axes accounted for 28% of the total variation in the data. It is apparent from Figure 2.9a that *R. repens* individuals from the Norfolk meres are very similar to each other but very different to the individuals from any of the Irish populations regardless of habitat type. Axis one clearly separates the Norfolk mere populations from the remaining populations. Figure 2.9b shows the Norfolk mere populations nestled among the wet pasture populations, intermediate in space between the turlough and dry pasture populations. Figure 2.9c again separates the Norfolk populations from the Irish populations showing that axis three accounts for only a small part of the variation in the data.

2.3.5 Non parametric multi – dimensional scaling (NMDS)

Scatterplots resulting from the non-parametric multidimensional scaling analysis trials showed that the best analysis options for this data set (i.e. results showing the lowest stress in the real data) were to use Jaccard's similarity coefficient, with three unrotated axes. The table below (Table 2.12) compares the stress obtained from the real data with stress obtained from randomised data, each of the 1-D to 3-D solutions provide significantly more reduction in stress than expected by chance, accepting a probability of Type I error < 0.05. This method allowed for the examination of significant structure in the data ($P = 0.01$).

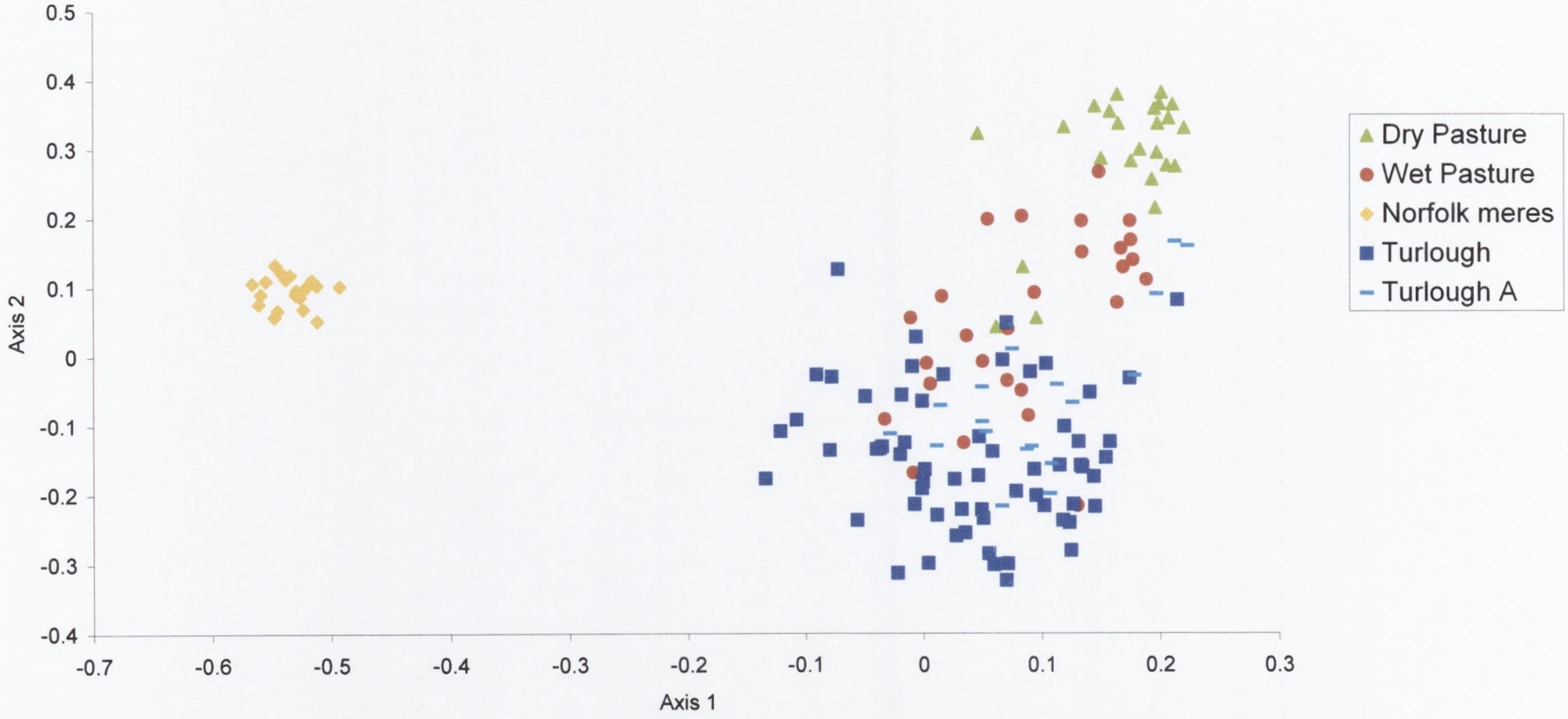

Figure 2.9a: Axes 1 and 2 of a PCO analysis from AFLP data for all individuals from 42 Irish populations, plus 4 UK populations of *R.repens*. Samples are coded according to one of five habitat types.

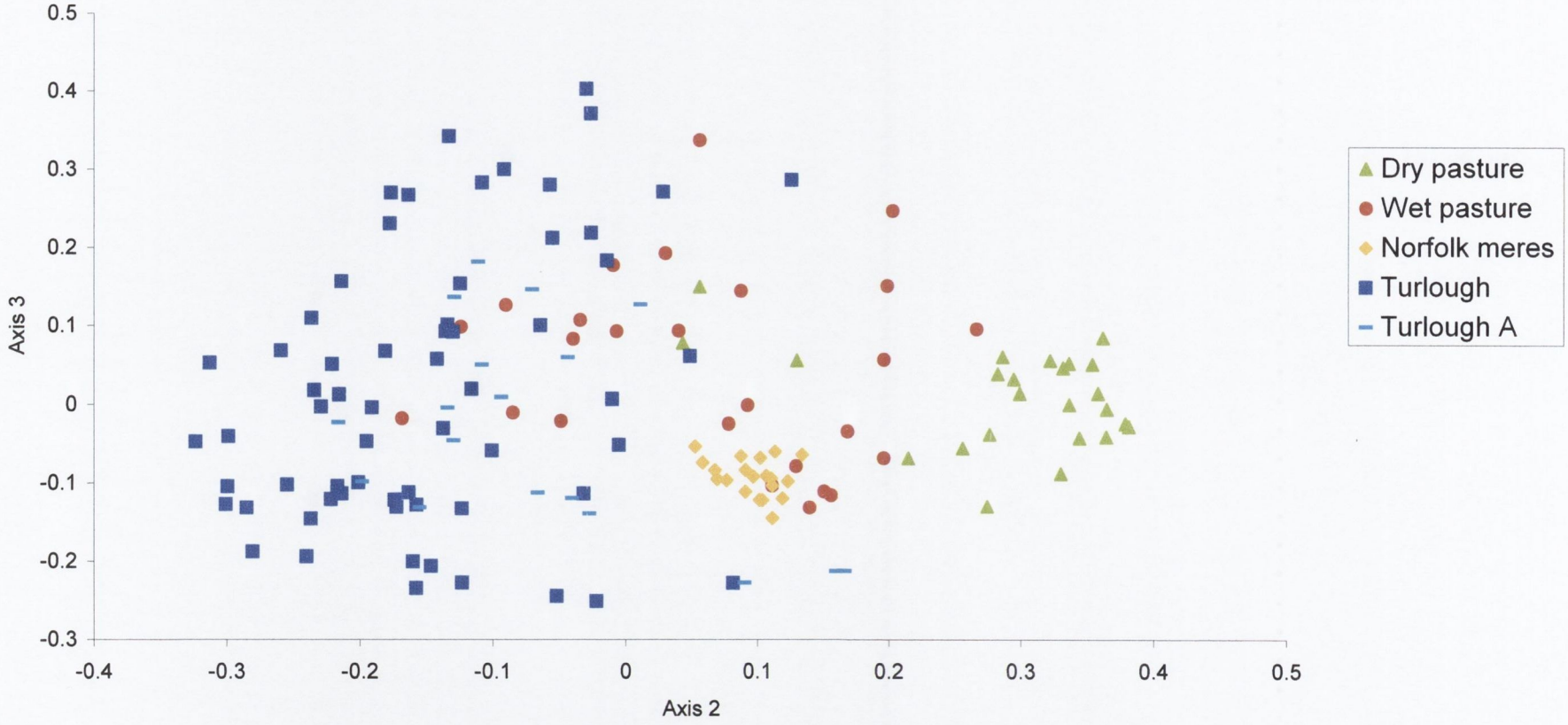

Figure 2.9b: Axes 2 and 3 of a PCO analysis from AFLP data for all individuals from 42 Irish populations, plus four UK populations of *R.repens*. Samples are coded according to one of five habitat types.

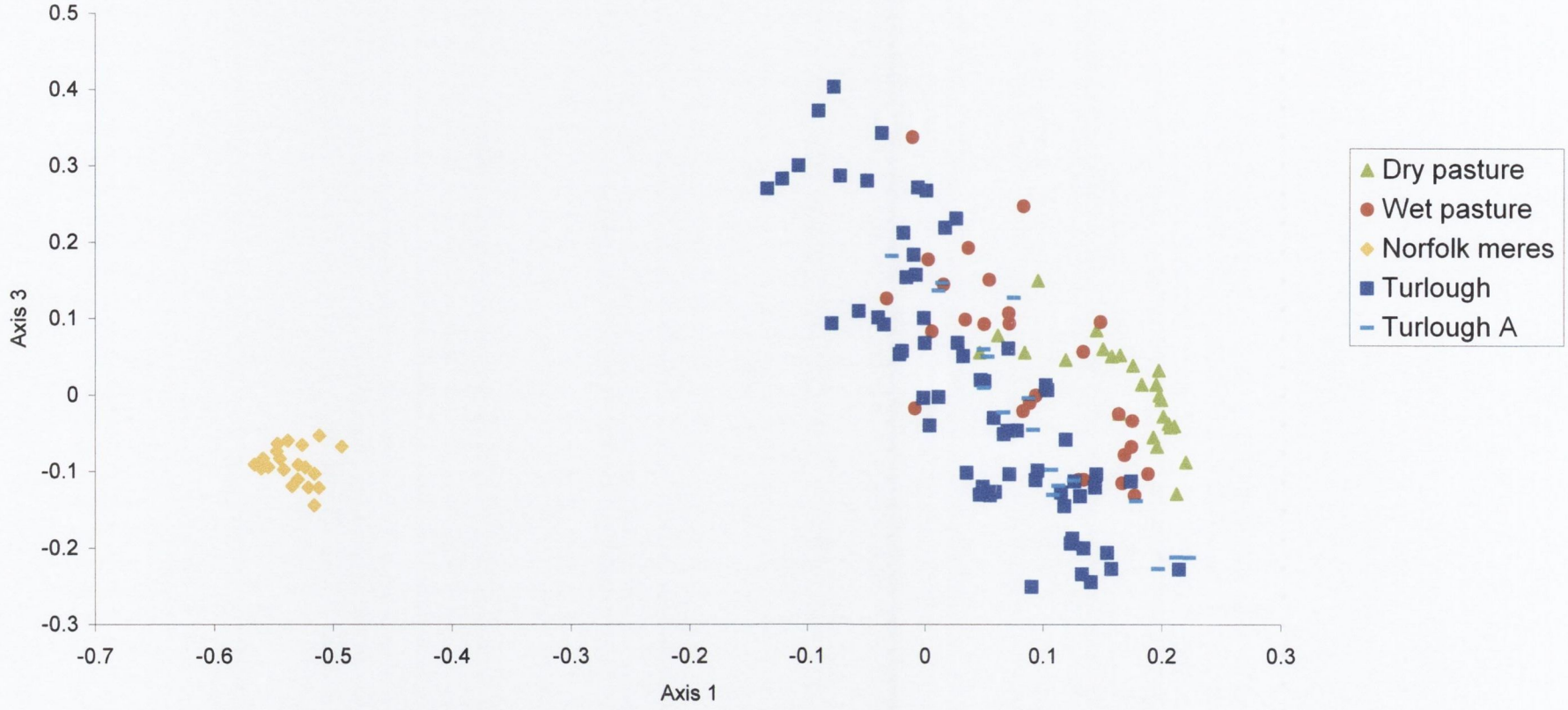

Figure 2.9c: Axes 1 and 3 of a PCO analysis from AFLP data for all individuals from 42 Irish populations, plus four UK populations of *R.repens*. Samples are coded according to one of five habitat types.

Table 2.12: Showing stress as a function of dimensionality, Stress in real data is compared with stress in randomized data (Monte Carlo test, 100 runs). p = proportion of randomized runs with stress < or = observed stress, i.e., p = (1 + number of permutations <= observed)/(1 + number of permutations).

Dimensions	Stress	Change	Mean	Minimum	Maximum	p
1	43.821	0	54.141	51.949	57.404	0.01
2	24.348	19.473	37.824	36.361	40.297	0.01
3	17.336	7.012	29.675	28.483	32.233	0.01

Generally, the results from this method of analysis were very similar to those obtained from the principal coordinate analysis. Following the results from the PCO, individuals were plotted according to population. As with the PCO analysis little structure can be found in the resulting scatterplots (Figures 2.10a, 2.10b and 2.10c), apart from the clustering of individuals into populations. As in the principal co ordinates analysis the first dimension separates turlough from dry pasture populations (Figure 2.10a). Individuals from the same populations are grouping together, and populations collected adjacent to turlough populations are generally grouping with their corresponding turlough site, with the same notable exceptions of BTA and MYA individuals. As in the PCO analysis the interpretation of the second and third dimensions are more difficult to decipher. There does not appear to be any clear groupings resulting from the scatterplots of these two dimensions (Figure 2.10b and 2.10c), although Figure 2.10b appears to separate certain populations from the rest (HK, FE and GK).

Figures 2.11a, 2.11b and 2.11c show scatterplots for each of the possible dimension combinations from the results when individuals were categorised in terms of one of four habitat types. The results for this set of analyses do not separate individuals from different habitat types as clearly as in the PCO analysis although the same general trends are apparent. Dimension 1 separates dry pasture individuals from turlough individuals with wet pasture samples being scattered between the two groups, although these wet pasture individuals do appear to be closer to dry pasture individuals in the ordination space. Figure 2.11a reveals that some turlough adjacent samples are nested among the turlough samples (FT and FTA, HK and HKA) with the

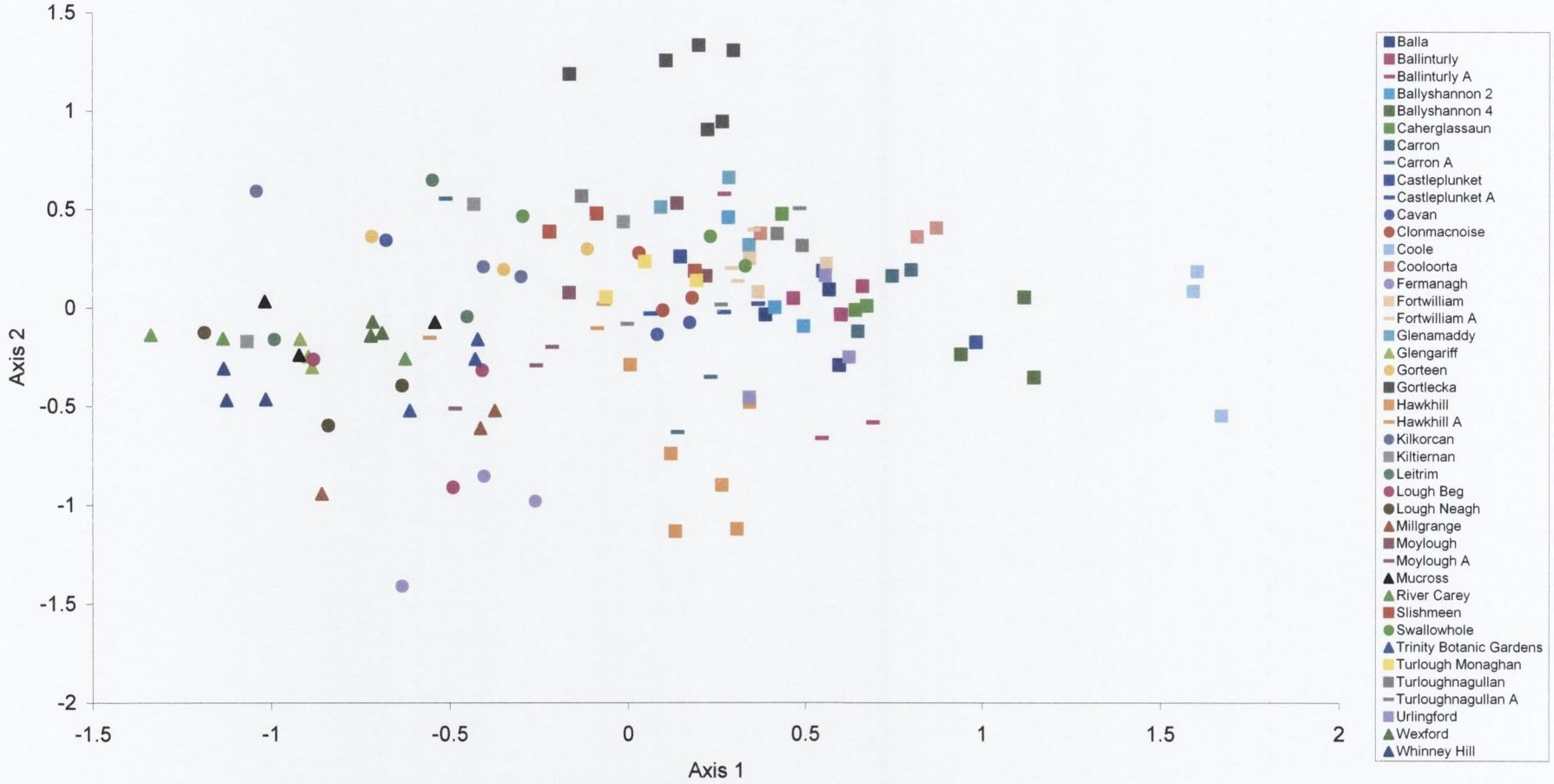

Figure 2.10a: Axes 1 and 2 from NMDS analysis obtained from the AFLP data for all individuals from 42 populations of *R.repens*. Each symbol corresponds to a different habitat type, and colours represent different populations within these habitat types.

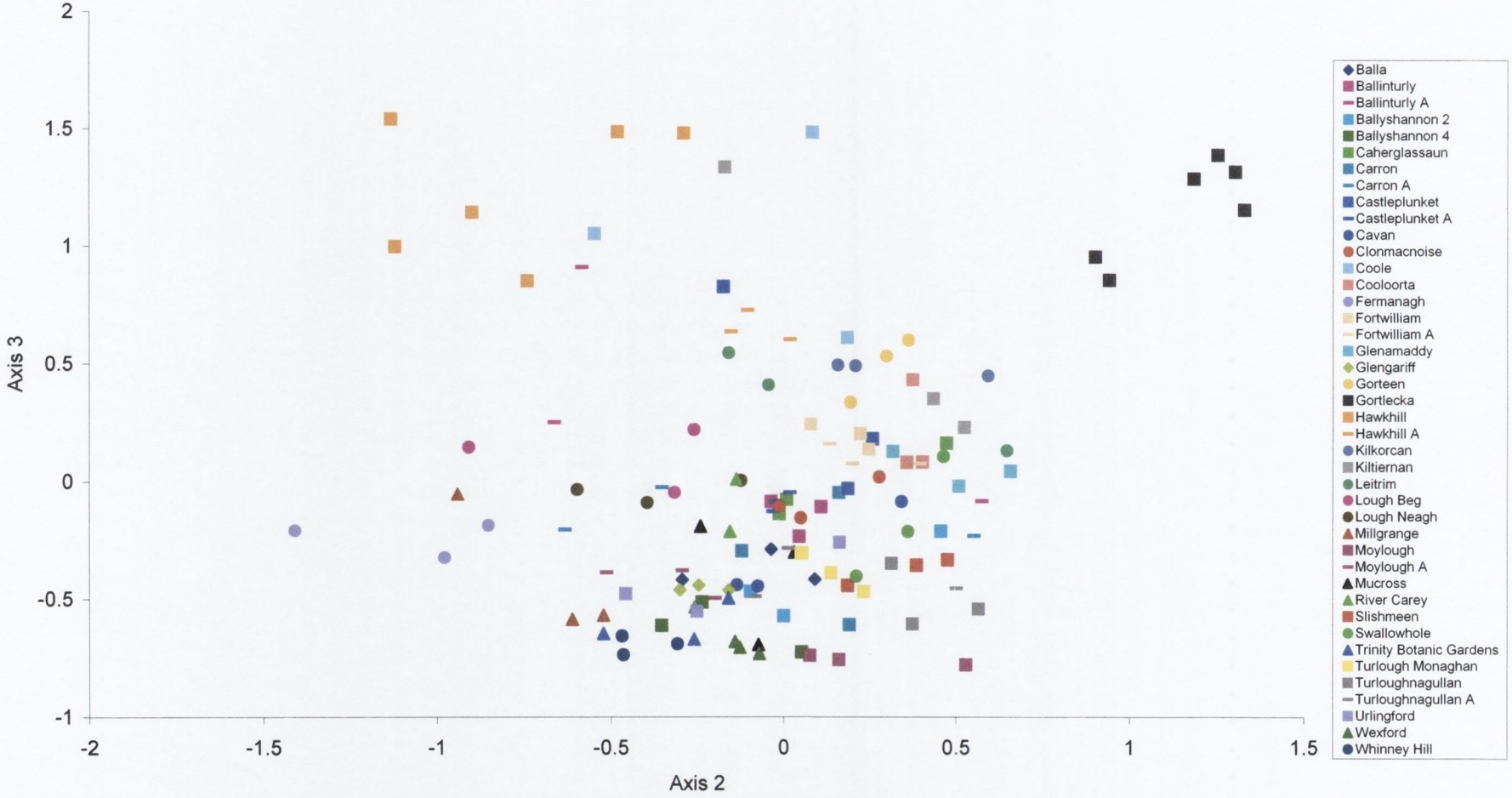

Figure 2.10b: Axes 2 and 3 from NMDS analysis obtained from AFLP data for all individuals from 42 populations of *R.repens*. Each symbol corresponds to a different habitat type, and colours represent different populations within these habitat types.

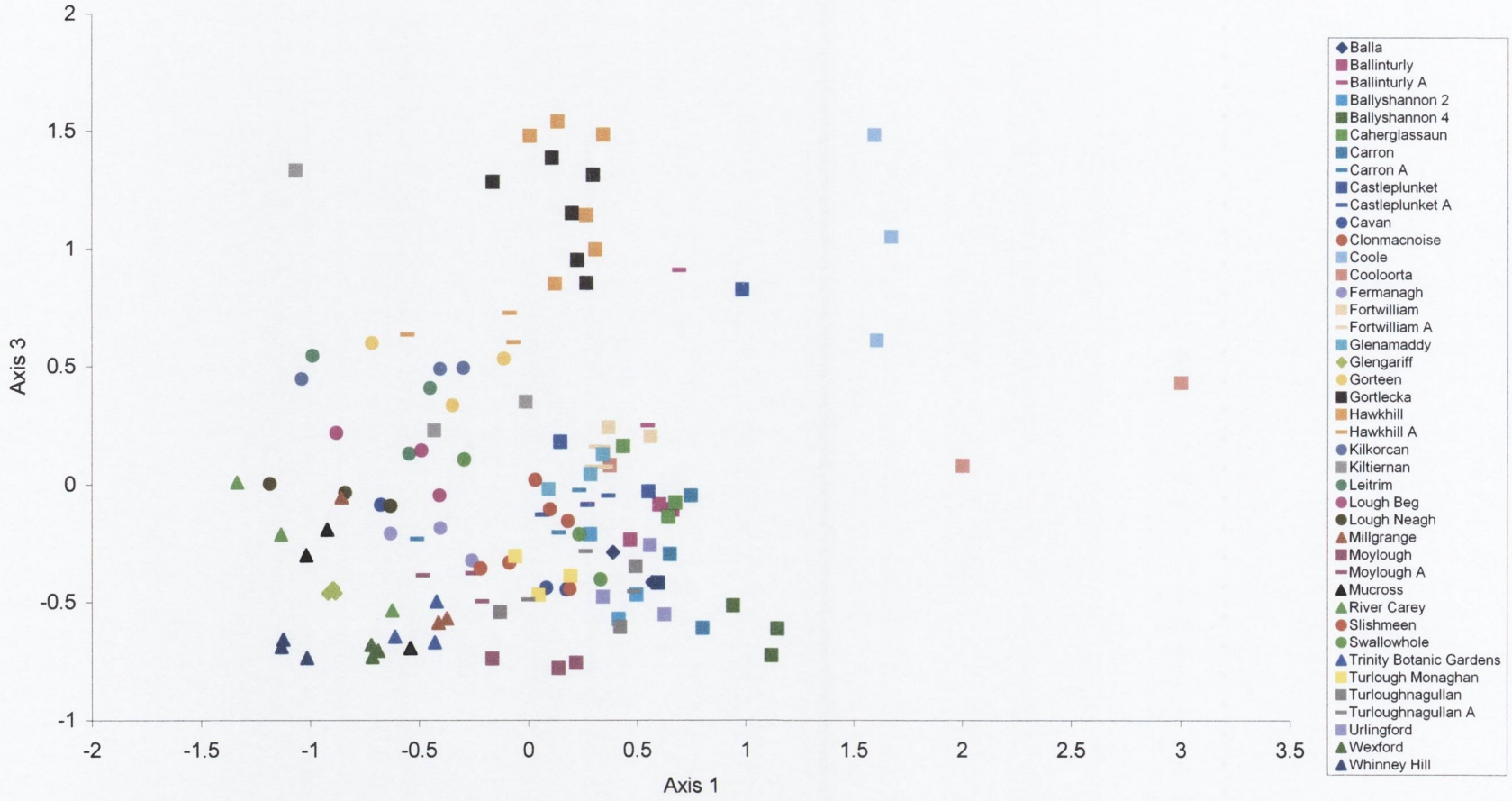

Figure 2.10c: Axes 1 and 3 from NMDS analysis obtained from AFLP data for all individuals from 42 populations of *R.repens*. Each symbol corresponds to a different habitat type, and colours represent different populations within these habitat types.

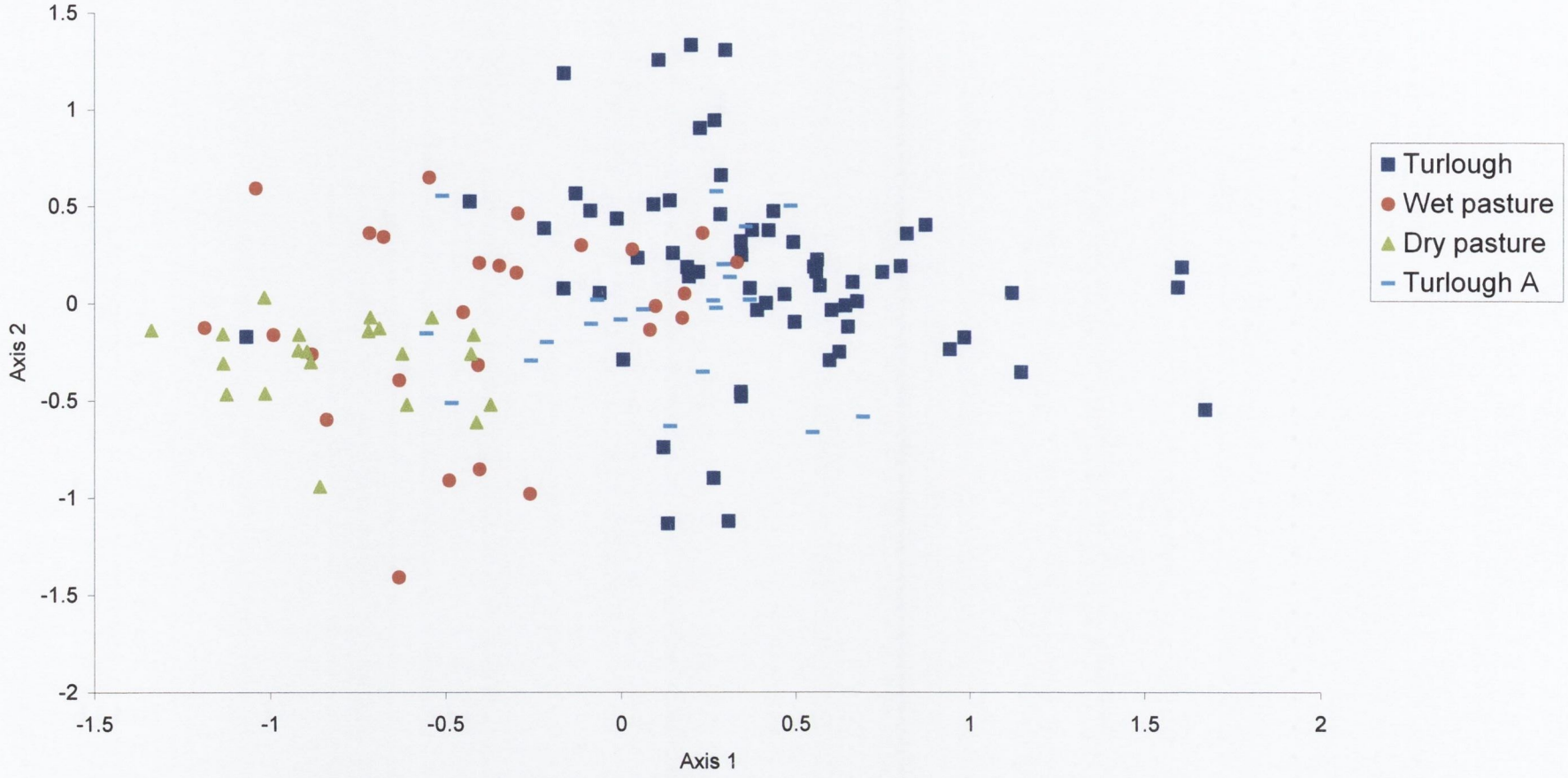

Figure 2.11a: Axes 1 and 2 of an NMDS analysis obtained from AFLP data for all individuals from 42 populations of *R.repens*. Samples are coded according to one of four habitat types.

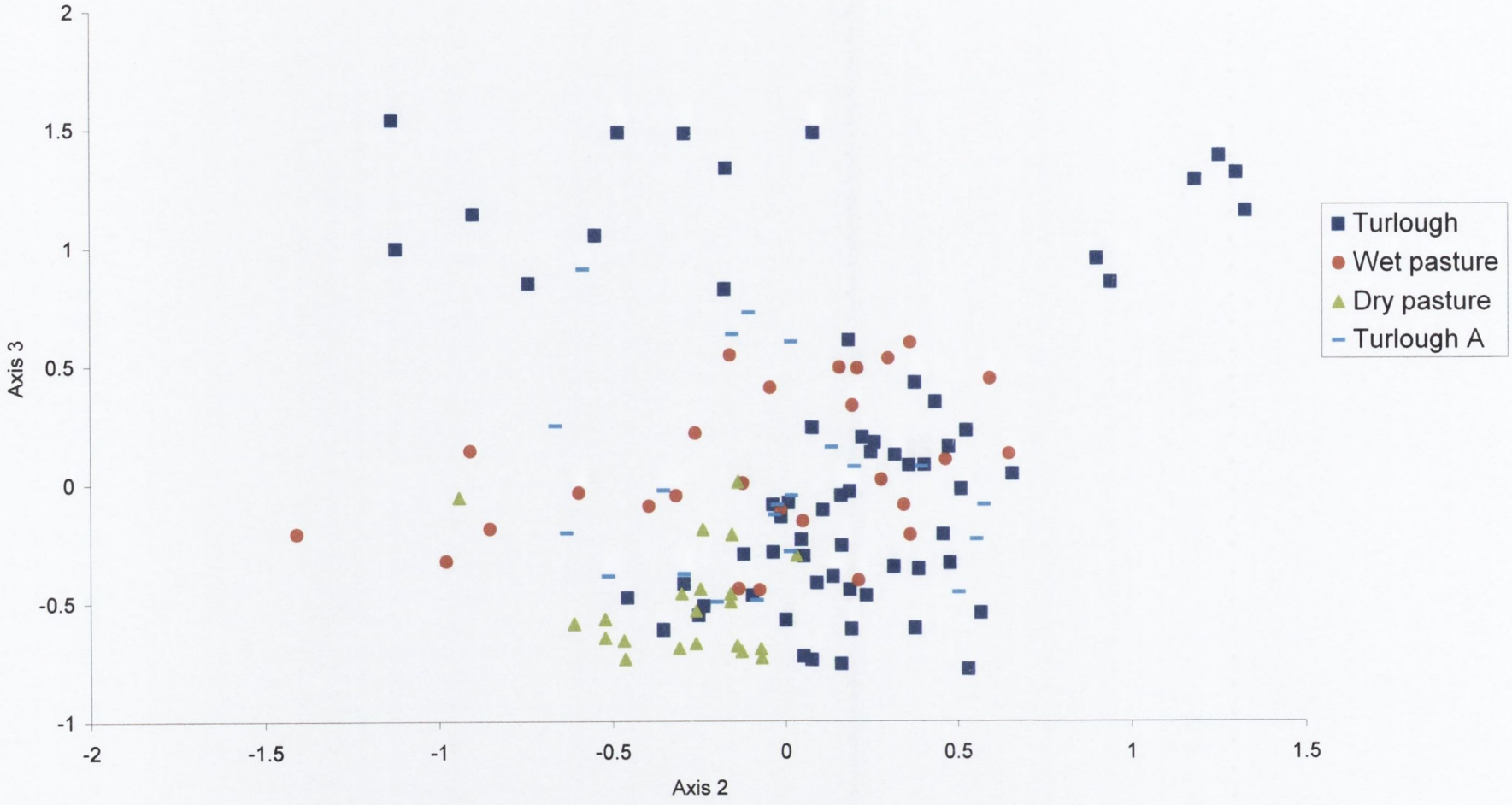

Figure 2.11b: Axes 2 and 3 of an NMDS analysis obtained from AFLP data for all individuals from 42 populations of *R.repens*. Samples are coded according to one of four habitat types.

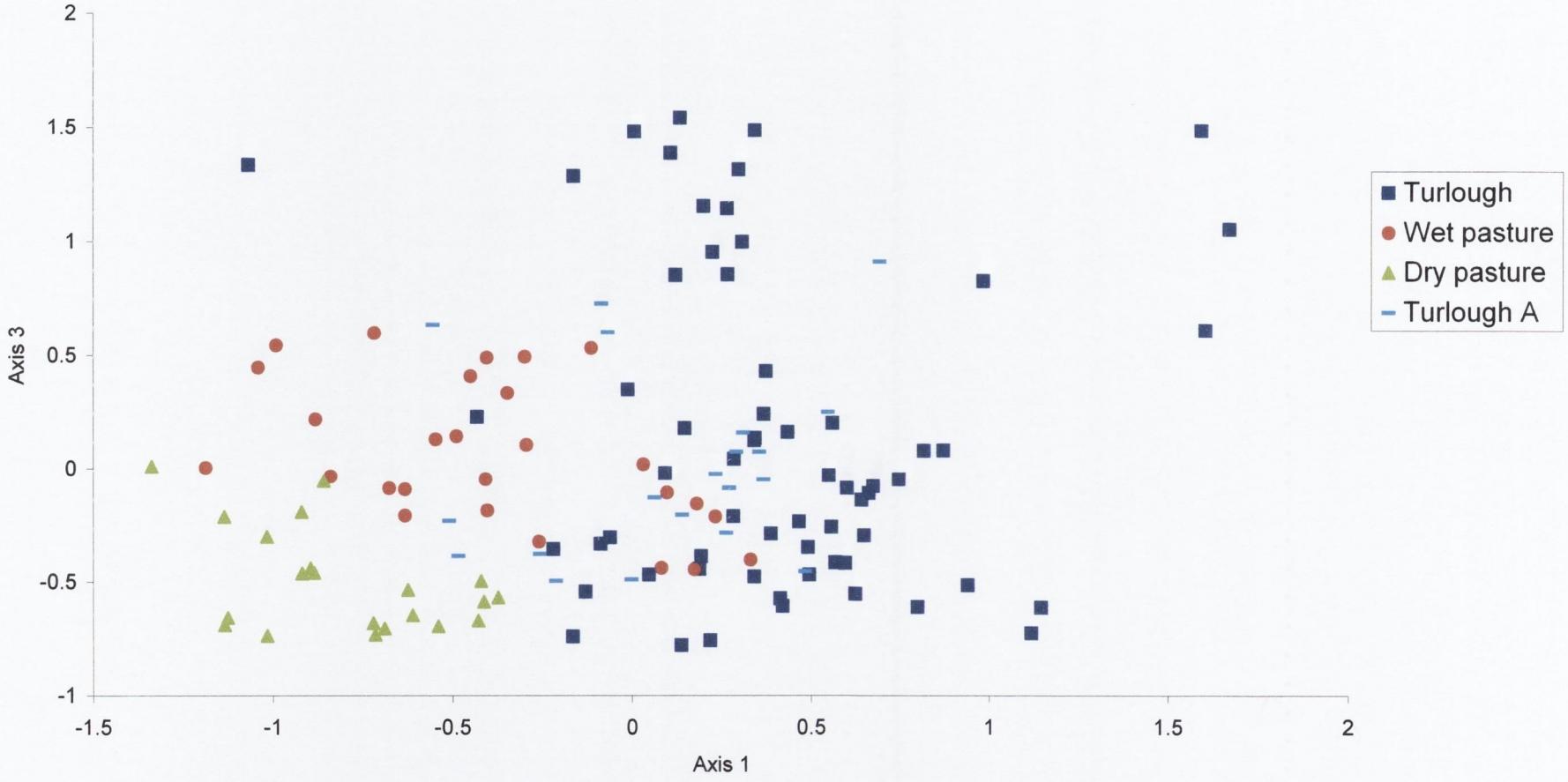

Figure 2.11c: Axes 1 and 3 of an NMDS analysis obtained from AFLP data for all individuals from 42 populations of *R.repens*. Samples are coded according to one of four habitat types.

same exceptions as found in the PCO analysis (BT and BTA, MY and MYA). NMDS dimensions 2 and 3 are more difficult to interpret. Figure 2.11b appears to separate a subgroup of turlough individuals on axis 3 that comprises of HK and GK individuals. Figure 2.11c makes more ecological sense than Figure 2.11b with dry pasture individuals being separate from turlough individuals, and wet pasture individuals in an intermediate position between these two extreme habitat types.

2.3.6 Diversity estimates

Data on the number and distribution of polymorphic loci detected with the combined three AFLP primer pairs and an estimation of Nei's genetic diversity (Nei 1987) for all loci in individual populations are given in Table 2.13. The percentage of polymorphic loci was generally lowest in the dry pasture populations. Populations with the lowest percentages were Wexford and Whinney Hill (5%), Muckross (11%), River Carey (12%), and Fermanagh (13%), which was the notable exception, being a wet pasture population. The Glengariff population had the lowest levels of polymorphism from all 42 populations included in the analysis, with only 4 out of a total of 176 loci being polymorphic (Table 2.13). Turlough populations generally revealed the highest percentage of polymorphic loci. Among the highest were Cooloorta (28%), Turloughnagullan (29%), Glenamaddy (32%), and Castleplunket (36%), one notable exception was a population adjacent to Ballinturly turlough (34%) which had the second highest percentage of polymorphic loci from the 42 populations included in the analysis.

The highest levels of genetic diversity were found in turlough populations (Table 2.13). Among the highest were Cooloorta (H=0.1166), Glenamaddy (0.1185), Ballyshannon 2 (0.1196), Turloughnagullan (0.1211), with the highest of all being found in the Castleplunket population (0.1443). One non-turlough population did exhibit a relatively high level of genetic diversity (Ballinturly Adjacent; 0.1214). The lowest levels of genetic diversity were found in dry pasture populations such as Muckross (0.0473), River Carey (0.0390), Whinney Hill (0.0200), and Wexford (0.0196) with the lowest of all being found in the Glengariff population (H = 0.0090).

Table 2.13: Mean diversity values detected with three AFLP primer pairs for *R. repens* populations (N = number of samples; n = number of polymorphic loci; H = Nei's (1973) genetic diversity; % P = % polymorphic loci per population).

Number	Code	Location	N	n	H	% P
1	BL	Balla	3	35	0.0896	19.89
2	BN	Ballinturly	3	47	0.1057	26.70
3	BNA	Ballinturly Adjacent	3	60	0.1214	34.09
4	B2	Ballyshannon 2	3	47	0.1196	26.70
5	B4	Ballyshannon 4	3	46	0.1029	26.14
6	CH	Caherglassaun	3	36	0.0752	20.45
7	CR	Carron	3	45	0.0991	25.57
8	CS	Castleplunket	3	64	0.1443	36.36
9	CSA	Castleplunket Adjacent	3	35	0.0767	19.89
10	CV	Lough Ramor, Virginia	3	41	0.0966	23.30
11	CFS	Carron Field Station	3	46	0.1019	26.14
12	CL	Clonmacnoise	3	29	0.0644	16.48
13	CE	Coole	3	31	0.0720	17.61
14	CO	Cooloorta	3	49	0.1166	27.84
15	FE	Fermanagh	3	22	0.0524	12.50
16	FT	Fortwilliam	3	37	0.0758	21.02
17	FTA	Fortwilliam Adjacent	3	38	0.0829	21.59
18	GM	Glenamaddy	3	57	0.1185	32.29
19	GG	Glengariff	3	4	0.0090	2.27
20	GT	Gorteen / Aglish	3	43	0.0968	24.43
21	HK	Hawkhill	6	46	0.0936	26.14
22	HKA	Hawkhill Adjacent	3	36	0.0795	20.45
23	KK	Kilkorcan	3	38	0.0872	21.59
24	KT	Kiltiernan	3	43	0.0946	24.43
25	GK	Gortlecka	6	33	0.0794	18.75
26	LT	Leitrim	3	31	0.0688	17.61
27	LB	Lough Beg	3	38	0.0775	21.59
28	LN	Lough Neagh	3	23	0.0477	13.07
29	ML	Millgrange	3	25	0.0650	14.20
30	MY	Moylough	3	34	0.0793	19.32
31	MYA	Moylough Adjacent	3	22	0.0546	12.50
32	MC	Muckross	3	19	0.0473	10.80
33	RC	River Carey, Antrim	3	21	0.0390	11.93
34	SL	Slishmeen	3	36	0.0838	20.45
35	SW	Swallowhole	3	47	0.1111	26.70
36	TBG	Trinity Botanic Gardens	3	24	0.0666	13.64
37	TM	Turlough Monaghan	3	27	0.0695	15.34
38	TG	Turloughnagullan	3	51	0.1211	28.98
39	TGA	Turloughnagullan Adjacent	3	48	0.0989	27.27
40	URL	Urlingford	3	34	0.0857	19.32
41	WX	Wexford	3	9	0.0196	5.11
42	WN	Whinney Hill	3	8	0.0200	4.55

The genetic distance matrix (Nei 1987) was used to establish the levels of genetic divergence between the populations (Table 2.14) with genetic similarity shown in the upper diagonal and genetic distance in the lower diagonal. Estimates of genetic distance from the AFLP data ranged from 0.04 for the most closely related

	BL	BN	BNA	B2	B4	CH	CR	CS	CSA	CV	CFS	CL	CE	CO	FE	FT	FTA	GM	GG	GT	HK	HKA	KK	KT	GK	LT	LB	LN	ML	MY	MYA	MC	RC	SL	SW	TBG	TM	TG	TGA	URL	WX	WN
BL	****	0.86	0.83	0.92	0.86	0.85	0.90	0.85	0.86	0.87	0.83	0.85	0.78	0.84	0.79	0.85	0.85	0.82	0.79	0.80	0.81	0.82	0.79	0.83	0.76	0.79	0.82	0.81	0.77	0.83	0.81	0.82	0.81	0.87	0.84	0.83	0.88	0.89	0.89	0.89	0.80	0.80
BN	0.15	****	0.81	0.84	0.81	0.83	0.81	0.83	0.82	0.80	0.77	0.80	0.69	0.82	0.74	0.87	0.83	0.80	0.72	0.75	0.72	0.78	0.72	0.78	0.74	0.73	0.75	0.75	0.73	0.79	0.75	0.76	0.74	0.82	0.80	0.76	0.80	0.81	0.82	0.85	0.74	0.72
BNA	0.19	0.22	****	0.82	0.86	0.79	0.85	0.90	0.84	0.86	0.86	0.81	0.85	0.83	0.85	0.85	0.85	0.83	0.83	0.89	0.89	0.91	0.88	0.92	0.85	0.88	0.87	0.86	0.82	0.84	0.80	0.87	0.87	0.82	0.81	0.84	0.81	0.86	0.85	0.84	0.81	0.81
B2	0.09	0.17	0.19	****	0.86	0.81	0.85	0.85	0.83	0.84	0.84	0.84	0.76	0.83	0.79	0.79	0.81	0.82	0.78	0.82	0.81	0.82	0.80	0.84	0.78	0.81	0.82	0.83	0.81	0.88	0.83	0.82	0.83	0.88	0.84	0.84	0.88	0.88	0.87	0.84	0.80	0.77
B4	0.16	0.22	0.15	0.15	****	0.83	0.90	0.87	0.83	0.84	0.85	0.80	0.82	0.85	0.82	0.82	0.83	0.80	0.82	0.83	0.85	0.84	0.81	0.87	0.85	0.84	0.84	0.85	0.80	0.84	0.82	0.84	0.85	0.84	0.81	0.83	0.83	0.88	0.87	0.90	0.82	0.85
CH	0.16	0.18	0.24	0.21	0.19	****	0.88	0.86	0.86	0.85	0.82	0.83	0.73	0.85	0.78	0.85	0.86	0.82	0.74	0.79	0.79	0.80	0.76	0.81	0.80	0.77	0.80	0.77	0.72	0.79	0.77	0.79	0.77	0.85	0.83	0.80	0.84	0.86	0.86	0.86	0.76	0.76
CR	0.10	0.21	0.16	0.16	0.11	0.13	****	0.88	0.86	0.87	0.89	0.84	0.81	0.88	0.82	0.87	0.88	0.83	0.82	0.84	0.84	0.86	0.85	0.87	0.84	0.84	0.86	0.84	0.79	0.85	0.83	0.84	0.85	0.86	0.83	0.84	0.85	0.91	0.91	0.91	0.81	0.83
CS	0.17	0.19	0.11	0.17	0.14	0.15	0.12	****	0.89	0.90	0.89	0.88	0.87	0.90	0.86	0.88	0.90	0.87	0.84	0.92	0.89	0.89	0.88	0.93	0.88	0.89	0.88	0.87	0.80	0.85	0.83	0.87	0.89	0.88	0.86	0.86	0.89	0.92	0.88	0.88	0.83	0.84
CSA	0.15	0.20	0.18	0.19	0.19	0.16	0.15	0.12	****	0.94	0.84	0.89	0.74	0.85	0.81	0.89	0.91	0.86	0.82	0.85	0.82	0.85	0.82	0.85	0.79	0.83	0.81	0.81	0.76	0.82	0.83	0.85	0.84	0.84	0.85	0.84	0.88	0.90	0.94	0.85	0.85	0.79
CV	0.14	0.22	0.15	0.18	0.18	0.16	0.13	0.11	0.06	****	0.90	0.86	0.80	0.85	0.89	0.87	0.89	0.88	0.89	0.88	0.86	0.88	0.87	0.89	0.85	0.87	0.88	0.89	0.86	0.88	0.86	0.92	0.92	0.90	0.88	0.92	0.91	0.92	0.93	0.87	0.90	0.86
CFS	0.18	0.27	0.15	0.18	0.17	0.20	0.12	0.12	0.18	0.11	****	0.85	0.85	0.83	0.84	0.82	0.84	0.84	0.88	0.87	0.85	0.88	0.88	0.90	0.85	0.89	0.88	0.88	0.84	0.82	0.82	0.88	0.90	0.86	0.83	0.87	0.84	0.88	0.87	0.85	0.86	0.87
CL	0.16	0.22	0.22	0.18	0.22	0.19	0.17	0.13	0.12	0.15	0.16	****	0.75	0.84	0.79	0.83	0.86	0.87	0.82	0.84	0.78	0.83	0.83	0.85	0.79	0.85	0.83	0.82	0.78	0.81	0.79	0.81	0.83	0.85	0.83	0.82	0.85	0.84	0.87	0.86	0.81	0.80
CE	0.25	**0.37**	0.16	0.27	0.19	**0.32**	0.21	0.14	**0.30**	0.23	0.17	0.29	****	0.83	0.81	0.78	0.78	0.75	0.80	0.84	0.87	0.84	0.85	0.89	0.89	0.85	0.82	0.84	0.78	0.77	0.74	0.82	0.85	0.80	0.73	0.79	0.77	0.82	0.79	0.79	0.77	0.81
CO	0.18	0.20	0.18	0.19	0.16	0.16	0.13	0.11	0.16	0.16	0.18	0.17	0.19	****	0.78	0.87	0.88	0.85	0.79	0.86	0.83	0.84	0.83	0.88	0.84	0.81	0.82	0.81	0.75	0.82	0.79	0.80	0.82	0.84	0.80	0.79	0.82	0.85	0.83	0.85	0.77	0.79
FE	0.23	**0.30**	0.16	0.24	0.19	0.25	0.20	0.15	0.21	0.12	0.18	0.24	0.21	0.25	****	0.80	0.80	0.76	0.89	0.88	0.89	0.86	0.88	0.89	0.86	0.87	0.88	0.92	0.85	0.87	0.87	0.92	0.92	0.86	0.80	0.90	0.85	0.87	0.83	0.83	0.86	0.86
FT	0.17	0.14	0.16	0.23	0.20	0.16	0.13	0.13	0.12	0.13	0.20	0.19	0.25	0.14	0.23	****	0.96	0.85	0.78	0.83	0.81	0.85	0.80	0.86	0.84	0.82	0.81	0.81	0.75	0.82	0.78	0.83	0.81	0.84	0.83	0.82	0.84	0.88	0.87	0.86	0.80	0.77
FTA	0.17	0.18	0.16	0.21	0.19	0.15	0.13	0.11	0.10	0.12	0.18	0.15	0.24	0.12	0.23	0.04	****	0.86	0.81	0.86	0.82	0.86	0.86	0.87	0.83	0.84	0.82	0.83	0.77	0.83	0.80	0.84	0.83	0.87	0.85	0.83	0.84	0.88	0.89	0.86	0.81	0.79
GM	0.19	0.23	0.18	0.20	0.23	0.20	0.19	0.14	0.16	0.13	0.18	0.14	0.29	0.16	0.28	0.16	0.15	****	0.77	0.87	0.77	0.82	0.82	0.86	0.81	0.83	0.82	0.82	0.77	0.79	0.77	0.80	0.82	0.84	0.86	0.79	0.83	0.86	0.86	0.80	0.77	0.77
GG	0.24	**0.33**	0.19	0.24	0.20	0.30	0.20	0.17	0.20	0.12	0.13	0.20	0.23	0.23	0.11	0.25	0.21	0.26	****	0.88	0.84	0.86	0.89	0.88	0.84	0.88	0.89	0.90	0.85	0.84	0.89	0.92	0.95	0.86	0.81	0.93	0.84	0.85	0.85	0.82	0.90	0.91
GT	0.22	0.28	0.12	0.20	0.19	0.23	0.17	0.08	0.17	0.12	0.14	0.17	0.18	0.15	0.13	0.18	0.16	0.14	0.12	****	0.90	0.94	0.94	0.95	0.90	0.93	0.93	0.93	0.84	0.85	0.86	0.90	0.94	0.88	0.85	0.87	0.86	0.89	0.85	0.86	0.86	0.88
HK	0.22	**0.33**	0.12	0.22	0.16	0.24	0.17	0.12	0.20	0.16	0.16	0.24	0.14	0.19	0.12	0.21	0.20	0.26	0.17	0.10	****	0.93	0.88	0.92	0.91	0.91	0.89	0.89	0.82	0.84	0.82	0.88	0.90	0.83	0.81	0.87	0.82	0.88	0.84	0.85	0.84	0.86
HKA	0.20	0.25	0.09	0.19	0.18	0.23	0.15	0.12	0.16	0.13	0.13	0.19	0.18	0.17	0.15	0.16	0.15	0.20	0.15	0.06	0.08	****	0.92	0.94	0.90	0.93	0.92	0.90	0.84	0.84	0.85	0.90	0.91	0.85	0.85	0.87	0.85	0.87	0.86	0.86	0.85	0.86
KK	0.24	**0.33**	0.13	0.22	0.21	0.28	0.17	0.13	0.20	0.14	0.13	0.19	0.16	0.19	0.12	0.22	0.15	0.20	0.12	0.06	0.13	0.09	****	0.95	0.89	0.92	0.91	0.91	0.85	0.86	0.87	0.91	0.92	0.85	0.80	0.86	0.85	0.86	0.86	0.82	0.84	0.85
KT	0.18	0.25	0.08	0.18	0.14	0.21	0.14	0.07	0.17	0.12	0.10	0.16	0.11	0.13	0.11	0.15	0.14	0.15	0.13	0.05	0.08	0.06	0.05	****	0.93	0.95	0.95	0.93	0.86	0.87	0.85	0.94	0.94	0.88	0.85	0.90	0.88	0.90	0.88	0.89	0.87	0.90
GK	0.27	**0.30**	0.17	0.25	0.17	0.23	0.17	0.13	0.23	0.16	0.16	0.24	0.12	0.17	0.15	0.17	0.19	0.21	0.18	0.10	0.10	0.11	0.12	0.08	****	0.90	0.88	0.88	0.82	0.83	0.80	0.87	0.90	0.84	0.80	0.84	0.82	0.87	0.82	0.81	0.82	0.84
LT	0.23	**0.31**	0.13	0.21	0.18	0.26	0.17	0.12	0.19	0.14	0.12	0.16	0.17	0.21	0.13	0.20	0.17	0.19	0.13	0.08	0.09	0.07	0.08	0.05	0.11	****	0.93	0.92	0.87	0.84	0.84	0.90	0.94	0.87	0.83	0.89	0.84	0.87	0.86	0.85	0.87	0.89
LB	0.20	0.28	0.13	0.20	0.17	0.23	0.15	0.13	0.20	0.13	0.13	0.19	0.19	0.20	0.12	0.21	0.20	0.20	0.12	0.08	0.12	0.08	0.10	0.05	0.13	0.07	****	0.95	0.91	0.85	0.90	0.93	0.95	0.86	0.81	0.92	0.84	0.87	0.85	0.86	0.87	0.91
LN	0.21	0.29	0.15	0.18	0.17	0.26	0.17	0.14	0.21	0.12	0.13	0.19	0.17	0.21	0.09	0.21	0.19	0.20	0.10	0.07	0.12	0.10	0.10	0.07	0.13	0.08	0.05	****	0.91	0.86	0.90	0.93	0.97	0.88	0.82	0.91	0.86	0.88	0.88	0.86	0.88	0.93
ML	0.26	**0.32**	0.20	0.22	0.23	**0.32**	0.24	0.22	0.27	0.16	0.17	0.24	0.25	0.29	0.17	0.29	0.27	0.26	0.16	0.17	0.20	0.17	0.16	0.15	0.20	0.15	0.09	0.10	****	0.81	0.85	0.88	0.90	0.81	0.76	0.88	0.80	0.81	0.80	0.79	0.84	0.87
MY	0.19	0.24	0.18	0.13	0.17	0.23	0.16	0.16	0.20	0.13	0.20	0.21	0.26	0.20	0.14	0.19	0.19	0.24	0.17	0.16	0.18	0.18	0.15	0.14	0.18	0.17	0.17	0.15	0.21	****	0.91	0.88	0.89	0.89	0.83	0.85	0.88	0.89	0.87	0.83	0.82	0.83
MYA	0.22	0.28	0.22	0.19	0.20	0.26	0.19	0.19	0.19	0.15	0.19	0.24	0.30	0.24	0.14	0.24	0.22	0.26	0.12	0.15	0.20	0.17	0.14	0.16	0.23	0.18	0.11	0.11	0.16	0.10	****	0.90	0.90	0.87	0.80	0.89	0.87	0.86	0.85	0.81	0.86	0.87
MC	0.20	0.27	0.14	0.19	0.17	0.24	0.18	0.14	0.16	0.08	0.13	0.20	0.19	0.22	0.09	0.19	0.18	0.22	0.09	0.11	0.12	0.11	0.10	0.07	0.14	0.10	0.07	0.07	0.13	0.13	0.10	****	0.95	0.88	0.85	0.95	0.87	0.89	0.88	0.87	0.91	0.90
RC	0.21	**0.30**	0.14	0.18	0.16	0.26	0.17	0.12	0.18	0.09	0.10	0.19	0.16	0.20	0.09	0.21	0.18	0.20	0.06	0.06	0.10	0.10	0.08	0.06	0.11	0.06	0.05	0.03	0.11	0.12	0.10	0.05	****	0.89	0.84	0.93	0.87	0.90	0.89	0.85	0.91	0.93
SL	0.14	0.20	0.19	0.13	0.17	0.16	0.15	0.13	0.17	0.11	0.15	0.16	0.23	0.17	0.15	0.18	0.14	0.18	0.15	0.13	0.19	0.16	0.16	0.13	0.18	0.14	0.15	0.13	0.20	0.12	0.14	0.13	0.12	****	0.90	0.90	0.90	0.90	0.89	0.89	0.86	0.86
SW	0.17	0.22	0.21	0.17	0.21	0.19	0.19	0.15	0.16	0.13	0.19	0.18	**0.32**	0.22	0.22	0.19	0.16	0.15	0.22	0.16	0.22	0.16	0.23	0.16	0.23	0.18	0.21	0.20	0.28	0.18	0.22	0.16	0.18	0.10	****	0.85	0.87	0.87	0.87	0.85	0.82	0.79
TBG	0.19	0.27	0.17	0.18	0.19	0.23	0.18	0.15	0.17	0.09	0.14	0.19	0.23	0.24	0.11	0.20	0.19	0.24	0.08	0.14	0.14	0.14	0.15	0.11	0.17	0.11	0.09	0.09	0.13	0.16	0.11	0.06	0.07	0.10	0.16	****	0.89	0.88	0.88	0.86	0.94	0.91
TM	0.12	0.23	0.21	0.12	0.19	0.18	0.16	0.12	0.13	0.09	0.17	0.17	0.26	0.19	0.16	0.18	0.18	0.19	0.17	0.15	0.19	0.16	0.16	0.13	0.20	0.18	0.17	0.15	0.22	0.13	0.14	0.14	0.14	0.11	0.14	0.12	****	0.93	0.90	0.86	0.85	0.82
TG	0.12	0.21	0.16	0.12	0.12	0.15	0.10	0.09	0.10	0.08	0.13	0.18	0.20	0.17	0.14	0.13	0.13	0.15	0.16	0.11	0.13	0.14	0.15	0.11	0.14	0.14	0.14	0.12	0.21	0.12	0.15	0.12	0.11	0.11	0.14	0.12	0.07	****	0.96	0.90	0.87	0.87
TGA	0.11	0.19	0.16	0.14	0.13	0.15	0.10	0.12	0.07	0.08	0.14	0.14	0.24	0.18	0.18	0.14	0.12	0.15	0.16	0.16	0.17	0.16	0.15	0.13	0.20	0.15	0.16	0.13	0.22	0.14	0.16	0.13	0.12	0.11	0.14	0.13	0.10	0.04	****	0.90	0.87	0.86
URL	0.12	0.16	0.17	0.17	0.11	0.15	0.10	0.12	0.16	0.14	0.17	0.15	0.23	0.16	0.18	0.15	0.15	0.22	0.20	0.15	0.16	0.15	0.20	0.12	0.21	0.16	0.15	0.15	0.24	0.18	0.21	0.14	0.16	0.12	0.16	0.15	0.15	0.10	0.10	****	0.83	0.86
WX	0.22	**0.31**	0.20	0.22	0.20	0.28	0.21	0.18	0.17	0.11	0.15	0.21	0.26	0.27	0.16	0.22	0.21	0.26	0.10	0.15	0.17	0.16	0.18	0.14	0.20	0.14	0.14	0.13	0.17	0.20	0.16	0.09	0.10	0.15	0.20	0.07	0.16	0.14	0.14	0.19	****	0.91
WN	0.22	**0.33**	0.21	0.26	0.16	0.27	0.18	0.18	0.24	0.15	0.14	0.23	0.21	0.23	0.15	0.26	0.24	0.26	0.10	0.13	0.15	0.15	0.16	0.11	0.18	0.11	0.09	0.07	0.14	0.19	0.14	0.10	0.08	0.15	0.24	0.10	0.20	0.14	0.15	0.15	0.09	****

Table 2.14 Genetic distance matrix based on Nei's unbiased diversity statistic, between 42 populations of *R. repens* sampled from various habitat types. Populations are coded as in Table 2.1. Distances are given in the lower diagonal and similarities are given in the upper diagonal. Highest distance values are denoted in bold and lowest are denoted in italics.

populations (Fortwilliam and Fortwilliam Adjacent, Turloughnagullan and Turloughnagullan Adjacent), to 0.37 in the most divergent populations (Ballinturly and Coole). Highest distance values are denoted in bold and lowest values are denoted in italics in Table 2.14. Most samples collected from populations adjacent to their neighbouring turlough population were found be genetically similar to them (low genetic distance values). Table 2.15 shows the genetic distance between these pairs of populations. Some turlough populations were found to be as genetically similar to each other as they were to populations sampled in close proximity to them, despite their geographic isolation.

Table 2.15 A comparison of genetic distances (Nei's unbiased diversity statistic) between a number of *R. repens* populations. The left hand column represents turlough - turlough adjacent pairwise comparisons, and the right hand column represents turlough - turlough pairwise comparisons.

Population pairs	Genetic distance	Population pairs	Genetic distance
T-TA		T-T	
BN-BNA	0.22	GK-HK	0.1
CS-CSA	0.12	GK-KT	0.08
FT-FTA	0.04	KT-HK	0.08
HK-HKA	0.08	HK-CS	0.12
MY-MYA	0.1	TG-CS	0.09
TG-TGA	0.04	TG-TM	0.07
GK-CE	0.12	TG-URL	0.1

The dendrogram (Figure 2.12) based on Nei's genetic distance (1973) is in agreement with both UPGMA and NJ analysis results. Generally, turlough populations are clearly more closely related to each other than they are to dry pasture populations, with the exception of some broadleaved populations that were collected adjacent to turlough populations, and to the turlough populations Ballinturly and Coole. There appear to be two main groups (denoted in different colours on the dendrogram); the first consisting of all turlough populations delimited with Balla at one end and Glenamaddy at the other, and a cluster which consists predominantly of non-turlough populations (wetland and dry pasture). Finally, Coole and Ballinturly were found to be ungrouped in this analysis.

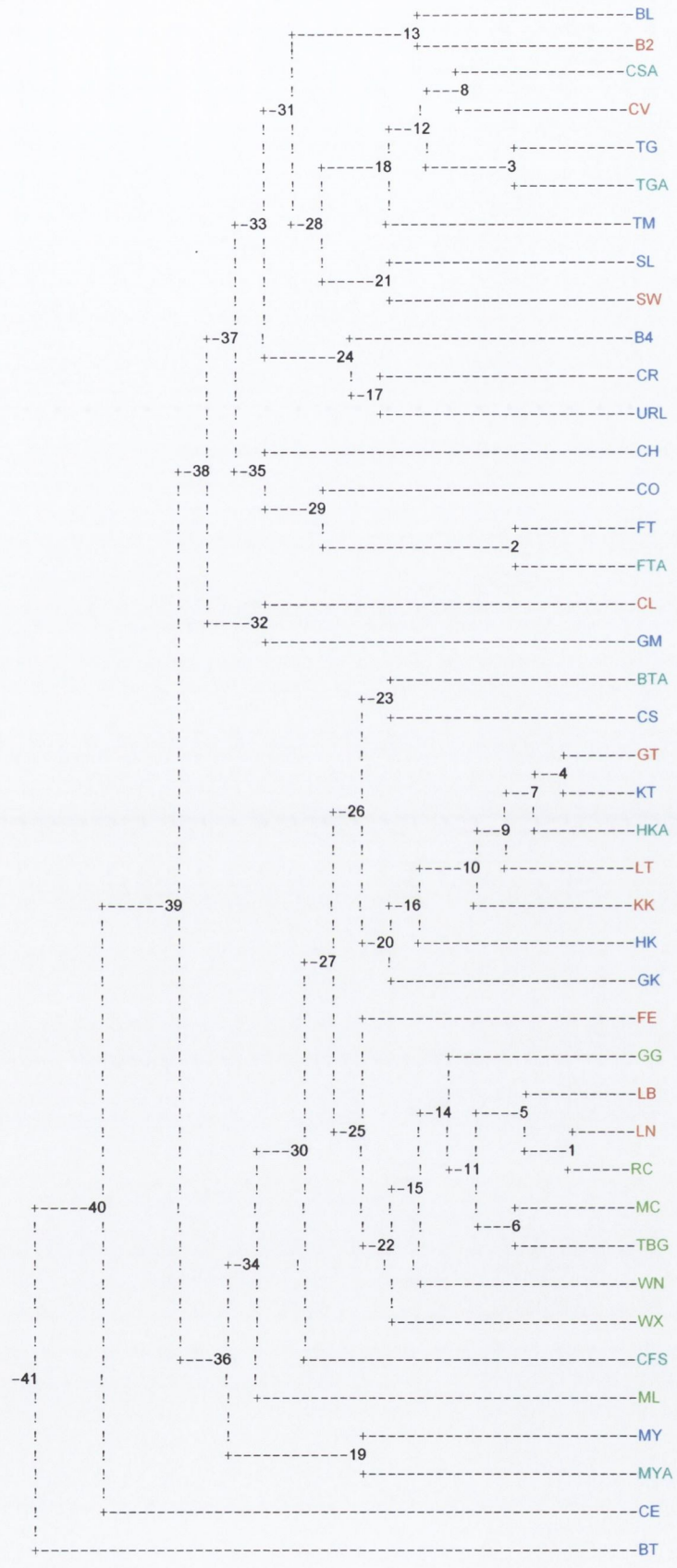

Figure 2.12: UPGMA dendrogram (Popgene) based on Nei's (1973) genetic distance from the AFLP data from 42 *R. repens* populations from 4 different habitat types; turlough populations are denoted in blue, turlough adjacent in light blue, wet pasture in red and dry pasture in green.

2.3.7 Universal chloroplast microsatellites

Five universal microsatellite primer pairs (Weising & Gardner 1999) were used to screen a number of *R. repens* from various habitat types in widely separated geographic regions (see Table 2.4). No variation was found to be present, with haplotypes for each primer pair being identical for each individual screened (Table 2.16). All fragments obtained were in the correct size range quoted for each primer pair according to analysis from a number of different plant species.

Table 2.16: Fragment size for each microsatellite amplified from five universal chloroplast microsatellite primers (Weising & Gardner 1999) on a number of *R. repens* samples from various habitat types and geographic locations.

Sample ID	Population code	ccmp 1 Size (bp)	ccmp 2 Size (bp)	ccmp 6 Size (bp)	ccmp 8 Size (bp)	ccmp 10 Size (bp)
113	GK	113	198	90	54	152
140	B2	113	198	90	54	152
152	TBG	113	198	90	54	152
170	WX	113	198	90	54	152
175	CV	113	198	90	54	152
188	RC	113	198	90	54	152
197	GG	113	198	90	54	152
304	P1	113	198	90	54	152
305	P1	113	198	90	54	152
309	P2	113	198	90	54	152
310	P2	113	198	90	54	152
314	LM	113	198	90	54	152
315	LM	113	198	90	54	152
319	DPB	113	198	90	54	152
320	DPB	113	198	90	54	152
42	URL	113	198	90	54	152
60	ML	113	198	90	54	152
71	FT	113	198	90	54	152
72	FT	113	198	90	54	152

2.3.8 Sequencing

All samples sequenced for the gene regions *trnL-F*, *rps*16 and *atpB-rbcl* showed very similar base pair sequence. The gene sequences for each individual for each gene region amplified are given in Appendix 2. The *trnL-F*, *rps*16 and *atpB-rbcl* sequences were approximately 923bp, 842bp, and 585bp long respectively. The search for microsatellite repeats within these sequences revealed a number of SSRs, which are highlighted in bold in Appendix 2. One microsatellite repeat of 7bp, and three of 6bp

length, were found in the *trnL-F* sequences. Two repeats were found in the *rps*16 sequences, one 6bp long and one 7bp long. Two SSRs were found in the *atpB-rbcl* sequences, one of 7bp and another of 9bp. No microsatellite repeat was found to be longer than 9bp, therefore the mononucleotide repeats were considered too short to use for designing *Ranunculus*-specific chloroplast microsatellite primers.

2.4 Discussion

2.4.1 Leaf dissection measurements

In agreement with previous studies (Lynn & Waldren 2002) leaf shape was found to differ between *R. repens* individuals from different habitat types. Regression analysis on the association between perimeter and √area was conducted for each of the three groups (turlough, wet pasture, and dry pasture) and they were found to have significantly different slopes. The results showed that turlough populations of the species had a more dissected leaf form than the wet pasture or dry pasture populations with a certain degree of gradation between the three groups; individuals sampled from turlough habitats had the most dissected leaf form, individuals from the wet pasture habitats had a somewhat intermediate leaf shape and those sampled from the dry pasture environments had the broadleaved form of the common weedy *R. repens*.

Leaf dissection was found to differ significantly between populations and between habitat types. From an ecological perspective these findings can be related to differences in the environmental factors associated with where these individuals were growing. The dissected leaf form of *R. repens* is associated with the turlough environment, where frequent prolonged periods of inundation have resulted in the populations adapting to their environment. It is evident from the field that the broadleaved form is absent from turlough basins. Lynn & Waldren (2001b) showed differences in leaf dissection to be highly heritable in five populations of *R. repens.* Lynn & Waldren (2002) postulated that a thinner boundary layer enhances gaseous exchange in submerged conditions, and that the dissected leaf form had evolved as a response to increasing susceptibility to inundation (Lynn & Waldren 2001b). Therefore the quantitative measure of leaf dissection appears to be a good indicator of habitat type. *R. repens* has subsequently been used as an indicator species for the extent of flooding in turlough environments (Lynn & Waldren 2003b).

2.4.2 Population sampling methods

One criticism of the findings of this chapter is the low sample size for each population studied. But the prime purpose of this study was to compare variation between as many populations as possible, and furthermore NJ and ordination techniques do not require large sample numbers. Pons & Petit (1995, 1996) emphasise the importance of

increased numbers of populations as opposed to increased within-population samples for studies of population subdivision. They state that optimal sampling for Nst is equal to 6.6 individuals per population, close to the value obtained for Gst which was 5.7. These results emphasise the necessity of sampling as many populations as possible, with homogenous sample sizes, since the sampling of the populations has a predominant influence on the accuracy of the differentiation estimate. Increasing the sample size within the population may improve only to a small extent the precision of the estimates (Pons & Petit 1996). Many studies, in particular those investigating the population structure of rare or endangered plant populations have similar sample sizes per population. Comes & Abbott (2000) sampled between 3 and 6 individuals per population for a study of *Senecio gallicus* using RAPDs. When many polymorphic loci are available, large numbers of individuals per population are not necessarily required to yield accurate estimates of average heterozygosity (Krauss 2000). In relation to the study discussed in this chapter, the objective was not a detailed investigation of population structure within and among turlough populations but to determine whether or not there was a genetic difference between the dissected and broadleaved populations of *R. repens*. Therefore it seemed logical to sample as many populations as possible, from various habitat types, in order to try to answer the problem at hand.

2.4.3 Patterns of genetic differentiation

Using AFLP analysis, individuals tend to cluster in their respective population units (Figures 2.4, 2.5, 2.7a, b, c, and 2.10a, b, c), there is no geographical pattern of the populations beyond the local scale. Even when ecological factors such as the quality of the turlough habitat i.e. whether or not its situated in agricultural area with adjacent pasture populations as opposed to isolated, is taken into account, there still does not appear to be a pattern to the grouping of some turlough populations with others on a broad geographic scale. For example Cooloorta and Gortlecka are two of the most oligotrophic turloughs sampled, they are also in relatively close geographic proximity to each other but they do not cluster in ordination space (Figure 2.7c). The absence of a nationwide pattern may be due to a number of reasons such as high levels of variation within and between each population and low sample size for each population sampled. Scoring error and uninformative bands causing noise in the data may also be factors contributing to these findings.

Andersson (1995) states the potential for adaptive change is greatly enhanced if a species can achieve the same adaptation by different genetic mechanisms, and that there are ample indications that an adaptation can have a different genetic basis from one population to another (Macnair 1977; Cohan 1984). This may be another contributing factor to the findings in this chapter that turlough populations are grouping together but are not generally grouping with wet pasture or dry pasture populations that have been sampled nearby. Although the exceptions to this are some adjacent turlough populations which were collected within approximately 200m of the turlough individuals. Some of these populations were found to be genetically similar to turlough populations (see Table 2.15). This being the case they were not found to be any more genetically similar than were some turloughs to each other (e.g. KT and HK, GK and CE). Other cases in which the genetic basis of the same adaptation differs between species or conspecific populations of plants include floral morphology (Prazmo 1965; Mayer & Charleworth 1992; Fenster & Barrett 1994), heavy metal tolerance (Macnair 1977; Schat *et al.* 1993) and herbicide resistance (Miller *et al.* 1973).

Conversely, it is evident that there is an ecotypic pattern emerging (Figures 2.8a, b, c, 2.11a, b, c) with *R. repens* populations grouping in terms of habitat type. Moore *et al.* (1998) found that geographically separate but similar ecotypes of *Prochlorococcus* were more closely related than different ecotypes in the same region. Therefore AFLP appears to be a suitable technique to separate populations according to habitat type. Differences in effective population size and restriction of local gene flow indicate the potential for very different evolutionary trajectories in each population (Hamilton 1997). Turlough populations are more closely related to each other than to non-turlough populations regardless of geographic factors. Populations sampled from the Norfolk meres, habitats that also have prolonged periods of flooding, did not group closely with turlough populations. Geographic distance may be the reason for this separation.

Historical relationships have been found to contribute to the genetic structure of most plant populations with those having recent common ancestry being genetically more similar than those having more distant common ancestry (Schaal *et al.* 1998). The data do seem to suggest that turlough populations are more related to each other than

to non-turlough populations. If this is true it could imply a different evolutionary history for the turlough populations, they could have colonised at a different time to dry pasture and other populations. Comes & Abbott (2000) found that variation at individual RAPD loci may be affected by linkage to quantitative trait loci subject to directional selection in response to similar environmental conditions. It is possible that the differences detected between turlough and non-turlough populations are due to some of the AFLP markers being linked to genes responsible for leaf dissection. Leaf dissection may be a good indicator of ecotypic differentiation. There are ecological factors present in the various habitat types that could lead to genetic structuring within the plant populations sampled. Norfolk populations had similar levels of leaf dissection to turlough populations sampled in Ireland. Despite this finding they were not found to be genetically similar. This would suggest that leaf morphology alone is not a good indicator of genetic history between mere and turlough populations but that it does have some basis when comparing turlough to other non-turlough populations in Ireland.

This may infer that *R. repens* may have an inherent 'dissected leafness' in its genetic makeup, with all populations of the species having the genes responsible at very low levels, but it has only been selected for in the turlough populations. It was apparent from the leaf dissection analysis that *R. repens* individuals from contrasting habitat types have significantly different leaf shape, with 'dissected leafness' not being selected for in dry pasture and to some degree wet pasture populations. Even so, it is evident from the results that there is a possibility that gene flow is occurring between turlough and adjacent non-turlough populations as many of these populations were found to be genetically similar (Figures 2.8a, b, c and 2.11a, b, c). Although this may be the case, turlough populations of *R. repens* still retain their dissected leaf form. This suggests that selective pressures are overcoming gene flow and the dissected leaf is being maintained. The exact relationship between turlough populations and adjacent non-turlough populations is still somewhat unclear. The examples in this chapter of these populations from different habitat types being genetically similar may be the exception rather than the rule. The sampling and analysis of more adjacent non-turlough populations would be required to clear up this matter.

The cpDNA analysis of *R. repens* from various habitat types using universal primers (Weising & Gardner 1999) did not show any patterns, with haplotypes in all individuals found to be identical. This finding is not altogether surprising as Weising and Gardner (1999) found few intraspecific polymorphisms in their series of experiments on a number of taxa. The low mutation rates associated with organelle genomes means that it is sometimes difficult to detect variation below the species level (Wolfe *et al.* 1998), and restriction site analyses have shown that cpDNA sequence divergence can vary considerably among species (Soltis *et al.* 1992). Therefore the identical sequence data from the chloroplast gene regions (*trn*L-F, *rps* 16, and *atp*B-*rbcl*) are not surprising. The search for microsatellites within these sequences regions did not prove to be fruitful, with no microsatellite repeat being longer than nine base pairs, a length considered short and less likely to produce useful SSR markers. Considering the time to stochastic lineage sorting among nuclear genes may be extended considerably because of the twofold larger N_e values for nuclear compared with cytoplasmic genes, this is not surprising. Differences between cpDNA and nuclear genes with respect to population differentiation could be compromised if classes of nuclear genes differ in their evolutionary rates and/or their response to natural selection (Comes & Abbott 2000). Therefore contrasting rates of evolution may be responsible for the difference in diversity found between the AFLP and cpDNA data, and for the evident lack of variation in microsatellite repeats in the regions sequenced in a number of *R. repens* samples from different habitat types. Comes & Abbott (2000) have documented that several plant studies, for example, Raybould *et al.* (1996a,b, 1997) have reported differences in the distribution of variation at allozyme loci vs. anonymous single copy nuclear DNA (scnDNA) loci in *Beta vulgaris*, and Le Corre *et al.* (1997) have shown that although overall patterns of allozyme and random amplified polymorphic DNA (RAPD) variation in *Quercus robur* were in good agreement, levels of genetic variation and fine-scale genetic structure sometimes differed. Similarly, Bonnin *et al.* (1996) reported that populations of *Medicago trunculata* were more differentiated for quantitative characters (e.g. growth rate, morphology) than for RAPDs, whereas Kolliker *et al.* (1998) observed that discrimination between populations of *Festuca pratensis* was greater for RAPDs than for agronomic traits. Finally, Keskitalo *et al.* (1998) found that morphological, quantitative relationships were similar to those of RAPDs in *Tanacetum vulgare*. The strong genetic differentiation of the turlough and non-turlough populations found with

AFLP suggests a separation of the lines. The contrasting results found from the universal cpSSR data are not surprising as the primers used were universal, and are evidently more suitable on an inter species level rather than an intra species level.

2.4.4 Levels of genetic diversity

The diversity measurements obtained from the data in this study show comparable levels of diversity in most populations with other studies. The terminal branch lengths of the NJ tree are large, indicating high levels of diversity between individuals (Figure 2.4). There is a general trend for the turlough populations to show higher levels of diversity than the dry pasture populations. Zawko *et al.* (2001) states that genetic diversity and its partitioning among and between populations reflect and influence breeding systems (Moran & Hopper 1987; Hamrick & Godt 1996). Outcrossing species commonly have higher levels of genetic diversity and lower differentiation between populations than selfing and clonal plants (Dawson *et al.*1993; Rosetto *et al.* 1995; Hamrick & Godt 1996; Palacios *et al.* 1999). Although *R. repens* is a clonal species it still retains a high level of genetic diversity, particularly in those populations sampled from turloughs. Therefore, other factors are possibly contributing to the diversity levels found in this study.

Within population diversity values were calculated using Nei's unbiased diversity statistic (Nei 1987), averaging over all AFLP products. The highest genetic diversity (H) (Nei 1973) was found in the Castleplunket population (0.1443), followed closely by Turloughnagullan (0.1211), Ballyshannon 2 (0.1196) and Glenamaddy (0.1185), all of these being turlough populations. Without exception, the lowest diversity levels were found in the dry pasture populations, with Glengariff having the lowest diversity value of all 42 populations sampled (0.0090), followed by Wexford (0.0196), and Whinney Hill (0.0200). The range of genetic diversity values over all populations was 0.1960 to 0.1443. The levels of genetic variation found in this population study of *R.repens* from contrasting habitat types are similar to those of many plant population studies. Muluvi *et al.* (1999) found values for Nei's average diversity within populations of the tropical tree species *Moringa oleifera* Lam. ranged from 0.04 to 0.122, (they included monomorphic bands), Cardoso *et al.* (1998) found genetic diversity within remnant populations of *Caesalpinia echinata* Lam. ranging from 0.031 to 0.157, Travis *et al.* (1996) describe diversity levels for populations of the

endangered species *Astragalus cremnophylax* var. *cremnophylax* Barneby between 0.0177 and 0.1337, and Despres *et al.* (2002) give diversity values between 0.148 and 0.270 for populations of the European globeflower (*Trollius europaeus* L.). These cited studies gave reasons such as geographical isolation, small population size, relict populations, and historical fragmentation for low levels of genetic diversity. Turlough populations were found to have higher levels of genetic diversity than populations sampled from dry pastures therefore certain factors may be influencing the different genetic diversity values between turlough and non-turlough populations such as selection pressures. If turlough populations are more genetically diverse than non-turlough does this mean they are source populations of 'newer' dry pasture populations? Or more realistically, are high levels of genetic diversity required to combat the fluctuating water regime, and changeable environment of the turlough habitat. This point will be discussed further in the following two chapters.

2.5 Conclusions

Leaf dissection is a good indicator for determining habitat type of *R. repens* individuals. This morphological trait has been shown to correlate with ecological preference and genetic relatedness of populations of the creeping buttercup within Ireland. AFLP proved to be an effective tool for distinguishing populations of *R. repens* from three different habitat types, based on susceptibility to inundation. The main aim of this study was to determine whether or not the turlough populations are genetically different from non-turlough populations. The objective was not to decipher within and among population differentiation between turlough populations but to determine whether there was an ecological component involved in discriminating individuals from different habitat types as is present from the morphological data. The high resolving power, and large number of polymorphic bands resulting from AFLP analysis make the technique appropriate at the ecotypic level. In terms of methods of analysis ordination techniques were more easily interpretable than were cluster techniques. In general NJ and UPGMA results were congruent and the reason for the low bootstrap levels in each were because of the high levels of variation that are inherently present in AFLP data. Both of the ordination techniques showed similar results but in general PCO seemed more appropriate than NMDS for explaining the variation in the data from this present study.

It was evident from the results obtained from all methods of analysis used in this study that turlough populations are more closely related to each other than they are to non-turlough populations, with the exception of several populations adjacent to turloughs that grouped closely with their respective turlough neighbours. The lack of a geographic pattern between the *R. repens* populations sampled throughout Ireland, and the grouping of turlough populations regardless of location would suggest that the turlough populations have a different evolutionary history to the non-turlough populations. Similar selection pressures will be exerted on all turlough populations and it is possible that several of the AFLP markers are closely linked to the genes responsible for leaf shape or flooding tolerance in the turlough form of the species. Presumably these genes are present in all populations of *R. repens* at low levels but are strongly selected for in turlough populations; rare occurrences of the dissected leaf form have been found in dry pasture habitats. The occasional grouping of turlough populations to the adjacently sampled non-turlough populations suggests that they may exchange genes. The lack of genetic similarity between the Irish turlough populations and the Norfolk mere populations suggests that the turlough form of *R. repens* is very different from that of the Breckland Mere form and suggests that parallel evolution has taken place in these similar habitat types.

This study has established that turlough populations of *R. repens*, with their dissected leaf form, are genetically different from non-turlough, broadleaved forms of the species. The next two chapters will focus on populations of the turlough buttercup in more detail; Chapter three investigates the genetic relationship between individuals sampled along a transect in a turlough basin, running through a strong ecological gradient, and Chapter four focuses on the clonal structure in a turlough population of *R. repens*.

Chapter 3

Genetic variation of *Ranunculus repens* across a turlough basin

Just as a sculptor creates a statue by removing chips from an amorphous block of marble, so natural selection creates new systems of adaptation to the environment by eliminating all but the favourable gene combinations out of the enormous diversity of random variants which could otherwise exist

Stebbins 1950

3.1 Introduction

3.1.1 Adaptation to heterogeneous environments

Plant populations are adapted for existence in an environment when their individuals are adequately equipped both physiologically and morphologically for maintenance, growth and reproduction. The population then has what is called immediate fitness (Cook & Johnson 1968). In reality this is found to be more the exception than the norm. No environments are constant, but instead are dynamic systems of change that are, more often than not, unpredictable. Therefore, in order to survive, individuals must be able to cope with heterogeneity both within space and time and between generations. This is done through individual and population adaptability. This can be achieved in many ways and has been referred to by a wide array of terminology such as plasticity, and phenotypic plasticity (Thoday 1953), homeostasis (Lewontin 1957), buffering (Allard & Bradshaw 1964), and the formation of ecotypes (Van Valen 1965).

3.1.2 Selection pressure and micro-differentiation

The study of natural populations (Stebbins & Daly 1961), as well as artificial selection experiments (Millicent & Thoday 1960; Thoday & Boam 1961), have all indicated that isolation is not a prerequisite for divergence under selection and that local differentiation in plant populations does not require geographical discontinuity (Antlfinger 1981). Ecological factors, in the form of strong selection pressure, can be adequate in bringing about these changes. Many studies have proved that differentiation can occur across strong selective gradients such as copper, (*Agrostis stolonifera)*, zinc (*Anthoxanthum oderatum*) and serpentine mine boundaries (*Silene paradoxa)* (Antonovics 1972; Wu *et al.* 1975; Mengoni *et al.* 2000). These studies prove that geographic isolation is not critical for the differentiation of plant populations; the key factor in this divergence is environmental change and the selective pressure it exerts.

The environment of a plant species may not only change in time, it may also change in space. Localised races or ecotypes may form through environmental heterogeneity over distances as little as 10 metres (Bradshaw 1963). Changes in environment however, can occur over much smaller distances than this, distances that may

preclude the formation of genetically different populations (Bradshaw 1965). Genetic differentiation is predicted for characters that are adaptations to environmental conditions that are likely to remain constant during the lifespan of the organism (Goldstein & Pollock 1997). Phenotypic plasticity should be high for characters that are adaptations to environmental conditions that are likely to change frequently during the lifespan of an organism (Bradshaw 1965). When different demes or lineages encounter consistently different selection pressures, genetic differentiation is likely to persist as subpopulations adapt to local conditions (Endler 1979; Gillespie & Turelli 1989). Adults essentially provide the population with a 'memory' of past selective events and therefore conserve variability. If the memory is too long (i.e. decay rate and turnover is slow) then the population will tend to have an increased genetic load (selective intensity) since the present environment may have changed yet the gene pool and hence newly establishing individuals may be receiving contributions from genotypes that were favoured at an earlier date. On the other hand, if the memory is too short then genetic variability that can be used, should a particular environmental extreme recur, will not be available (Antonovics 1972). Theoretical analyses of patterns of micro-differentiation suggest that: (1) clines can be maintained by strong selection in the face of gene flow over short distances, (2) genetic divergence and reproductive isolation can occur between genetically contiguous populations and (3) perennial populations differ from annual populations in the rate of gene frequency change and response to pollen flow during the initial stages of colonisation (Antlfinger 1981).

3.1.3 The turlough habitat as a model system

The turlough habitat provides an excellent model system for the study of differentiation in plant populations. This strong ecological gradient in hydrology and inundation exists from the top to the bottom of a turlough basin. Previous studies (Lynn & Waldren 2001b, 2002, 2003a,b,c) indicated a high level of morphological and physiological variation in turlough populations of *R. repens* along these turlough gradients. This variation is retained when plants were grown in uniform conditions, indicating that these traits are not strongly influenced by variation in environmental conditions, and implies a strong genetic basis for the variability. Morphological traits were also shown to be highly heritable when plants were grown from seed (Lynn & Waldren 2001b). Within a turlough basin there are varying degrees of leaf dissection

in *R. repens*, which are associated with the levels of inundation that plants are exposed to. Leaves become more dissected as one moves further down a turlough basin, corresponding to increased periods of inundation (Plate 3.1). Between the lower zone of finely dissected individuals and the upper zone of more broad-leaved individuals occurs what could be coined an intermediate zone. Individuals in this area vary greatly in relation to degree of leaf dissection; this region suffers from the greatest levels of water fluctuation. Lynn & Waldren (2002) showed that in addition to the morphological differences, extreme populations of *R. repens* are physiologically different.

3.1.4 Aims

The aims of this study were to investigate genetic variation present in a population of *R. repens* within a turlough basin. Many individuals in the 'intermediate zone' are not of the dissected leaf form; all zones along the transect are changeable but the intermediate zone is likely to be more unpredictable (Lynn & Waldren 2001b, 2002). By sampling a cross section through the population (transect) and also along a strong ecological gradient, it was anticipated that the genetic relationship between and among individuals subjected to different selection pressures, in terms of period of inundation, would be obtained. Therefore, in conjunction with the morphological data available, ISSR and AFLP were used to examine patterns of genetic variation along an obvious ecological gradient, spanning a short spatial distance, and to determine whether the genetic distance between individuals was in any way correlated with geographic distance.

Plate 3.1: *Ranunculus repens* individuals sampled from the top and bottom of a transect along Hawkhill turlough basin.

Plate 3.2: Hawkhill turlough basin.

3.2 Materials and Methods

3.2.1 Site description

Hawkhill turlough basin (Plate 3.2), situated in Co. Galway (grid reference – M411025) is a small relatively deep turlough with a permanent pool at the base, which is under tidal influence and therefore experiences slight daily fluctuations in water levels (Lynn & Waldren in 2003b). A 60 metre transect was set up which ran from the middle of the turlough basin to the edge of the turlough basin which becomes inundated following extensive periods of rainfall. The elevation of each quadrat was calculated relative to a temporary benchmark within the basin (Lynn & Waldren 2003b). The difference in elevation along the transect was 7 metres from top to bottom. Aquatic species such as *Alisma aquatica* and *Eleocharis acicularis* flourish in the permanent aquatic community at the base of the turlough. Behind the permanent pool lies an area of open mud that is colonized by annuals such as *Polygonum persicaria* and *Rorippa islandica.* This area is likely to be subjected to prolonged bouts of flooding which thus reduces the chance of the formation of a closed community. There is an obvious change in species composition at around 4m up the slope evident by the presence of dry pastureland species such as *Lotus corniculatus, Plantago lanceolata*, and *Trifolium repens*, and damp grassland species such as *Leontodon autumnalis*. A transition also occurs in the grassland species with the dominance of the damp grassland species *Agrostis stolonifera* in the lower parts of the basin replaced by drier pasture grassland species such as *Elymus repens, Agrostis capillaris, Festuca rubra* and *Lolium perenne* (Lynn & Waldren 2003b).

3.2.2 Plant collections

Six *R. repens* rosettes were randomly collected from $1m^2$ quadrats at 5m intervals along the 60m transect in Hawkhill. The sampled plants of *R. repens* were potted in equal parts sand:compost:loam and artificially fertilised with 1.7g/l "Osmocote" slow release fertiliser. The plants were left to establish in an unheated glasshouse (minimum 12 ^{o}C) and then transferred to growth room conditions (14 hr day, PAR 60-100 μmoles $/m^{-2}$ s^{-1}, 25°C day, 22°C night). Clean leaf material was collected for DNA analysis from thirty of the individuals kept in cultivation chosen from Q1 to Q12 that ran from the top to the bottom of the basin.

3.2.3 Breeding experiment

Breeding experiments were carried out in the year 2000 using a series of *R. repens* plants from various positions along the Hawkhill transect as well as individuals from a Trinity College Botanic Gardens (TBG) population. These TBG individuals were collected randomly from a dry pasture habitat within the garden grounds. Breeding experiments testing for self-sterility in *R. repens* resulted in seed development in 10% of the cases, but each seed head produced only one or two achenes, indicating that a small degree of selfing may occur (Coles 1977). He also indicates that there appear to be no signs of even a partial sterility barrier to crossing between plants from widely differing geographical regions. Attempts were made to interbreed turlough individuals from the top and bottom of a turlough basin and individuals from the bottom of a turlough basin with individuals from a non-turlough population (TBG) in order to examine the leaf dissection of the progeny. The rationale behind these reciprocal crosses was to test whether or not the 'intermediate' form of *R. repens* was a result of hybridisation between a broad-leaved and dissected leaf form of *R. repens.*

The reciprocal crosses between individuals involved emasculation of the anthers before they dehisced to avoid selfing, and then covering with a 15-dernier-nylon mesh to prevent contamination with foreign pollen. When the stigmas were receptive pollen was brushed onto the ripe stigmas of the mother flower, and the flower heads were again covered with nylon mesh bags until seeds were set. Seeds were subsequently collected and stored in brown paper envelopes at room temperature.

In the Spring 2001 seeds were germinated and propagated in compost in growth room conditions (14hr day, PAR 60-100μmoles/m/s, 25°C day, 22°C night) on moist filter paper in glass Petri dishes. When the cotyledons emerged the seedlings were transferred to equal part sand:peat:loam. Ramets of *R. repens* individuals were potted in equal parts sand:peat:loam. The ramets were left to establish in a heated glasshouse (minimum 12°C) with no supplementary light and subsequently transferred to outdoor conditions.

3.2.4 Leaf dissection measures

Leaf dissection measures were previously analysed for individuals collected from along the transect following a year in cultivation. Progeny leaf dissection measures were conducted in the same manner as those described in section 2.2.3. Samples were grouped together in terms of cross type (turlough - turlough or turlough – non-turlough) and regression analysis was carried out for each of these and the slopes were derived from the association between perimeter and √area and compared. The slopes of the association between both perimeter and √area were obtained for the progeny type (turlough - turlough or turlough – non-turlough) and were statistically compared using the interaction of perimeter and √area derived from ANOVA with √area as a continuous independent variable.

3.2.5 DNA extraction, clean up and quantification

DNA extraction, clean up and quantification were carried out on thirty individuals sampled from along the transect using the same method employed in section 2.2.4, 2.2.5, and 2.26.

3.2.6 ISSR procedure

3.2.6.1 ISSR-PCR

Inter simple sequence repeat PCR reactions were of a volume of 50µl, and used 0.5µl primer at a concentration of 100ng/µl, 1µl of template DNA (approximately 200ng/µl), 5µl PCR buffer x 10 [10mM Tris-HCl (pH 9.0), 50mM KCl Promega], 3mM $MgCl_2$ (Promega), 250µM each of dATP, dCTP, dGTP, and dTTP, and 0.5 units *Taq* polymerase. Reactions were made up over ice and all samples were centrifuged at approximately 5000g in an MSE microcentrifuge and overlaid with 20µl of mineral oil. Amplification was carried out in a Perkin Elmer 480 thermal cycler. See Table 3.1 for cycling parameters.

Table 3.1: Cycling parameters for Inter simple sequence repeat reactions.

Step	Temperature (°C)	Time	Cycles
Denature	93	20 secs	40
Annealing	52	1 min	40
Strand extension	72	1 min	40
Final strand extension	72	7 min	1
Incubation	4	infinite	

After optimisation the following standard profile in Table 3.1 was used. Negative controls, lacking template DNA, were included in each PCR and each sample/primer combination was repeated at least once. Duplicate samples were also included in each PCR reaction to evaluate reproducibility and to estimate scoring error in the analysis. PCR products were separated by gel electrophoresis (110 volts for 50 minutes) on 1.5% agarose gels (11x14cm) in 1xTBE buffer and visualised using ethidium bromide staining on a Horizon® 11.14 gel rig (Life Technologies™). Successful reactions were re-run on 2% agarose gels (20x25cm) stained with ethidium bromide at 80 volts for 5 hours on a Horizon® 20.25 gel rig. ISSR bands were visualised on a UV trans illuminator and were photographed using a Kodak Digital Science DC120 Zoom Digital Camera. Images were imported into Kodak Digital Science ID 2.0.2 image analysis package and saved as a TIFF file for subsequent printing using an Apple Macintosh Laser writer.

3.2.6.2 ISSR primer screening

ISSR primers (Gibco BRL) were screened using a number of *R. repens* samples. Only those primers showing reproducible, polymorphic fingerprints were selected for further analysis. Table 3.2 shows primers that were screened for use on *R. repens* samples.

Table 3.2: ISSR primers used in suitability trials for *R. repens*, suitability ranges from poor *, to moderate **, and good *. Anchors were at 3' end of primer, meaning they will amplify any repeat designated by the primer at the 3' end of the sequence.**

Repeat	Anchor	Suitability
$(CA)_7$	NNN	*
$(CA)_8$	RG	***
$(GATA)_4$		*
$(GACA)_4$		***
$(CA)_8$	RY	***
$(CAC)_5$		**

Four suitable ISSR primers were used in single-primer reactions: $(CAC)_5$, $(CA)_8RY$, $(CA)_8RG$, and $(GACA)_4$

3.2.6.3 ISSR band scoring

Agarose gels (2%) were scored visually for the presence or absence of bands, with only strong and unambiguous bands considered in the analysis. Bands of equal fragment size were interpreted as homologous and a difference in staining intensity of the bands was not considered as a difference. The ISSR fingerprint of each individual for each primer was expressed in the form of a 0/1 matrix, 0 for the absence of a band and 1 for the presence of a band.

3.2.7 AFLP optimisation, reaction procedures and band scoring

All AFLP reactions were carried out using the same procedure outlined in section 2.2.7.1. Primer selection and optimum DNA concentration procedures were identical to those described in section 2.2.7.2.

3.2.8 Data analysis

3.2.8.1 Cluster analysis

Neighbor – joining (NJ) analysis was applied to a distance matrix based on Nei and Li distances (Nei & Lei 1979) (PAUP 4.0; Swofford 1998) for both the ISSR and AFLP

data. Internal support for grouping was assessed using 1000 bootstrap replicates (Felsenstein 1985).

3.2.8.2 Ordination methods

Principal coordinates analysis (PCO) was conducted with The R Package v4.0d (Casgrain 1999) using the Jaccard similarity measure (Jaccard 1908) (see section 2.2.8.2). Ordination of individuals is displayed in three different ways. Firstly, individuals were assigned to three arbitrary categories depending on elevation along the transect and colour coded accordingly.

- Top (quadrat 1 to quadrat 3, elevation 7m – 6.4m) Red
- Middle (quadrat 5 to quadrat 8, elevation 5.2m – 3.4m) Blue
- Bottom (quadrat 10 to quadrat 12, elevation 2m – 0.8m) Green

This category was further subdivided by quadrat so that each quadrat was given a different shade within the position category colour. Secondly individuals were also categorised in terms of leaf dissection index. Genotypes were ranked according to leaf dissection and placed in one of three categories, each of which was given a different symbol. These arbitrarily assigned categories did not necessarily mirror position of the plants along the transect with some individuals from the middle of the transect being placed in the broadleaf category, and some individuals from the bottom of the turlough basin being placed in the intermediate leaf dissection category.

- Broad-leaved (Dissection Index range 2.56 to 3.21) Triangle
- Intermediate (Dissection Index range 3.24 to 4.09) Square
- Dissected (Dissection Index range 4.21 to 6.61). Circle

Ordinations for all combinations of the first three axes were graphed in Microsoft Excel.

3.2.8.3 Marker frequency variation

The frequency of each band in each of the thirty individuals was calculated as the proportion of plants along the transect sampled with the band present. The individuals

were assigned to three arbitrary categories depending on elevation along the transect as described in section 3.2.8.2. Individuals were also categorised in terms of leaf dissection index as described in section 3.2.8.2. A contingency χ^2 test was employed to assess the statistical significance of differences in marker frequency between categories. By comparing observed and expected frequencies this test allows one to determine whether or not the values obtained deviate from those determined by the null hypothesis.

3.2.8.4 Mantel test for relationship between genetic and geographic distances

Mantel tests using Jaccard distances and geographic distance between quadrats were carried out using PC ORD version 3.04. The Mantel test evaluates the null hypothesis of no relationship between two dissimilarity (distance) or similarity matrices. The Mantel test is an alternative to regressing distance matrices that circumvents the problem of partial dependence in these matrices. Two methods are available in the PC ORD program: Mantel's asymptotic approximation method and the randomisation (Monte Carlo), both were used in the analysis.

3.3 Results

3.3.1 Breeding experiment

Table 3.3 provides information on the experimentally produced F1 hybrids maintained in cultivation in Trinity College Botanic Gardens and it can be seen that some of the crosses were more successful than others. Out of the 60 plants in propagation, 14 flowered, only 8 successful crosses were made and only 2 of these were a complete reciprocal cross (Q1.2 and 10.3, Q1.5 and 11.6). There are many reasons for this; the genotypes that occur at the bottom of the turlough basin (Q10-Q12) often do not produce many flowers. As a result it was difficult to obtain plants that could act as both a recipient and donor of pollen. A second factor that influenced the outcome of this experiment was that many of the flowerheads enclosed with nylon mesh bags were infested with woolly aphids.

Table 3.3: Mean leaf dissection index (LDI) for each of the *R. repens* pollen recipients and donors used in the reciprocal crosses. The number of F1 generation hybrids as well as their mean LDI produced from these crosses between turlough population individuals of *R. repens* from different positions along Hawkhill transect, and between Hawkhill individuals and dry pasture population individuals from Trinity College Botanic Gardens. All quadrats (Q) were taken from along the transect through Hawkhill turlough basin. FAT, FAG, and Tunnel are all genotypes from the Trinity College Botanic Gardens.

Pollen recipient	LDI of recipient	Pollen donor	LDI of donor	Number of Progeny	Mean LDI of progeny
Q1.2	2.66	Q10.3	5.72	26	4.24
Q1.4	3.18	Q12.1	6.61	14	4.56
Q1.5	3.28	Q11.6	4.52	18	4.96
Q10.3	5.72	Q1.2	2.66	12	4.25
Q10.5	4.10	FAT	4.59	5	3.49
Q11.6	4.52	Q1.5	3.28	52	5.58
Tunnel	4.46	Q10.2	3.93	52	4.58
FAG	3.19	Q11.1	4.25	68	3.77

3.3.2 Leaf dissection measures

Individuals from the top and bottom of the transect running through Hawkhill turlough (turlough – turlough cross), as well as individuals from the bottom of the transect and a non-turlough population (turlough – non-turlough cross) were crossed

and the leaf dissection of the resulting progeny examined. The mean leaf dissection indices for the parents and progeny are given in Table 3.3. Leaf dissection for parents sampled at the top of the turlough basin (Q1) were found to be very similar to those of the parents used at Trinity College Botanic Gardens. Individuals sampled from the top of the turlough basin (Q1) had a much lower dissection index than those sampled from the bottom of the basin (Q10, 11 and 12) with one exception (Q10.2) which had an index comparable to those found at the top of the turlough basin.

The highest dissection index was found in the progeny from a turlough-turlough cross. These individuals, produced from a cross between a genotype from the bottom of the turlough basin (Q11.6) as a pollen recipient and an individual from the top of the turlough basin (Q1.5) as pollen donor, had a mean leaf dissection index of 5.58. This LDI was higher than that found in either of the parents. The lowest mean leaf dissection index (3.49) found in the offspring resulted from a cross between a pollen recipient from the bottom of the turlough basin (Q10.5) and a pollen donor from the Trinity College Botanic Gardens (FAT). The breeding experiment yielded just one reciprocal cross (Q1.5 with Q11.6). Mean leaf dissection for the resulting progeny was found to be higher when the pollen recipient was from the bottom of the turlough basin (Q11.6) as opposed to the top of the turlough basin (Q1.5).

The perimeter and √area values for each type of cross (turlough - turlough and turlough - non-turlough) were grouped and the resulting data examined. The relationship between perimeter and √area for the progeny are presented in Figure 3.1. There was a certain degree of overlap between the turlough – non-turlough and the turlough - turlough crosses. The relationship between perimeter and √area was different between the crosses, the full turlough cross progeny had a greater slope than the turlough - non-turlough hybrid progeny (26.37 and 12.62 respectively) indicating that the perimeter was relatively greater for a given √area in the pure turlough cross (Table 3.4). The progeny derived from a turlough pollen donor and a turlough pollen recipient had a significantly greater slope, and therefore leaf dissection index than the progeny derived from a cross between a turlough and non-turlough parent ($F = 48.42$, $df = 257,1$ $P<0.0001$).

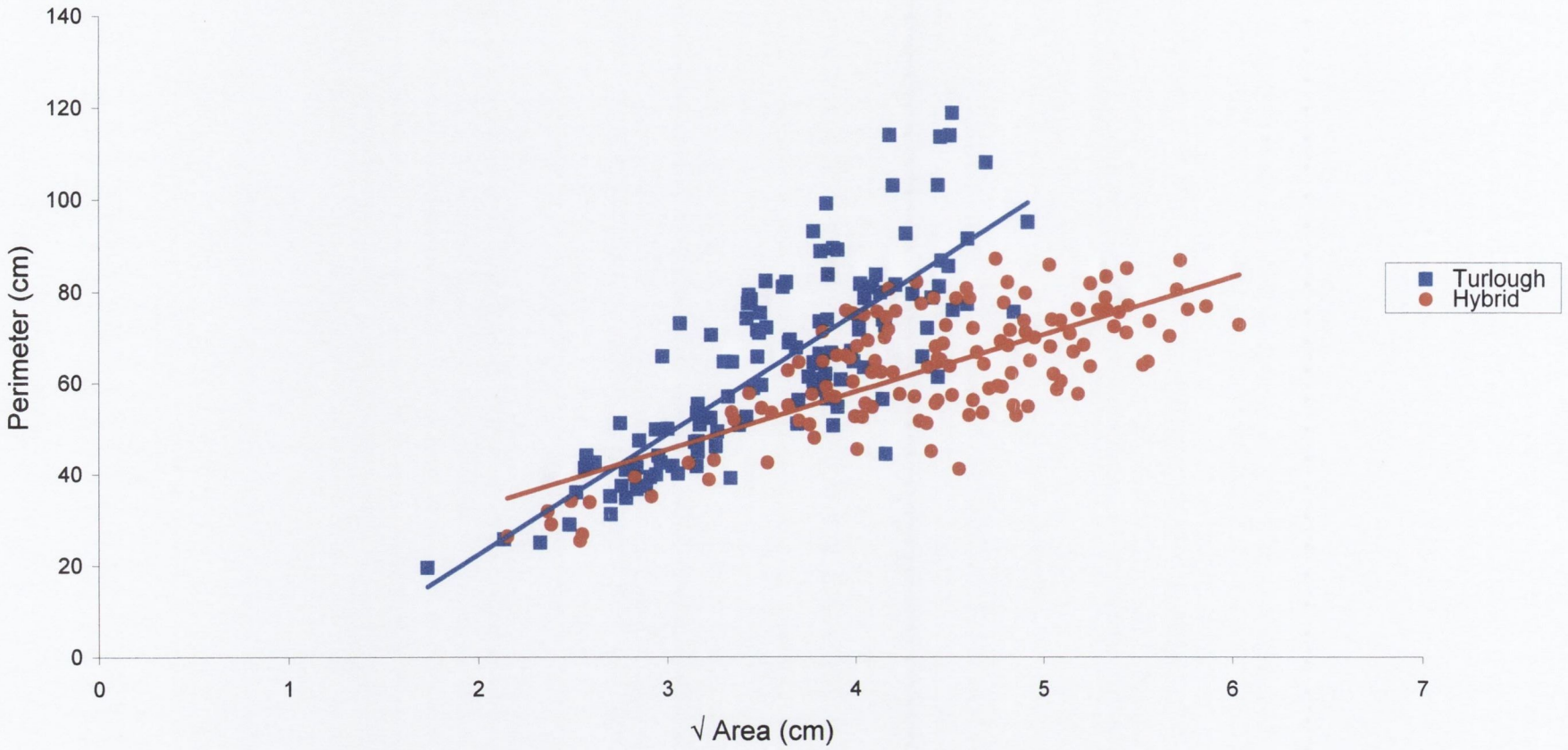

Figure 3.1: The relationship between √area and perimeter for progeny derived from experimental crosss between turlough and non-turlough individuals (hybrid) and turlough individuals (turlough) of *R.repens*. Regression equations are given in Table 3.4.

Table 3.4 Regression equations for the relationship between perimeter and √area for each of the experimental crosses and pooled means of leaf dissection indices for turlough, and hybrid cross progeny of *R. repens*. n = sample size.

Cross	Mean LDI ± se	n	Regression equation	F value	P value	df
T – T	4.98 ± 0.008	123	Y=26.37X – 30.21	209	<0.0001	1, 121
T – NT	4.06 ± 0.006	135	Y=12.62X + 7.61	161	<0.0001	1, 132

Differences in leaf dissection between the pure turlough cross and hybrid cross were examined. The pooled data for the dissection index of the pure turlough crosses had a significantly higher dissection index than the hybrid cross (Table 3.5). The progeny derived from a turlough pollen donor and a turlough pollen recipient had a significantly higher dissection index than the progeny derived from a cross between a turlough and non-turlough parent ($P< 0.0001$).

3.3.3 DNA extraction, and quantification

Extracted, cleaned DNA concentration varied greatly between samples, ranging from 174ng/µl to 1360ng/µl.

3.3.4 ISSR analysis

3.3.4.1 ISSR patterns

A total of 48 bands resulted from the ISSR analysis of individuals from the Hawkhill transect using 4 primers (Table 3.5). Primers varied in their ability to detect differences in individuals sampled. Primers that produced most polymorphic bands were $(CA)_8RY$ and $(GACA)_4$, the least suitable primer was $(CAC)_5$ which produced the lowest number of bands and showed only 60% polymorphism among samples. Each reaction was run twice and duplicates were included to check for scoring error, which was calculated at approximately 7% by comparing the same plants twice.

Table 3.5: Primers used and size range of bands detected for the analysis of 40 individuals from Hawkhill transect, Co. Clare.

Primer	Total number of bands	Number of polymorphic bands	% Polymorphism	Size range (bp)
$(CAC)_5$	5	3	60	1800 - 3700
$(CA)_8RY$	15	14	93	350 - 3000
$(CA)_8RG$	13	12	92	350 – 2750
$(GACA)_4$	15	14	93	300 - 1700

3.3.4.2 Cluster analysis

Figure 3.2a displays the unrooted phylogram obtained from the NJ analysis of the ISSR data from 34 *R. repens* individuals along the transect. There is evidence of clonal identity from this analysis with four individuals from quadrat one grouping as two genotypes and two individuals from quadrat eleven having the same banding pattern. Samples from quadrat eight also had similar banding patterns and grouped closely in the unrooted phylogram. Individuals sampled from the same quadrat along the transect group together, although there is no separation or grouping of individuals from the top and the bottom of the transect; For example, some individuals from Q1 and Q12 group closely together. Figure 3.2b shows the bootstrap consensus tree for the NJ analysis. Not all groupings were given bootstrap support, and some branches were given higher bootstrap support than others in the analysis, such as the grouping of individuals from quadrat two and individuals from quadrat 11. High bootstrap values were mainly given to individuals sampled from the same quadrats along the transect. For example the grouping of individuals from quadrat 2 had bootstrap support of 95%. Table 3.6 shows the leaf dissection index for all individuals used in the ISSR analysis. Some individuals sampled from the top of the turlough basin have a relatively high dissection index (Q1.6, Q3.1), and some individuals from the bottom of the turlough basin have a relatively low dissection index (Q10.2, Q10.5) when compared to others (Q1.2 and Q10.3 respectively).

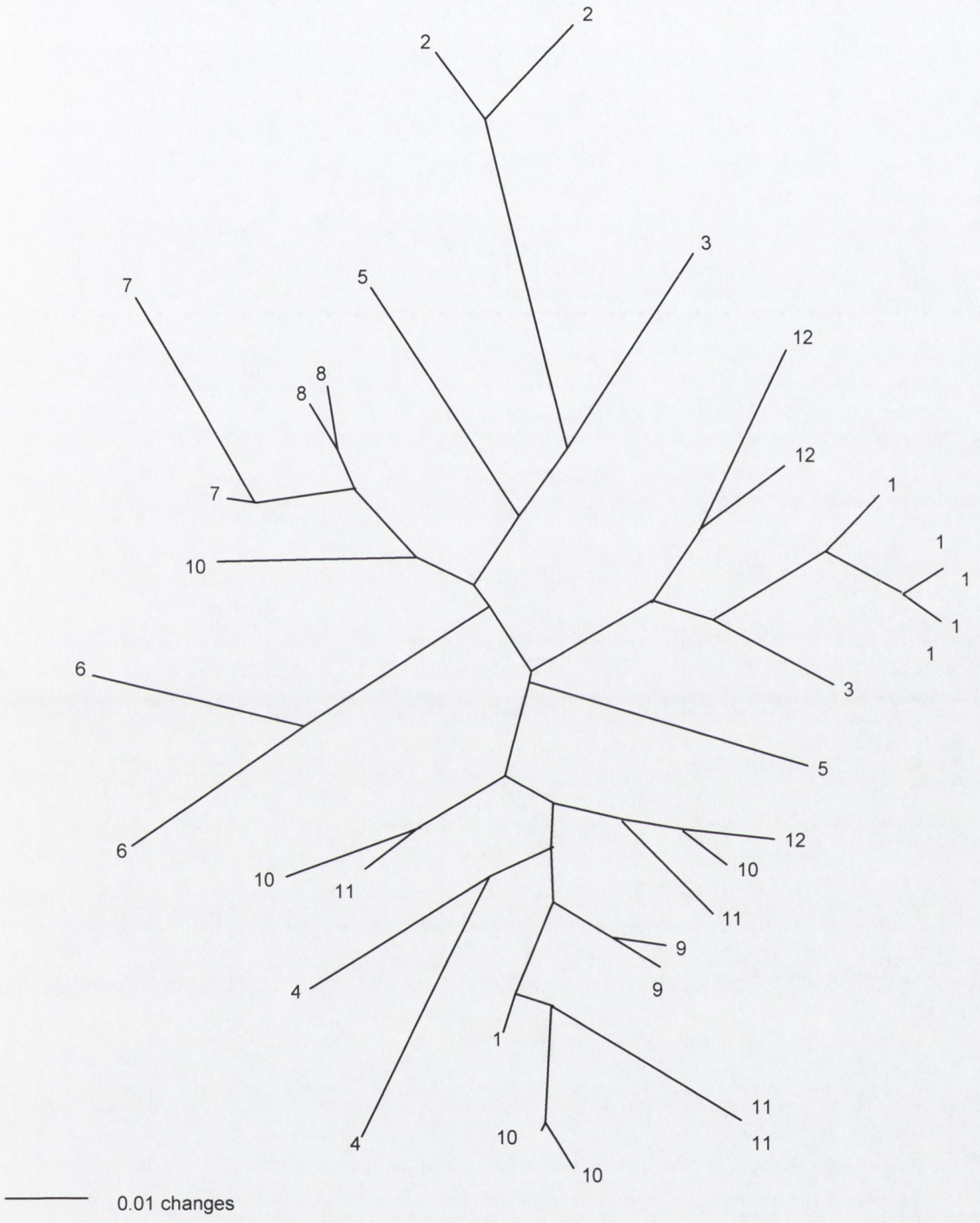

Figure 3.2a: A NJ tree based on Nei & Li (1979) distance estimated from ISSR data for *R.repens* individuals sampled along a transect through Hawkhill turlough basin. Numbers at the end of branches represent quadrat numbers; One at the top of the basin running through to 12 at the bottom of the turlough basin, an increase in number corresponds to an increase in period of inundation.

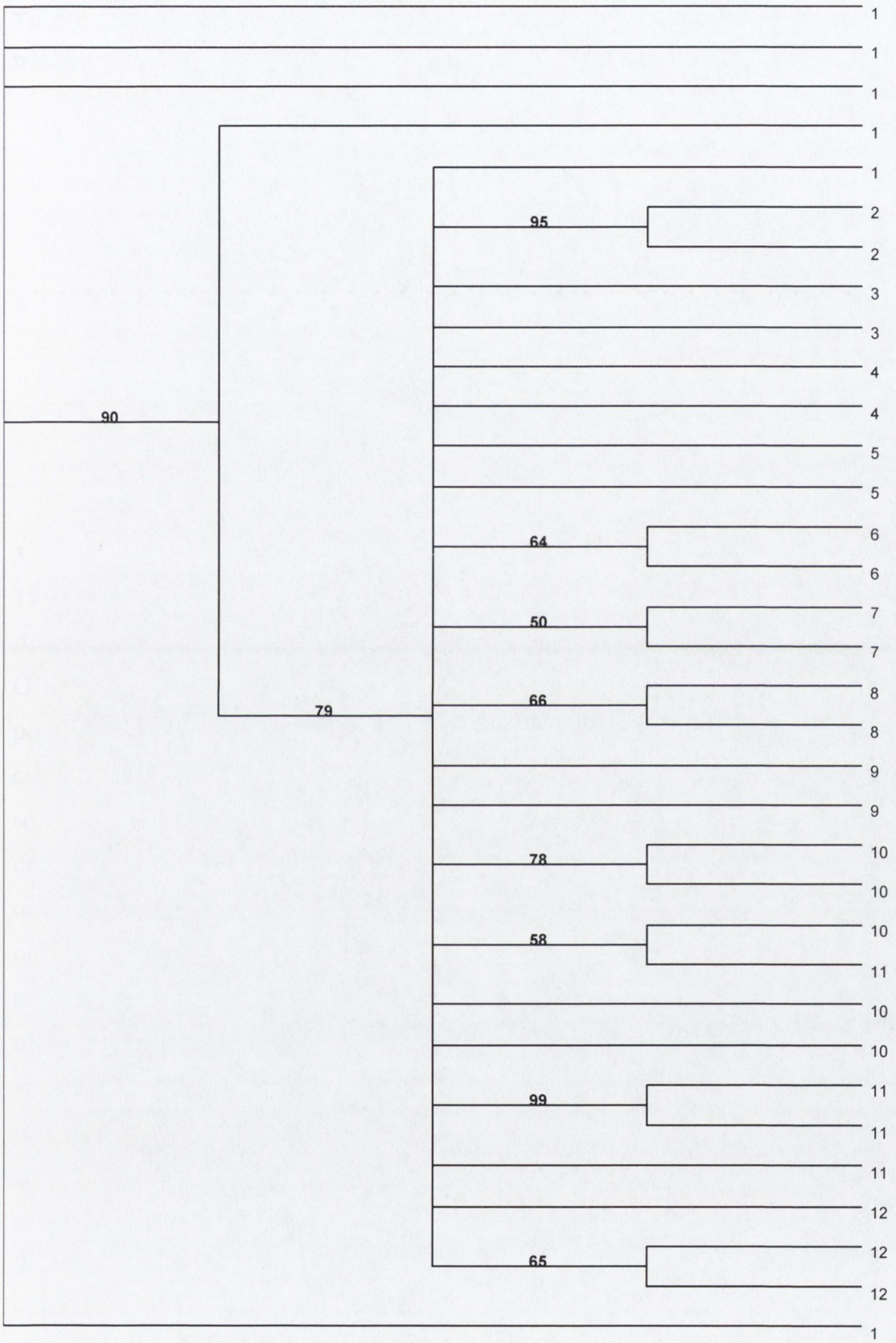

Figure 3.2b: A NJ BS tree based on Nei & Li (1979) distance estimated from ISSR data for *R.repens* individuals sampled along a transect through Hawkhill turlough basin. Numbers at the end of branches represent quadrat numbers; One at the top of the basin running through to 12 at the bottom of the turlough basin, an increase in number corresponds to an increase in period of inundation. Numbers on the branches represent bootstrap support.

Table 3.6: Leaf dissection indices for the 34 *R. repens* samples from Hawkhill transect used in the ISSR analysis.

Quadrat	LDI	Quadrat	LDI	Quadrat	LDI
1.1	2.99	5.3	3.06	10.2	3.93
1.2	2.66	5.5	3.33	10.3	5.72
1.3	2.70	6.1	3.14	10.5	4.10
1.4	3.18	6.3	3.45	10.6	4.76
1.5	3.28	7.1	3.22	11.1	4.25
1.6	3.72	7.3	2.87	11.2	4.42
2.3	3.24	8.3	3.19	11.5	4.52
2.4	3.16	8.5	3.66	11.6	4.21
3.1	3.67	9.2	4.06	12.1	6.61
3.2	3.21	9.6	3.26	12.4	4.46
4.3	3.34	10.1	5.51	12.6	4.21
4.5	2.71				

3.3.4.3 Ordination

The first three principal co-ordinates accounted for 43% of the variation in the data. Axes 1, 2 and 3 accounted for 19, 15, and 9% of the variation respectively. Ordinations were plotted as described in section 3.2.8.2, denoting the category for position along the transect in different colours, quadrats in different shades of these colours and leaf dissection categories denoted by different symbols. Axis one separates those individuals from quadrats 1, 2, 3, 5, 6, and 7 from those collected from quadrats 8, 9, 10, and 11 (Figure 3.3a). In terms of position along the transect, axis one separates samples from the top of the transect from the rest with the exception of one individual from quadrat one and one individual from quadrat three being notably separate from this group. Axis one also appears to group individuals from the middle of the transect and from the bottom of the transect in the ordination space. In terms of spatial separation of samples this category gives a comprehensive view of the genetic relationship between individuals. This trend is not seen as clearly in terms of leaf dissection (Table 3.6). Some individuals in each of the leaf dissection categories appear to cluster in the ordination space but these grouping are weak. For example there is a general trend for broadleaved individuals to group at the negative end of axis one, whereas the majority of individuals with an intermediate or dissected leaf form cluster at the positive end of axis one. Axis two and three are more difficult to interpret, the general trend is for axis two to separate individuals from the middle of the transect from those individuals positioned at the top and bottom of the transect (Figures 3.3a, b, and c).

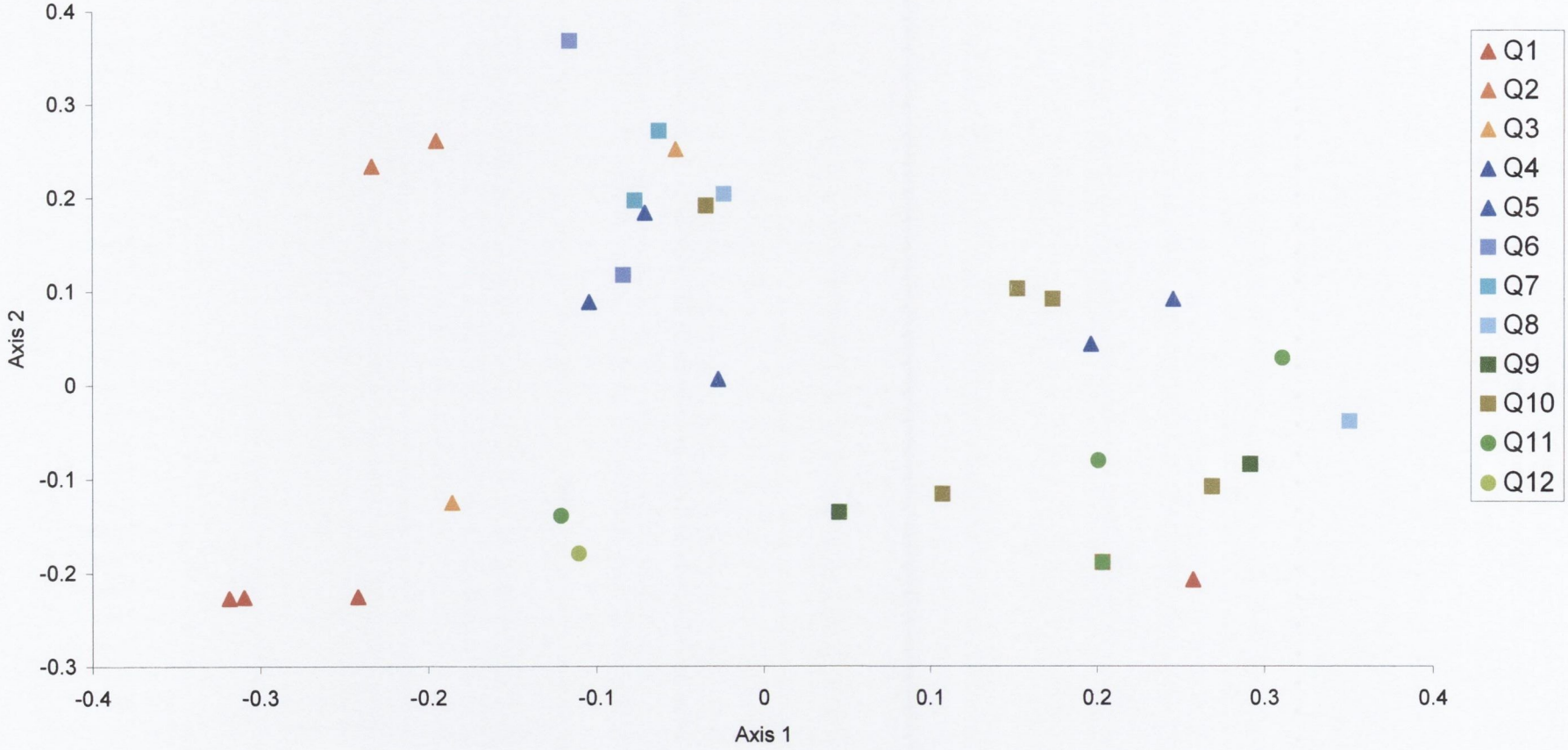

Figure 3.3a: Axis 1 and 2 of a PCO analysis of ISSR data obtained for *R.repens* samples along a transect through Hawkhill turlough basin. Triangles, squares, and circles represent broad, intermediate and dissected leaved individuals respectively.

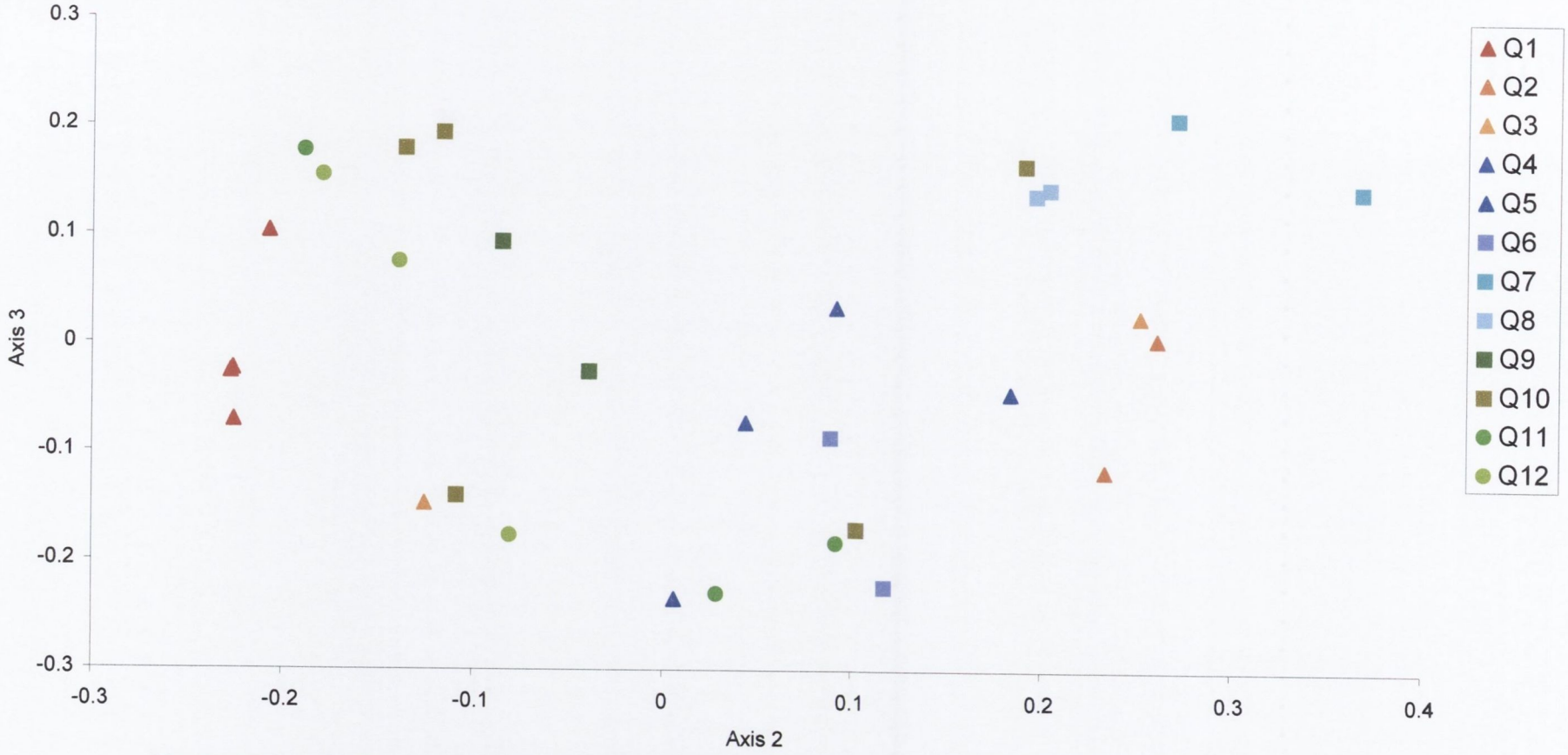

Figure 3.3b: Axis 2 and 3 of a PCO analysis of ISSR data obtained for *R.repens* samples along a transect through Hawkhill turlough basin. Triangles, squares, and circles represent broad, intermediate and dissected leaved individuals respectively.

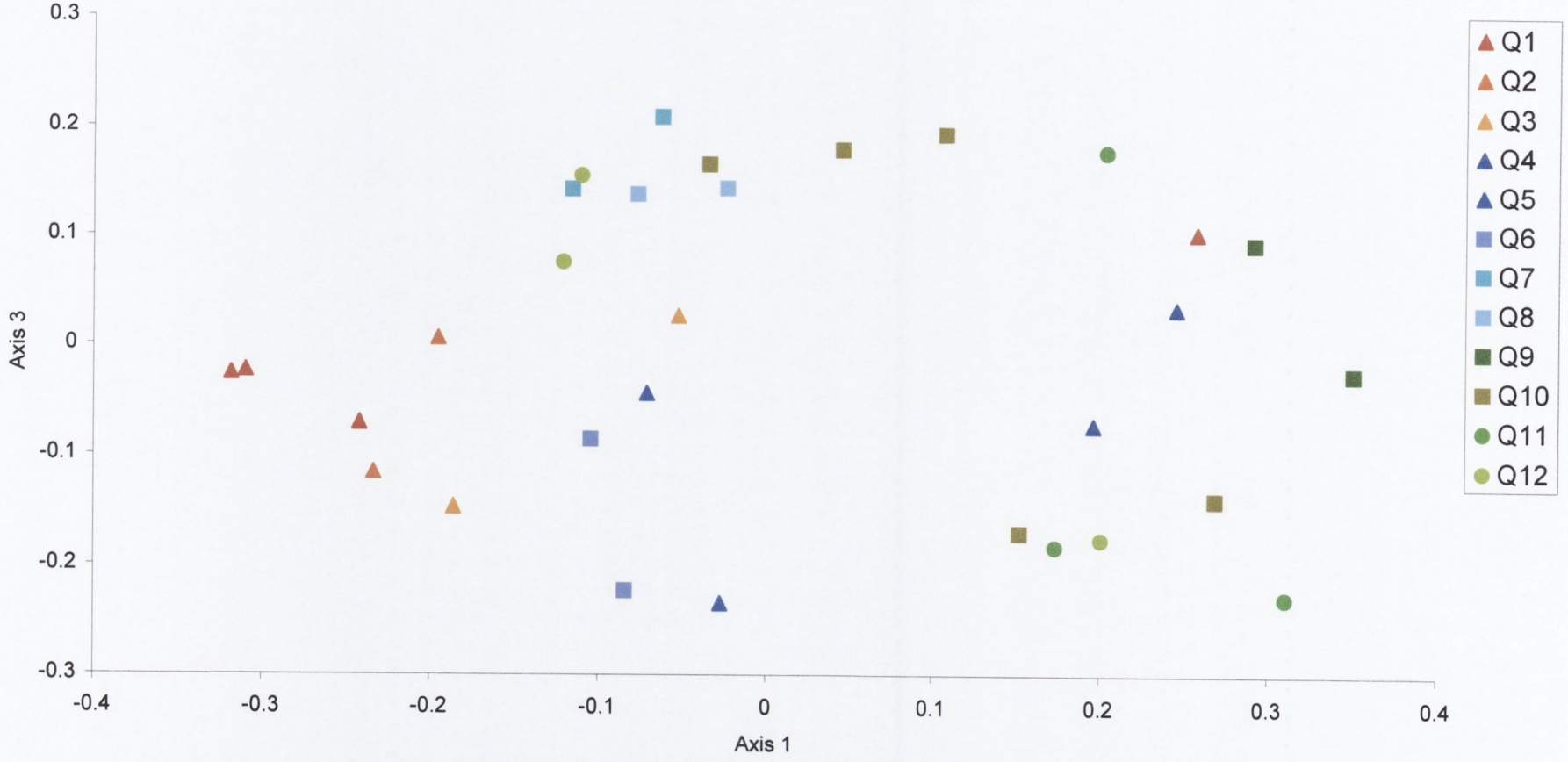

Figure 3.3c: Axis 1 and 3 of a PCO analysis of ISSR data obtained for *R.repens* samples along a transect through Hawkhill turlough basin. Triangles, squares, and circles represent broad, intermediate and dissected leaved individuals respectively.

3.3.5 AFLP analysis

3.3.5.1 AFLP patterns

Initial scoring of electropherograms from the AFLP analysis of 30 individuals from Hawkhill transect using three pairs of primers revealed a total of 151 different markers. Some bands were ignored following the criteria for the removal of unreproducible, monomorphic bands as described in section 2.3.2. A total of 40 markers were removed from the analysis. Therefore the final analysis consisted of 111 different markers (74% polymorphism). Scoring error was calculated from the distance matrix and averaged at 5% over all individuals.

The AFLP primer pairs differed in their ability to detect polymorphism within samples. The mean number of polymorphic bands amplified over all samples per primer pair combination varied from 24 with AGG-CTC, to 50 with AAC-CAG, to a mid value of 37 with ACT-CAG. The frequency of individual markers also varied between primers with ACT-CAG marker presence ranging from 2 to 12 individuals, AGG-CTC marker presence ranging from 6 to 13 individuals and, AAC-CAG marker presence ranging from 6 to 25 individuals. On average ACT-CAG markers occurred in 5 individuals, AGG-CTC bands occurred in 11 individuals, and AAC-CAG bands occurred on average in 9 individuals. The mean number of total polymorphic markers (all three primers) per individual ranged from 19 in an individual from quadrat 12, to 42 in an individual from quadrat 10. The mean number of polymorphic bands per individual is given below in Table 3.7.

Table 3.7: Mean number of polymorphic AFLP markers per *R. repens* individual sampled along Hawkhill transect.

Quadrat	Markers / Individual	Quadrat	Markers / Individual	Quadrat	Markers / Individual
1.1	28	5.3	34	10.3	39
1.2	27	5.5	27	10.5	42
1.3	34	6.1	29	10.6	34
1.4	27	6.3	26	11.1	27
1.5	23	7.1	35	11.2	33
1.6	25	7.3	22	11.5	21
2.3	34	8.3	28	11.6	33
2.4	30	8.5	26	12.1	19
3.1	37	10.1	40	12.4	20
3.2	29	10.2	40	12.6	23

3.3.5.2 NJ analysis

Patterns in terms of clinal variation along the transect were not apparent in the unrooted phylogram based on the AFLP data (Figure 3.4a). Although individuals collected from proximal positions along Hawkhill transect tended to group together, overall separation was unclear. The bootstrap consensus tree (Figure 3.4b) shows strong support for some groupings and, as in the ISSR analysis, this may imply the presence of clones along the transect. For example the grouping of individuals from quadrats 2, 6 and 8 had bootstrap values of 94%, 91% and 87% respectively. However the branch lengths on the tree possibly refutes the idea of clonality. It is also apparent that individuals from Q10 are closely allied.

3.3.5.3 Ordination

The first three principal coordinates accounted for 33% of the variation in the AFLP data. Axes 1, 2 and 3 accounted for 15, 10, and 8% of the variation respectively. Ordination of individuals is displayed in the manner as for the ISSR data and is described in section 3.2.8.2. It is apparent from the scatterplots obtained from the AFLP data that there is some degree of population genetic structuring.

When individuals were split according to quadrat those from the same quadrat generally grouped together in the same ordination space. Axis 1 separates individuals sampled from Q1 – 3 from those along the remainder of the transect (Figure 3.5a). Axis 2 reveals fewer patterns in the data, but does appear to separate Q1 from Q10 and axis 3 generally separates individuals from the top six quadrats from the bottom six quadrats. In terms of position along the transect, axis one separates samples from the top of the transect from the rest. Axis one also appears to group individuals from the bottom of the transect in ordination space and those individuals found in the middle of the turlough basin are somewhat scattered in the ordination space. This trend is followed when individuals are categorised in terms of leaf dissection with a few notable exceptions. Axis one separates all individuals in the broadleaved category from the rest with the exception of one individual from quadrat five (Figure 3.5a). This trend is not followed through in the intermediate and dissected leaf categories with individuals being generally scattered in the ordination space. As with the ISSR data axes two and three are more difficult to interpret, the general trend is for axis two

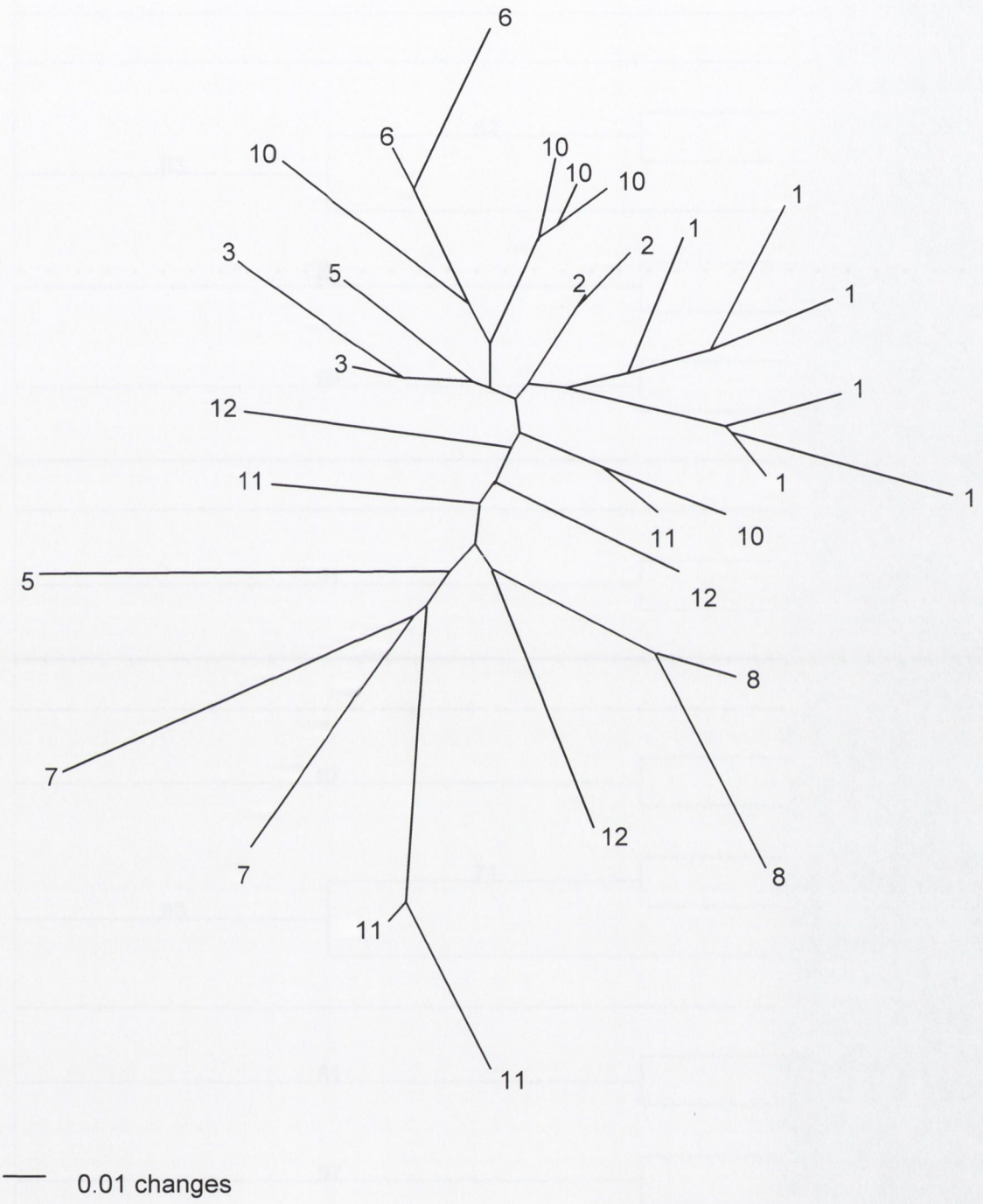

Figure 3.4a: A NJ tree based on Nei & Li (1979) distance estimated from AFLP data for *R.repens* individuals sampled along a transect through Hawkhill turlough basin. Numbers at the end of branches represent quadrat numbers; One at the top of the basin running through to 12 at the bottom of the turlough basin, an increase in number corresponds to an increase in period of inundation.

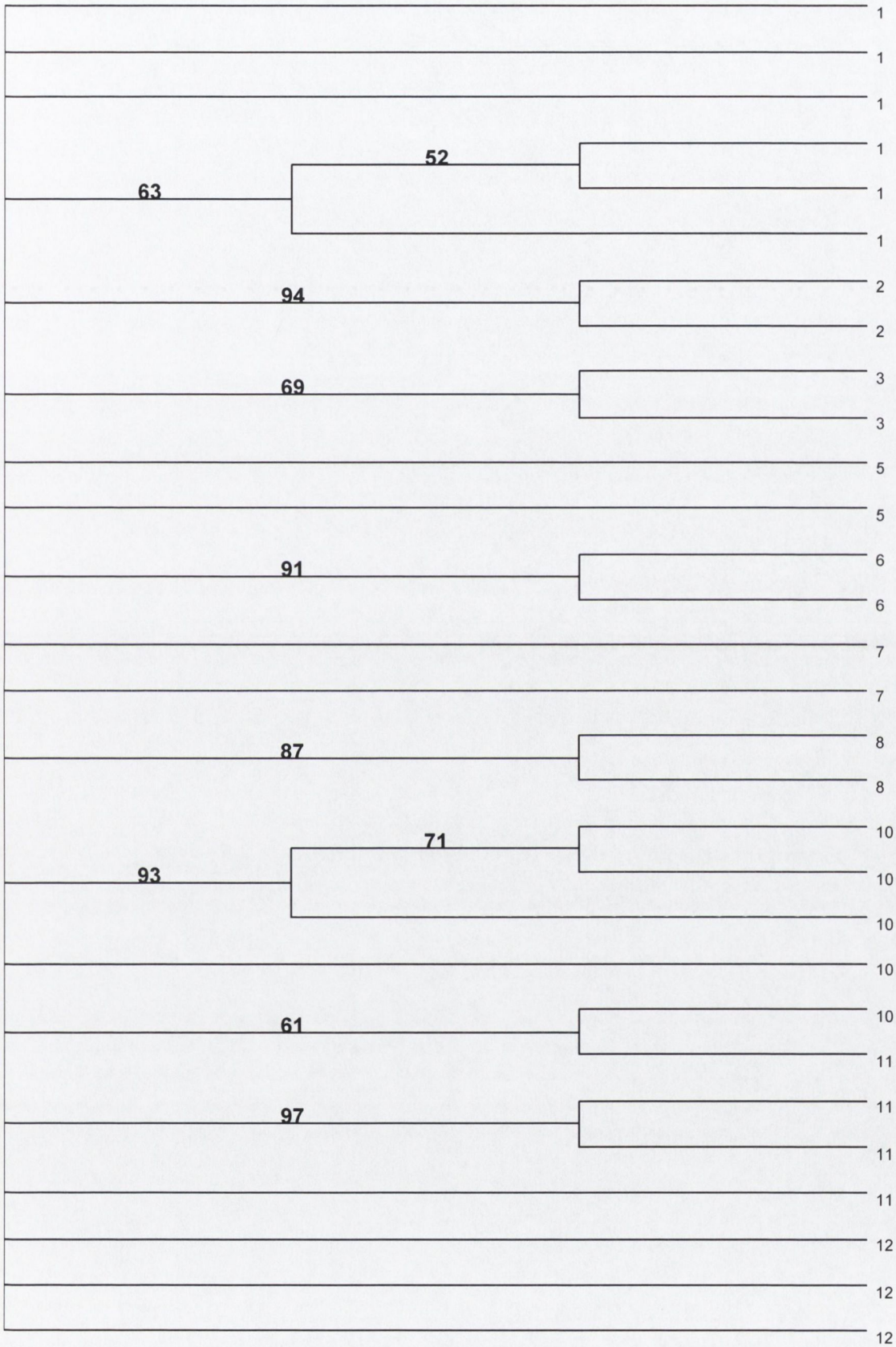

Figure 3.4b: A NJ tree based on Nei & Li (1979) distance estimated from AFLP data for *R.repens* individuals sampled along a transect through Hawkhill turlough basin. Numbers at the end of branches represent quadrat numbers. Numbers on the branches represent bootstrap support.

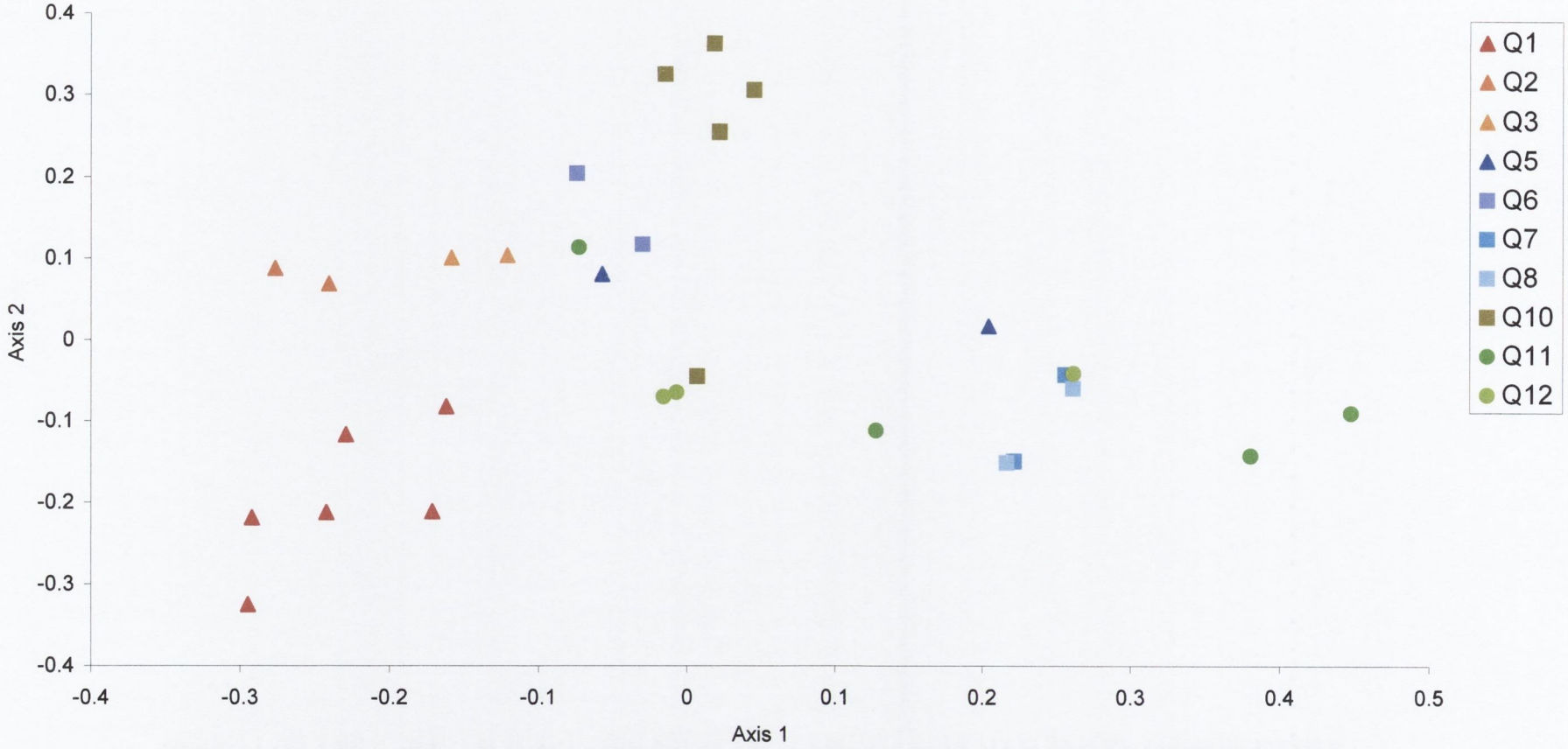

Figure 3.5a: Axis 1 and 2 of a PCO analysis of AFLP data obtained for *R.repens* samples along a transect through Hawkhill turlough basin. Triangles, squares, and circles represent broad, intermediate and dissected leaved individuals respectively.

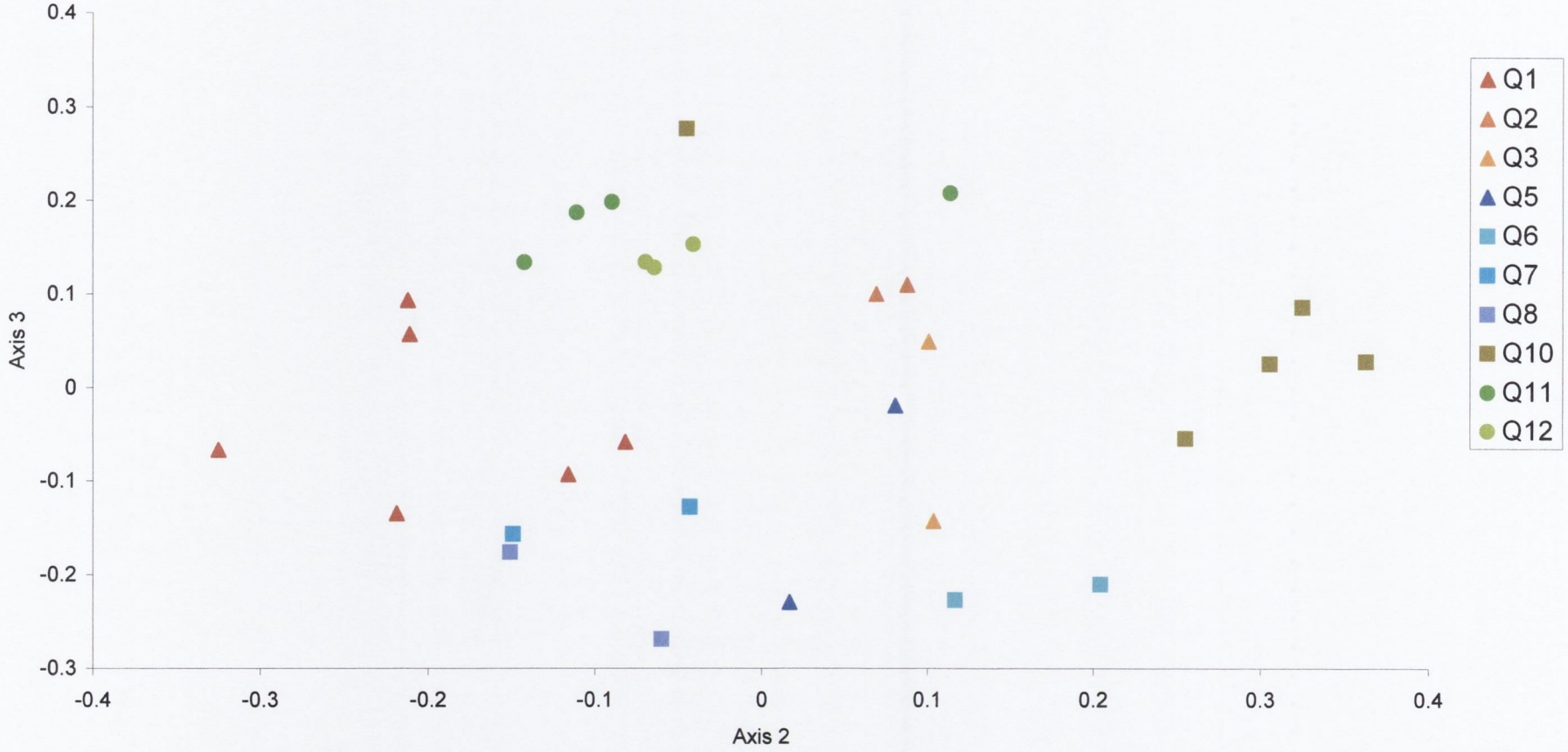

Figure 3.5b: Axis 1 and 2 of a PCO analysis of AFLP data obtained for *R.repens* samples along a transect through Hawkhill turlough basin. Triangles, squares, and circles represent broad, intermediate and dissected leaved individuals respectively.

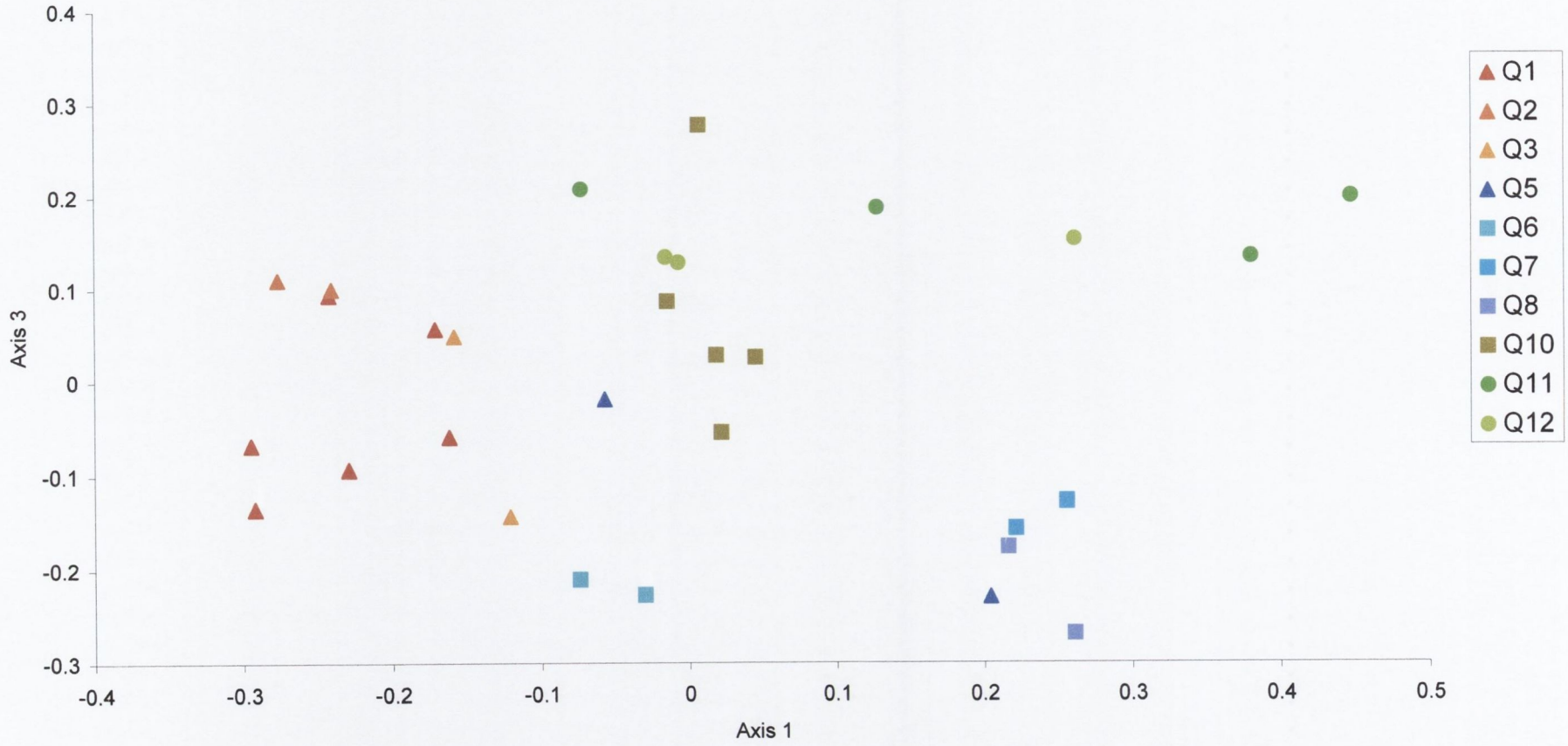

Figure 3.5c: Axis 1 and 3 of a PCO analysis of AFLP data obtained for *R.repens* samples along a transect through Hawkhill turlough basin. Triangles, squares, and circles represent broad, intermediate and dissected leaved individuals respectively.

to separate individuals according to quadrat and for axis three to separate individuals from the bottom of the transect from the remainder of the samples (Figures 3.5b, c).

The general trend, found from PCO analysis of the ISSR and AFLP data is for individuals from the same position along the transect to group together in the ordination space but this finding is not without exceptions; one individual sampled from each of Q 1, 3, and 5 cluster with individuals sampled from the bottom of the transect (Q10 – 12). It appears from both sets of data that there are more groupings of individuals according to quadrat and position along the transect than for leaf dissection category.

3.3.5.4 AFLP marker frequencies

Individuals were categorised into groups according to elevation along Hawkhill transect and 9 markers were chosen from the AFLP data that appeared to show heterogeneous distribution. Four of these showed a significantly (Chi sq. with 2 degrees of freedom) heterogeneous distribution at the 5% level, and four more showed heterogeneous distribution at the 1% level. These markers are listed in Table 3.8. Markers 1 and 4 were present in 60% of individuals from the top of the transect and completely absent from individuals from either the middle or bottom of the transect. In contrast, markers 5, 6, 7, and 8 were present in 90%, 40%, 40%, and 50% respectively, of individuals at the bottom of the turlough basin, being completely absent from individuals in the middle or at the top of the basin. All of these markers, with the exception of marker 2 showed significantly heterogeneous distribution in frequency along the transect. The lack of significance of marker 2 was most likely due to small sample size. Figure 3.6 shows frequencies for each of the nine AFLP markers given in Table 3.8. Markers 1, 4 and 5 show the extremes of selected marker distribution along the transect, these findings are revealed by their significance in a Chi square test. Other markers such as 2 and 3 are absent from only one of the three position categories arbitrarily assigned.

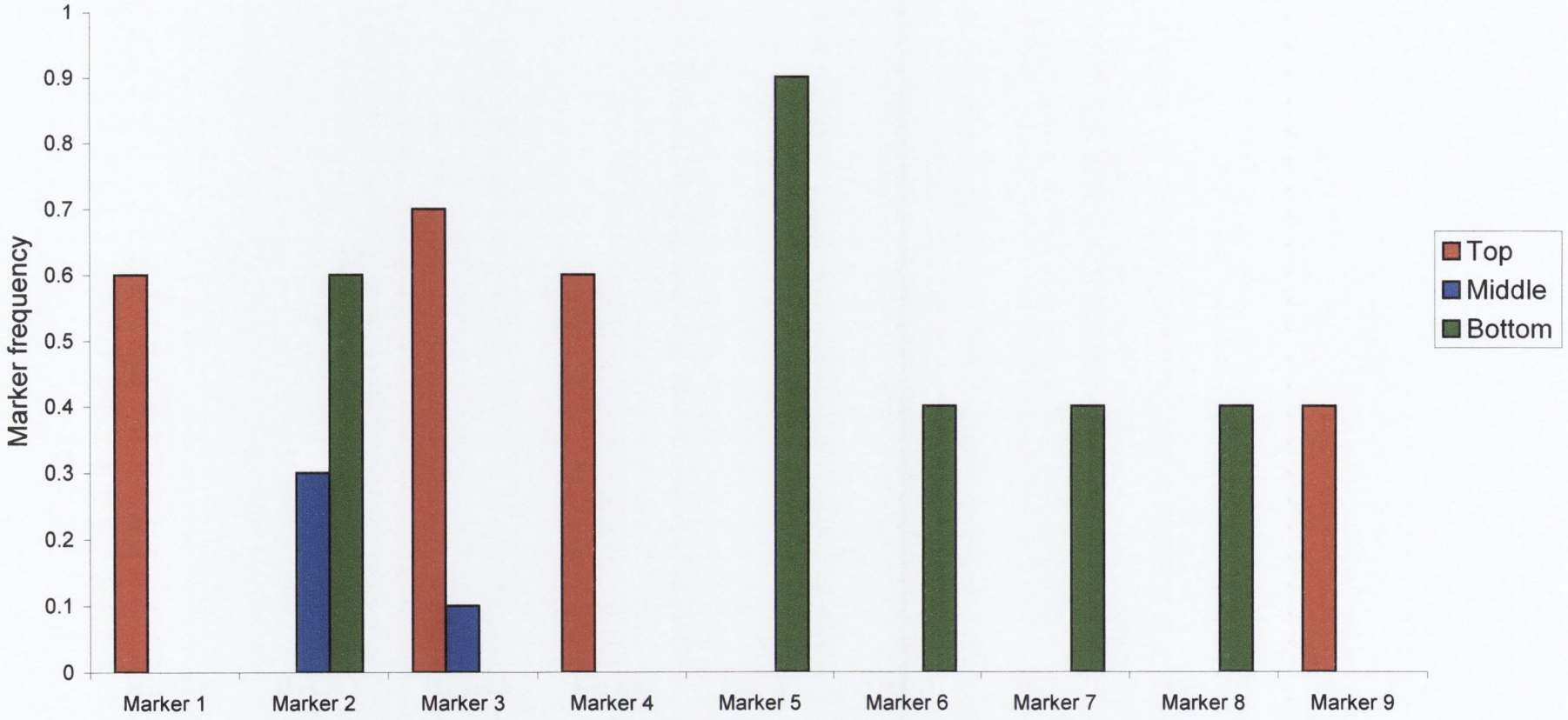

Figure 3.6: Frequencies of AFLP markers with heterogeneous distribution in *R. repens* samples along a transect through Hawkhill turlough basin. Individuals are categorised according to position along the transect. Chi sq. values are given in Table 3.8.

Table 3.8: Contingency χ^2 analysis for nine AFLP markers from *R. repens* individuals positioned along Hawkhill transect. Samples are categorised into position along transect.

	Position				
	Top	Middle	Bottom	$\Sigma(O-E)^2/E$	χ^2 significance
Marker 1					
O	6	0	0		
E	2	1.6	2.4		
$(O-E)^2/E$	8	1.6	2.4	12	<0.01
Marker 2					
O	0	3	6		
E	3	2.4	3.6		
$(O-E)^2/E$	3	0.15	1.6	4.75	ns
Marker 3					
O	7	1	0		
E	2.7	2.1	3.2		
$(O-E)^2/E$	6.8	0.6	3.2	10.6	<0.01
Marker 4					
O	6	0	0		
E	2	1.6	2.4		
$(O-E)^2/E$	8	1.6	2.4	12	<0.01
Marker 5					
O	0	0	9		
E	3	2.4	3.6		
$(O-E)^2/E$	3	2.4	8.1	13.5	<0.01
Marker 6					
O	0	0	4		
E	1.3	1.1	1.6		
$(O-E)^2/E$	1.3	1.1	3.6	6	<0.05
Marker 7					
O	0	0	4		
E	1.3	1.1	1.6		
$(O-E)^2/E$	1.2	1.1	3.6	6	<0.05
Marker 8					
O	0	0	5		
E	1.7	1.3	2		
$(O-E)^2/E$	1.7	1.3	4.5	7.5	<0.05
Marker 9					
O	4	0	0		
E	1.3	1.1	1.6		
$(O-E)^2/E$	5.6	1.1	1.6	8.3	<0.05

Legend: χ^2 with two degrees of freedom; 5.99: P=0.05, 9.21: P=0.01. Markers 1 – 7 correspond to 126, 158, 173, 181bp (ACT CAG), 38, 108bp (AGG CTC), and 35, 67, 195bp (AAC CAG) respectively.

When individuals were categorised in terms of leaf dissection, which did not necessarily correlate with spatial distribution along the transect, 3 markers showed significant variation in marker frequency between groups, all at the 5% level (Table

3.9). No marker was found to be strictly diagnostic of any of the three groups in the sense that no band was present at a frequency of 100% in one group and absent from the remaining groups. However Marker 4 was found in only 30% of individuals of the broadleaf form, and in contrast, Marker 6 was found in 40% of dissected leaf individuals and was absent from individuals from either of the other two leaf shape categories (broadleaf, intermediate).

Table 3.9: Contingency χ^2 analysis for seven AFLP markers from *R. repens* individuals positioned along Hawkhill transect. Samples are categorised according to level of leaf dissection.

	Leaf shape				
	Broad leaf	Intermediate	Dissected	$\Sigma(O-E)^2/E$	χ^2 significance
Marker 1					
O	4	2	0		
E	2	2	2		
$(O-E)^2/E$	2	0	2	4	ns
Marker 2					
O	5	3	0		
E	2.7	2.7	2.7		
$(O-E)^2/E$	1.97	0.03	2.7	4.7	ns
Marker 3					
O	4	2	0		
E	2	2	2		
$(O-E)^2/E$	2	0	2	4	ns
Marker 4					
O	3	0	0		
E	1	1	1		
$(O-E)^2/E$	4	1	1	6	<0.05
Marker 5					
O	0	2	7		
E	3	3	3		
$(O-E)^2/E$	3	0.3	5.3	8.6	<0.05
Marker 6					
O	0	0	4		
E	1.3	1.3	1.3		
$(O-E)^2/E$	1.3	1.3	5.6	8.2	<0.05
Marker 7					
O	8	5	2		
E	5	5	5		
$(O-E)^2/E$	1.8	0	1.8	3.6	ns

Legend: χ^2 with two degrees of freedom; 5.99: $P<0.05$, 9.21: $P<0.01$. Markers 1 – 7 correspond to 126, 173, 181, 208bp (ACT CAG), 38, 108bp (AGG CTC), and 195bp (AAC CAG) respectively.

Figure 3.7 shows frequencies for each of the nine AFLP markers given in Table 3.9. Markers 4 and 6 were the least widespread, only occurring in the broadleaved and dissected leaf individuals respectively. The remaining markers were present in two of the three categories based on leaf shape, with the exception of marker 5; they did not show significant heterogeneous distribution in terms of leaf shape.

3.3.5.5 Spatial and genetic distances among individuals

Mantel tests using both Mantel's asymptotic approximation method and the randomisation (Monte Carlo) test in which the distances between individuals sampled along the transect were used to construct a second matrix for comparison with the AFLP distance matrix indicated that there was a significant relationship between genetic distance and geographic distance ($P = 0.0000012$) and ($P = 0.001$) respectively. This provides strong evidence that samples that were geographically close (i.e. were collected a small distance apart) were more likely to show lower genetic distance and thus higher genetic similarity in terms of the AFLP information.

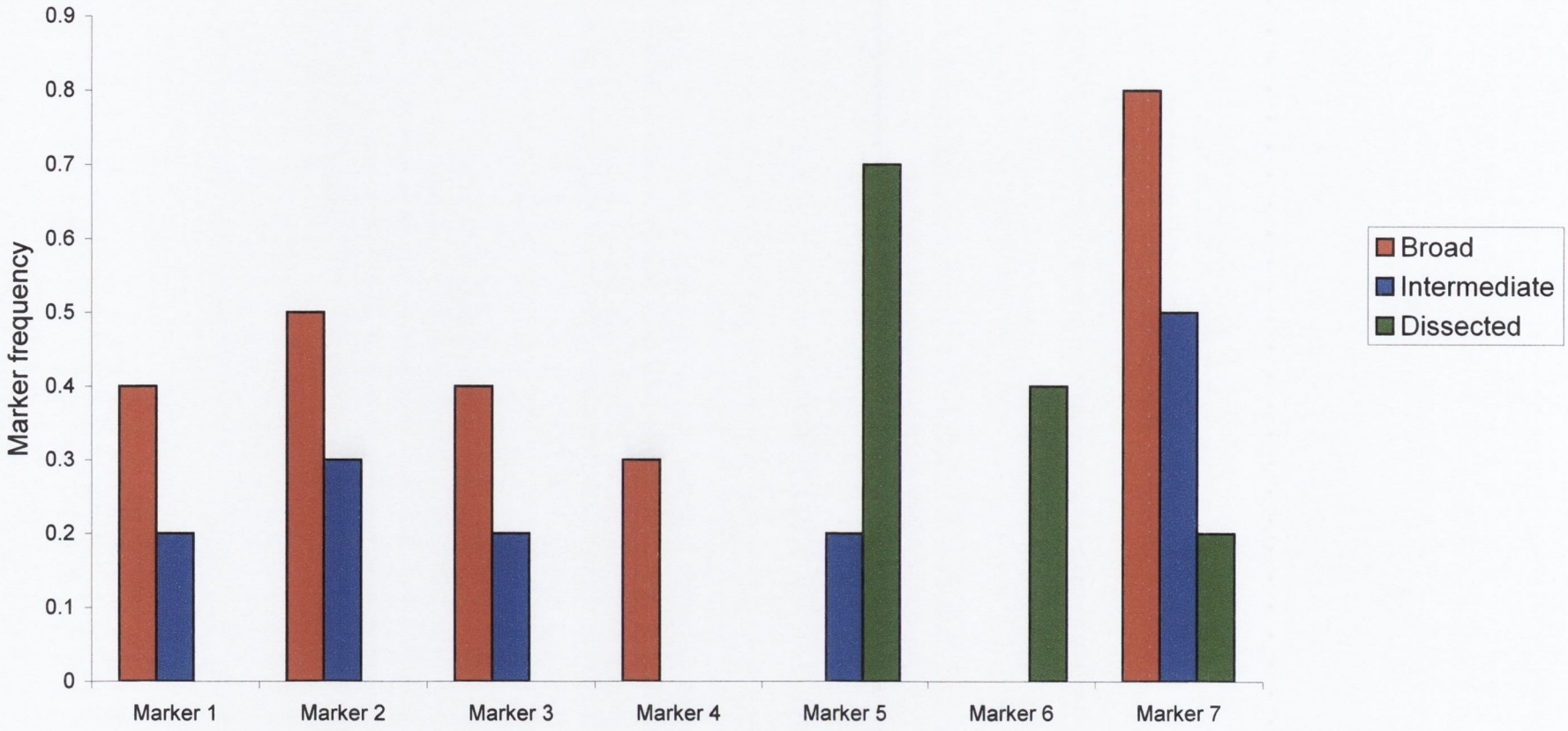

Figure 3.7: Frequencies of AFLP markers showing heterogeneous distribution in *R. repens* samples along a transect through Hawkhill turlough basin. Individuals are categorised according to leaf shape. Chi sq. significance values are given in Table 3.9.

3.4 Discussion

3.4.1 Morphological gradient along Hawkhill transect

Lynn & Waldren (2001a) found that the different leaf morphologies of *R. repens* individuals across Hawkhill turlough basin from broad to dissected were spatially separated. The broad form occurs at the top of a turlough basin where periods of inundation are short and infrequent, the intermediate form occurs in those areas where flooding time is most unpredictable, and the dissected form is found at the bottom of the turlough basin where periods of submergence are prolonged. It is apparent that there is a link between increased dissection and increased susceptibility to submergence (Lynn & Waldren 2001b). The presence of little overlap between the areas in terms of morphology would suggest that selection pressures are acting against a particular form in a particular area.

3.4.2 Origin of the intermediate form

Lynn (1998) discusses possible theories on the presence of individuals with an intermediate dissection index. Firstly, the form may be derived from gene flow between broadleaved and dissected forms; secondly, this intermediate form may be a result of directional selection that has produced the individuals with different optima adapted to the different micro-sites. As stated in the introduction to this chapter, spatial separation is not necessarily a pre-requisite for adaptation. The breeding experiment results described in this chapter have shown that a less dissected leaf form is produced when dissected leaf turlough individuals are crossed with broadleaved non-turlough individuals, than when turlough individuals from the top and bottom of a turlough basin are crossed. Individuals from the top of the turlough basin have a broader leaf than those situated at the bottom of the turlough basin, even so they are rarely found to be as broadleaved as individuals found in a dry pasture habitat. This may be a reason for the turlough cross (T – T) progeny having a more dissected leaf than the hybrid cross (T – NT) progeny.

Unfortunately, only two full reciprocal crosses were achieved so the results of this breeding experiment must be interpreted with a degree of caution. There are many reasons why this experiment was not altogether successful; turlough individuals tended to produce fewer flowers than the non-turlough individuals in the experiment,

and flowering time between the groups differed considerably, this meant that artificial pollination proved difficult. Many of the turlough individuals were also infested with woolly aphids, which rendered them unsuitable for inclusion. Even so, from this preliminary study it is apparent that hybridisation between broadleaved and dissected forms of *R. repens* produced similar dissection indices as those intermediately dissected in the field. It would be beneficial to conduct more reciprocal crosses between turlough and non-turlough individuals and to further cross the F1 generations of these crosses to give an experimentally produced F2 generation to determine any segregation. It must be noted that the breeding experiment described in this chapter was a preliminary investigation, with molecular techniques employed in this chapter as the dominant tools for investigation of the individuals sampled along the transect through the turlough basin.

3.4.3 Appraisal of the ISSR and AFLP marker techniques

ISSRs are easy to apply once the specific conditions have been determined. AFLP on the other hand requires both more capital and time to achieve results. Because SSR sequences are the binding sites for ISSR primers, their distribution across the genomes is important for an ISSR study (Barth *et al.* 2002). Some possible explanations for the lack of genetic diversity of related individuals based on ISSR data could be

(I) Mis-scoring of polymorphic fragments, i.e. scoring error.

(II) Low variability of regions amplified by ISSR primers.

(III) Unsuitable primer choice

(IV) Relatively fewer markers produced by the ISSR method

Hodkinson *et al.* (2002) state that the non-automated ISSR method is less efficient than AFLPs for screening large numbers of accessions, one problem being the manual scoring of bands on agarose gels in which the sizing of fragments is not as accurate as automated genotyping methods. Another problem with the ISSR technique is that it is often difficult to compare across different agarose gels, which increases the scoring error of the procedure. Automated AFLP can differentiate fragments of 1bp, whereas for ISSR that are run on 2% agarose this distinguishing power decreases to approximately 20bp (T. Hodkinson pers. comm.). Fewer polymorphic loci were detected per ISSR primer (mean of 11) than per corresponding AFLP primer pair

(mean of 50). However, ISSR is less expensive and requires less technical support than AFLP. Considering all these factors the AFLP results revealed more variation and also made more ecological sense with individuals along the transect grouping in some logical and scientifically explainable manner.

3.4.4 Morphological and genetic variation combined

A comparison of the leaf dissection indices for individuals before and after cultivation demonstrates the genetic basis of the variation found in turlough populations of *R. repens* (Lynn & Waldren 2001b). This implies that the phenotypic expression of leaf dissection is under relatively direct genotypic control and that the morphological differences between samples exhibited in cultivation agree closely with those pertaining under natural conditions. In addition broadleaved *R. repens* individuals situated at the top of a turlough basin do not survive when transplanted to the bottom of a turlough basin indicating that tolerance to submergence is under genetic control. Thus, the obvious progressive step was to use molecular techniques to complement these findings. Although AFLP is a neutral marker technique it has been revealed in previous studies that markers linked to genes can be detected by AFLP (Pakniyat *et al.* 1997).

Plants from the lower part of the transect had considerably more dissected leaves than those from the upper part, with some intermediacy in the centre of the transect. ISSR and AFLP analyses show some structure to the Hawkhill population that partly complements the morphological analysis of leaf dissection. Individuals from the bottom of Hawkhill turlough basin were found to be more closely related to each other than they are to individuals from the top and vice versa. Several factors may be contributing to the inconsistencies that arise from comparing morphological and molecular data that are as follows.

Although it was a transect that was sampled, this is in essence a continuum of one population. Therefore it is not surprising that there is no distinct population genetic structure, which can be correlated to leaf morphology. The AFLP technique produced over 150 polymorphic loci that are likely to be randomly positioned throughout the genome. It is probable that the expression of leaf shape is under the control of a few genes, and AFLP samples predominantly neutral DNA from the entire genome,

therefore it should prove difficult, if not impossible to detect the genetic variation for leaf shape with this technique but the results were never intended to be interpreted in terms of leaf shape alone.

There is no doubting the usefulness of AFLP in plant population genetic studies. The method revealed copious amounts of molecular information although it is likely that many of the genetic markers are not informative in showing genetic relatedness (i.e. shared bands may not be due to inheritance from a shared ancestor, but may be due to parallel evolution and hence be homoplasious), thus creating noise in the data. There are other factors that may be contributing to the pattern that has emerged from the molecular data. McNeilly and Bradshaw (1968) state that very high levels of selection are needed to maintain genetically adapted populations against the smothering of gene flow from surrounding populations. Selection has created distinct morphological differences in the turlough form of *R. repens* but they are likely to be due to only a few genes. Selection maintains these genes but allows gene flow to occur with other non-adaptive DNA. Therefore gene flow may have been sufficiently extensive to prevent the divergence of highly distinct local genotypes. Although there is evidence of spatial structure within this population of *R. repens,* there are no sharp boundaries present, it is more a case of clinal change from one end of the transect to the other.

Ranunculus repens is a clonal species but several genotypes exist along the transect studied. A large amount of clonal spread could be expected from a species such as *R. repens* but the data presented in this chapter suggest that there are numerous genotypes present which show genetic variation graded along the transect. This may suggest some ecological specialisation along the transect, probably governed by periods of inundation. If selection did not play a role in the genetic structure of this population then presumably fewer clones would be present which would spread more profusely along the transect. This idea of ecologically specialised clones is further developed in the following chapter.

Gene flow via pollen or seed may also be a contributing factor for the high number of genotypes present. Gene flow may be taking place between individuals from the top and bottom of the turlough basin as well as from neighbouring ruderal populations. During the summer months the dry turlough basin has areas available for colonisation,

it is possible that more ruderal forms of the species from nearby populations above the high water mark invade and establish in these temporary windows of opportunity and thus are incorporated into the gene pool. They may not be able to survive the winter flood but the six months of dryness in the summer is ample time for them to flower, be pollinated and set seed. Therefore gene flow may be obscuring some of the morphological and genetic patterns observed, and perhaps explain the apparent clinal nature of the variation.

The molecular data appear to make more sense when looked at in terms of the environmental conditions along the ecological gradient present in the turlough basin. The general trend in the molecular results was for individuals sampled from a particular position along the transect to be more genetically similar to other individuals sampled in close proximity than they were to individuals collected from disparate locations along the transect. The use of two marker techniques (ISSR and AFLP) provided similar interpretations of the results meaning conclusions about the data can be strengthened. Unlike the studies of Antonovics and Bradshaw (1970) and Wu *et al.* (1975), this Hawkhill transect study did not use an environment with sharp discontinuities. All individuals sampled along the transect are subjected to inundation, the only difference is the period for which these individuals are submerged. This suggests that all individuals have adapted to the flooding regime present in the turlough basin to differing degrees, with individuals from the top of the basin only being submerged occasionally.

Principal co-ordinate analysis was found to be a more readily interpretable tool than the unrooted phylogram resulting from cluster analysis in explaining the molecular variation present. Axis one of the PCO in both sets of analyses to some extent mirrors the leaf dissection data. Despite the lack of association or distinct patterns between the morphological and molecular data, several AFLP markers were revealed to show heterogeneous distribution among individuals along the transect, which can be related to the ecological gradient present along the transect. A Chi square test conducted on a restricted number of AFLP markers. This test revealed that certain markers showed significant heterogeneous distribution when categorised according to position along the transect and according to level of leaf dissection. This could imply that the patterns emerging from the AFLP data in terms of population structure are based on

spatial genetic and morphological differentiation. This theory is strengthened by the results of the Mantel test (section 3.3.5.5), which showed that samples that were geographically close (i.e. were collected a small distance apart) were likely to be more genetically similar in terms of the AFLP information. Care must be taken when inferring that these differences are adaptive in nature as dissimilarity could be due to isolation by distance.

Very little clonal growth was detected along Hawkhill transect. This would infer that genotypes are adapted to a small range of environmental conditions with edaphic factors playing a major role in the spatial genetic structuring of this turlough population of *R. repens*. This can be explained by looking at the way in which the population was sampled. If genotypes are adapted to a small range of environmental conditions they will tend to spread along the contours of flooding susceptibility (main selective pressure), whereas the transect was sampled vertically through the turlough. The results obtained from the PCO analyses would suggest such an event is occurring, therefore selective forces on a small spatial scale appear to be over riding the influence of migration to favour a genetically adapted form rather than a plastic one. If selection coefficients are sufficiently high, local pockets of distinct genotypes can be maintained as little as 10cm apart, even in outbreeding species (Snaydon 1970; Davies & Snaydon 1974).

3.4.5 Genetic diversity within the turlough population of *R. repens*

Levels of genetic distance between pairs of individuals reached a maximum value of 93% for the ISSR data and 67% for the AFLP data. This may be a result of high recruitment from outside the population during the summer months. High genetic variation may be needed to combat the fluctuating conditions in the turlough habitat, the time and duration of flooding is not very predictable therefore some genotypes may not be able to tolerate change. There is also the possibility that those individuals sampled were not true turlough individuals (i.e. had established in the turlough environment from adjacent population seed during the summer period when the turlough was dry) but pasture population derivatives that established in this environment during the summer months.

Micro-environment and narrow range adaptation of individuals along the transect is another probable contributing factor to this lack of clonal detection using molecular techniques and may also be the reason that there does not appear to be micro-spatial dominance of one clone. Genotypes successful at one spatial point in the field may not be so at a different position, or at any other point in time. Differential growth and competition among clones combined with somewhat restricted gene exchange could contribute to the maintenance of local differentiation (Antlfinger 1981). Adaptation to ecological gradients is a classic hypothesis for the cause of spatial heterogeneity in plant communities (Davies & Snaydon 1974; Antlfinger 1981; Silander 1985; Prentice *et al.* 1995). It could be the case that disruptive selection, in conjunction with gene flow, is maintaining the genetic variation necessary to combat the changeable turlough conditions. In the turlough habitat, genotypes may not be guaranteed to prosper once established as conditions change from season to season and from year to year. Burdon (1980) gives a simple explanation for the observed levels of genetic diversity in a population of *Trifolium repens*, stating that a highly diverse physical environment, in which selection pressures are present on a micro-scale, had resulted in the occurrence of a large number of distinctive ecotypes in close proximity to each other.

3.5 Conclusions

Analysis of the genetic variation along this environmental gradient was a useful approach in the study of possible selective forces involved in micro evolutionary processes. It was found that the genetic structure of the population did not completely mirror that revealed from the morphological data, although only a single phenotypic trait was quantitatively compared. Individuals did show some degree of spatial separation along the transect and a number of potentially useful molecular markers were found to show heterogeneous distribution among individuals sampled across the ecological gradient. Therefore, although AFLP is in essence a neutral molecular technique it appears that certain markers that were found in this analysis may be linked to genes responsible for leaf dissection in the turlough buttercup. This molecular information may be useful for possible future QTL investigations.

Many individuals were delimited along the transect with little clonal growth apparent. Genetic variability is being retained in this population running through a strong

ecological gradient. It appears that the periodic flooding in the turlough habitat has acted as an effective selection force that has brought about the evolution of this turlough population of *R. repens*. The coexistence and distribution of multiple genotypes may permit the survival of the population as a whole over a broader range of environmental conditions than would be possible for a homogenous population. Whether these spatial genetic differences disappear when the environmental conditions are homogeneous will be tackled in the following chapter.

Chapter 4

Using AFLP to reveal the genetic structure in a turlough population of *Ranunculus repens*

Nature only uses the longest threads to weave her patterns, so each small piece of her fabric reveals the organization of the entire tapestry

Feynman 1980

4.1 Introduction

4.1.1 The evolution of clonal growth

Clonal growth can be seen as an ecological adaptation that influences the population biology and evolution of organisms. Individuals can be recognised at two different organisational levels: genets and ramets (Kays & Harper 1974). A genet, (i.e. the developmental product of a single zygote), is an inclusive unit comprised of functional units called ramets, which are potentially physiologically independent (Eriksson & Bremer 1993). Clonal plants present special challenges for analyses of populations. Identification of clones, determination of genet number and visualisation of the spatial distribution of clones are crucial to study the dynamics and evolution of populations (Escaravage *et al.* 1998). In multiclonal populations, the spatial distribution and the degree of intermingling will depend on ecological heterogeneity, selection, the clonal growth strategy (Harper 1957) and the efficiency of seed dispersal (Cook 1983). Asexual reproduction, and local clonal growth in particular, exploits the fact that a genotype can be successful repeatedly in both time and space (Sebens & Thorne 1985). To be successful, genets must possess not only a suitable set of general fitness characters, but also a set of local fitness characters, and be lucky enough to establish in a complementary environment (Burdon 1980).

The ramets or vegetative propagules represent tested genotypes that can expand clonally or contract depending on environmental conditions. They will be represented in the populations and be ready to expand when conditions are amenable for a given genotype (Sarukhan 1976). Indirect evidence suggests that mortality acts on certain individuals of the population, particularly weak, nonaggressive plants, and selective pressures like competition or grazing certainly do affect the amount of progeny left by different genets in a population (Sarukhan 1976). Ramets are exposed to a constant annual death risk; this constant annual risk is, however, unevenly spread within the year. The seasonal cycle that is superimposed on the constant annual decay rate provides some evidence of the way in which plant numbers are regulated (Sarukhan & Harper 1973).

Mortality, birth rates and longevity are critical elements in the life cycles of a species. A balance, resulting from complex interactions between the three, must be obtained if

populations of a species are to maintain stability (Sarukhan & Harper 1973). Seedlings are clearly at a highly vulnerable stage in the life cycle of *R. repens* but represent the only way in which new genets are recruited to populations; the high risk of death at this stage may reflect not only the innate risks in establishing a viable root and shoot system but also part of the genetic load carried by the species. New genotypes are by nature experimental and elimination of most of the unfit genotypes might be expected to occur early in their life (Sarukhan & Harper 1973).

4.1.2 Environment as a diversifying factor

A particular environment provides a diversity of challenges to the plants that inhabit it. During establishment, the local environment acts as a series of sieves or filters that are permeable to only some individuals (Galen & Stanton 1999). Generalising from adult to seedling stages is unwise; seedlings are not merely small adults, but have unique patterns of gene expression, resource allocation, and development (Lawson & Poethig 1995). Little is known about the processes that determine the number and relative sizes of genets in clonal plants (Watkinson & Powell 1993). Through simulation models Watkinson and Powell (1993) discovered that there is rapid loss of genets from populations in which there is no further seedling recruitment following an initial colonisation event. Such populations can, therefore be expected to become increasingly dominated by a few, large clones, especially in those populations establishing from a small number of seedlings, unless there is some form of frequency or diversifying selection to maintain diversity. Regular disturbance and the occasional establishment of seedlings can be seen as a powerful mechanism generating clonal diversity (Soane & Watkinson 1979; Sebens & Thorne 1985).

Turloughs provide a challenging combination of biotic factors for the turlough buttercup. It is unclear what effect these conditions have on sexual reproduction, clonal growth, survival and mortality. For example, summer flash flooding in the turlough habitat may interfere with flowering time of the species and lead to the genetic isolation of turlough populations from other broadleaved populations. As a consequence, higher genetic differentiation, in the form of directional selection, may occur. Long periods of inundation, as well as the fact that many turlough populations are geographically separated from non-turlough populations of the species, means that

the rate and degree of genetic separation of these turlough populations of *R. repens* may be augmented.

4.1.3 Clonal growth in *R. repens*

The usual growth cycle of *R. repens* is for leaves on a parent rosette to die at about the same time as daughter ramets become established (Lovett-Doust 1981b). Lovett-Doust (1981a) concluded that the placement of ramets in a stoloniferous species would be influenced by several constraints. Some of these are related to the internal organisation of the clone, for example: (1) the avoidance of inter-ramet competition, (2) the cost of synthesising long stolons, and (3) the advantage gained by obtaining resources directly from the parent rosette. Selective pressures should influence the resulting internal compromise, and can result in differences in growth form between locally specialised populations (Lovett-Doust 1981a). Lynn and Waldren (2001a) also found different growth forms of *R. repens* populations from contrasting habitat types when grown in uniform conditions. The safety of vegetative reproduction as against the risky sexual reproduction in plants is illustrated with the probabilities of survival of seedlings and daughter plants of *R. repens* to the year next to their "birth"; 0.12 for a seedling and 0.77 for a daughter plant (Sarukhan 1976). This is not surprising as a fit genotype generally survives to adulthood, its ramets having in some way already been selected for as they are identical to the parent plant. Therefore daughter ramet survival should be almost as high as parent survival. Sarukhan and Harper (1973) suggest that the great mortality risks involved at the seedling stages in sexually reproducing species, may be more the result of the genetic load of unfit genotypes than the problems of metabolic adjustment between the stages of the food-dependent seedling and the self-sufficient established plant.

Until recently individuals could only be identified by comparative morphology, with numerous characters being measured, but problems arise due to environmental influence in the form of phenotypic plasticity. Molecular markers provide a more stringent technique for the identification of genets, with less subjective data being obtained in the form of DNA fingerprints. Although the technique of AFLP produces dominant markers preventing the discrimination of homozygosity or heterozygosity at a locus, such molecular techniques generally provide a greater quantitative accuracy

than morphological measures. A description of techniques available and the suitability of these methods has already been discussed in Chapter 1.

4.1.4 Aims

The aim of this study was to examine the clonal structure of *R. repens* within a turlough basin to see how many genotypes are present. It has been established in the preceding chapters that the turlough populations of *R. repens* are different to the common broadleaved populations of the species (Chapter 2), and that these differences are likely to be due to inundation tolerance (Chapter 3). The question remains as to whether or not these unique turlough populations are genetically diverse enough to be maintained given that the turlough environment is susceptible to environmental change. Living in these unique environments is risky, therefore it is critical to assess the viability of these populations. A depauparate gene pool may render the turlough populations susceptible to extinction if changes were to occur in the turlough environment, whether anthropogenic or due to climate change.

There are two questions associated with this study: Firstly, are the turlough populations dominated by very few genotypes that are specifically adapted to the conditions imposed and therefore show high levels of fitness? Soane and Watkinson (1979) claims that it takes many years to produce a genetically homogenous population, and that there is usually local dominance by a few clones or genets. Secondly, what kind of growth strategy is present in these turlough populations of *R. repens*? These questions will be tackled by using molecular tools and an assessment of leaf morphology to investigate a known area of one turlough basin, which harbours a prolific population of *R. repens*.

Two techniques for the identification of clones were used, one based on morphology (Leaf Dissection Index, LDI), and the other based on molecular analysis (AFLP). Individual identification using DNA fingerprinting methods is emerging as a critical tool in conservation genetics and molecular ecology (Waits *et al.* 2001). When using molecular techniques for the purpose of identification of clones there is no need to carry out a detailed population genetic analysis of the variation, it is sufficient to show the definite identity of each individual ramet sampled. Molecular techniques were also used to assess the genetic diversity present within the population. Thus the aim of this

study is to investigate the broader population biology of the turlough form of *R. repens*.

4.2 Materials and Methods

4.2.1 Field mapping and site details

In late July 2001, a rectangular sampling grid of 20x5m was selected in the middle of a homogeneously sloped area of Gortlecka turlough basin (grid reference R 313 938) (Plate 4.1). This grid was segregated into one hundred $1m^2$ quadrats using twine and plastic tent pegs (Plate 4.2). Within the hundred square metre grid 10 random quadrats were chosen and the number of ramets in each were counted. The initial plan was to count the number of ramets in each of the 100 sampled quadrats in the grid, but on discovering the extremely high density of ramets it was considered unfeasible in terms of time to quantify the entire 100 square metres covered by the sampling grid. Vegetation was recorded for all species present within a subset of $1m^2$ quadrats using Domin scores (Kent & Coker 1992).

4.2.2 Plant collection

It was not possible to identify genets in the field, therefore clean, uncontaminated leaf material from the ramet positioned at the baricentre of each $1m^2$ quadrat was collected and preserved in silica gel by the same method described in section 2.2.2.

4.2.3 Morphological measures

One mature leaf was taken from each ramet sampled. Leaf dissection indices were calculated for all one hundred individuals sampled. Methods used were identical to those described in Section 2.2.3. Differences in leaf dissection between clones were analysed using a one-way ANOVA.

4.2.4 DNA extraction, clean up, and quantification

DNA extraction, clean up and assessment of DNA concentration followed the methods described in Sections 2.2.4, 2.2.5, and 2.2.6 respectively.

4.2.5 Molecular analysis and clone identification

AFLP reactions, optimisation and band scoring procedures employed were identical to those described in Section 2.2.7. An important consideration when using a set of markers is the likelihood of observing a unique genotype when looking at different clones (Cheliak & Pitel 1984; Waits *et al.* 2001). Statistically, the larger the sample of

Plate 4.1: Gortlecka turlough basin.

Plate 4.2: Sampling grid in Gortlecka turlough basin.

loci, the greater is the probability of observing a unique genotype. Ramets with identical banding patterns in this study were considered to belong to the same genet. It is important to recognise that this assumption may result in the true genetic diversity being under estimated. A map showing the spatial distribution of genets according to their AFLP fingerprint within the plot was constructed. The sampling plot did not delimit the full degree of clonal spread, therefore the exact size of each genet could not be determined.

4.2.6 Data analysis

4.2.6.1 NJ analysis

Neighbor – joining (NJ) analysis was applied to a distance matrix based on Nei and Li distances (Nei & Lei 1979) (PAUP 4.0; Swofford 1998) for the AFLP data. See section 2.2.8.1 for methods and equations. Internal support for grouping was assessed using 1000 bootstrap replicates (Felsenstein 1985). This method was used to test whether or not the position in the grid was related to genetic similarity, which would be important for future sampling protocols.

4.2.6.2 Ordination methods

Principal coordinates analysis (PCO) was conducted with The R Package v4.0d (Casgrain 1999) using the Jaccard similarity measure (Jaccard 1908) (see Section 2.2.8.2) and the ordinations for all combinations of the first three axes were graphed in Microsoft Excel. This method was employed to give another means of graphically representing the data on genetic similarity between clones.

4.2.6.3 Diversity measures

Two measures of genotypic diversity appropriate for clonal material were calculated following Ellstrand and Roose (1987).

(I) The "proportion distinguishable" was calculated as the number of clones detected (G) divided by the number of samples (ramets) (N).

(II) The complement of the Simpson Index corrected for finite samples, D (Pielou 1969; Peet 1974):

$$D = 1-[\Sigma n_i (n_i - 1) / N (N - 1) \quad \text{[Equation 4.1]}$$

Where n_i is the number of samples of genotype i and N is the total number of samples. D can range from zero in a population composed of a single clone to one in a population where every individual sampled has a different genotype (Escaravage *et al.* 1998).

A note of caution must be taken when using this type of analysis as the strategy with which ramets are sampled influences estimates of genotypic variation (Escaravage *et al.* 1998). Genotypic variation usually correlates positively with the scale and negatively with the size of the sampling mesh on a given surface, because collecting more ramets at finer spatial scales increases the probability that genets are sampled repeatedly (Widen *et al.* 1994; Mc Lellan *et al.* 1997). Within-population diversity values were calculated using Nei's unbiased diversity statistic (Nei 1987), averaging over individual AFLP products, using POPGENE version 1.31 (Yeh *et al.* 1997).

4.3 Results

4.3.1 The density of ramets in a turlough population of *R. repens*

Ramet density per square metre ranged from 419 in quadrat 80 to 881 in quadrat 27 (Figure 4.1), therefore the range was large with the highest density per m^2 being approximately twice that found in the lowest. This was a much higher density than originally expected based on visual examination of the site.

Figure 4.1: *R. repens* ramet density in ten randomly sampled $1m^2$ quadrats within a $100m^2$ grid in Gortlecka turlough basin.

			595													671			
					881														
	559																		857
							457												
495		430										465	419						

4.3.2 AFLP band scoring

The concentration of extracted DNA samples ranged from 66ng/µl to 1117ng/µl. AFLP analysis of 100 individuals from a 5m x 20m plot in Gortlecka turlough basin using three pairs of primers revealed a total of 91 markers, 69 (76%) of which were polymorphic. The primers differed in their ability to detect polymorphism among clones. Over all clones the average number of polymorphic bands amplified per primer pair combination were 12 (ACT-CAG), 23 (AGG-CTC) and 34 (AAC-CAG). Scoring error was calculated from the Jaccard distance matrix, by comparing two plants known to be identical, and averaged at 4% over all individuals. Amplification of the 100 DNA samples with the same primers listed in section 2.2.7.2 gave a range of between 20 and 38 detectable peaks per sample, depending on both primer and clone, which ranged from 39 to 240 bp. All unreliable bands were excluded from the analyses according to the marker selection criteria described in section 2.2.7.2; in total 69 reliable polymorphic markers were retained.

AFLP efficiently identified different clones; the study revealed that in the $100m^2$ grid studied in Gortlecka turlough basin, *R. repens* formed a multiclonal population with 14 separable genotypes present (i.e. there were only 14 different genetic fingerprints

from the sample of 100 plants). The spatial distribution of the clones from the sampled grid can be seen in Figure 4.2. Primers were analysed separately to confirm the multilocus genotypes and all repeated analyses revealed that the same genets were present. Each clone was given an arbitrary number from 1 to 14 for ease of discussion. The largest clone within the $100m^2$ grid covered an area of at least $20m^2$ (clone 14). The limit of clones was distinct, a clear pattern of clonal delimitation emerged from the results. In this population grid, individuals appear to spread more profusely in the North-South direction (top to bottom of grid) (Figure 4.2). Clone size varied from $1m^2$ (genotype 7 and 9) to $20m^2$ (genotype 14). Among all the clones in the population, clone 14 appears to be the most prolific, and clone 4 appears to be the most "intrusive", showing a "guerrilla" type growth strategy as opposed to "phalanx". There was a moisture gradient along this grid, with an area prone to longer periods of inundation to the left, following through to shorter periods of inundation to the right of the grid.

Figure 4.2: Spatial distribution of *R. repens* genets within Gortlecka turlough basin, as identified by AFLP analysis. Each number corresponds to a single genet. A moisture gradient was found to occur from left to right of the grid, with the area to the left being prone to longer periods of inundation.

North

1	1	2	2	3	4	4	5	5	8	8	10	10	10	12	12	14	14	14	14
1	1	2	2	3	3	4	5	6	8	8	10	10	11	12	13	14	14	14	14
1	2	2	2	3	3	4	5	6	8	8	10	10	11	12	13	14	14	14	14
1	2	2	2	3	3	4	5	6	8	8	10	10	11	12	13	14	14	14	14
1	2	2	2	4	4	4	5	7	8	9	10	10	11	12	13	14	14	14	14

South **Decreasing moisture gradient** ➡

4.3.3 Leaf Dissection measures

Mean LDI for each clone, standard errors, and the number of ramets sampled from each clone are given in Table 4.1. No standard error values are available for clones 7 and 9 as only one ramet of each of these clones was sampled. Leaf dissection varied greatly within a genet; from 5.57 for the most dissected ramet to 2.99 for the least dissected (clone 14). Mean clone leaf dissection index ranged from 3.98 (clone 2) to 4.75 (clone 12). Neither within-clone or between-clone variation in leaf dissection

was significant. Therefore, leaf dissection could not be used as a means of clone identification.

Table 4.1: Mean leaf dissection index (LDI) and standard error (se) for each *R. repens* clone sampled in Gortlecka basin identified by AFLP.

Clone	Mean LDI	n	se
1	4.06	7	0.10
2	3.98	13	0.06
3	4.47	7	0.13
4	4.39	8	0.08
5	4.24	6	0.10
6	4.23	3	0.26
7	4.57	1	-
8	4.04	9	0.07
9	4.23	1	-
10	4.14	11	0.08
11	4.16	4	0.14
12	4.75	6	0.08
13	4.31	4	0.21
14	4.13	20	0.04

4.3.4 NJ analysis

Figure 4.3a displays the unrooted phylogram obtained from the NJ analysis of AFLP data from 14 *R. repens* clones. In general, clones that were positioned adjacent to each other on the grid clustered together, although these groupings were not strongly supported by the bootstrap analysis with only clusters of individuals 4 and 6, 11, 12, and 13, and 9 and 14 having bootstrap support over 55% (Figure 4.3b).

4.3.5 Ordination

The mean similarity between pairs of genets was 0.40 (range from 0.28 to 0.57, 19 to 39 shared bands in common). Figure 4.4a, b and c show the clones positioned in PCO ordination space according to their genetic similarity. Axis 1, 2 and 3 accounted for 17%, 16% and 13% of the variation in the data respectively, giving a total of 46% for the first three ordination axes. There did not appear to be any distinct pattern from the PCO analysis. Figure 4.4a shows that certain clones (11, 12 and 13) did appear to cluster together in the ordination space. Clones 2, 3 4 and 6 were also more genetically similar to each other than to the rest of the clones (axis 2). Table 4.2 shows pairwise similarity values for all pairwise combinations of genets.

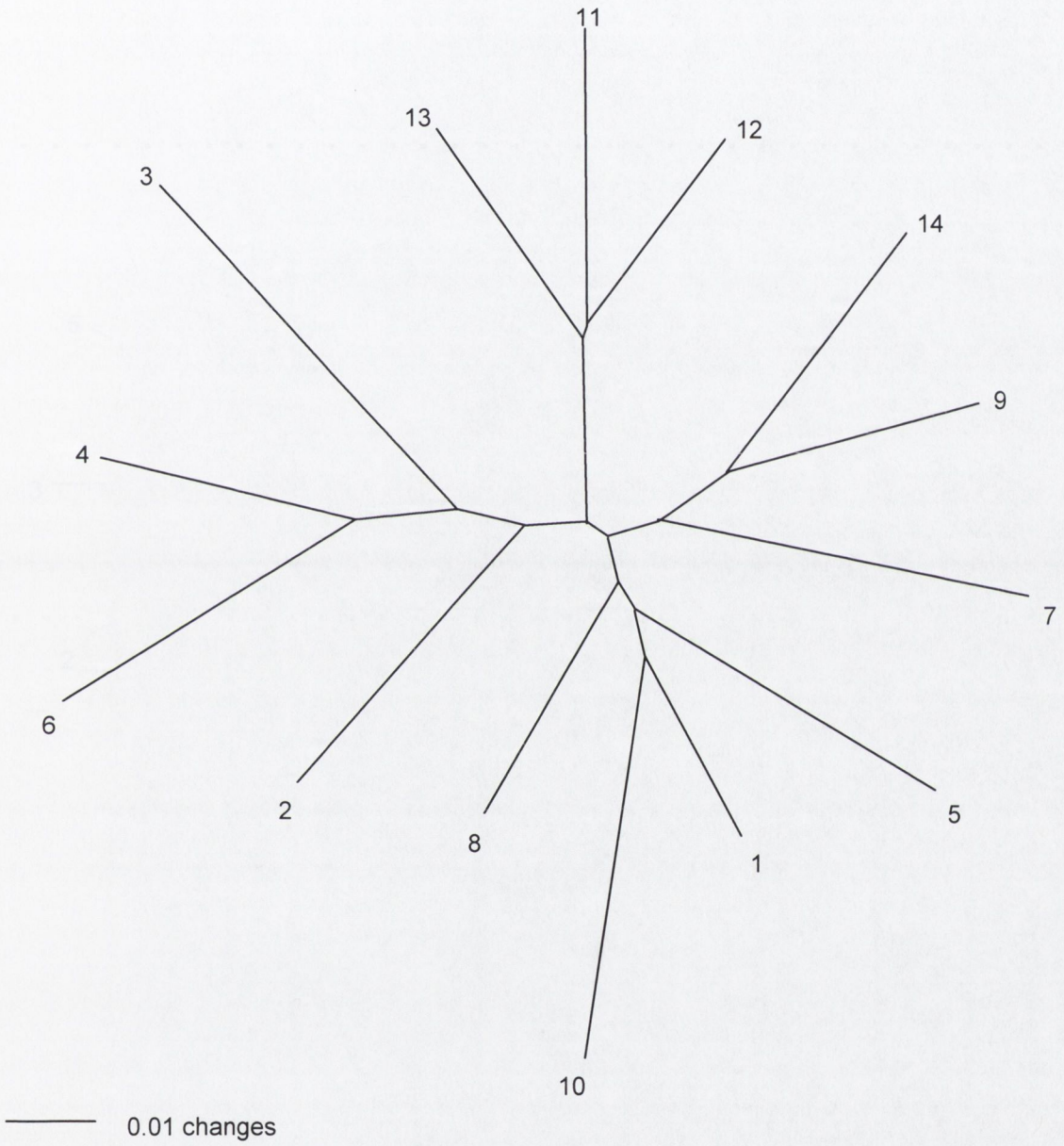

Figure 4.3a: A NJ tree based on Nei & Li (1979) distance estimated from AFLP data for *R.repens* clones from a sampling grid in Gortlecka turlough basin. Numbers at the end of branches represent individual clones, with numbers increasing from one side of the grid to the other, (i.e. clones 1 and 2 are more spatially proximal as are clones 13 and 14).

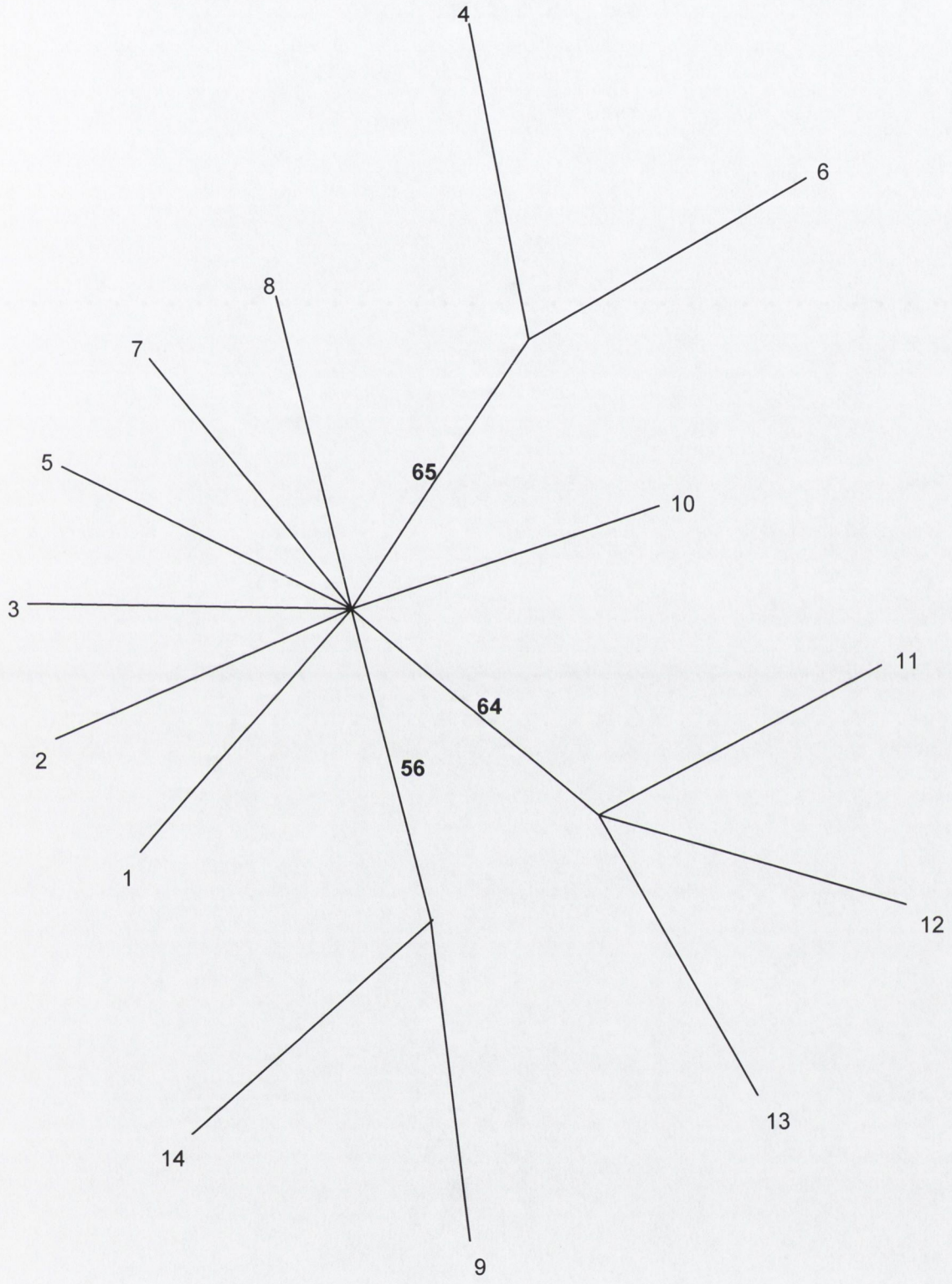

Figure 4.3b: A NJ BS tree based on Nei & Li (1979) distance estimated from AFLP data for *R.repens* clones from a sampling grid in Gortlecka turlough basin. Numbers at the end of branches represent individual clones, with numbers increasing from one side of the grid to the other, (i.e. clones 1 and 2 are more spatially proximal, as are clones 13 and 14). Numbers on the branches represent percentage bootstrap support.

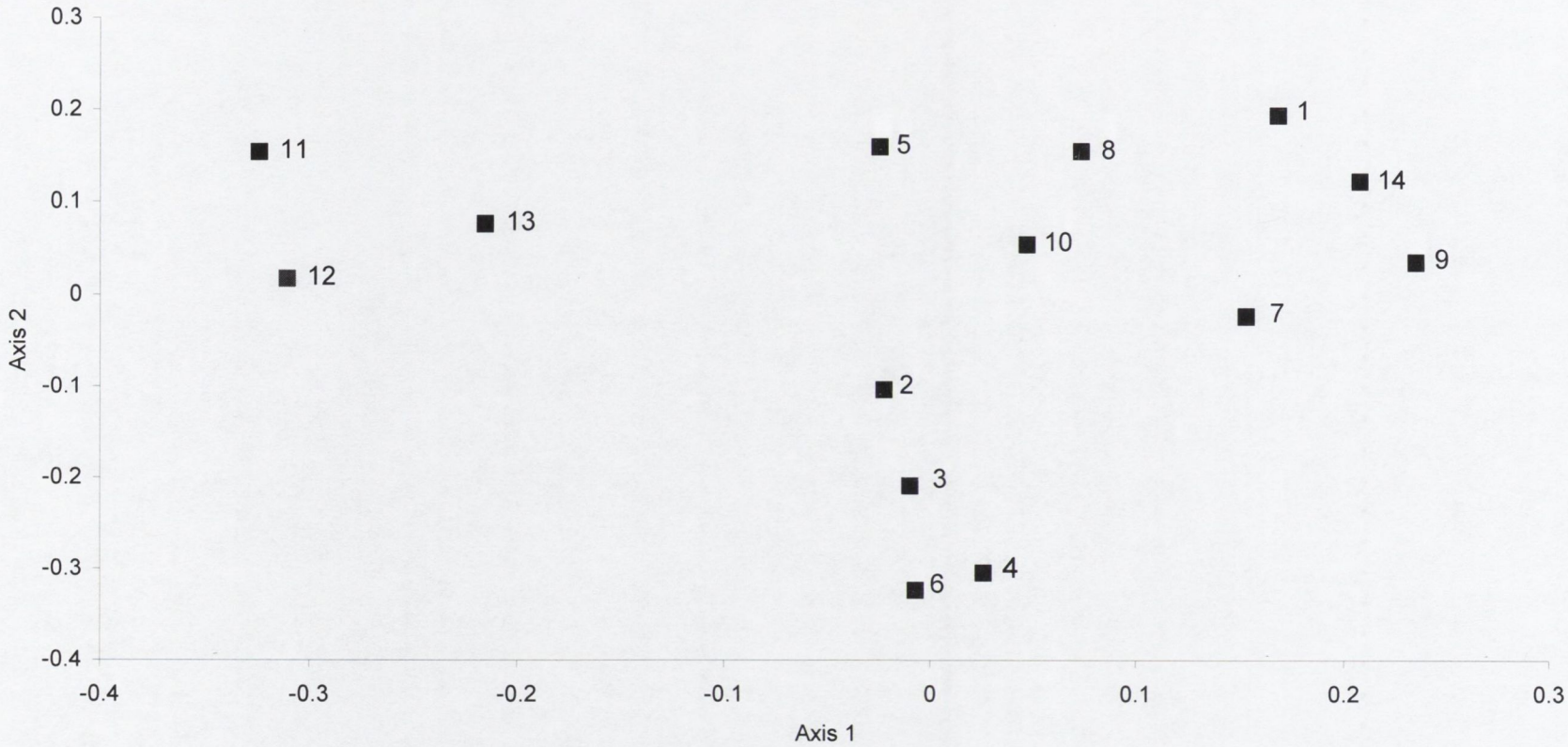

Figure 4.4a: Axes 1 and 2 of a PCO analysis from AFLP data for *R.repens* clones from a sampling grid in Gortlecka turlough basin. Numbers represent individual clones identified by AFLP.

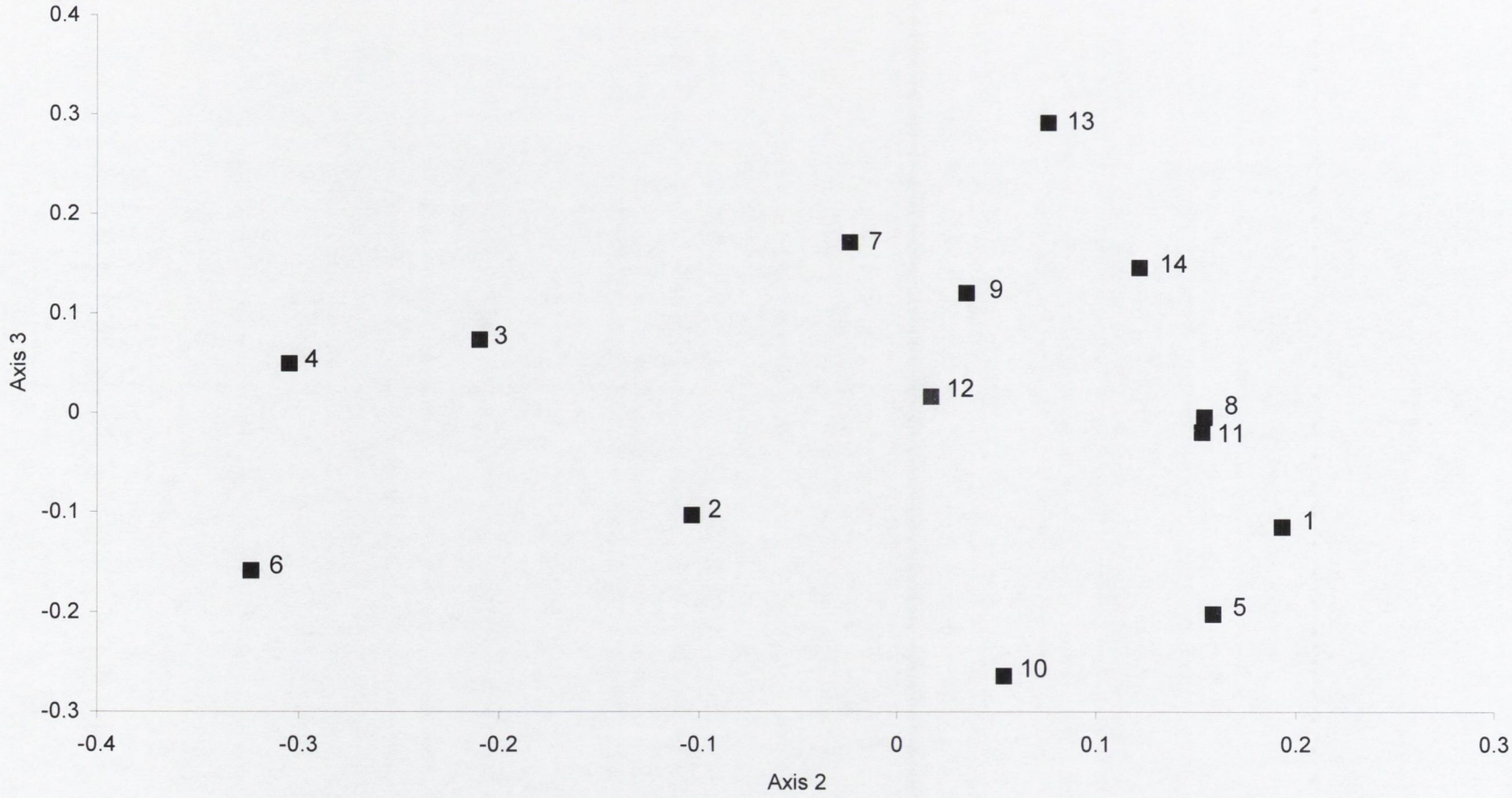

Figure 4.4b: Axes 2 and 3 of a PCO analysis from AFLP data for *R.repens* clones from a sampling grid in Gortlecka turlough basin. Numbers represent individual clones identified by AFLP.

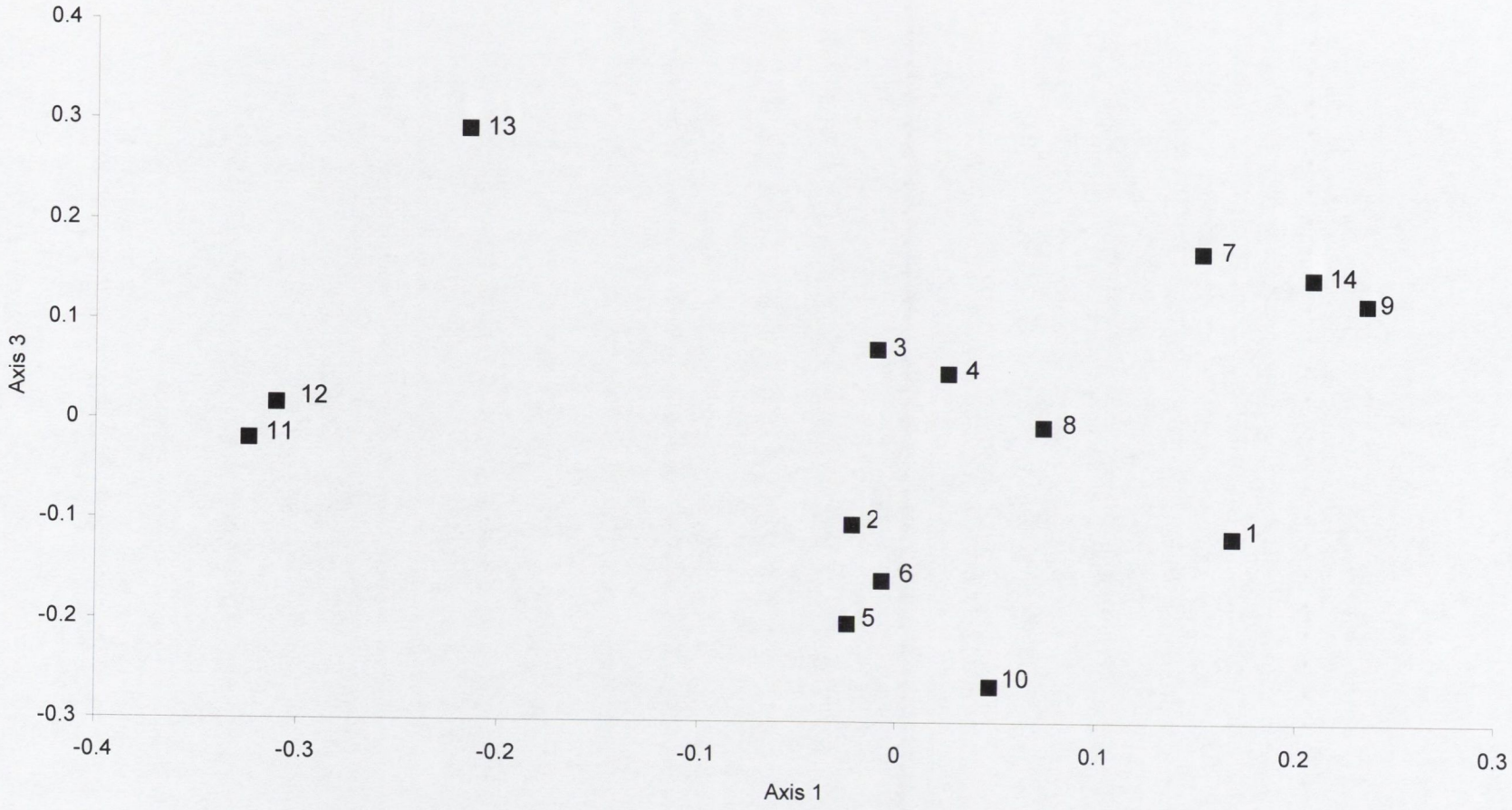

Figure 4.4c: Axes 1 and 3 of a PCO analysis from AFLP data for *R.repens* clones from a sampling grid in Gortlecka turlough basin. Numbers represent individual clones identified by AFLP.

Table 4.2: Matrix of Nei & Li (1979) similarity indices between clones sampled in Gortlecka turlough basin. Similarities greater than 50% are highlighted in bold font.

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14
C1	1													
C2	0.43	1												
C3	0.40	0.40	1											
C4	0.31	0.41	0.41	1										
C5	**0.50**	0.40	0.30	0.38	1									
C6	0.34	0.39	0.39	**0.50**	0.37	1								
C7	0.46	0.44	0.32	0.42	0.33	0.34	1							
C8	**0.57**	0.44	0.32	0.39	0.49	0.37	0.45	1						
C9	0.46	0.41	0.37	0.42	0.41	0.33	0.45	0.44	1					
C10	**0.50**	0.39	0.35	0.33	0.42	0.37	0.36	0.39	0.36	1				
C11	0.40	0.40	0.34	0.33	0.48	0.31	0.28	0.40	0.35	0.35	1			
C12	0.34	0.43	0.36	0.38	0.42	0.38	0.40	0.40	0.31	0.44	**0.54**	1		
C13	0.38	0.35	0.40	0.38	0.35	0.31	0.44	0.47	0.38	0.30	**0.52**	0.56	1	
C14	0.46	0.30	0.38	0.36	0.41	0.31	0.42	0.45	**0.53**	0.40	0.33	0.35	0.41	1

It can be seen from Table 4.2 that certain clones are genetically more similar than others. Among the most closely related are clones 11, 12 and 13, clones 4 and 6, clones 1 and 5, and clones 1 and 8. Clones that appear to be most genetically separate are clones 2 and 14, and clones 3 and 5.

4.3.6 Clonal diversity statistics

Estimation of Nei's genetic diversity (Nei 1987) for all loci within the population gave an average genetic diversity value of 0.3575. The "proportion distinguishable" or the number of genotypes divided by the number of samples (G/N) was 0.14 and the estimated genotypic diversity (D) was 0.9.

4.4 Discussion

4.4.1 Identification of clones

Identification of clones on the basis of phenotypic characteristics can be highly subjective; differences obvious to one observer may not be recognized by another (Cheliak & Pitel 1984). Furthermore, many useful phenotypic characters can only be measured on the mature plant (Cheliak & Pitel 1984). The level of leaf dissection was found to be a distinguishing trait between turlough and non-turlough populations (Lynn & Waldren 2001b) but the results reported here suggest this is not the case within a single turlough population. Therefore the leaf dissection index is not a suitable method to use to distinguish *R. repens* clones. However this study has shown that this problem can be easily overcome through the use of the molecular technique of AFLP. The results have also added to the growing body of evidence that molecular identification, delineation, and "certification" of clones in natural populations is a tool of considerable power which can be used in the study of plant populations. Advantages of these techniques, relative to and in combination with conventional approaches, are that they are objective and can be used on plants of essentially any age (Cheliak & Pitel 1984).

There were high levels of variation in leaf shape, and leaf dissection index differences between clones were not large enough to separate genets (Figure 4.3a). While inter-clonal differences can be marked, environmentally induced intra-clonal variation may often obscure these differences. That is, phenotypic differences among ramets within a clone may be as large as (or larger than) differences among clones (as seen from the morphological data). The molecular approach of AFLP fingerprinting however, proved to be appropriate for the identification of clones in Gortlecka turlough basin. There have been similar findings with a variety of molecular techniques, each stating that genetic analysis of samples leads to identification of clones (Cheliak & Pitel 1984 on *Populus tremula*; Nybom *et al.* 1990 on *Malus, Prunus and Rubus*; Parker & Hamrick 1992 on *Lophocercus schotii;* Eriksson & Bremer 1993 on *Rubus saxatilis*; Miller *et al.* 1996 on *Arrhenatherum elatius*; Escaravage *et al.* 1998 on *Rhododendron ferrugineum*; Kreher *et al.* 2000 on *Vaccinium stamineum*), but among these studies some techniques have been found to be more suitable that others. For example the RAPD technique revealed no variation in alpine populations of *Saxifraga cernua*

(Bauert *et al.* 1998), whereas Dewey and Heywood (1988) successfully revealed spatial genetic structure in a population of *Psychotria nervosa* using RAPDs. The previous chapter revealed that ISSR showed much less variation in individuals sampled along a transect through a turlough basin than AFLP, therefore it is questionable whether this result found by Bauert *et al.* (1998) was because of the marker technique used rather than complete lack of genetic variation.

4.4.2 The spatial distribution of clones

Growth and replacement of *R. repens* clones in the turlough basin appears to be occurring more in one direction than the other. It is possible that there is a moisture gradient running from East to West along the grid, this is suggested by the Domin scores for quadrats within the grid, with *Carex spp.* being more common at the left of the grid, decreasing as one moves right along the grid (Appendix 3). This could result in genotypes being adapted to a certain level of water tolerance, and thus growth of these individuals in a micro-environment best suited to their tolerance levels.

Maddox *et al.* (1989) used allozyme genotypes to map the micro-spatial distributions of *Solidago altissima* clones in fields of various ages. Patterns of dispersion of clones differed depending on the age of the fields, with younger fields having more localised clones than the older fields which showed more intermingling of clones and fewer rhizomic connections among ramets. This was not found to be the case with *R. repens* in Gortlecka basin, daughter ramets are produced each growing season and at the time of collection numerous stolons of varying lengths were seen to connect ramets. It appears that the prolific vegetative growth of these buttercups that have, over time, adapted to the turlough conditions, has been a considerable force in their successful establishment and survival. Although the turlough habitat can be almost described as a harsh environment due to the degree of change, which occurs within a year, it is albeit quite stable with long periods of drying and long periods of inundation. Total elimination of the founder genets without selection may be a slow process, as they will tend to be numerically dominant in the representation of ramets (Soane & Watkinson 1979). It is likely that the turlough genotypes of *R. repens* have evolved over many years, thus representing a reasonably old, well-established population.

4.4.3 Ramet density and clonal growth form of the turlough buttercup

The results of this study revealed that the maximum number of ramets recorded in the sampling grid was 857 per m^2. Lynn and Waldren (2001a) found that a turlough clone produced only 30 ramets in a uniform garden experiment whereas a ruderal clone produced over 300. Two possible reasons for the incongruence in ramet density are that firstly the ruderal clone was at a selective advantage in the uniform garden conditions and secondly that there is an inherent difference in the clones capacity to produce ramets. Lynn and Waldren (2001a) state that these turlough clones may have expressed alternative phenotypes induced by the conditions imposed by the uniform experiment, resulting in a relatively lower production potential that would be expressed under more optimal conditions for the particular genotype, but they considered it conceivable that a vigorous genet could establish a colony of identical clones over a relatively large area within a short time span, providing there is space available for expansion. In the present study one clone covered an area of at least $20m^2$ emphasising the clonal capabilities of the species. Sarukhan (1974) reports a mean number of vegetative offspring recruited for *R. repens* as $115/m^2$ and Lovett-Doust (1981a) found that the peak number of ramets in one growing season reached approximately 325 ramets/m^2. In another study, Sarukhan and Harper (1973) found the density of *R. repens* ramets to vary between 38 and 385/m^2. It is evident from these studies on ramet density of *R. repens* that ramet production is variable and these findings are in congruence with the present study (Figure 4.1). Variation in ramet density may be due to a number of factors such as degree of competition between ramets and between clones, and suitability of clones to their environment, as well as recruitment success.

Recruitment to the adult population is largely through the successful establishment of new ramets from rooted stolon nodes and rarely through the establishment of seedlings. Soane & Watkinson (1979) found through a simulation model based on population structure of *R. repens* that when new genet input is taken into account it could be seen that each site maintained a surprisingly high level of clonal diversity, unless there was strong directional selection. Asexual reproduction, and local clonal growth in particular, exploits the fact that a successful genotype can be successful repeatedly in both time and space (Sebens & Thorne 1985). Field mapping studies have shown some examples of very large, widespread clones (Kemperman & Barnes

1976, (American aspen); Harberd 1962 (*Festuca rubra*)). The vegetative spread of a species in a community has many genealogical implications (Harberd 1958). Harberd (1961) has shown that single clones of the common grass *Festuca rubra* can be spread over an area of grassland 220m in diameter; in this instance 51% of the tillers from an $80m^2$ (c. 100sq. yards) quadrat were of a single genotype, and 82% were accounted for by the commonest genotypes; he estimated that the time for such a clone to develop was of the order of 400 years. An even more impressive example of clonal longevity is provided by Oinonen (1967) who dated individual clones of *Pteridium aquilinum* in Finland back to about 550-570 A.D., some of these clones extended over areas more than 470m in diameter (Sarukhan 1974).

In multiclonal populations, the spatial distribution and the degree of intermingling will depend on the clonal growth strategy ("phalanx" versus "guerilla", Harper 1957; Lovett-Doust 1981a,b) and the efficiency of seed dispersal (Cook 1983; Handel 1985; Escaravage *et al.* 1998). In the "phalanx" growth form, plants producing shorter internodes tend to form an advancing front of closely packed ramets, and produce clones that are juxtaposed (Lovett-Doust 1981a). In the "guerilla" growth form longer internodes have more widely spaced ramets (Lovett-Doust 1981a) and will be more likely to intermingle with each other (Harper 1957).

Lynn and Waldren (2001a) on studying a turlough population in experimental garden conditions, found the clones to have a "guerrilla" like mode of clonal growth, with long unbranched stolons with widely spaced ramets but mode of growth may have been influenced by a possible selective disadvantage in the uniform garden experiment. The mode of growth was variable and highly clone specific, to the extent that individual clones could often be recognised by their pattern of stolon development (Lynn & Waldren 2001a). Therefore it may not be wise to compare this uniform garden experiment with the study discussed in this chapter, as conditions were obviously different. Turlough *R. repens* ramets appear to spread more profusely in their native habitat than when forced to grow in conditions which are not necessarily favourable to their spread. The Gortlecka population appears to be well adapted to the habitat in which it grows, showing both high levels of clonal spread and genetic diversity.

4.4.4 Population structure

The number of ramets of *R. repens* in a subset of quadrats within the sampling grid in Gortlecka turlough basin was quantified (10 x $1m^2$ quadrats). There were on average 583 *R. repens* ramets per m^2. This could suggest that the population is well established and that the conditions imposed by the turlough environment are amenable to the individuals present. Clonal growth was found to be prolific and genetic diversity levels high. It may be a case that these genets present in the grid surveyed are long established, and could be considered as clones with high fitness in relation to the turlough environment. Clones may have survived for many years in Gortlecka turlough basin, years of selective pressures exerting forces on the population so that the population has adapted to the inundation regime, with non-fit individuals being eliminated. Numerous studies that have been previously discussed based on both morphological and molecular methods have shown that populations of clonal plants are in fact inhabited by single or very few genets (Anderson & Beare 1983; Huenneke 1985; Murawski & Hamrick 1990). Despite these findings, in many cases clonal populations have considerable genetic diversity (Ellstrand & Roose 1987; Aspinwall & Christian 1992). The fact that such a relatively large number of genets were discovered in a relatively small area, (i.e. 14 genotypes among 100 sampled ramets), indicates that clonal spread of genetically suited individuals has occurred, and that high levels of genetic diversity are being maintained. Similar levels of genetic diversity were found by Escavarage *et al.* (1998); using the technique of AFLP they identified 32 *Rhododendron ferrugineum* genotypes in a $200m^2$ plot.

4.4.5 Origin of genetic diversity

Predominantly selfing and/or clonal species often show a much higher proportion of monomorphic RAPD or AFLP loci, typically between 45% and 80%, (Travis *et al.* 1996; Sharma *et al.* 1996; Winfield *et al.* 1998; Bussell 1999; Hogbin & Peakall 1999; Palacio *et al.* 1999; Zhivotovsky 1999). In this present study, genotypes sampled from a single turlough basin had similarity values as low as 28% between clones (ranging from 28 to 57%). At this low level of similarity many markers discriminated one genotype from another. These similarity values are relatively low compared to the studies mentioned above. However studies in which similarity was high between individuals from clonal plant populations were largely based on isozymes and other techniques such as RAPD. It was revealed in the previous chapter

that higher levels of genetic variation are detected by the method of AFLP than for ISSR. Therefore, care must be taken when making comparisons about the clonal diversity of a species when different molecular techniques have been employed. One study, assessing the differentiation of Bermuda grass genotypes using AFLP, found similarity measures between genotypes to be as low as 0.33 (33%), (Zhang *et al.* 1999). Therefore genetic diversity measures may be influenced by the technique used. In order to overcome this it would be advisable to carry out analyses using more than one molecular technique.

There are numerous factors which may be contributing to the low levels of genetic similarity between clones of *R. repens* which have been revealed by this present study. Population development can lead to the selection of genetically dissimilar genotypes. Several hypotheses have been formulated for the mechanisms underlying the maintenance of high levels of genotypic diversity in clonal plants

I. Micro-site heterogeneity, which promotes the coexistence of clones through diversifying selection (Antonovics & Ellstrand 1984; Ellstrand & Roose 1987).
II. Occasional seedling recruitment (Saone & Watkinson 1979).
III. Symmetric competition among clones (Hartnett & Bazazz 1985).
IV. Mutation

The environment in which this study was conducted may not show enough heterogeneity to concur with the first hypothesis. The remaining hypotheses may prove to be move plausible in this turlough environment. With approximately 600 ramets per $1m^2$ competition is likely to be occurring, and in such a large basin with so many *R. repens* individuals present, it is not surprising that genetic diversity is high. In addition, populations of many clonal species are founded from seeds, but show increasing vegetative reproduction over time (Erikkson 1993). These populations can maintain genetic diversity through clonal growth so long as mortality of genotypes is not too high. The input of genetic variability through seedling recruitment and mutation will increase the possibility of attaining adapted genotypes within populations.

Another factor to consider when looking at similarity between clones is population isolation. Pornon *et al.* (2000) in their population genetic study of *Rhododendron ferrugineum* using AFLP found that the more isolated a population, the more similar genetically the genotypes were. There is evidence to suggest that Gortlecka is not an isolated population of *R. repens*; broadleaf forms of the species have been found in neighbouring pastures. One possible explanation for the high levels of genetic diversity revealed in this study of a turlough population of *R. repens* is that gene flow is occurring between individuals from other areas of the turlough and from ruderal populations that are found surrounding the turlough. From the initial time when the water in the turloughs subsides there are often patches of bare ground, which are available for colonisation. These "windows of opportunity" create sites for local seedling establishment, which can occur from individuals within and outside the turlough population (nearby broad-leaved populations). These broad-leaved seedlings may not survive the winter floods, but during the period between April and September these individuals may breed with the established turlough genotypes thus adding genetic variation to the source gene pool. New genotypes may also be formed from recombination between existing turlough individuals.

The proliferation of a few aggressive clones is thought to impoverish genetic diversity in clonal plant populations. However, recent studies (Ellstrand & Roose 1987; Widen *et al.* 1994; Hamrick & Godt 1996) concluded that this is not necessarily true and that clonal plant populations may have high levels of genotypic diversity. Such high levels of genetic diversity have often been attributed to micro-site heterogeneity that promotes the coexistence of clones through diversifying selection (Antonovics & Ellstrand 1984; Ellstrand & Roose 1987). Ecological specificity of *R. repens* clones to the micro-environment in Gortlecka turlough may be contributing to the apparent high levels of genetic variation. Evidence of this was also found in the previous chapter. In addition, population maturation may lead to biotic interactions between genets changing with time, and even very episodic recruitment may be sufficient to compensate for mortality and maintain a high level of genetic diversity in the population (Pornon *et al.* 2000). The diversity reported in this study (Simpson Index: 0.9) is similar to that given in some studies on clonal species (0.96 Escavarage *et al.* 1998; and 0.85-0.93 Pornon *et al.* 2000 both based on *Rhododendron ferrugineum* populations) but was higher than for others (0.62 Ellstrand & Roose 1987; 0.61

Widen *et al.*1994, on clonal species in general). The "proportion distinguisable" (0.14) was higher than that found by Escavarage *et al.* 1998 (0.08) but similar to those values reported by Pornon *et al.* 2000 (0.157-0.183). It is obvious from these examples that it is difficult to generalise in terms of population diversity across species and habitats, but overall the findings from this study are congruent with those published on clonal species diversity.

From the simulation models of Soane and Watkinson (1979) based on clonal variation in populations of *R. repens*, the authors state that high genet diversity may be maintained for long periods within populations which spread clonally. Tests on the model have shown that the introduction of any element of selection quickly results in the populations departing significantly from the structure observed in similar populations where there is no selection; selection is likely to increase the rate of loss of genets from the population. Local dominance of a few clones is to be expected unless new genotypes are recruited into the population or unless apostatic and diversifying selection favours the maintenance of genet diversity within the population (Soane & Watkinson 1979). The simulation models of Watkinson and Powell (1993) appear relevant to this turlough population of *R. repens*, where occasional seedling recruitment both from within and outside the turlough basin may be maintaining high levels of genetic variation. The contribution of seedlings to the total number of individuals in populations of plants which spread clonally may be very small, the occasional establishment of seedlings may nevertheless play a significant role in determining the number of genets, and thus in maintaining genotypic variation within these populations (Soane & Watkinson 1979). Both of these factors are likely to be contributing to the relatively high levels of genetic diversity found in the Gortlecka population of *R. repens.* Maintenance of population densities by clonal growth, as opposed to sexual reproduction and frequent establishment of seedlings, is a means of preserving intact a successful genotype in a favourable, stable environment (Soane & Watkinson 1979).

4.4.6 Conclusions

The spatial distribution of clones mapped here is comparable to previous studies on the clonal growth of *R. repens*. The quantitative method of measuring leaf dissection was not a sufficient means of identifying clones of *R. repens*. However, the molecular

technique of AFLP proved to be a highly suited tool for the identification of genotypes and the differentiation of clones. The density of *R. repens* ramets revealed in this study was relatively high compared to other studies, thus competition in the form of density dependence may be an important factor influencing the structure of this population. It must be remembered that because *R. repens* can survive long periods of inundation it has a "head start" over other species. *R. repens* overwinters as submerged leafy rosettes, with high levels of starch storage in the roots (Lynn & Waldren 2002), and so growth can begin rapidly as soon as the water recedes. The bare ground aids its profuse spread, although it does not appear to out-compete other species (Lynn *et al.* in prep).

Fourteen genotypes were found within the $100m^2$ sampling grid. There appeared to be a structured spatial pattern of clones that may possibly be related to the moisture gradient present in the turlough basin. Diversity values and percentage similarity between clones were comparable to studies on other clonal species using the AFLP technique. Considering all of these points, the population structure appears to be heterogeneous and should remain so once conditions in the turlough habitat are not dramatically altered. Many turloughs have been subjected to anthropogenic disruption including draining programmes, eutrophication, and high levels of grazing, as discussed in Chapter 1. Fortunately Gortlecka has escaped these events and is currently under a light-grazing regime during the summer months. Sarukhan (1974) stated that rosette density of *R. repens* increases with increased grazing therefore the light grazing regime in this turlough environment may be enhancing the growth of the clones present. Turloughs are priority habitats, protected under EU law; unfortunately many of these habitats have been irreparably damaged by the factors mentioned above. Gortlecka is one of the more pristine turloughs found in Ireland and it is important that it remains so. If management practices remain the same in this turlough the *R. repens* populations as well as the remainder of the plant community present should remain intact and continue to prosper.

Chapter 5 General Discussion

From macro to micro: an evaluation of the population biology and evolutionary history of the turlough form of *Ranunculus repens*

If we fully apprehend the pattern of things of the world will it not be found that everything must have a reason why it is as it is?

Hsu Heng (1209-1281)

5.1 Introduction

This Chapter aims to present an overview of the findings of the previous three Chapters. The objective is to discuss and compare the results of the preceding analyses with the idea of drawing broader ecological trends from the independent yet connected studies conducted on the turlough buttercup. The first chapter of this thesis gave an introduction to some of the concepts involved, and included a discussion of the study organism *R. repens*, and the habitat in which it was studied, the turloughs of the West of Ireland. The thesis had a three-tiered aim; beginning with the upper level, a broad scale, inter-population, nationwide study of *R. repens* in various habitat types was undertaken. This was then narrowed down, in Chapter 3, which investigated the phenomenon of the effect of ecological change in terms of both morphology and genetic structure on a turlough population of the creeping buttercup. Finally the lower tier of the project, (Chapter 4) focussed on the population structure of the turlough form of *R. repens*, tackling issues such as clonal growth, and genetic diversity. It was hoped that by tying all of these studies together in a logical progression, from macro to micro, that it would be possible to elucidate an overall picture of this unique form of *R. repens* that can survive in the amphibious environment of a turlough basin.

5.2 Technique appraisal

In the light of the present day explosion of advances and accessibility of molecular techniques it is becoming increasingly common for research to be conducted that appears to neglect the real purpose of its existence. Molecular markers are a means to an end, tools that should be used in plant population biology to answer ecological questions. The research carried out in this thesis demonstrates the importance of not relying on one technique but to use a number of complementary methods to enhance the understanding of the species or system being studied. The work presented in this thesis stemmed from the obvious morphological and physiological differences that are found within populations of *R. repens*. The morphological analyses were very useful for identifying levels of population differentiation. Because of this morphological differentiation between turlough and non-turlough forms molecular studies were conducted to determine whether genetic differences were also present between the two morphs. The molecular analyses proved useful for ecotypic distinction of *R. repens* populations. However, due to the three-tiered approach of this work, some

techniques were more suitable at certain levels than others. In the next section, the suitability of these techniques at different levels will be discussed.

5.3 Molecular and morphological analysis of the turlough form of *R. repens*

The morphological research carried out in this study showed leaf dissection to be a distinguishing trait that separates turlough populations of *R. repens* from non-turlough populations of the species (Chapter 2). Statistical analysis showed that leaf shape was significantly different in contrasting habitat types. This finding is in agreement with the study carried out by Lynn & Waldren (2001b). On a finer scale, leaf shape was found to vary, not only between populations from different ecological systems, but within a turlough basin, albeit through an ecological gradient of inundation (Lynn & Waldren 2002). Susceptibility to submergence was suggested as a key factor in the variation and expression of this phenotypic character (Chapter 3). The variation in leaf dissection across a turlough basin illustrated differences that could in turn be related to genetic differences in terms of heterogeneous marker frequency, thus adding more depth to the significance of the findings. The breeding experiment results described in this chapter have shown that a less dissected leaf form is produced when dissected leaf turlough individuals are crossed with broadleaved non-turlough individuals, than when turlough individuals from the top and bottom of a turlough basin are crossed. In Chapter four it was discovered that leaf dissection, although variable, did not differ significantly among clones of *R. repens* growing in a turlough basin. Quantitative morphological methods have thus proved successful in distinguishing the turlough form of *R. repens* from the more common broadleaved form. Morphological analysis was a useful tool at the interpopulation level (turlough and non-turlough), but not at the intra-population level due to high intra-individual variation in leaf dissection. However, this obvious visual difference found in the turlough buttercup allowed the formation of a more intricate molecular study based on the morphological findings, and this molecular analysis was the basis for this thesis.

Many studies have documented the usefulness of molecular techniques in elucidating trends in plant population biology (Bachmann 1994; Karp *et al.* 1996; Parker *et al.* 1998; Ouborg *et al.* 1999; Sunnacks 2000). A number of molecular techniques were employed in this research, each with its advantages and pitfalls (See section 1.10 for a discussion of the techniques). Initially ISSRs were chosen to assess the genetic

diversity of *R. repens* individuals along an ecological gradient across a turlough basin (Chapter 3). In hindsight this was probably not the best place to start as genetic relatedness among individuals showed complex interactions that were beyond the resolving power of the technique. The use of AFLP throughout all three molecular studies presented in this thesis proved to be much more suitable. From the broad scale of inter-population relationships, to the intermediate scale of an ecological gradient, and finally to the fine scale of the identification of genets within a turlough basin this method proved to be fruitful. AFLP enabled the detection of ecotypic genetic differences in Chapter 2. From this study it was concluded that turlough populations of *R. repens* were more similar to each other than they were to non-turlough populations regardless of geographic distribution. This may infer a separate evolutionary lineage. Significant differences in AFLP marker frequency between individuals of *R. repens* along the ecological gradient studied in Chapter 3 were also revealed by the technique. Several markers showed a heterogeneous distribution among individuals along the turlough gradient, which could be related to differences in leaf dissection among individuals. Finally, in Chapter 4, AFLP was successful in identifying genets of the species spreading clonally within a turlough basin. However, as discussed in detail in Chapter 1, this DNA fingerprinting technique is not flawless, but a combination of stringent primer selection trials and the development of equally stringent laboratory protocols makes the method useful in plant population studies from an inter-population to an intra-population level.

Other molecular techniques used in this thesis did not prove to be so successful in elucidating genetic patterns in the data. The use of universal chloroplast microsatellite primers did not reveal any patterns among populations of *R. repens* from turlough and non-turlough populations, with no variation detected in any of the screened microsatellites. This may be due to the fact that these primers were deemed "universal"; designed to be applicable to a range of taxa, and also because the taxonomic level at which they were used in this study was too low. They would be more suitably used to compare variation between species in the genus *Ranunculus*, a task that was not part of this thesis. The search for variable microsatellite repeats in various regions of the chloroplast genome in *R. repens* was not altogether successful. Considering the conserved nature of the chloroplast genome, and its uniparentally-inherited nature, this result was not surprising. However, similar approaches have

proven successful in other plant groups such as in Oaks and Yews (T. Hodkinson, pers. comm.). The key to getting results from these techniques would be to sequence other regions of the genome, in particular nuclear regions which have different rates of evolution, and to design SSR primers specific to *R. repens*. Unfortunately, time and money did not allow this.

Among the various methods of analysis used for the molecular data throughout the thesis PCO (Section 2.3.4) appeared to show the clearest patterns and give the most interpretable results. In Chapter 2, the cluster methods of UPGMA and NJ proved to be visually difficult to interpret on the inter population level due to the high levels of variation and large sample size. When these techniques were used on a smaller set of samples (Chapter 3) the results made more sense, even in the light of the complex molecular findings. The genetic statistics used in Chapter 2 (Nei's genetic diversity and percentage polymorphic loci) gave comparable results to other similar studies in plant population genetics thus rendering them suitable to elucidate the genetic structure of populations of *R. repens*.

5.4 An overview of the findings

When the molecular results from each of the three Chapters in this thesis are brought together some overall conclusions can be made. Chapter 2 revealed ecotypic differences in the species, with turlough populations clustering together and thus being more similar to each other than to non-turlough populations regardless of geographic location. The general trend found from this study was that populations separated in ordination space according to habitat type. This in turn could be related to leaf shape with all turlough populations having a dissected leaf, dry pasture populations having a broadleaf shape and wet pasture being somewhat intermediate. On the finer scale the story becomes more complex. When the technique of ISSRs was used in Chapter 3 a somewhat blurred picture of genetic population structure was revealed with individuals from the top and bottom of a turlough basin not appearing genetically distinct. This finding may not in fact be due to a lack of structure but may have been a result of the low levels of variation and low resolving power of the technique. When this study was repeated using the technique of AFLP, patterns emerged. There appear to be genetic differences present in turlough populations

depending on the environmental conditions. Although variation was very high, certain markers were shown to have uneven distribution along the transect.

Thus in Chapter 3, it was discovered that *R. repens* individuals differed genetically across an ecological gradient and that this variation was not randomly distributed. This finding was backed up statistically by both Chi square and Mantel tests that were conducted on the data. It was also apparent that relatively low levels of clonal growth were occurring along the gradient. Within each of the twelve quadrats sampled only two samples were identified to be of identical genetic makeup. In contrast, the results from Chapter 4 show that high levels of clonal growth were present, with a total of 14 genotypes inhabiting an area of $100m^2$. It must be noted that the sampling strategies in these two studies were different. Despite this, it could be postulated that the Hawkhill population of *R. repens* (Chapter 3) is maintaining a higher level of genetic diversity compared to that of Gortlecka population (Chapter 4). Disruptive selection, and gene flow from nearby broadleaved populations are among the reasons suggested for these differences. *R. repens* clones appear to have adapted to their environment on a very small spatial scale. Thus at Hawkhill, where micro-environmental change is rapid and frequent, a large number of genotypes are present, whereas at Gortlecka these changes are not as drastic and environmental changes are less severe, allowing individual clones to spread more profusely. The main factor that appeared to be affecting the clonal growth pattern of *R. repens* in Gortlecka was a moisture gradient which had an influence on the direction of spread of the clones (Chapter 4). This may also explain the high number of genets present along the pronounced moisture gradient at Hawkhill.

One problem with using random, dominant molecular marker techniques such as RAPD and AFLP is that they primarily reflect neutral genetic variation and do not accurately reflect adaptive evolutionary processes and differentiation brought about by natural selection (Palacios & Gonzales-Candelas 1997). Despite this, several AFLP markers were found to show heterogeneous distribution between turlough and non-turlough populations of *R. repens* (Chapter 2) and among individuals within a turlough basin that were subjected to differing environmental factors. However, no turlough-specific markers were discovered through these analyses. In this sense, the morphological trait of leaf dissection is more congruent with the ecological

differences between the populations from different habitat types, whereas the molecular analyses sometimes showed a blurred pattern. This is probably because the morphological trait of leaf dissection is just one character, whereas hundreds of markers or characters are produced from the technique of AFLP. It would be beneficial to screen many more turlough populations of *R. repens* at higher sampling intensity per population to try to discover ecotype-specific molecular markers.

5.5 Evolution of the turlough buttercup ecotype: a taxonomic perspective

Based on morphology (leaf shape, leaf hairiness) alone, the turlough buttercup is visually different to the more common weedy form. In terms of physiology, the turlough form was found to have higher rates of aerial and submerged photosynthesis, and higher rates of respiration in submerged conditions than the dry pasture form (Lynn & Waldren 2002). From the current study it was discovered that the turlough populations are more closely related to each other than they are to non-turlough populations of the species. Two theories on the evolutionary history of the turlough form of *R. repens* were postulated in Chapter 1. The first theory suggested that the turlough form of the species evolved many times, independently, from dry pasture populations of the species, to give rise to the dissected leaf form. This theory was refuted, following the findings of Chapter two, in favour of the opposing theory that postulated that the turlough populations of the species are of a different evolutionary lineage to the broadleaf populations of the species, and may share a common ancestry.

The potential geographic isolation of the turlough populations together with the different ecological preferences have not resulted in breeding barriers as seen in Chapter 3, where experimental crosses between turlough and non-turlough populations produced viable seed. The high level of genetic diversity is possibly due to gene flow between turlough and adjacent broadleaved populations of the species. This latter theory was not proved, and all experimental crosses were conducted out of the turlough environment, therefore it is not clear whether breeding does in fact occur between different morphs in the field. It is possible that breeding occurs between turlough and non-turlough types during the summer months when turlough basins are free of water, but summer flash floods, differing ecological requirements, and possibly differing flowering times of the two morphs may hinder this phenomenon from occurring.

Stebbins (1950) defines a subspecies as a series of populations having certain morphological and physiological characters in common, inhabiting a geographic subdivision of the range of the species or a series of ecological habitats. These populations must differ in several characteristics from typical members of other subspecies although be connected with one or more of them by a series of intergrading forms. Lynn & Waldren (2001b, 2002) demonstrated that morphological and physiological differences occur between turlough and non-turlough forms of the species. This present study has shown that turlough populations of the species are more similar to each other genetically than they are to non-turlough populations of the species, and although the Norfolk mere populations showed similar levels of leaf dissection to the Irish turlough populations they were not found to be genetically similar. It would seem that the turlough form in Ireland is an ecotype of different ancestry to the similar ecotype found in Norfolk. In combination, the morphological, physiological and molecular studies appear to fulfill the criteria for subspecies status, although a more in-depth screening of populations would be necessary before an attempt to sub-rank the species could be made.

If the turlough form of *R. repens* is to be described as a subspecies it is imperative that the extent of the dissected leaf form is examined. Variation is likely to occur in this broad ranging species, but whether this variation can be correlated with ecological parameters throughout its range is unclear. Hintikki (1975) and Coles (1977) refer to heterophylly in *R. repens*. Briggs (1962) and Menadue and Crowden (1988) have observed variation in other members of the genus. Unfortunately very little ecological data appears to have been collected for these studies. It was suggested from the morphological data presented in Chapter 2 that the degree of leaf dissection was related to one of three habitat types but further comprehensive research is needed. Reporting variation is useless if the conditions that impose the changes are not documented also.

5.6 Implications for turlough conservation

A combination of geological, hydrological, and geographical factors determines the uniqueness of the turlough habitat in Ireland (Coxon 1986). Turloughs, the temporary limestone lakes unique to Ireland, hold Priority Habitat status under EU Law. It is thus Ireland's duty and obligation to protect and manage these ecosystems and all that

they entail, including flora and fauna. The dissected leaf form of *R. repens* is one of the many unusual inhabitants of these temporary lakes. Others include the rare plant species *Potentilla fruticosa*, *Teucrium scordium*, *Frangula alnus* and the fairy shrimp *Tanymastix stagnalis*. The fluctuating water levels that are an intergral part of the turlough ecosystem are likely to control the composition of the vegetation community. Thus any changes that occur due to anthropogenic activities, such as drainage, will most likely have a strong impact on the plant communities present. It has been suggested that other plant species that inhabit the turlough environment have adapted to the fluctuating water conditions such as *Potentilla anserina*, *Leontodon autumnalis* and *Agrostis stolonifera* (S. Waldren pers. comm.). These species occur throughout a turlough basin and in the pastures surrounding so it may be a case that they too have developed a tolerance to submergence, although no formal scientific studies have been conducted. The information on population genetic structure of the turlough buttercup documented in Chapters 3 and 4 is fundamental to the development of a greater understanding of turlough plant communities. Although turlough populations of *R. repens* are often isolated, the study documented in Chapter 2 revealed high levels of genetic diversity. These finding are fundamental for such issues as conservation and management of plant species because if genetic diversity is somehow lost, the ability to withstand change is also lost. A plant population is not one fixed unit that is generally adapted, but a dynamic complexity of genotypes that interact and adapt to a particular suite of conditions, or micro-environment. It would therefore be useful to broaden this molecular study to other plant species that occur in the turlough habitat.

5.7 Further studies

Preceding the study of Lynn (1998) very little work had been carried out on turlough populations of *R. repens.* Prior to her work, observed differences in leaf dissection between turlough and non-turlough populations were the only information available. Her results raised many questions of interest about the turlough form of *R. repens* and a molecular analysis of these populations appeared to be the next logical step towards a greater understanding of the mechanisms responsible for the presence of this dissected leaf form of the species which is able to survive prolonged periods of submergence in the turlough habitat. The work presented in this thesis lays the foundations for an area of study with great potential for development. The turlough

form of *R. repens* presents many interesting problems and thus merits more research. A short time frame limited the present study and thus only a few studies were possible. Further directions of research are outlined below.

Developing *R. repens*-specific microsatellites, both nuclear and chloroplast, for further work on more in-depth population studies would be beneficial as they would be of considerable value in identifying levels of differentiation and may help elucidate further the evolutionary events which have lead to the existence of the turlough form of the species. SSRs would also be useful in examining gene flow into turlough populations and between different parts of a turlough population. Development could be achieved in numerous ways, for example database and enriched library searches for nuclear microsatellites as well as database searches of published sequences from the chloroplast genome. This study could include a more intense sampling strategy in which higher numbers of samples per population would be collected and analysed. This sort of study, through phylogenetic analysis, may help to elucidate the origin of the turlough form as it is still unclear whether the turlough form is the source of broadleaved populations or vice versa.

In addition to this molecular tool, AFLP could be used to discover whether or not the quality of a turlough has an effect on the genetic diversity present. A reduced number of populations from oligotrophic and eutrophic turloughs with a greater number of individuals per population should be sampled. Diversity levels could then be compared among and within populations in order to determine whether or not management practices influenced the profuseness of the turlough form of *R. repens.* By adding non-turlough populations to this study the genetic structure and diversity levels in this present study could be confirmed or rejected.

The breeding experiment conducted in this thesis was not altogether successful due to propagation difficulties. Previous studies have shown the F1 progeny to be of an intermediate leaf shape (Lynn 1998), It would be beneficial to repeat these experiments on a larger scale, over many generations to determine the mode with which the dissected leaf form is revealed through generations and to determine the level of fitness in the progeny with regards tolerance of submergence.

Many demographic studies on clonal growth have been conducted (Sarukhan 1974; Soane & Watkinson 1979; Lovett-Doust 1981a; Lynn & Waldren 2001a). These studies, which are largely based on morphology, have many problems associated with them such as the difficulty of identifying genets in the field. The experiment documented in Chapter 4 of this thesis is the first molecular study on the clonal growth strategies of *R. repens* to have been conducted. Therefore it would be interesting to undertake more of these studies, to determine the clonal diversity and population structure of a number of turlough and non-turlough populations to decipher whether the conditions imposed in the different ecosystems have an effect on the mode of spread of the species. To follow on from the investigations documented in Chapter 3 it would be of particular interest to sample down a gradient and also along the various moisture contours of a turlough basin to look at any ecological specialisation of clones.

It is still largely unclear what the relationship is between turlough populations of *R. repens* and mere populations of the species found in Norfolk, UK. Only four Norfolk populations were used in this present study and the results showed that these mere populations were not genetically similar to the Irish turlough populations despite having similar leaf morphology. These mere populations have a dissected leaf form that is suggested to be a result of the populations adapting to long periods of inundation. The results from the present study seem to infer that this phenomenon is due to parallel evolution. It would be beneficial to sample mere populations to a greater extent and to add them to further molecular analyses being conducted. It would also be of use to sample broadleaf populations of *R. repens* growing in close proximity to the meres to determine the genetic relationship between them. A more detailed molecular study using AFLPs and SSRs may help to elucidate whether this similarity is a result of parallel evolution or whether in fact they are part of the same evolutionary lineage.

If both time and money were no object, a search to determine the gene or genes responsible for changes in the leaf morphology of the turlough form of *R. repens* could be conducted. This could be achieved by constructing a genetic map based on techniques such as AFLP and SSRs to identify quantitative trait loci (QTL) associated

with morphological, physiological and submergence tolerance traits in populations of *R. repens.* See Ellis *et al.* (2002) for a discussion of the techniques available.

R. repens is the only species found in the turlough environment, apart from *Frangula alnus*, with an evident morphological trait that makes it different from the more common weedy form. This phenotypic character is proposed to be a result of adaptation to inundation. Other species that inhabit this environment have presumably adapted to the turlough conditions and it would be beneficial to conduct a genetic analysis on species present such as *Potentilla anserina* to determine whether they too have evolved genetic differences in order to survive in this environment. A turlough population of this species (Gortlecka) showed much more rapid senescence and cessation of leaf production compared to a non-turlough population that was never subject to submergence. The latter continued to produce leaves, albeit slowly, when experimentally submerged (S. Waldren pers. comm.).

5.8 Concluding remarks

The turlough form of *Ranunculus repens* was shown to be both genetically and morphologically distinct from the more common weedy, broadleaved form of the species. These differences are likely to be related to ecological preferences of the different forms, the susceptibility to inundation being the most obvious factor in the case of the turlough buttercup. The hypothesis of different evolutionary lineages of the two forms was put forward and supported by this population genetics study of the turlough form of *R. repens*. The one exception to this finding was the occasional broadleaved population sampled adjacent to a turlough grouping closely with its turlough neighbour. This result may be due to the exchange of genes between these proximal populations but further work would be required in order to test this hypothesis. The origin and evolutionary history of the turlough buttercup is still unclear but the work presented in this thesis would suggest that the turlough form is of a separate evolutionary lineage to the more common broadleaved form and that the turlough form is an ecotype that has resulted from the conditions imposed in the turlough environment. This story is far from complete, and hopefully this research will lay down the foundations for future investigations into the plant populations that occur in the unique turlough habitats of Ireland, and in particular to the development

of a greater understanding of this unique form of *Ranunculus repens* which has been named, by those who hold it near and dear, the turlough buttercup.

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Appendix 1 Site details

Site details for the *Ranunculus repens* populations collected. Detailed site descriptions were obtained from Coxon (1986), Goodwillie (1992), Goodwillie *et al.* (1998), and Lynn (1998); associated species were assessed directly in the field at the time of sample collection. Nomenclature for the associated species follows Webb *et al.* (1996). The populations were all placed in one of three categories, depending on susceptibility to inundation; turlough, collected from turlough basins, wet pasture, collected from a variety of habitats such as river callow, lakeshores, marshes, and water logged fields, habitats susceptible to occasional flooding, and dry pasture which again were collected from a variety of habitats such as roadsides, and gardens, habitats rarely, if ever subjected to periods of flooding. Collection sites from the UK are listed at the end of the Irish sites. Populations are described in terms of habitat type, beginning with turloughs, followed by wet pasture and finally dry pasture. Population codes described are those used throughout the study. All grid references were obtained from GPS, described in Section 2.2.1.

Population code: CFS

Location: Carron Field Research Station, Carron, Co. Clare

Grid reference: R288991

Habitat type: Ruderal / Dry pasture

Collection date: 15.06.00

Site details: Roadside habitat adjacent to Carron turlough.

Associated Species:

Achillea nullefolia, Anthoxanthum oderatum, Arrenatherum elata, Bellis perennis, Briza media, Centauria nigra, Corylus avellana, Crataegus monogyna, Cynosurus cristata, Dactylis glomerata, Festuca rubra, Geranium lucidum, Geranium sanguineum, Hedera helix, Holcus lanatus, Lotus corniculatus, Plantago lanceolata, Plantago major, Poa trivialis, Potentilla anserina, Pteridium aquilinum, Ranunculus acris, Ranunculus bulbosus, Rhinanthus minor, Rubus fruticosa, Stellaria holostea, Trifolium pratensis, Trifolium repens, Urtica dioica, Veronica chamaedris, Vicia sp.

Population code: HK

Location: Hawkhill turlough, Co. Clare

Grid reference: M411025

Habitat type: Turlough

Collection date: 16.06.00

Site description: A small relatively deep turlough with a permanent pool at the base, which is under tidal influence and therefore experiences daily fluctuations. The turlough is surrounded by pastureland and there is evidence of eutrophication. The vegetation is a marshy community and there is a permanent aquatic community at the base of the turlough. The vegetation is lightly grazed by cattle and a horse with stumpy legs.

Associated species:

Agrostis stolonifera, Carex panicea, Filipendula ulmeria, Leontodon autumnalis, Plantago major, Rumex crispus, Trifolium repens, Lotus corniculatus, Potentilla anserina, Leontodon autumnalis, Mentha aquatica, Plantago lanceolata, Potentilla reptans, Ranunculus flammula, Galium boreale, Plantago major, Carex nigra, Potentilla erecta, Ophioglossum vulgatum, Viola canina.

Population code: HKA

Location: Hawkhill turlough, Co. Clare

Grid reference: M413025

Habitat type: Ruderal / Dry pasture

Collection date: 16.06.00

Site description: Wooded laneway, NW corner of Hawkhill turlough basin.

Associated species:

Agrostis stolonifera, Bellis perennis, Cardamine pratensis, Craetagus monogyna, Filipendula ulmeria, Fraxinus excelsior, Galium aparine, Geranium robertianum, Geum urbanum, Lolium perenne, Plantago major, Poa pratensis, Poa trivialis, Potentilla reptans, Prunus domestica, Pteridium aquilinum, Ranunculus acris, Rubus fruticosa, Rumex sanguineum, Rumex obtusifolius, Stellaria media, Taraxacum officinalis, Trifolium repens, Urtica dioica, Veronica chemaedrys.

Population code: IN

Location: Inistoige West, Nore valley, Co. Kilkenny

Grid reference: S629384

Habitat type: Wet pasture

Collection date: 4.07.00

Site description: River callow on the edge of the river Nore, damp pasture vegetation with patches of *Iris pseudocarpus*

Associated species:

Agrostis stolonifera, Alopecurus geniculatus, Apium rodiflorum, Cerastium fontanum, Circium arvense, Circium palustre, Cynosaurus cristata, Dactylis glomerata, Filipendula ulmeria, Galium uliginosum, Glyceria fluitans, Holcus lanatus, Iris pseudocarpus, Lolium perenne, Myosotis scorpioides, Gnaphalium sylvaticum, Phalaris arundinacea, Polygonum persicaria, Ranunculus flammula, Rorippa nasturium – aquaticum, Rorippa palustre, Trifolium repens.

Population code: URL

Location: Urlingford, Co. Kilkenny

Grid reference: S310640

Habitat type: Turlough

Collection date: 4.07.00

Site description: Slightly undulating turlough basin with banks and hummocks of glacial drift around which the water rises. The turlough has suffered significant drainage but it still continues to flood for long periods during the year. In summer it retains a central permanent pond. There is high intensity grazing by sheep and cattle.

Associated species:

Agrostis stolonifera, Bellis perennis, Carex nigra, Crepis paludosa, Equisetum arvense, Filipendula ulmeria, Galium palustre, Hydrocotyle vulgaris, Leontodon autumnalis, Lotus corniculatus, Mentha aquatica, Ophioglossum vulgatum, Plantago lanceolata, Plantago major, Polygonum ambhibium, Potentilla anserina, Potentilla erecta, Potentilla reptans, Ranunculus flammula, Rumex crispus, Stellaria media, Taraxacum palustre, Trifolium repens.

Population code: FT

Location: Fortwilliam turlough, Co. Longford

Grid reference: N020630

Habitat type: Turlough

Collection date: 13.07.00

Site description: The surrounding countryside is flat with a thin cover of drift. The floor of the oval turlough basin is at two levels, a lower central area with several lakes and ponds and a higher surrounding level of till with scattered rocks extending into flat fields and woodland. The turlough appears unaffected by drainage and standing water may persist throughout the summer in the reed beds. Both cattle and sheep graze the turlough.

Associated species:

Agrostis stolonifera, Baldellia ranunculoides, Cardamine pratense, Carex nigra, Circium dissectum, Eleocharis palustre, Galium palustre, Glyceria fluitans, Hydrocotyle vulgaris, Lotus corniculatus, Mentha aquatica, Myosotis scorpioides, Polygonum persicaria, Potentilla anserina, Potentilla reptans, Prunella vulgaris, Ranunculus flammula, Rumex crispus, Samolus valerendi, Trifolium repens.

Population code: BT

Location: Ballinturly, Co. Roscommon

Grid reference: M840600

Habitat type: Turlough

Collection date: 13.07.00

Site description: A very large predominantly flat bedded turlough except for a line of depression at the centre of the northern limb. There is a boggy area to the SE where the main flow of water comes from and this eventually flows into a lake. There is no evidence of external drainage. The drier parts of the basin are grazed intensively by sheep as well as by cattle. Ballinturly is the largest turlough found in Co. Roscommon.

Associated species:

Agrostis stolonifera, Alopecurus pratensis, Bellis perennis, Calium palustre, Carex sp., Cerastium fontanum, Eleocharis acicularis, Festuca pratensis, Filipendula ulmaria, Hydrocotyle vulgaris, Iris pseudacorus, Leontodon autumnalis, Lotus

corniculatus, Mentha aquatica, Myosotis scorpioides, Phalaris arundinacea, Poa trivialis, Potentilla anserina, Potentilla reptans, Rumex conglomeratus, Rumex crispus, Rumex sanguineus, Trifolium repens.

Population code: SW

Location: Swallow hole, Co. Sligo

Grid reference: G740174

Habitat type: Wet pasture

Collection date: 13.07.00

Site description: Presence of permanent pool, *Veronica, Alisma, Typha* Swamp, which changes to wet grassland with Iris patches. Fenced off from rest of pasture so little or no grazing.

Associated species:

Agrostis stolonifera, Alisma plantago–aquatica, Eleocharis palustris, Equisetum fluviatile, Glyceria fluctans, Hippuris vulgaris, Iris pseudacorus, Juncus articulatus, Juncus conglomeratus, Mentha arvensis, Myosotis scorpioides, Poa trivialis, Polygonum persicaria, Potentilla anserina, Ranunculus acris, Rorippa nasturtium-aquaticum (Nasturtium officinale), Rumex crispus, Senecio aquatalis, Trifolium repens, Typha latifolia, Veronica beccabunga.

Population code: CS

Location: Castleplunket, Co. Roscommon

Grid reference: M780770

Habitat type: Turlough

Collection date: 13.07.00

Site description: The turlough runs into bog land on the east and into higher sloping ground on the south. The turlough floor is flat and evenly sloping with a few shallow depressions and channels in the centre. Both cattle and sheep graze the turlough.

Associated species:

Agrostis stolonifera, Carex sp., Festuca pratensis, Filipendula ulmeria, Iris pseudacorus, Leontodon autumnalis, Phalaris arundinacea, Plantago major,

Polygonum persicaria, Potentilla anserina, Potentilla reptans, Rumex crispus, Rumex obtusifolia X *crispus, Trifolium repens.*

Population code: CE

Location: Coole, outside Gort, Co. Galway

Grid reference: M430030

Habitat type: Turlough

Collection date: 16.07.00

Site description: A flat floored turlough lying West of Gort. The turlough is contiguous with Coole Lough. There is no evidence of drainage. Cattle, sheep and horses graze the area.

Associated species:

Agrostis stolonifera, Carex sp., Filipendula ulmeria, Leontodon autumnalis, Phalaris arundinacea, Plantago major, Polygonum persicaria, Rumex crispus, Rumex obtusifolia, Trifolium repens, Lotus corniculatus, Stellaria media, Ceratium fontanum, Viola spp., Potentilla anserina, Leontodon autumnalis, Hydrocotyle vulgaris, Mentha aquatica, Plantago lanceolata, Potentilla reptans, Ranunculus flammula, Galium palustre, Plantago major, Carex nigra, Potentilla erecta.

Population code: GK

Location: Gortlecka turlough, Co. Clare

Grid reference: R313938

Habitat type: Turlough

Collection date: 12.07.00

Site description: An unnamed turlough consisting of three connected basins with basins becoming deeper from SW to NE which is situated in the flat limestone pavement SW of Mullagh Mor. Many areas of the upper and middle basin are composed of bare areas of limestone pavement. The turlough is surrounded mostly by limestone pavement but there is an area of farmland to the west of the lower basin. The turlough is totally dependent on groundwater and there is no inflow into the basin.

Associated species:

Agrostis stolonifera, Carex spp., Filipendula ulmeria, Leontodon autumnalis, Phalaris arundinacea, Plantago major, Polygonum persicaria, Rumex crispus, Rumex obtusifolia, Trifolium repens, Lotus corniculatus, Stellaria media, Ceratium fontanum, Viola spp., Potentilla anserina, Leontodon autumnalis, Mentha aquatica, Plantago lanceolata, Potentilla reptans, Ranunculus flammula, Galium palustre, Plantago major, Carex nigra, Potentilla erecta, Potentilla reptans, Carex hostiana, Carex flacca, Molinia caerulea, Cirsium dissectum.

Population code: CO

Location: Cooloorta turlough, Co. Clare

Grid reference: R337979

Habitat type: Turlough

Collection date: 12.07.00

Site description: Turlough fringes of a permanent lake. Relatively shallow basin with evidence of local peat accumulation. The whole water body is surrounded by limestone pavement. Soil remains marshy even when the basin is empty. Horses, goats and, occasionally cattle, lightly graze the vegetation.

Associated species:

Agrostis stolonifera, Carex spp., Filipendula ulmeria, Leontodon autumnalis, Phalaris arundinacea, Plantago maritima, Potentilla anserina, Leontodon autumnalis, Mentha aquatica, Plantago lanceolata, Potentilla reptans, Stellaria media, Ranunculus flammula, Galium palustre, Plantago major, Carex nigra, Potentilla erecta, Potentilla reptans, Carex hostiana, Carex flacca, Glyceria fluitans, Baldellia ranunculoides, Veronica scutellata, Crepis paludosa, Littorella uniflora, Galium boreale.

Population code: KY

Location: Killaturly, Co. Mayo

Grid reference: M410980

Habitat type: Turlough

Collection date: 13.07.00

Site description: Killaturly Lough is a permanent lake set in a hollow between moraines and surrounded by much bog. The sampling site was damp pastureland, with a peat base. Peat cutting has ceased in practically the whole area. Evidence of drainage, moderate grazing

Associated species:

Agrostis stolonifera, Alisma plantago–aquatica, Caltha palustris, Cirsium vulgare, Eleocharis palustre, Filipendula ulmeria, Galium palustre, Glyceria fluitans, Hydrocotyle vulgare, Juncus conglomeratus, Leontodon autumnalis, Mentha, Myosotis scorpioides, Polygonum persicaria, Potentilla anserina, Potentilla reptans, Prunella vulgaris, Rumex crispus, Senecio aquatalis, Trifolium repens

Population code: MY

Location: Moylough, Co. Mayo

Grid reference: G540080

Habitat type: Turlough

Collection date: 14.07.00

Site description: Permanent pool fringed with *Carex elata* and Eleocharis, merging into damp pasture. Cattle grazing occurs but at low densities.

Associated species:

Agrostis stolonifera, Apium inundatum, Carex elata, Carex nigra, Cerastium fontanum, Eleocharis acicularis, Filipendula ulmeria, Galium palustre, Glyceria fluitans, Leontodon autumnalis, Gnaphalium sylvaticum, Phalaris arundinacea, Polygonum minus, Polygonum persicaria, Polygonum hydropiper, Potentilla anserina, Rorippa palustris, Rumex crispus, Rumex obtusifolius, Senecio aquaticus, Stellaria media.

Population code: MYA

Location : Field adjacent to Moylough turlough, Co. Mayo

Grid reference: G544080

Habitat type: Ruderal / Dry pasture

Collection date: 14.07.00

Site description: Lightly grazed pasture with a diverse vegetation community

Associated species:

Achillea millefolium, Agrostis capillaris, Anthoxantheum oderatum, Bellis perennis Cerastium fontanum, Cirsium arvense, Cynosorus cristatus, Dactylis glomerata, Festuca pratense, Filipendula ulmeria, Heracleum sphondylium, Holcus lanatus, Lolium perenne, Phleum pratense, Plantago lanceolata, Poa trivialis, Prunella vulgaris, Ranunculus acris, Rumex acetosa, Senecio jacobea, Trifolium pratense, Trifolium repens , Veronica chamaedrys.

Population code: BL

Location : Balla, Co. Mayo

Grid reference : M260850

Description : Turlough

Collection date: 14.07.00

Site description : The turlough lies in a N-S hollow, and along each side of the basin are elongated drumlinoid ridges. The floor is probably flat but it has become covered with a dome of peat. A large number of drainage ditches are present. *Equisetum, Glyceria, Carex* marsh, with *Carex* dominating, moderate levels of grazing. Peat cutting occurred in the past but has presently ceased.

Associated species:

Agrostis stolonifera, Balldellia ranunculoides, Caltha palustris, Cardamine pratense, Eleocharis acicularis, Equisetum fluviatile, Filipendula ulmeria, Galium palustre, Glyceria fluitans, Hydrocotyle vulgare, Juncus articulatus, Leontodon autumnalis, Mentha aquatica, Menyanthes trifoliata, Myosotis scorpioides, Oenanthe fistilosa, Potentilla anserina, Potentilla erecta, Potentilla reptans, Ranunculus flammula, Rorripa nasturium–aquaticum, Senecio aquatalis, Trifolium repens, Veronica scutellata.

Population code: SL

Location: Slishmeen, Co. Mayo

Grid reference: M220790

Habitat type: Turlough

Collection date: 14.07.00

Site description: Small even basin overlooked by drift-covered fields. The landscape consists of drumlinoid ridges. The floor of the basin is flattish and descends to a pond in the west centre. Drainage ditches present, most of the turlough is heavily grazed but an area fenced off from the cattle consists of open mud that has been colonised by *R. repens, Polygonum persicaria* and *Potentilla anserina* dominated.

Associated species:

Agrostis stolonifera, Carex spp., Cerastium fontanum, Filipendula ulmeria, Mentha aquatica, Myosotis scorpioides, Polygonum minus, Polygonum persicaria, Potentilla anserina, Ranunculus tripartitus, Rumex crispus, Trifolium repens, Veronica beccabunga but with pink flowers

Population code: CHV

Location: Cahervoostia, Co. Mayo

Grid reference: M260650

Habitat type: Turlough

Collection date: 14.07.00

Site description: The floor of the turlough basin is very flat and the surrounding topography gently rolling. A semi permanent lake exists in the middle of the main basin associated with deep ditches. Cattle are numerous in the turlough and sheep grazing also occurs.

Associated species:

Agrostis stolonifera, Alisma plantago–aquatica, Alopecurus geniculatus, Apium nodiflorum, Balldellia ranunculoides, Carex spp., Cerastium fontanum, Eleocharis palustre, Equisetum fluviatile, Filipendula ulmeria, Glyceria fluctans, Hydrocotyle vulgaris, Mentha aquatica, Myosotis scorpioides, Oenanthe aquatica, Plantago lanceolata, Poa annua, Polygonum amphibium, Polygonum minus, Potentilla anserina, Potentilla reptans, Ranunculus flammula, Ranunculus tripartitus, Rumex crispus, Trifolium repens

Population code: RL

Location: Ramolin townland , Co. Mayo

Grid reference: M260540

Habitat type: Turlough

Collection date: 14.07.00

Site description: Locally referred to as The Lough, this turlough is surrounded by gently undulating farmland. There is much peat development in the basin that consequently appears almost flat. Most of the turlough is very lightly grazed.

Associated species:

Agrostis stolonifera, Bellis perennis, Carex sp., Filipendula ulmeria, Galium palustre, Hydrocotyle vulgaris, Juncus sp., Lotus corniculatus, Mentha aquatica, Myosotis scorpioides, Plantago lanceolata, Polygonum persicaria, Potentilla anserina, Potentilla reptans, Prunella vulgaris, Ranunculus flammula, Rumex crispus, Trifolium repens.

Population code: CR

Location: Carron, Co. Clare

Grid reference: R290990

Habitat type: Turlough

Collection date: 24.07.00

Site description: Occurs in the high Burren just below the village of the same name. It occupies an immense polje, a collapsed canyon dissolved out by the partially underwater river which flows south. The base of the turlough is mostly covered by peat. There has been no attempt at drainage. Cattle occur in large free-ranging herds that are concentrated in the south where most of the farms are.

Associated species:

Agrostis stolonifera, Caltha palustris, Cardamine pratensis, Carex spp., Eleochoris palustre, Equisetum fluviatale, Eriophorum latifolium, Filipendula ulmeria, Galium palustre, Glyceria maxima, Hydrocotyle vulgaris, Juncus articulatus, Leontodon autumnalis, Mentha aquatica, Menyanthus trifoliata, Myosotis scorpioides, Phalaris arundinacea, Potentilla anserina, Ranunculus flammula, Rorippa nasturium-aquaticum, Senecio aquaticus, Succisa pratense, Trifolium repens.

Population code: GM

Location: Glenamaddy, Co. Galway

Grid reference: M640610

Habitat type: Turlough

Collection date: 26.07.00

Site description : A vast turlough covering the junction of three valleys, the land surrounding is gently undulating, bogland stretches away to the south-east. The turlough is relatively deep and the sides slope down to a flattish floor which is uncovered by water for a short season if at all. The unenclosed parts of the turlough are grazd by cattle.

Associated species:

Agrostis stolonifera, Alisma plantago-aquatica, Carex nigra, Eleochoris palustre, Equisetum fluviatale, Galium palustre, Glycerium fluitans, Hydrocotyle vulgare, Juncus articulatus, Leontodon autumnalis, Lotus corniculatus, Mentha arvensis, Molinia coerulea, Myosotis scorpioides, Ophioglossum vulgatum, Plantago aquatica, Polygonum amphibium, Potentilla anserina, Ranunculus flammula, Salix repens, Sparganium angustifolium, Viola persicifolia.

Population code: LG

Location: Lough Gash, Co. Clare

Grid reference: R390680

Habitat type: Wet pasture

Collection date: 27.07.00

Site description: A very flat poached basin that is slightly raised in the middle. The basin is used as a discharge for the sewage works of Newmarket upon Fergus and is too wet for any grazing. There is an *Apium nodiflorum* dominated centre, and very little *Ranunculus repens* present.

Associated species:

Agrostis stolonifera, Alisma plantago aquatica, Alopecurus geniculatus, Apium inundatum, Apium nodiflorum, Eleocharis acicullariss, Glyceria fluitans, Hippuris vulgaris, Mentha arvensis, Myosotis scorpioides, Polygonum persica, Potentilla anserina, Rorippa palustris, Rumex crispus, Sparganium angustifolium, Veronica beccabunga.

Population code: KK

Location: Kilkorcan, Co. Galway

Grid reference: R350941

Habitat type : Wet pasture

Collection date: 23.07.00

Site description : Two adjacent fields form a deep basin outline. The deeper part often becomes inundated following periods of particularly wet weather. The vegetation is grass dominated woith *Iris pseudacorus* in the wettest parts. Grazed by cattle.

Associated species:

Iris pseudacorus, Agrostis stolonifera, Cardamine pratensis, Festuca rubra, Leontodon autumnalis Myosotis scorpioides, Potentilla anserina, Potentilla erecta, Rumex crispus, Plantago major.

Population code: CL

Location: Clonmacnoise, Co. Offaly

Grid reference: N013312

Habitat type : Wet pasture

Collection date: 21.07.00

Site description : A flat expanse at the east side of the Shannon river. Inundation occurs following periods of prolonged rainfall when the banks become flooded. The vegetation is composed of a diverse damp grassland sward. There is evidence of improvement with fertilisers in some areas. The sward is cut for hay at the end of the summer.

Associated species:

Anthoxanthum odoratum, Agrostis stolonifera, Cardamine pratensis, Filipendula ulmaria, Lotus corniculatus, Plantago lanceolata, Juncus conglomeratus, Juncus inflexus, Ranunculus acris, Potentilla anserina, Potentilla erecta, Rumex acetosa, Plantago major, Carex nigra, Poa pratensis, Poa trivialis.

Population code: GT

Location: Gorteen, Co. Sligo

Grid reference: G675055

Habitat type : Wet pasture

Collection date: 27.07.00

Site description : Boggy, large drainage ditches

Associated species:

Agrostis capillaris, Agrostis sp., Agrostis stolonifera, Carex spp., Cirsium dissectum, Filipendula ulmeria, Galium palustre, Hydrocotyle vulgare, Juncus articulatus, Leontodon autumnalis, Linum catharticum, Lotus corniculatus, Mentha aquatica, Molinia coerulea, Myosotis scorpioides, Phalaris arundinacea, Phleum pratense, Phragmites communis, Potentilla anserina, Potentilla erecta, Prunella vulgaris, Ranunculus flammula, Schoenoplectus lacustris, Scirpus maritimus, Succisa pratense, Teucrium scordium, Vicia cracca

Population code: CH

Location: Caherglassaun, Co. Clare

Grid reference: M410060

Habitat type : Turlough

Collection date: 9.08.00

Site description: The turlough surrounds a lake and is therefore tidal. There is no evidence of drainage. Cattle and sheep graze most of the turlough. There are patches in the wetter areas dominated by *Eleocharis acicularis.*

Associated species:

Agrostis stolonifera, Carex sp., Cerastium fontanum, Eleocharis acicularis, Filipendula ulmeria, Gnaphalium uliginosum, Polygonum hydropiper, Leontodon autumnalis, Plantago major, Polygonum aviculare, Polygonum minus, Rorippa islandica, Rorippa palustre, Rumex crispus, Stellaria media, Trifolium repens

Population code: TG

Location: Turloughnagullan, Co. Clare

Grid reference: M282046

Habitat type : Turlough

Collection date: 10.08.00

Site description: Situated at the head of a valley in the eastern Burren. The turlough is divided into two basins by a road. The main basin is deeply sunk in the surroundings with a line of depressions of varying depths separated and surrounded by stone covered slopes. The basin floods deeply, up to 4-5 metres from a number of swallow holes. There is no evidence of drainage. Cattle heavily graze the turlough.

Associated species:

Agrostis stolonifera, Filipendula ulmaria, Trifolium repens, Rumex crispus, Potentilla anserina, Leontodon autumnalis, Hydrocotyle vulgaris, Mentha aquatica, Plantago lanceolata, Potentilla reptans, Stellaria media, Ranunculus flammula, Ophioglossum vulgatum, Galium palustre, Bellis perennis, Plantago major, Carex nigra, Crepis paludosa, Potentilla anserina, Potentilla erecta.

Population code: TGA

Location: Pasture adjacent to Turloughnagullan, Co. Clare

Grid reference: M282046

Habitat type : Ruderal/ Dry pasture

Collection date: 10.08.00

Site description: Grazed, improved pasture

Associated species:

Agrostis stolonifera, Bellis perennis, Cirsium arvense, Cynosurus cristata, Heracleum sphondylium, Holcus lanatus, Lolium perenne, Plantago lanceolata, Pteridium aquilinum, Ranunculus acris, Rumex crispus, Rumex obtusifolius, Taraxacum officinale, Trifolium pratense, Trifolium repens

Population code: KT

Location: Kiltiernan, Co. Galway

Grid reference: M430410

Habitat type : Turlough

Collection date: 11.08.00

Site details: A flattish basin which includes about eight depressions which are joined at times of high water. A relatively dry turlough with a limited though regular flood in winter. There is no evidence of drainage present. Cattle and sheep intensively graze the turlough.

Associated species:

Agrostis stolonifera, Cerastium fontanum, Filipendula ulmeria, Galium boreale, Leontodon autumnalis, Plantago major, Poa pratensis, Polygonum aviculare, Potentilla anserina, Potentilla reptans, Rumex crispus, Trifolium repens.

Population code: KT

Location: Killtullagh, Co. Mayo

Grid reference: M370300

Habitat type : Turlough

Collection date: 11.08.00

Site description: A flat linear basin running NE – SW that deepens at the SW end. The turlough is relatively dry and may empty more completely in the summer months than many others. The basin has been disrupted by bulldozing and piles of rubble are evident at its edges. Cattle and sheep grazing are at relatively intense levels.

Associated species:

Agrostis stolonifera, Alopecurus geniculatus, Bellis perennis, Carex spp., Leontodon autumnalis, Lolium perenne, Lotus corniculatus, Myosotis scorpioides, Phleum pratensis, Plantago lanceolata, Potentilla anserina, Potentilla erecta, Prunella vulgaris, Rorripa islandica, Trifolium repens

Population code: TM

Location: Turlough Monaghan, Co. Galway

Grid reference: M330460

Habitat type : Turlough

Collection date: 11.08.00

Site description: This turlough has a flat floor in most places apart from a rocky rise that projects from the SW side. It is a relatively shallow basin that floods regularly. There are no drainage activities visible although it may have occurred in the past. The turlough is grazed by cattle, horses and sheep at varying levels of intensity.

Associated species:

Festuca pratensis, Filipendula ulmeria, Phleum bertolonii, Potentilla anserina, Rumex crispus, Trifolium repens, Potamogeton natans, Matricaria recutita, Coronopus didymus, Potentilla reptans, Mentha arvensis, Molinia coerulea, Carex hostiana, Leontodon hispidus, Leontodon taraxacoides.

Population code: FTA

Location: Pasture population adjacent to Fortwilliam turlough, Co. Longford

Grid reference: N014627

Habitat type : Ruderal / Dry pasture

Collection date: 12.09.00

Site description: Pasture adjacent to Fortwilliam turlough. There is intense cattle grazing and evidence of artificial enrichment.

Associated species:

Agrostis capillaris, Agrostis stolonifera, Bellis perennis, Capsella bursa-pastoris, Cardamine flexuosa, Centaurea nigra, Cerastium fontanum, Cirsium arvense, Crateagus monogyna, Cynosurus cristata, Dactylis glomeratus, Galium verum, Geranium robertianum, Heracium pilosella, Hypochaerus radicata, Lolium perenne, Lotus corniculatus, Matricaria recutita, Medicago lupulina, Phleum pratense, Plantago lanceolata, Plantago major, Poa annua, Poa trivialis, Polygonum minus, Potentilla anserina, Potentilla reptans, Ranunculus acris, Rubus fruticosa, Rumex acetosa, Rumex obtusifolius, Taraxacum officinale, Trifolium pratense, Trifolium repens, Ulex europaeus, Urtica dioica, Veronica hederifolia

Population code: BTA

Location: Pasture adjacent to Ballinturly turlough, Co. Roscommon

Grid reference: M841599

Habitat type : Ruderal / Dry pasture

Collection date: 12.09.00

Site description: Pasture adjacent to turlough, may occasionally flood, sheep grazing, fertilised.

Associated species:

Achillea milifolium, Agrostis capillaris, Agrostis stolonifera, Cardamine flexuosa, Cerastium fontanum, Cirsium arvense, Deschampsia caespitosa, Geranium robertianum, Holcus lanatus, Hypochoerus radicata, Leontodon autumnalis, Lolium perenne, Lotus corniculatus, Medicago lupulina, Odontites verna, Plantago lanceolata, Plantago major, Poa annua, Poa trivialis, Potentilla anserina, Ranunculus acris, Rubus fruticosa, Rumex acetosa, Rumex obtusifolius, Trifolium pratense, Trifolium repens, Urtica dioica, Veronica officinalis

Population code: CSA

Location: Pasture adjacent to Castleplunket turlough, Co. Roscommon

Grid reference: M785779

Habitat type : Ruderal / Dry pasture

Collection date: 12.09.00

Site description: Pasture adjacent to turlough, wasteground that is grazed by cattle

Associated species:

Agrostis capillaris, Agrostis stolonifera, Bellis perennis, Cerastium fontanum, Cirsium arvense, Cirsium vulgare, Cynosurus crisatus, Deschampsia caespitosa, Holcus lanatus, Juncus conglomeratus, Leontodon autumnalis, Lolium perenne, Matricaria recutita, Phleum pratense, Plantago lanceolata, Plantago major, Poa annua, Poa trivialis, Poa trivialis, Polygonum minus, Potentilla anserina, Prunella vulgaris, Ranunculus acris, Rorripa nasturium-aquaticum, Rumex acetosa, Rumex

obtusifolius, Taraxacum officinale, Trifolium pratense, Trifolium repens, Urtica dioica, Veronica hederifolia

Population code: CV

Location: Lough Ramor, Virginia, Co. Cavan

Grid reference: N603865

Habitat type : Wet pasture

Collection date: 10.11.00

Site description: Damp lakeshore dominated with repens and Dactylis Holly , Oak and Beech plantation behind

Associated species:

Agrostis capillaris, Agrostis stolonifera, Dactylis glomerata, Festuca pratense, Juncus conglomeratus, Lolium perenne, Plantago lanceolata, Potentilla anserina, Ranunculus acris, Trifolium repens, Urtica dioica

Population code: LBG

Location: Lough Baile na gCea, Co. Clare

Grid reference: O230370

Habitat type : Wet pasture

Collection date: 10.07.00

Site description: Marshy area surrounded on one side with a *Cladium* dominated fen and on the other with limestone pavement. Diverse marshy lightly grazed vegetation.

Associated species:

Agrostis capillaris, Agrostis stolonifera, Deschampsia flexuosa, Festuca rubra, Juncus conglomeratus, Lolium perenne, Plantago lanceolata, Potentilla anserina, Ranunculus acris, Trifolium repens, Succisa pratense, Bellis perennis, Hydrocotyle vulgaris, Baldellia ranunculoides, Cardamine pratense, Taraxacum palustre, Carex flacca, Eleocharis palustre, Briza media, Leontodon taraxacoides, Plantago major, Ranunculus flammula, Filipendula ulmaria.

Population code: MN

Location: Balladian townland, Whitelough, Co. Monaghan

Grid reference: H728206

Habitat type : Wet pasture

Collection date: 10.11.02

Site description: River flood plain

Associated species:

Agrostis stolonifera, Cynosurus cristata, Dactylis glomerata, Juncus conglomeratus, Lolium perenne, Ranunculus acris, Senecio jacobea, Urtica dioica

Population code: LN

Location: Lough Neagh South, Derrymacash Townland, Co. Armagh

Grid reference: J034599

Habitat type : Wet pasture

Collection date: 11.11.00

Site description: Flooded field

Associated species:

Agrostis stolonifera, Capsella bursa-pastoris, Cirsium arvense, Geranium molle, Lolium perenne, Plantago major, Poa annua, Ranunculus acris, Rumex crispus, Stellaria media, Trifolium repens, Urtica dioica

Population code: LB

Location: Lough Beg, East of Castledawson Mullach townland, Co. Tyrone

Grid reference: J578927

Habitat type : Wet pasture

Collection date: 11.11.00

Site description: Flooded field

Associated species:

Agrostis stolonifera, Cardamine hirsuta, Carex spp., Juncus conglomeratus, Lolium perenne, Ranunculus flammula, Rorippa nasturtia-aquaticum, Senecio aquaticus, Spergula arvense, Trifolium repens

Population code: MG

Location: Magilligan Point, Co. Derry

Grid reference: C675355

Habitat type : Wet pasture

Collection date: 12.11.00

Site description: Reclaimed land, wet pasture with drainage ditch, stream Agricultural land

Associated species:

Agrostis stolonifera, Cirsium vulgare, Dactylis glomerata, Lolium perenne, Rumex obtusifolia, Senecio jacobea, Sonchus asper, Trifolium repens, Urtica dioica

Population code: RC

Location: River Carey, Ballycastle, Co. Antrim

Grid reference: D140408

Habitat type : Ruderal / Dry pasture

Collection date: 12.11.00

Site description: Wooded River bank

Associated species:

Acer pseudoplatanus, Alnus glutinosa, Angelica sylvestris, Anthriscus sylvaticum, Brachipodium sylvaticum, Dactylis glomerata, Equisetum telmeteia, Filipendula ulmeria, Geranium robertianum, Geum urbanum, Glechoma hederacea, Hedera helix, Heracleum sphondylium, Impatiens glandulifera, Iris pseudacorus, Larix decidua, Petasites hybridis, Phyllitis scolopendrium, Polystidium setiferum, Ranunculus acris, Rubus sp., Salix cineria ssp. atrocinerea, Silene dioica, Tussilago farfara, Urtica dioica

Population code: WN

Location: Whinney Hill, Holywood, Co. Down

Grid reference: J795425

Habitat type : Ruderal / Dry pasture

Collection date: 13.11.00

Site description: Pasture lightly grazed by cattle

Associated species:

Agrostis capillaris, Cirsium vulgare, Lamium purpureum, Lolium perenne, Poa annua, Rumex obtusifolia, Senecio vulgaris, Stellaria media, Taraxacum officinale, Trifolium repens

Population code: TBG

Location: Trinity College Botanic Gardens, Co. Dublin

Grid reference: O160305

Habitat type : Ruderal / Dry pasture

Collection date: 13.11.00

Site description: Flat tended garden

Associated species:

Agrostis capillaris, Bellis perennis, Stellaria media, Taraxacum officinale, Trifolium repens, Rumex obtusifolius, Galium aparine, Festuca rubra, Veronica filiformis, Cardamine hirsuta.

Population code: WX

Location: Outside Wexford town, Co. Wexford

Grid reference: T040201

Habitat type : Ruderal / Dry pasture

Collection date: 13.11.00

Site description: Damp sloping tended garden

Associated species:

Trifolium repens, Prunella vulgaris, Holcus lanatus, Dactylis glomerata, Agrostis capillaris, Lolium perenne, Taraxacum officinale.

Population code: MC

Location: Mucross, Killarney National Park, Co. Kerry

Grid reference: V963864

Habitat type : Ruderal / Dry pasture

Collection date: 27.11.00

Site description: Wasteground adjacent to path leading through Killarney National Park

Associated species:

Agrostis capillaris, Alnus glutinosa, Angelica sylvestris, Carex flacca, Cirsium palustre, Dactylis glomerata, Eupatorium cannabinum, Juncus effusus, Molinia caerulea, Plantago lanceolata, Ranunculus acris, Succisa pratensis, Trifolium repens

Population code: GG

Location: Glengariff Woods, Co. Cork

Grid reference: V920568

Habitat type : Ruderal / Dry pasture

Collection date: 27.11.00

Site description: Path verge in woodland

Associated species:

Blechnum spicant, Carex sp., Crocosmia x crocosmiiflora, Dryopteris aemula,, Geranium robertianum, Hedera helix, Ilex aquifolia, Juncus effusus, Polypodium integutum, Quercus petraea, Ranunculus acris, Rhododendron ponticum, Rubus sp., Saxifraga spathularis, Succisa sp., Trifolium pratense, Trifolium repens, Ulex europeae

Population code: B2

Location: Ballyshannon, Co. Donegal

Grid reference: G853601

Habitat type : Turlough

Collection date: 01.09.02

Site description: A wet hollow that may have been grazed or even cut. Area of permanent marsh vegetation present which is heavily dominate by bog bean, bottle sedge, and water horsetail, along with other sedge species

Associated species:

Agrostis stolonifera, Alisma lanceolata, Carex disticha, Carex sp., Carex rostrata, Equisetum fluviatile, Festuca pratensis, Filipendula ulmeria, Galium palustre, Menyanthes trifoliata, Phleum pratense, Potentilla anserina, Potentilla reptans, Ranunculus flammula, Ranunculus repens, Rumex crispus, Trifolium repens.

Population code: B4

Location: Ballyshannon, Co. Donegal

Grid reference: G860603

Habitat type : Turlough

Collection date: 01.09.02

Site description: A wet hollow in a limstone area which resembles a turlough

Associated species:

Alisma lanceolata, Carex disticha, Carex rostrata, Equisetum fluviatile, Festuca pratensis, Filipendula ulmeria, Menyanthes trifoliata, Phleum pratense, Potentilla anserine, Ranunculus repens, Rumex crispus, Trifolium repens,

Population code: FE

Location: Knockininny Quay, Co. Fermanagh

Grid reference:

Habitat type : Wet pasture

Collection date: 02.09.01

Site description: Lake shore vegetation

Associated species:

Agrostis stolonifera, Carex elata, Eleocharis acicularis, Equisetum palustre, Festuca aurundinacea, Hydrocotyle vulgaris, Iris pseudacorus , Mentha arvensis, Myosotis scorpioides, Phalaris arundinacea, Plantago major, Ranunculus repens, Rorippa sylvatica, Salix cineria ssp. atrocineria, Schoenoplectus lacustris

Population code: LT

Location: Leitrim

Grid reference:

Habitat type : Ruderal / Dry pasture

Collection date: 02.09.01

Site description: Improved wet fertilised field cut for silage

Associated species:

Agrostis capillaris, Agrostis stolonifera, Cynosurus cristata, Lolium perenne, Trifolium repens, Stellaria media, Rumex obtusifolia, Rumex acetosa, Carex sp., Juncus effusus, Carex nigra, Holcus lanata, Agropogon repens, Deschampsia caespitosa, Ranunculus repens

List of Turloughs surveyed which are of ecologocal value according to Goodwillie (1992).

International	National	Regional	Local
Coole	LoughGash	Castleplunket	Cahervoostia
Carron	Balla	Fortwilliam	Kiltiernan
Caherglassaun		Turloughnagullan	Turlough Monaghan
Ramolin		Killaturly	Slishmeen
Gortlecka		Killtullagh	Moylough
Ballinturly			
Glenamaddy			

The Breckland Meres and Norfolk Pingos

In this area of Norfolk are found a chain of twelve meres with annually fluctuating water levels. Meres generally flood in summer and empty in winter, which is the complete opposite water regime to turloughs. In May 2002 the meres had been full for at least 15 months, and are now fuller than they have been for at least 20 years (pers comm., Ranger for Norfolk Wildlife Trust). Four populations were sampled in the Norfolk region; two populations were from the edges of meres and two populations were collected from the edges of Pingos. 'Pingo' is an Eskimo word meaning 'hill'. These permanent ponds are over 10,000 years old having been fformed at end of last ice age when ice mounds in the topsoil finally melted and collapsed to form irregular ponds.

Population code: P1

Location: Great Eastern Pingo trail, Thompson Common, Stow Bedon, Norfolk, England

Grid reference: TL936966

Habitat type : Wet pasture

Collection date: 15.05.02

Site description: Plants collected from edge of Pingo pond. Wet but plants not submerged, very little water fluctuation, reed swamp at edge of pond. No dissected *R.repens*, plenty of *R. acris*. All plants collected were hairy and quite broadleaved but not as broad as common ruderals.

Associated species:

Agrostis capillaris, Agrostis stolonifera, Apium nodiflorus, Bellis perennis, Cardamine pratensis, Carex elata, Carex nigra, Carex spp., Eleocharis palustris, Epilobium parviflora, Epilobium sp., Equisetum palustris, Eupatorium cannabinum, Glyceria sp., Hottonia palustris, Hydrocotyle vulgaris, Juncus articulatus, Juncus inflexus, Lemna trisulca, Mentha aquatica, Polygonum amphibium, Potentilla anserina, Potamogeton natans, Prunella vulgaris, Ranunculus acris, Ranunculus repens, Rorippa sylvatica, Salix cineria spp. atrocineria, Schoenoplectus lacustris, Sparganium sp., Stellaria palustris, Trifolium repens, Typha latifolia.

Population code: P2

Location: Great Eastern Pingo trail, Thompson Common, Stow Bedon, Norfolk, England

Grid reference: TL937965

Habitat type : Wet pasture

Collection date: 15.05.02

Site description: Small permanently wet hollow adjacent to path through forest. *R. repens* growing in water, more dissected than at edge of P 1 but still not as dissected as the turlough populations.

Associated species:

Agrostis capillaris, Agrostis stolonifera, Calltriche sp., Cardamine pratensis, Carex elata, Cratageus monogyna, Dactylis glomerata, Eupatorium cannabinum, Fraxinus excelsior, Galium palustre, Glyceria sp., Glycoma hederacea, Ilex aquifolium, Juncus effuses, Juncus inflexus, Lonicera periclymenum, Mentha aquatica, Potentilla anserina, Quercus petraea, Rubus fruticosus, Rumex crispus, Stellaria neglecta, Taraxacum officinale, Urtica dioica.

Population code: LM

Location: Langmere, East Wretham Heath, 3 miles NE of Thetford, Norfolk

Grid reference: TL 909885

Habitat type : Wet pasture

Collection date: 15.05.02

Site description: Mere surrounded by grass / heath land, heavily grazed by sheep and rabbits. Sandy soil. Plants collected from flood area around the mere. Plants extremely small, some still had cotyledons present, not very dissected.

Associated species:

Carex spp., Cirsium spp., Cirsium vulgare, Galium palustre, Plantago major, Poa annua, Polygonum persicaria, Potentilla anserina, Prunella vulgaris, Ranunculus repens, Ranunculus sarduous, Rumex acetosella, Urtica dioica.

Population code: DPB

Location: Devils Punchbowl, East Wretham Heath, Thetford, Norfolk

Grid reference: TL 878892

Habitat type : Wet pasture

Collection date: 15.05.02

Site description: Mere surrounded by woodland, *R. repens* samples collected from the waters edge.

Associated species:

Agrostis stolonifera, Cratageus monogyna, Eleocharis palustris, Galium palustre, Geum urbanum, Holcus lanatus, Littorella uniflora, Poa annua, Polygonum amphibia, Potentilla anserina, Prunella vulgaris, Quercus petraea, Ranunculus repens, Rosa sp., Rumex acetosella, Salix cineria spp. atrocineria, Salix spp., Urtica dioica, Veronica beccabunga,

Appendix 2

Sequence data for the gene regions *trnL-F*, *rps*16 and *atpB-rbcl* amplified in a number of *R. repens* individuals from various habitat types. A description of each region is given in Table 2.6 and code numbers for individuals are explained in Table 2.7. Microsatellite repeats within these sequences are highlighted in bold.

TrnL-F alignment

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                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                    5           15          25          35          45          55
197             CTAAGTGATA ACTTTCAAAT TCAGAGAAAC CCTGGAATTA AAAATGGGCA ATCCTGGAGC
42              CTAAGTGATA ACTTTCAAAT TCAGAGAAAC CCTGGAATTA AAAATGGGCA ATCCT-GAGC
74              CTAAGTGATA ACTTTCAAAT TCAGAGAAAC CCTGGAATTA AAAATGGGCA ATCCTGGAGC
140             CTAAGTGATA ACTTTCAA-T TCAGAGAAAC CCTGGAATTA AAAATGGGCA ATCCTG-AGC
315             CTAAGTGATA ACTTTCAAAT TCAGAGAAAC CCTGGAATTA AAAATGGGCA ATCCTG-AGC
72              CTAAGTGATA ACTTTC-AAT TCAGAGAAAC CCTGGAATTA AAAATGGGCA ATCCTG-AGC
319             CTAAGTGATA ACTTTC-AAT TCAGAGAAAC CCTGGAATTA AAAATGGGCA ATCCTG-AGC
170             CTAAGTGATA ACTTTC-AAT TCAGAGAAAC CCTGGAATTA AAAATGGGCA ATCCTG-AGC
305             CTAAGTGATA ACTTTC-AAT TCAGAGAAAC CCTGGAATTA AAAATGGGCA ATCCTG-AGC
309             CTAAGTGAT- ACTTTC-AAT TCAGAGAATC CCTGGAATTA AAAATGGGCA ATCCTG-AGC

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                    65          75          85          95          105         115
197             CAAATCCTGC TTTCAGAAAA CAAAAAGAGG GTTCAGAAAG CAAAGGGATA GGTGCAGAGA
42              CAAATCCTGC TTTCAGAAAA CAAAAAGAGG GTTCAGAAAG CAAAGGGATA GGTGCAGAGA
74              CAAATCCTGC TTTCAGAAAA CAAAAAGAGG GTTCAGAAAG CAAAGGGATA GGTGCAGAGA
140             CAAATCCTGC TTTCAGAAAA CAAAAAGAGG GTTCAGAAAG CAAAGGGATA GGTGCAGAGA
315             CAAATCCTGC TTTCAGAAAA CAAAAAGAGG GTTCAGAAAG CAAAGGGATA GGTGCAGAGA
72              CAAATCCTGC TTTCAGAAAA CAAAAAGAGG GTTCAGAAAG CAAAGGGATA GGTGCAGAGA
319             CAAATCCTGC TTTCAGAAAA CAAAAAGAGG GTTCAGAAAG CAAAGGGATA GGTGCAGAGA
170             CAAATCCTGC TTTCAGAAAA CAAAAAGAGG GTTCAGAAAG CAAAGGGATA GGTGCAGAGA
305             CAAATCCTGC TTTCAGAAAA CAAAAAGAGG GTTCAGAAAG CAAAGGGATA GGTGCAGAGA
309             CAAATCCTGC TTTCAGAAAA CAAAAAGAGG GTTCAGAAAG CAAAGGGATA GGTGCAGAGA

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   125         135         145         155         165         175
197             CTCAATGGAA GCTGTTCTAA CGAATAGAGT TAACT-GCGT TGATCGAGGA ATCCTTCTAT
42              CTCAATGGAA GCTGTTCTAA CGAATAGAGT TAACT-GCGT TGATCGAGGA ATCCTTCTAT
74              CTCAATGGAA GCTGTTCTAA CGAATAGAGT TAACT-GCGT TGATCGAGGA ATCCTTCTAT
140             CTCAATGGAA GCTGTTCTAA CGAATAGAGT TAACTTGCGT TGATCGAGGA ATCCTTTTAT
315             CTCAATGGAA GCTGTTCTAA CGAATAGAGT TAACT-GCGT TGATCGAGGA ATCCTTTTAT
72              CTCAATGGAA GCTGTTCTAA CGAATAGAGT TAACT-GCGT TGATCGAGGA ATCCTTTTAT
319             CTCAATGGAA GCTGTTCTAA CGAATAGAGT TAACT-GCGT TGATCGAGGA ATCCTTTTAT
170             CTCAATGGAA GCTGTTCTAA CGAATAGAGT TAACT-GCGT TGATCGAGGA ATCCTTCTAT
305             CTCAATGGAA GCTGTTCTAA CGAATAGAGT TAACT-GCGT TGATCGAGGA ATCCTTTTAT
309             CTCAATGGAA GCTGTTCTAA CGAATAGAGT TAACT-GCGT TGATCGAGGA ATCCTTTTAT
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TrnL-F alignment continued

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                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   185         195         205         215         225         235
197             TTTAATTCCC GAAAGGATGA ACCCATATAC GTATGTACAA ATACGTAATA AAATAGGAAA
42              TTTAATTCCC GAAAGGATGA ACCCATATAC GTATGTACAA ATACGTAATA AAATAGGAAA
74              TTTAATTCCC GAAAGGATGA ACCCATATAC GTATGTACAA ATACGTAATA AAATAGGAAA
140             TTTAATTCCC GAAAGGATGA ACCCATATAC GTATGTACAA ATACGTAATA AAATAGGAAA
315             TTTAATTCCC GAAAGGATGA ACCCATATAC GTATGTACAA ATACGTAATA AAATAGGAAA
72              TTTAATTCCC GAAGGGATGA ACCCATATAC GTATGTACAA ATACGTAATA AAATAGGAAA
319             TTTAATTCCC GAAGGGATGA ACCCATATAC GTATGTACAA ATACGTAATA AAATAGGAAA
170             TTTAATTCCC GAAAGGATGA ACCCATATAC GTATGTACAA ATACGTAATA AAATAGGAAA
305             TTTAATTCCC GAAGGGATGA ACCCATATAC GTATGTACAA ATACGTAATA AAATAGGAAA
309             TTTAATTCCC GAGGGGATGA ACCCATATAC GTATGTACAA ATACGTAATA AAATAGGAAA

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   245         255         265         275         285         295
197             GAGGCGACCC AAATCTTTAT TTTTTATGTT ATA-GCAAAA CAAATAGAAG AATTCTTGCG
42              GAGGCGACCC AAATCTTTAT TTTTTATGTT ATATGCAAAA CAAATAGAAG AATTCTTGCG
74              GAGGCGACCC AAATCTTTAT TTTTTATGTT ATATGCAAAA CAAATAGAAG AATTCTTGCG
140             GAGGCGACCC AAATCTTTAT TTTTTATGTT ATATGCAAAA CAAATAGAAG AATTCTTGCG
315             GAGGCGACCC AAATCTTTAT TTTTTATGTT ATATGCAAAA CAAATAGAAG AATTCTTGCG
72              GAGGCGACCC AAATCTTTAT TTTTTATGTT ATATGCAAAA CAAATAGAAG AATTCTTGCG
319             GAGGCGACCC AAATCTTTAT TTTTTATGTT ATATGCAAAA CAAATAGAAG AATTCTTGCG
170             GAGGCGACCC AAATCTTTAT TTTTTATGTT ATATGCAAAA CAAATAGAAG AATTCTTGCG
305             GAGGCGACCC AAATCTTTAT TTTTTATGTT ATATGCAAAA CAAATAGAAG AATTCTTGCG
309             GAGGCGACCC -ACTCTTTAT TTTTTATGTT ATATGCAAAA CAAATAGAAG AATTCTTGCG

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   305         315         325         335         345         355
197             ATTCCAAGTT GAAGGAATAA TATTCAATAT TCATTGATCA ?AATCAGTTA TTCTATAACC
42              ATTCCAAGTT GAAGTAATAA TATTCAATAT TCATTGATCA ?AATCAGTTA TTCTATAACC
74              ATTCCAAGTT GAAGTAATAA TATTCAATAT TCATTGATCA ?AATCAGTTA TTCTATAACC
140             ATTCCAAGTT GAAGTAATAA TATTCAATAT TCATTGATCA ?AATCAGTTA TTCTATAACC
315             ATTCCAAGTT GAAGTAATAA TATTCAATAT TCATTGATCA ?AATCAGTTA TTCTATAACC
72              ATTCCAAGTT GAAGTAATAA TATTCAATAT TCATTGATCA ?AATCAGTTA TTCTATAACC
319             ATTCCAAGTT GAAGTAATAA TATTCAATAT TCATTGATCA ?AATCAGTTA TTCTATAACC
170             ATTCCAAGTT GAAGTAATAA TATTCAATAT TCATTGATCA ?AATCAGTTA TTCTATAACC
305             ATTCCAAGTT GAAGTAATAA TATTCAATAT TCATTGATCA ?AATCAGTTA TTCTATAACC
309             ATTCCAAGTT GAAGTAATAA TATTCAATAT TCATTGATCA ?AATCAGTTA TTCTATAACC

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   365         375         385         395         405         415
197             TGGTAGATTT TTTAAAGAAC TGACTGGTTG GACGAGAATA AAGATAGAGT CCCATTATAC
42              TGGTAGATTT TTTAAAGAAC TGACTGGTTG GACGAGAATA AAGATAGAGT CCCATTATAC
74              TGGTAGATTT TTTAAAGAAC TGACTGGTTG GACGAGAATA AAGATAGAGT CCCATTATAC
140             TGGTAGATTT TTTAAAGAAC TGACTGGTTG GACGAGAATA AAGATAGAGT CCCATTATAC
315             TGGTAGATTT TTTAAAGAAC TGACTGGTTG GACGAGAATA AAGATAGAGT CCCATTATAC
72              TGGTAGATTT TTTAAAGAAC TGACTGGTTG GACGAGAATA AAGATAGAGT CCCATTATAC
319             TGGTAGATTT TTTAAAGAAC TGACTGGTTG GACGAGAATA AAGATAGAGT CCCATTATAC
170             TGGTAGATTT TTTAAAGAAC TGACTGGTTG GACGAGAATA AAGATAGAGT CCCATTATAC
305             TGGTAGATTT TTTAAAGAAC TGACTGGTTG GACGAGAATA AAGATAGAGT CCCATTATAC
309             TGGTAGATTT TTTAAAGAAC TGACTGGTTG GACGAGAATA AAGATAGAGT CCCATTATAC
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***TrnL-F* alignment continued**

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   425        435        445        455        465        475
197             ATGTCAATAT CAATACCGGC AAAAATGAAA TTTATAGTAA GAGGAAAATC CGTCGACTTT
42              ATGTCAATAT CAATACCGGC AAAAATGAAA TTTATAGTAA GAGGAAAATC CGTCGACTTT
74              ATGTCAATAT CAATACCGGC AAAAATGAAA TTTATAGTAA GAGGAAAATC CGTCGACTTT
140             ATGTCAATAT CAATACCGGC AAAAATGAAA TTTATAGTAA GAGGAAAATC CGTCGACTTT
315             ATGTCAATAT CAATACCGGC AAAAATGAAA TTTATAGTAA GAGGAAAATC CGTCGACTTT
72              ATGTCAATAT CAATACCGGC AAAAATGAAA TTTATAGTAA GAGGAAAATC CGTCGACTTT
319             ATGTCAATAT CAATACCGGC AAAAATGAAA TTTATAGTAA GAGGAAAATC CGTCGACTTT
170             ATGTCAATAT CAATACCGGC AAAAATGAAA TTTATAGTAA GAGGAAAATC CGTCGACTTT
305             ATGTCAATAT CAATACCGGC AAAAATGAAA TTTATAGTAA GAGGAAAATC CGTCGACTTT
309             ATGTCAATAT CAATACCGGC AAAAATGAAA TTTATAGTAA GAGGAAAATC CGTCGACTTT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   485        495        505        515        525        535
197             -AAAA---AT CGTGAGGGTT CAAGTCCCTC CTATCCCCAA TAAAAGGTTC GATCCACTCT
42              AAAAATGGAT CGTGAGGGTT CAAGTCCCT- CTATCCCCAA TAAAAGGTTC GATCCACTCT
74              -AAAA---AT CGTGAGGGTT CAAGTCCCT- CTATCCCCAA TAAAAGGTTC GATCCACTCT
140             -AAAA---AT CGTGAGGGTT CAAGTCCCT- CTATCCCCAA TAAAAGGTTC GATCCACTCT
315             -AAAA---AT CGTGAGGGTT CAAGTCCCT- CTATCCCCAA TAAAAGGTTC GATCCACTCT
72              -AAAA---AT CGTGAGGGTT CAAGTCCCT- CTATCCCCAA TAAAAGGTTC GATCCACTCT
319             -AAAA---AT CGTGAGGGTT CAAGTCCCT- CTATCCCCAA TAAAAGGTTC GATCCACTCT
170             -AAAA---AT CGTGAGGGTT CAAGTCCCT- CTATCCCCAA TAAAAGGTTC GATCCACTCT
305             -AAAA---AT CGTGAGGGTT CAAGTCCCT- CTATCCCCAA TAAAAGGTTC GATCCACTCT
309             -AAAA---AT CGTGAGGGTT CAAGTCCCT- CTATCCCCAA TAAAAGGTTC GATCCACTCT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   545        555        565        575        585        595
197             CTACCTATTT TTCTAACTTT TTTATTTTTA GCGGTTCCAC ATTAGTTATG TTTCTCAGCC
42              CTACCTATTT TTCTAACTTT TTTATTTTTA GCGGTTCCAC ATTAGTTATG TTTCTCAGCC
74              CTACCTATTT TTCTAACTTT TTTATTTTTA GCGGTTCCAC ATTAGTTATG TTTCTCAGCC
140             CTACCTATTT TTCTAACTTT TTTATTTTTA GCGGTTCCAC ATTAGTTATG TTTCTCAGCC
315             CTACCTATTT TTCTAACTTT TTTATTTTTA GCGGTTCCAC ATTAGTTATG TTTCTCAGCC
72              CTACCTATTT TTCTAACTTT TTTATTTTTA GCGGTTCCAC ATTAGTTATG TTTCTCAGCC
319             CTACCTATTT TTCTAACTTT TTTATTTTTA GCGGTTCCAC ATTAGTTATG TTTCTCAGCC
170             CTACCTATTT TTCTAACTTT TTTATTTTTA GCGGTTCCAC ATTAGTTATG TTTCTCAGCC
305             CTACCTATTT TTCTAACTTT TTTATTTTTA GCGGTTCCAC ATTAGTTATG TTTCTCAGCC
309             CTACCTATTT TTCTAACTTT TTTATTTTTA GCGGTTCCAC ATTAGTTATG TTTCTCAGCC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645        655
197             ATTCGACTCT TTCACAAATG GATCGAATT? GTGGGAGAAA AGTTTCTCTC TTATCACAAG
42              ATTCGACTCT TTCACAAATG GATCGAATT? GTGGGAGAAA AGTTTCTTTC TTATCACAAG
74              ATTCGACTCT TTCACAAATG GATCGAATT? GTGGGAGAAA AGTTTCTCTC TTATCACAAG
140             ATTCGACTCT TTCACAAATG GATCGAATT? GTGGGAGAAA AGTTTCTCTC TTATCACAAG
315             ATTCGACTCT TTCACAAATG GATCGAATT? GTGGGAGAAA AGTTTCTCTC TTATCACAAG
72              ATTCGACTCT TTCACAAATG GATCGAATT? GTGGGAGAAA AGTTTCTCTC TTATCACAAG
319             ATTCGACTCT TTCACAAATG GATCAAATT? GTGGGAGAAA AGTTTCTCTC TTATCACAAG
170             ATTCGACTCT TTCACAAATG GATCGAATT? GTGGGAGAAA AGTTTCTCTC TTATCACAAG
305             ATTCGACTCT TTCACAAATG GATCGAATT? GTGGGAGAAA AGTTTCTCTC TTATCACAAG
309             ATTCGACTCT TTCACAAATG GATCGAATT? GTGGGAGAAA AGTTTCTCTC TTATCACAAG
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***TrnL-F* alignment continued**

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                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   665         675         685         695         705         715
197             TCTTTTGGAT CTAGATATAA ATATATATAC AAATCAACAT CTATGAATAC CTATTTGAAT
42              TC-TTGGGAT CTAGATATAA ATATATATAC AAATCAACAT CTATGAATAC CTATTTGAAT
74              ACTTTT-GAT CTAGATATAA ATATATATAC AAATCAACAT CTATGAATAC CTATTTGAAT
140             TCTTGTTGAT CTAGATATAA ATATATATAC AAATCAACAT CTATGAATAC CTATTTGAAT
315             TCTT-TTGAT CTAGATATAA ATATATATAC AAATCAACAT CTATGAATAC CTATTTGAAT
72              TCTT-TTGAT CTAGATATAA ATATATATAC AAATCAACAT CTATGAATAC CTATTTGAAT
319             TCTT-TTGAT CTAGATATAA ATATATATAC -CATCAACAT CTATGAATAC CTATTTGAAT
170             TCTT-TTGAT CTAGATATAA ATATATATAC AAATCAACAT CTATGAATAC CTATTTGAAT
305             TCTT-TTGAT CTAGATATAA ATATATATAC AAATCAACAT CTATGAATAC CTATTTGAAT
309             TCTT-TTGAT CTAGATATAA ATATATATAC AAATCAACAT CTATGAATAC CTATTTGAAT

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   725         735         745         755         765         775
197             CAGTCA?CAG TTAACATAAA TTTTTTTAGT TAAACTTACA AAGTTTTCTT TTTGAAGATC
42              CAGTCA?CAG TTAACAAAAA TTTTTTTAGT TAAACTTACA AAGTTTTCTT TTTGAAGATC
74              CAGTCA?CAG TTAACAAAAA TTTTTTTAGT TAAACTTACA AAGTTTTCTT TTTGAAGATC
140             CAGTCA?CAG TTAACATAAA TTTTTTTAGT TAAACTTACA AAGTTTTCTT TTTGAAGATC
315             CAGTCA?CAG TTAACATAAA TTTTTTTAGT TAAACTTACA AAGTTTTCTT TTTGAAGATC
72              CAGTCA?CAG TTAACATAAA ATTTTTTAGT TAAACTTACA AAGTTTTCTT TTTGAAGATC
319             CAGTCA?CAG TTAACATAAA ATTTTTTAGT TAAACTTACA AAGTTTTCTT TTTGAAGATC
170             CAGTCA?CAG TTAACATAAA TTTTTTTAGT TAAACTTACA AAGTTTTCTT TTTGAAGATC
305             CAGTCA?CAG TTAACATAAA TTTTTTTAGT TAAACTTACA AAGTTTTCTT TTTGAAGATC
309             CAGTCA?CAG TTAACATAAA TTTTTTTAGT TAAACTTACA AAGTTTTCTT TTTGAAGATC

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   785         795         805         815         825         835
197             CAAGCCAAAC CAAGAAATTC TAAATTACAA GGTTTGCGTA AGACTTTTTA ATACTATGCT
42              CAAGCCAAAC CAAGAAATTC TAAATTACAA GGTTTGCGTA AGACTTTTTA ATACTATGCT
74              CAAGCCAAAC CAAGAAATTC TAAATTACAA GGTTTGCGTA AGACTTTTTA ATACTATGCT
140             CAAGCCAAAC CAAGAAATTC TAAATTACAA GGTTTGCGTA AGACTTTTTA ATACTATGCT
315             CAAGCCAAAC CAAGAAATTC TAAATTACAA GGTTTGCGTA AGACTTTTTA ATACTATGCT
72              CAAGCCAAAC CAAGAAATTC TAAATTACAA GGTTTGCGTA AGACTTTTTA ATACTATGCT
319             CAAGCCAAAC CAAGAAATTC TAAATTACAA GGTTTGCGTA AGACTTTTTA ATACTATGCT
170             CAAGCCAAAC CAAGAAATTC TAAATTACAA GGTTTGCGTA AGACTTTTTA ATACTATGCT
305             CAAGCCAAAC CAAGAAATTC TAAATTACAA GGTTTGCGTA AGACTTTTTA ATACTATGCT
309             CAAGCCAAAC CAAGAAATTC TAAATTACAA GGTTTGCGTA AGACTTTTTA ATACTATGCT

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   845         855         865         875         885         895
197             TAGTCTATTT AATTGACTGA CATATACCCA ACAAGTCTTC GAAATAGTAT GGGGCTGGTG
42              TAGTCTATTT AATTGACTGA CATATACCCA ACAAGTCTTC GAAATAGTAT GGGGATGGTG
74              TAGTCTATTT AATTGACTGA CATATACCCA ACAAGTCTTC GAAATAGTA- GGGGCTGGTG
140             TAGTCTATTT AATTGACTGA CATATACCCA ACAAGTCTTC GAAATAGTA- GGGGATGGTG
315             TAGTCTATTT AATTGACTGA CATATACCCA ACAAGTCTTC GAAATAGTAT GGGGATGGTG
72              TAGTCTATTT AATTGACTGA CATATACCCA ACAAGTCTTC GAAATAGTAT GGGGC-TGGG
319             TAGTCTATTT AATTGACTGA CATATACCCA ACAAGTCTTC GAAATAGAAT GGGGA-GGTG
170             TAGTCTATTT AATTGACTGA CATATACCCA ACAAGTCTTC GAAATAGTAT GGGGA-TGGG
305             TAGTCTATTT AATTGACTGA CATATACCCA ACAAGTCTTC GAAATAGTAT GGGGCTGGTG
309             TAGTCTATTT AATTGACTGA CATATACCCA ACAAGTCTTC GAAATAGTAT GGGGA-GGTG
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***TrnL-F* alignment continued**

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                ....|....| ....|....| ...
                   905        915
197             CATCGGGAAA AGTCGGGAGG CTC
42              CATCGGGAAA AGTCGGGGAG CTC
74              CATCGGGAAA AGTCGGGGAG CTC
140             CATCGGGAAA AGTCGGGAAG CTC
315             CATCGGGAAA AGTCGGGAAG CTC
72              CATCGGGAAA AGTC------ ---
319             CATCGGGAAA AGTC------ ---
170             CATCGGGAAA AGTCGGGAAG CTC
305             CATCGGGAAA AGTCGGGAAG CTC
309             CATCGGGAAA AGTCGGGAAG CTC
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***rps*16 alignment**

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          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
               5         15         25         35         45         55
170       GTGACGGTTG TGGTTCTACA TCCCCATTCT AGGAATTGTG AGGTGCTCTT GGCTCGACAT
140       ~~~~~~~~~G TGGATCTCCA TCCCCATTCT AGGAATTGTG AGGTGCTCTT GGCTCGACAT
315       ---------- -GGATCTCCA TCCCC-TTCT AGGAATTGTG AGGTGCTCTT GGCTCGACAT
72        ---------- ---------A TCCCC-TTCT AGGAATTGTG AGGTGCTCTT GGCTCGACAT
305       ---------- ---------A TCCCCATTCT AGGAATTGTG AGGTGCTCTT GGCTCGACAT
309       ---------- ---------- TCCCC-TTCT AGGAATTGTG AGGTGTTCTT GGCTCGACAT
319       ---------- ---------- TCCCC-TTCT AGGAATTGTG AGGTGTTCTT GGCTCGACAT

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
               65         75         85         95        105        115
170       AGT-TTGTTC TACACGTAAC CCTCATTTAG TTGGGTTGTG GAGTTAAAGT AAATAGTACA
140       AGT-TTGTTC TACACGTAAC CCTCATTTAG TTGGGTTGTG -AGTTAAAGT AAATAGTACA
315       AGT-TTGTTC TACACGTAAC CCTCATTTAG TTGGGTTGTG -AGTTAAAGT AAATAGTACA
72        AGT-TTGTTC TACACGTAAC CCTCATTTAG TTGGGTTGTG -AGTTAAAGT AAATAGTACA
305       AGT-TTGTTC TACACGTAAC CCTCATTTAG TTGGGTTGTG -AGTTAAAGT AAATAGTACA
309       ACCCTTGTTC TACACGTAAC CCTCATTTAG TTGGGTTGTG -AGTTAAAGT AAATAGTACA
319       ACCCTTGTTC TACACGTAAC CCTCATTTAG TTGGGTTGTG -AGTTAAAGT AAATAGTACA

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
              125        135        145        155        165        175
170       CG-ATGTAGC TCGAGCAAAA AGGATTCATT TCTTTTTCAG AGGTAAGGAT CTAGGGTTAA
140       C-GATGTAGC TCGAGCAAAA AGGATTCATT TCTTTTTCAG AGGTAAGGAT CTAGGGTTAA
315       CG-ATGTAGC TCGAGCAAAA AGGATTCATT TCTTTTTCAG AGGTAAGGAT CTAGGGTTAA
72        CG-ATGTAGC TCGAGCAAAA AGGATTCATT TCTTTTTCAG AGGTAAGGAT CTAGGGTTAA
305       CGGATGTAGC TCGAGCAAAA AGGATTCATT TCTTTTTCAG AGGTAAGGAT CTAGGGTTAA
309       CG-ATGTAGC TCGAGCAAAA AGGATTCATT TCTTTTTCAG AGGTAAGGAT CTAGGGTTAA
319       CG-ATGTAGC TCGAGCAAAA AGGATTCATT TCTTTTTCAG AGGTAAGGAT CTAGGGTTAA

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
              185        195        205        215        225        235
170       TATCAATCAA TAATTTGGAA CAACTTTGTG AGCATATCTT CGATATAGAA ATTTGAAAGA
140       TATCAATCAA TAAGTTGGAA CAACTTTGTA AGCATATCTT CGATAGAGAA ATTTGAAAGA
315       TATCAATCAA TAATTTGGAA CAACTTTGTA AGCATATCTT CGATAGAGAA ATTTGAAAGA
72        TATCAATCAA TAATTTGGAA CAACTTTGTA AGCATATCTT CGATATAGAA ATTTGAAAGA
305       TATCAATCAA TAAGTTGGAA CAACTTTGTA AGCATATCTT CGATATAGAA ATTTGAAAGA
309       TATCAATCAA TTTTTTGGAA CAACTTTGTA GGCGTATCTT CGATAGAGAA ATTTGAAAGA
319       TATCAATCAA TTTTTTGGAA CAACTTTGTA GGCGTATCTT CGATAGAGAA ATTTGAAAGA

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
              245        255        265        275        285        295
170       TTCAATTTGA ACAAAACCCC TTCTTGGATT TGAAACTTTT GTTGGAATTG GAAAAACTAT
140       TTCAATTTGA ACAAAACCCC TTCTTGGATT TGAAACTTTT GTTGGAATTG GAAAAACTAT
315       TTCAATTTGA ACAAAACCCC TTCTTGGATT TGAAACTTTT GTTGGAATTG GAAAAACTAT
72        TTCAATTTGA ACAAAACCCC TTCTTGGATT TGAAACTTTT GTTGGAATTG GAAAAACTAT
305       TTCAATTTGA ACAAAACCCC TTCTTGGATT TGAAACTTTT GTTGGAATTG GAAAAACTAT
309       TTCAATTTGA ACAAAACCCC TTCTTGGATT TGAAACTTTT GTTGGAATTG GAAAAACTAT
319       TTCAATTTGA ACAAAACCCC TTCTTGGATT TGAAACTTTT GTTGGAATTG GAAAAACTAT
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***rps*16 alignment continued**

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345        355
170          TTCGATCAAA AGTGTATCAC ACGGGAATCA AGCATTCACA TGATTTTTCG ATAGTAAATA
140          TTCGATCAAA AGTGTATCAC ACGGGAATCA AGCATTCACA TGATTTTTCG ATAGTAAATA
315          TTCGATCAAA AGTGTATCAC ACGGGAATCA AGCATTCACA TGATTTTTCG ATAGTAAATA
72           TTCGATCAAA AGTGTATCAC ACGGGAATCA AGCATTCACA TGATTTTTCG ATAGTAAATA
305          TTCGATCAAA AGTGTATCAC ACGGGAATCA AGCATTCACA TGATTTTTCG ATAGTAAATA
309          TTCGATCAAA AGTGTATCAC ACGGGAATCA AGCATTCACA TGATTTTTCG ATAGTAAATA
319          TTCGATCAAA AGTGTATCAC ACGGGAATCA AGCATTCACA TGATTTTTCG ATAGTAAATA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   365        375        385        395        405        415
170          AATCACAAAA GGTATGTTGC TGCCATTTTG AAATGATTCA GGATCACCGA AGTAATGTCT
140          AATCACAAAA GGTATGTTGC TGCCATTTTG AAATGATTCA GGATCACCGA AGTAATGTCT
315          AATCACAAAA GGTATGTTGC TGCCATTTTG AAATGATTCA GGATCACCGA AGTAATGTCT
72           AATCACAAAA GGTATGTTGC TGCCATTTTG AAATGATTCA GGATCACCGA AGTAATGTCT
305          AATCACAAAA GGTATGTTGC TGCCATTTTG AAATGATTCA GGATCACCGA AGTAATGTCT
309          AATCACAAAA GGTATGTTGC TGCCATTTTG AAATGATTCA GGATCACCGA AGTAATGTCT
319          AATCACAAAA GGTATGTTGC TGCCATTTTG AAATGATTCA GGATCACCGA AGTAATGTCT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   425        435        445        455        465        475
170          AAACCCAATG ATTCAAAACA AAGATAAACG ATCCTGGAAC AAGAAAACGC CAATTTAAAT
140          AAACCCAATG ATTCAAAACA AAGATAAACG ATCCTGGAAC AAGAAAACGC CAATTTAAAT
315          AAACCCAATG ATTCAAAACA AAGATAAACG ATCCTGGAAC AAGAAAACGC CAATTTAAAT
72           AAACCCAATG ATTCAAAACA AAGATAAACG ATCCTGGAAC AAGAAAACGC CAATTTAAAT
305          AAACCCAATG ATTCAAAACA AAGATAAACG ATCCTGGAAC AAGAAAACGC CAATTTAAAT
309          AAACCCAATG ATTCAAAACA AAGATAAACG ATCCTGGAAC AAGAAAACGC CAATTTAAAT
319          AAACCCAATG ATTCAAAACA AAGATAAACG ATCCTGGAAC AAGAAAACGC CAATTTAAAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   485        495        505        515        525        535
170          TATCTCAACA ATTTGAGCAG AAAAAAAGAA ATAAAAATAA GGAATAAAGG GTGTATTTTA
140          TATCTCAACA ATTTGAGCAG AAAAAAAGAA ATAAAAATAA GGAATAAAGG GTGTATTTTA
315          TATCTCAACA ATTTGAGCAG AAAAAAAGAA ATAAAAATAA GGAATAAAGG GTGTATTTTA
72           TATCTCAACA ATTTGAGCAG AAAAAAAGAA ATAAAAATAA GGAATAAAGG GTGTATTTTA
305          TATCTCAACA ATTTGAGCAG AAAAAAAGAA ATAAAAATAA GGAATAAAGG GTGTATTTTA
309          TATCTCAACA ATTTGAGCAG AAAAAAAGAA ATAAAAATAA GGAATAAAGG GTGTATTTTA
319          TATCTCAACA ATTTGAGCAG AAAAAAAGAA ATAAAAATAA GGAATAAAGG GTGTATTTTA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   545        555        565        575        585        595
170          ATTTAAATGA GACAAAAATT GGTTAGAGAG AGCTCAATAA AGAAAATGTC AAAGATTCCG
140          ATTTAAATGA GACAAAAATT GGTTAGAGAG AGCTCAATAA AGAAAATGTC AAAGATTCCG
315          ATTTAAATGA GACAAAAATT GGTTAGAGAG AGCTCAATAA AGAAAATGTC AAAGATTCCG
72           ATTTAAATGA GACAAAAATT GGTTAGAGAG AGCTCAATAA AGAAAATGTC AAAGATTCCG
305          ATTTAAATGA GACAAAAATT GGTTAGAGAG AGCTCAATAA AGAAAATGTC AAAGATTCCG
309          ATTTAAATGA GACAAAAATT GGTTAGAGAG AGCTCAATAA AGAAAATGTC AAAGATTCCG
319          ATTTAAATGA GACAAAAATT GGTTAGAGAG AGCTCAATAA AGAAAATGTC AAAGATTCCG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645        655
170          GGTTTTGAAC TCTTTGAGAA TTATCTAACT TGAGTTATAA GTACGAATGT TTTTTTACTT
140          GGTTTTGAAC TCTTTGAGAA TTATCTAACT TGAGTTATAA GTACGAATGT TTTTTTACTT
315          GGTTTTGAAC TCTTTGAGAA TTATCTAACT TGAGTTATAA GTACGAATGT TTTTTTACTT
72           GGTTTTGAAC TCTTTGAGAA TTATCTAACT TGAGTTATAA GTACGAATGT TTTTTTACAA
305          GGTTTTGAAC TCTTTGAGAA TTATCTAACT TGAGTTATAA GTACGAATGT TTTTTTACTT
309          GGTTTTGAAC TCTTTGAGAA TTATCTAACT TGAGTTATAA GTACGAATGT TTTTTT-CAT
319          GGTTTTGAAC TCTTTGAGAA TTATCTAACT TGAGTTATAA GTACGAATGT TTTTTT-CAT
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***rps*16 alignment continued**

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   665        675        685        695        705        715
170             GTCAGGTTTT TCGGTAAGGA AGAAAAACGA ACAAAATCAT AGTTTAATTG ATGATTTTAT
140             GTCAGGTTTT TCGGTAAGGA AGAAAAACGA ACAAAATCAT AGTTTAATTG ATGATTTTAT
315             GTCAGGTTTT TCGGTAAGGA AGAAAAACGA ACAAAATCAT AGTTTAATTG ATGATTTTAT
72              GTCAGGTTTT TCGGTAAGGA AGAAAAACGA ACAAAATCAT AGTTTAATTG ATGATTTTAT
305             GTCAGGTTTT TCGGTAAGGA ATAAAAACGA ACAAAATCAT AGTTTAATTG ATGATTTTAT
309             GTCAGGTTTT TCGGTAAGGA AGAAAAACGA ACAAAATCAT AGTTTAATTG ATGATTTTAT
319             GTCAGGTTTT TCGGTAAGGA AGAAAAACGA ACAAAATCAT AGTTTAATTG ATGATTTTAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   725        735        745        755        765        775
170             TTTATAGATC TATATTGTCA CTCTATCATA GTATGATCTA AATTCGAATC ATTCTTTCTC
140             TTTATAGATC TATATTGTCA CTCTATCATA GTATGATCTA AATTCGAATC ATTCTTTCTC
315             TTTATAGATC TATATTGTCA CTCTATCATA GTATGATCTA AATTCGAATC ATTCTTTCTC
72              TTTATAGATC TATATTGTCA CTCTATCATA GTATGATCTA AATTCGAATC ATTCTTTCTC
305             TTTATAGATC TATATTGTCA CTCTATCATA GTATGATCTA AATTCGAATC ATTCTTTCTC
309             TTTATAGATC TATATTGTCA CTCTATCATA GTATGATCTA AATTCGAATC ATTCTTTCTC
319             TTTATAGATC TATATTGTCA CTCTATCATA GTATGATCTA AATTCGAATC ATTCTTTCTC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   785        795        805        815        825        835
170             GAGCCGTACG AGGAAAAAGC CTCCTATACG TTTCTAAGGG GGGAATTTTT CA--------
140             GAGCCGTACG AGGAAAAAGC CTCCTATACG TTTCTAAGGG GGGAATTTTT CGGCTTCCCA
315             GAGCCGTACG AGGAAAAAGC CTCCTATACG TTTCTAAGGG GGGAATTTTT CATCTTCCCA
72              GAGCCGTACG AGGAAAAAGC CTCCTATACG TTTCTAAGGG GGGAATTTTT C---------
305             GAGCCGTACG AGGAAAAAGC CTCCTATACG TTTCTAAGGG GGGAATTTTT CATCTTCCC-
309             GAGCCGTACG AGGAAAAAGC CTCCTATACG TTTCTAAGGG GGGAATTTTT C---------
319             GAGCCGTACG AGGAAAAAGC CTCCTATACG TTTCTAAGGG GGGAATTTTT CGG-------
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***atpB-rbcL* alignment**

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     5         15         25         35         45         55
140             GACAAGGTCT ACTCGAC-T- AG-ATTAGGC TTTAATG-AA ACCTTTCACA GG-AATCTTT
188             GACAAGGTCT ACTCGACATT AGTATTAGGC TTTAATGGAA ACCTTTCACA GGGAATCTTT
42              GACAAGGTCT ACTCGACAT- AG-ATTAGGC TTTAATG-AA ACCTTTCACA GG-AATCTTT
74              GACAAGGTCT ACTCGACAT- AGTATTAGGC TTTAATG-AA ACCTTTCACA GG-AATCTTT
319             GACAAGGTCT ACTCGACAT- AG-ATTAGGC TTTAATG-AA ACCTTTCACA GG-AATCTTT
197             GACAAGGTCT ACTCGACAT- AGTAT-AGGC TTTAATG-AA ACCTTTCACA GG-AATCTTT
113             GACAAGGTCT ACTCGACAT- AG-ATTAGGC TTTAATG-AA ACCTTTCACA GG-AATCTTT
72              GACAAGGTCT -CTCGACAT- AG-ATTAGGC TTTAATG-AA ACCTTTCACA GG-AATCTTT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
140             CACAAAATTA TCAACAAATC TTATCAATTA ATTAGAATGG TTCGTTATTA CACCATGC-G
188             CACAAAATTA TCAACAAATC TTATCAATTA ATTAGAATGG TTCGTTATTA CACCATGCCG
42              CACAAAATTA TCAACAAATC TTATCAATTA ATTAGAATGG TTCGTTATTA CACCATGC-G
74              CACAAAATTA TCAACAAATC TTATCAATTA ATTAGAATGG TTCGTTATTA CACCATGC-G
319             CACAAAATTA TCAACAAATC TTATCAATTA ATTAGAATGG TTCGTTATTA CACCATGC-G
197             CACAAAATTA TCAACAAATC TTATCAATTA ATTAGAATGG TTCGTTATTA CACCATGC-G
113             CACAAAATTA TCAACAAATC TTATCAATTA ATTAGAATGG TTCGTTATTA CACCATGC-G
72              CACAAAATTA TCAACAAATC TTATCAATTA ATTAGAATGG TTCGTTATTA CACCATGC-G

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
140             CCAAATCTAT CATTCATTAT TGTATACTAT TTTATATGTA TGGCGCAACC CAATCCCTTT
188             CCAAATCTAT CATTCATTAT TGTATACTAT TTTATATGTA TGGCGCAACC CAATCCCTTT
42              CCAAATCTAT CATTCATTAT TGTATACTAT TTTATATGTA TGGCGCAACC CAATCCCTTT
74              CCAAATCTAT CATTCATTAT TGTATACTAT TTTATATGTA TGGCGCAACC CAATCCCTTT
319             CCAAATCTAT CATTCATTAT TGTATACTAT TTTATATGTA TGGCGCAACC CAATCCCTTT
197             CCAAATCTAT CATTCATTAT TGTATACTAT TTTATATGTA TGGCGCAACC CAATCCCTTT
113             CCAAATCTAT CATTCATTAT TGTATACTAT TTTATATGTA TGGCGCAACC CAATCCCTTT
72              CCAAATCTAT CATTCATTAT TGTATACTAT TTTATATGTA TGGCGCAACC CAATCCCTTT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
140             TTTTGCAATT TTATAATCGC TTACTTTGTA AAAAAGAATA TAAATTAAGA TTAAGTAAAT
188             TTTTGCAATT TTATAATCGC TTACTTTGTA AAAAAGAATA TAAATTAAGA TTAAGTAAAT
42              TTTTGCAATT TTATAATCGC TTACTTTGTA AAAAAGAATA TAAATTAAGA TTAAGTAAAT
74              TTTTGCAATT TTATAATCGC TTACTTTGTA AAAAAGAATA TAAATTAAGA TTAAGTAAAT
319             TTTTGCAATT TTATAATCGC TTACTTTGTA AAAAAGAATA TAAATTAAGA TTAAGTAAAT
197             TTTTGCAATT TTATAATCGC TTACTTTGTA AAAAAGAATA TAAATTAAGA TTAAGTAAAT
113             TTTTGCAATT TTATAATCGC TTACTTTGTA AAAAAGAATA TAAATTAAGA TTAAGTAAAT
72              TTTTGCAATT TTATAATCGC TTACTTTGTA AAAAAGAATA TAAATTAAGA TTAAGTAAAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   245        255        265        275        285        295
140             ATCGAACGAA TAAGAAATTC ACTCTTGACA GTGATATATG TTGTAGTTGT ATATGGAAAT
188             ATCGAACGAA TAAGAAATTC ACTCTTGACA GTGATATATG TTGTAGTTGT ATATGGAAAT
42              ATCGAACGAA TAAGAAATTC ACTCTTGACA GTGATATATG TTGTAGTTGT ATATGGAAAT
74              ATCGAACGAA TAAGAAATTC ACTCTTGACA GTGATATATG TTGTAGTTGT ATATGGAAAT
319             ATCGAACGAA TAAGAAATTC ACTCTTGACA GTGATATATG TTGTAGTTGT ATATGGAAAT
197             ATCGAACGAA TAAGAAATTC ACTCTTGACA GTGATATATG TTGTAGTTGT ATATGGAAAT
113             ATCGAACGAA TAAGAAATTC ACTCTTGACA GTGATATATG TTGTAGTTGT ATATGGAAAT
72              ATCGAACGAA TAAGAAATTC ACTCTTGACA GTGATATATG TTGTAGTTGT ATATGGAAAT
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***atpB-rbcL* alignment continued**

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345        355
140          CCTCGATGTG AAAATACGTG GAAACAAAAA GGTATAACTA TATAACAAAG AAGAAGTGTA
188          CCTCGATGTG AAAATACGTG GAAACAAAAA GGTATAACTA TATAACAAAG AAGAAGTGTA
42           CCTCGATGTG AAAATACGTG GAAACAAAAA GGTATAACTA TATAACAAAG AAGAAGTGTA
74           CCTCGATGTG AAAATCCGTG GAAACAAAAA GGTATAACTA TATAACAAAG AAGAAGTGTA
319          CCTCGATGTG AAAATCCGTG GAAACAAAAA GGTATAACTA TATAACAAAG AAGAAGTGTA
197          CCTCGATGTG AAAATCCGTG GAAACAAAAA GGTATAACTA TATAACAAAG AAGAAGTGTA
113          CCTCGATGTG AAAATACGTG GAAACAAAAA GGTATAACTA TATAACAAAG AAGAAGTGTA
72           CCTCGATGTG AAAATCCGTG GAAACAAAAA GGTATAACTA TATAACAAAG AAGAAGTGTA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   365        375        385        395        405        415
140          AATGGATATG ATAAAATAAG AAACAATGGC CGATGTCCTA AAAATGATGA ATCATAAATA
188          AATGGATATG ATAAAATAAG AAACAATGGC CGATGTCCTA AAAATGATGA ATCATAAATA
42           AATGGATATG ATAAAATAAG AAACAATGGC CGATGTCCTA AAAATGATGA ATCATAAATA
74           AATGGATATG ATAAAATAAG AAACAATGGC CGATGTCCTA AAAATGATGA ATCATAAATA
319          AATGGATATG ATAAAATAAG AAACAATGGC CGATGTCCTA AAAATGATGA ATCATAAATA
197          AATGGATATG ATAAAATAAG AAACAATGGC CGATGTCCTA AAAATGATGA ATCATAAATA
113          AATGGATATG ATAAAATAAG AAACAATGGC CGATGTCCTA AAAATGATGA ATCATAAATA
72           AATGGATATG ATAAAATAAG AAACAATGGC CGATGTCCTA AAAATGATGA ATCATAAATA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   425        435        445        455        465        475
140          GAGTTCAGGT TCGAATTCCA TAGATAATAT GGGCGGGGTT GTTTATAATA ATAGATAAAT
188          GAGTTCAGGT TCGAATTCCA TAGATAATAT GGGCGGGGTT GTCTATAATA ATAGATAAAT
42           GAGTTCAGGT TCGAATTCCA TAGATAATAT GGGCGGGGTT GTCTATAATA ATAGATAAAT
74           GAGTTCAGGT TCGAATTCCA TAGATAATAT GGGCGGGGTT GTCTATAATA ATAGATAAAT
319          GAGTTCAGGT TCGAATTCCA TAGATAATAT GGGCGGGGTT GTCTATAATA ATAGATAAAT
197          GAGTTCAGGT TCGAATTCCA TAGATAATAT GGGCGGGGTT GTCTATAATA ATAGATAAAT
113          GAGTTCAGGT TCGAATTCCA TAGATAATAT GGGCGGGGTT GTCTATAATA ATAGATAAAT
72           GAGTTCAGGT TCGAATTCCA TAGATAATAT GGGCGGGGTT GTCTATAATA ATAGATAAAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   485        495        505        515        525        535
140          AAAACACTTC CTCCAAATTC TTATTCATCA AAATAATTTT ATATTTTGAA AAGAGTTTGG
188          AAAACACTTC CTCCAAATTC TTATTCATCT ACTTAATTTT ATATTTTGAA AAGAGTTTGG
42           AAAACACTTC CTCCAAATTC TTATTCATCT ACTTAATTTT ATATTTTGAA AAGAGTTTGG
74           AAAACACTTC CTCCAAATTC TTATTCATCT ACTTAATTTT ATATTTTGAA AAGAGTTTGG
319          AAAACACTTC CTCCAAATTC TTATTCATCT ACTTAATTTT ATATTTTGAA AAGAGTTTGG
197          AAAACACTTC CTCCAAATTC TTATTCATCT ACTTAATTTT ATATTTTGAA AAGAGTTTGG
113          AAAACACTTC CTCCAAATTC TTATTCATCT ACTTAATTTT ATATTTTGAA AAGAGTTTGG
72           AAAACACTTC CTCCAAATTC TTATTCATCT AAATAATTTT ATATTTTGAA AAGAGTTTGG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   545        555        565        575        585        595
140          TTGCGTTTGA AAATACACTC ATTGAATGAG TAAACAATTG AATTGGATTC AGTTAGACGG
188          TTGCGTTTGA AAATACACTG ATTGAATGAG TAAACAATTG AATTGGATTC AGTTAGACGG
42           TTGCGTTTGA AAATACACTC ATTGAATGAG TAAACAATTG AATTGGATTC AGTTAGACGG
74           TTGCGTTTGA AAATACACTC ATTGAATGAG TAAACAATTG AATTGGATTC AGTTAGACGG
319          TTGCGTTTGA AAATACACTC ATTGAATGAG TAAACAATTG AATTGGATTC AGTTAGACGG
197          TTGCGTTTGA AAATACACTC ATTGAATGAG TAAACAATTG AATTGGATTC AGTTAGACGG
113          TTGCGTTTGA AAATACACTC ATTGAATGAG TAAACAATTG AATTGGATTC AGTTAGACGG
72           TTGCGTTTGA AAATACACTC ATTGAATGAG TAAACAATTG AATTGGATTC AGTTAGACGG
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***atpB-rbcL* alignment continued**

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645        655
140             TACCAACGAA ATTTGGTACT AACTCCCATT TCTTATTGAA TTAACCGATC TACTTGCAAT
188             TACCAACGAA ATTTGGTACT AACTCCCATT TCTTATTGAA TTAACCGATC TACTTGCTAT
42              TACCAACGAA ATTTGGTACT AACTCCCATT TCTTATTGAA TTAACCGATC TACTTGCTAT
74              TACCAACGAA ATTTGGTACT AACTCCCATT TCTTATTGAA TTAACCGATC TACTTGCTAT
319             TACCAACGAA ATTTGGTACT AACTCCCATT TCTTATTGAA TTAACCGATC TACTTGCTAT
197             TACCAACGAA ATTTGGTACT AACTCCCATT TCTTATTGAA TTAACCGATC TACTTGCTAT
113             TACCAACGAA ATTTGGTACT AACTCCCATT TCTTATTGAA TTAACCGATC TACTTGCTAT
72              TACCAACGAA ATTTGGTACT AACTCCCATT TCTTATTGAA TTAACCGATC TACTTGCTAT

                ....|....| ....|....| ....|.
                   665        675        685
140             CGGACATTTT TTTTTGTTTG CAAACC
188             CGGACATTTT TTTTTGTTTG CAAACC
42              CGGACATTTT TTTTTGTTTG CAAACC
74              CGGACATTTT TTTTTGTTTG CAAACC
319             CGGACA--TT TTTTTGTTT- CAAACC
197             CGGACATTTT TTTTTGTTTG CAAACC
113             CGGACATTTT TTTTTGTTTG CAAACC
72              CGGACA-TTT TTTTTGTTT- CAAACC
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Appendix 3

Domin scores (Kent & Coker 1992) for species recorded from 10 quadrats in a sampling grid ($100m^2$) in Gortlecka turlough basin.

	Agrostis stolonifera	*Carex spp.*	*Filipendula ulmaria*	*Galium palustre*	*Hydrocotyle vulgaris*	*Leontodon autumnalis*	*Lotus corniculatus*	*Potentilla anserina*	*Ranunculus repens*	*Rhamnus spp.*	Rock	*Trifolium repens*
Q1	9	4	3	3		3	6	5	9	+	3	
Q2	9	5	3	3		4	3	5	8		3	
Q3	9	7	4	3		4	3	6	9			+
Q4	9	7	5	3		3	5	3	7		3	
Q5	8	8	5	3		3	3	5	7		3	
Q6	8	8	7	3		3	3	7	7		3	
Q7	8	6	4	3		3	3	4	9		3	
Q8	8	7	3	3		3	5	5	7		3	+
Q9	8	6	5	3	3	3	3	4	8			3
Q10	8	6	3	3		5	3	5	9		3	3