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Conservation Biology of *Colchicum autumnale* L. and *Campanula trachelium* L. in the Nore Valley, Southeast Ireland

Rhian Jane Smith

Submitted to the University of Dublin, Trinity College, for the degree of Doctor of Philosophy

September 2004

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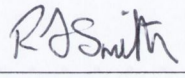

Rhian Jane Smith

This thesis is dedicated to my grandparents, Cyril and Daisy Smith, and to the memory of May Dulson (Nana Grampy), who was a constant source of enthusiasm and inspiration.

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List of Abbreviations and terms

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
BSBI	Botanical Society of the British Isles
CEFE	Centre d'Ecologie Fonctionnelle et Evolutive
CNRS	Centre National de la Recherche Scientifique
df	Degrees of freedom
DNA	Deoxyribonucleic Acid
IUCN	World Conservation Union (International Union for the Conservation of Nature and Natural Resources)
ISSRs	Inter-Simple Sequence Repeats
MVP	Minimum Viable Population
N_e	Genetic effective population size
NPWS	National Parks and Wildlife Service
pers. comm.	Personal communication
pers. obs.	Personal observation
PCR	Polymerase Chain Reaction
PVA	Population Viability Analysis
RAPD	Random Amplified Polymorphic DNA
RCF	Relative Centrifugal Force
REPS	Rural Environment Protection Scheme
SSRs	Simple Sequence Repeats (Microsatellites)
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

Additional comments

Plant names follow the nomenclature of Stace, 1997.
Basic statistical analyses were carried out using Datadesk 6.0 or SPSS 11.0 unless otherwise specified.

Summary

The conservation of plant biodiversity can only proceed effectively if it is informed by a detailed biological knowledge of the target species and their constituent populations. The overall aim of this study was to assess the Irish conservation status of *Colchicum autumnale* and *Campanula trachelium* in the Nore Valley through the collection, analysis and review of population data relevant to the survival of the species in Ireland. Demography and reproductive biology were investigated using permanent quadrats or by randomly sampling the populations. Genetic diversity within and between populations was investigated using the AFLP DNA fingerprinting technique.

Five of the six Irish populations of *Colchicum autumnale* occur in the Nore Valley and were estimated to range in size from 68 to 15,618 genets and from 270 to 45,741 ramets. Population size was significantly negatively correlated with mean vegetation height, suggesting that the spread of genets is restricted by tall vegetation. Recruitment and mortality were less than 10% in all populations over all years for both ramets and genets. Seedlings were recorded and seedling survival at Thomastown and Inistioge was 100% after one year but decreased to 40% at Thomastown and 50% at Inistioge at the end of the study period. Ramets were long-lived, with between 80 and 90% surviving the entire study period. Approximately 4% of corms were found to be dormant and dormancy lasted one year in all detected cases. The percentage of ramets producing fruit ranged from 0 to 62% and individual ramets produced between 1 and 4 fruits. The number of seeds per ramet ranged from 69 to 141.

Genetic variation in Irish populations of *Colchicum autumnale* was evaluated in a European context by collecting samples from twenty populations distributed through Ireland, Britain, France and Spain. Nei's total genetic diversity within the sampled populations was 0.2741 and within-population diversity was 0.0928. 90% of the AFLP loci were polymorphic. Genetic diversity in Irish populations was moderate; total gene diversity was 0.1162 and within-population diversity was 0.0901, using a square root estimation of null allele frequency. Using a Bayesian estimate of allele frequency, genetic diversity estimates were higher (0.1503 and 0.1358 respectively). Neighbour-joining, UPGMA and ordination analyses of the AFLP fingerprints revealed a major

biogeographic division between populations, with British and Irish populations separated from French populations. The Iberian populations were split between the two major groups, with the populations from the western Pyrenees showing an affinity to the British and Irish populations and the populations from the eastern side of the Pyrenees grouping with the French populations. Significant genetic differentiation occurred between countries, biogeographic regions and between populations within these groups. At a European level AMOVA showed 68.25% of variation to be distributed among populations and 31.75% within populations. In Ireland, 10.82% of the variation was distributed among populations and 89.18% within populations. Mantel tests revealed significant correlations between genetic and geographic distance overall, and within biogeographic groups, but not among populations within Ireland. The relative level of genetic diversity in Irish populations and the genetic relationships between populations suggest that *C. autumnale* is native to Ireland and that post-glacial migration from an Iberian refugium is the likely origin of the native British and Irish populations.

The twenty-one Irish study populations of *Campanula trachelium* were estimated to range from 1 to 600 flowering individuals. Three of the six populations monitored over the study period showed a decline, while the other three showed an increased in the number of flowering plants. Significant differences occurred between populations with respect to flowering stem height, number of capsules per stem, number of seeds per capsule, number of seeds per plant and mean seed mass. Stem height, seed output per capsule and seed output per plant showed a significant positive linear dependence on population size. Pollen supplementation did not significantly change seed output per capsule indicating that pollen limitation is not responsible for lower seed output in smaller populations. Gynodioecy was recorded in Irish populations and its estimated frequency within populations ranged from 0.08 to 0.26.

Genetic variation in *C. trachelium* was evaluated by sampling nineteen Irish populations, one garden population, two Welsh populations and one French population. Nei's total genetic diversity within the sampled populations was 0.2191 and within-population diversity was 0.1297. 89 % of the AFLP loci were polymorphic. Genetic diversity in Irish populations was high; total gene diversity was 0.2042 and within-population diversity was 0.1363, using a square root estimation of

null allele frequency. Using a Bayesian estimate, genetic diversity estimates were higher (0.2509 and 0.2062 respectively). However, gene diversity showed a significant positive linear dependence on log population size and the smallest Irish populations were also shown to have lower reproductive output than larger populations, suggesting the possible occurrence of inbreeding depression. Neighbour-joining, UPGMA and ordination analyses of the AFLP fingerprints separated French and Welsh populations from the Irish populations, which may therefore represent a unique component of the European diversity of the species, but did not reveal any significant genetic structuring of populations within Ireland. Mantel tests revealed no correlation between genetic and geographic distance. Population differentiation was significant at all hierarchical levels: between populations, between Irish populations and between Nore Valley populations. AMOVA revealed that the greater proportion of molecular variation is found within populations. In Irish populations, 72.7% of the molecular variation is found within populations while 27.3% is distributed among populations.

The degree of genetic variation within populations of the study species and the genetic relationships between populations led to the conclusion that both are native components of the Irish flora. The demographic, reproductive and genetic data on the species were integrated to produce management recommendations to ensure their survival on both an immediate and evolutionary time scale.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 A Brief Introduction To Conservation Biology

The conservation of global biodiversity is dependent upon the conservation of individual species and their inherent genetic variation. It is therefore necessary for conservation strategies to be based upon a fundamental scientific knowledge of the species they aim to conserve (Falk & Holsinger, 1991). This is at the core of the science of conservation biology, which aims to utilise biological knowledge of a species and its constituent populations as a tool in their conservation.

As a discipline, conservation biology is a relatively recent addition to the ranks of the biological sciences and therefore has yet to establish a formal definition for itself. The foundations for its development were laid during the mid-twentieth century when a growing social and political interest in environmental issues directed the attentions of scientists towards the problems associated with the survival of threatened species and it has since drawn on a variety of disciplines to achieve its aims. Consequently, a variety of different definitions are in use to describe conservation biology, the most concise of which is given by Hunter (1996) who describes it as "*the applied science of maintaining the earth's biological diversity*". Fundamental to all definitions is the commitment to the preservation of biodiversity through the scientific assessment of conservation practice.

Scientific interest in the field of conservation came about initially through developments in ecological theory and the application of this theory to the particular problems associated with rarity. For example, the fragmentation of habitats and the resulting isolation of small populations of plant and animal species drew immediate parallels with island populations, and thus MacArthur and Wilson's Theory of Island Biogeography (MacArthur & Wilson, 1967) was soon applied to the 'islands' of habitats created by increased development and their inherent fragmented populations of animals and plants. The potential applications were initially identified by the authors themselves, who recognised that the 'encroachment of civilisation' into natural areas would necessitate an accelerated use of the theory into the future. In particular, the concepts of reduced area and barriers to reproductive interaction and dispersal were pertinent to the problems

of isolated habitats and these were addressed with regard to their effects on species diversity and abundance. Although there are problems associated with the application of this theory to fragmented mainland habitats, it nevertheless provided an important stimulus to the academic development of conservation biology and was also the first work to explicitly relate extinction probability to population size (Simberloff, 1988).

Many other branches of biology also fed into conservation biology and some of the most important contributions came from the development of population genetic theory. The study of the genetics of small populations led to the identification of specific genetic threats to both short-term and long-term survival of populations and emphasised another dimension to the preservation of biodiversity: the preservation of the genetic diversity inherent within populations and species. The short-term importance of this added dimension was brought home with the documentation of inbreeding depression (the lowered fitness of inbred compared to outbred individuals) in small populations. This was one of the earliest identified genetic threats to the survival of rare species and first appeared in the literature in the second half of the last century (Moore, 1962; Hooper, 1971). Subsequent years saw the escalation of studies concerned with the genetic aspects of conservation and they have now increased to such a degree that 'conservation genetics' is establishing itself as a distinct discipline, with the recent release of the journal 'Conservation Genetics' in 2003 and the existence of many texts testifying to its independent scientific integrity (e.g. Falk & Holsinger, 1991; Karp *et al.,* 1998; Frankham *et al.*, 2002).

As genetic considerations came to the forefront, the long-term consequences of the loss of genetic diversity of species became apparent. The ability to change and to adapt to a continuously changing environment is determined by the wealth of genetic variability available to a species and erosion of this diversity therefore reduces evolutionary potential. It is this evolutionary dimension to conservation that distinguishes the modern discipline of conservation biology from previous approaches to conservation management. This fundamental distinction represents a conceptual shift from the simple prevention of extinction to the conservation of biodiversity on an

evolutionary time-scale. This is the defining feature of conservation biology, which essentially aims to conserve species whilst preserving the raw material for their continued evolution.

The multiple disciplines involved in modern-day conservation biology are consolidated by this common objective and were formally brought together with the formation of the Society for Conservation Biology in 1978, which now has over 7000 members. The Society defined the scope of the science and its mission statement encapsulates the fundamental aims and direction of scientists working in the field. As a society, it is committed to developing "*the scientific and technical means for the protection, maintenance, and restoration of life on Earth: species, ecosystems, and the processes that sustain them*" (Society for Conservation Biology, 2004). This has drawn together researchers from the fields of biogeography, ecology, environmental ethics, environmental law, genetics, population biology, systematics, wildlife ecology and many more. The two publications of the society: *Conservation Biology* and *Conservation in Practice*, provide a lively forum for debate and serve as the principle platform for the presentation and discussion of the most recent research in the field. Furthermore, these journals have been instrumental in the development of practical applications of published research (Keel *et al.*, 1993; Hogbin & Peakall, 1999; Milton *et al.*, 1999).

The emergence of conservation biology as a discipline was a direct product of scientific theory and development, but it must also be remembered that the seed for its establishment and growth was set by changes in society and in ethics. Although the utilitarian value of biodiversity conservation is now an important motivating factor, the fundamental basis for conservation biology is essentially a value judgement based on the assumption that species have an intrinsic value that is independent of their utilitarian value. This ethic has its roots in the romantic-transcendentalist writings of Ralph Waldo Emerson (1836) and Henry David Thoreau (1862;1863) and was later adapted by John Muir to make it the basis of a national campaign to preserve Yosemite Valley in the Sierra Nevada mountains of California (Van Dyke, 2003). The political success of this campaign led to the formation of the Sierra Club in 1892, which provided a permanent establishment dedicated to the preservation of wilderness areas.

The 'preservationism' heralded by Muir was an important development in environmental thinking but it was still a step away from conservation, which requires a more active role in species and ecosystem management. Aldo Leopold, a forester in the US Forest Service in the early 1900s, was concerned with game management within forests and was one of the leading proponents of the use of science to advise both ethical and practical conservation considerations. His most influential publication was '*A Sand County Almanac*' (Leopold, 1989) first published in 1949, and in this he argued that successful conservation required an acknowledgment of our ethical responsibility to nature. He became the father of 'wildlife ecology' and it is this mode of conservation that has prevailed partly due to the academic respectability gained when Leopold was appointed to the University of Wisconsin at Madison. He bridged the gap between theoretical and practical conservation at a time when the science of ecology was increasingly publishing studies with conservation implications, and thus created an academic environment in which conservation biology could develop (Van Dyke, 2003).

With the academic framework in place, the final factor contributing to the formation of the discipline was the birth of environmentalism. During the 1960s the pollution and degradation of the natural environment was thrust into the public domain by popular science writers such as Rachel Carson whose landmark book '*Silent Spring*', published in 1962, warned of the devastating effects on natural ecosystems caused by the release of pesticides into the environment. She was the first writer to bring the concept of biological amplification to public attention and described the exponential accumulation of toxins between stages in the food chain leading to lethal levels in predatory animals such as birds of prey (Carson, 1962). In this same publication she identified the need for a shift in perception from anthropocentrism to ecocentrism, emphasising the value in the biological integrity of the Earth and its living systems (Sessions, 1995). The testimony of such writers, combined with escalating habitat destruction and extinctions, particularly in the tropics, provoked the movement of environmental activism but also inspired a new generation of scientists determined to reverse the trends through the application of science to the problems faced by threatened species and their ecosystems.

The extinction crisis was the spur needed to consolidate the scientific movement into an active body of conservation biologists and in 1978 the Society for Conservation Biology was born. Its founder, Michael Soulé, has referred to it as a crisis discipline as its existence would not have been necessary without the unfolding extinction crisis and the threat to global biodiversity (Soulé, 1985). It arose as a discipline when "*a critical mass of people decided they were conservation biologists*" (Soulé, 1986) and has grown to be a powerful force leading the way in the application of scientific knowledge to the conservation of species and ecosystems and the preservation of biodiversity.

1.2 CONCEPTS IN CONSERVATION BIOLOGY

1.2.1 Biodiversity

The concept of biodiversity arose out of a need to define the sum total of the variety of life in one all-encompassing term and the word is a recent fusion of the term 'biological diversity' (Wilson & Peter, 1989). It is defined by the International Union for the Conservation of Nature (IUCN) as '*The variety of life in all its forms, levels and combinations. Includes ecosystem diversity, species diversity, and genetic diversity*' (IUCN, UNEP and WWF, 1991). A more recent definition is more concise and describes it as '*the structural and functional variety of life-forms at genetic, population, community, and ecosystem level*' (Sandlund, Hindar and Brown, 1992). In studies based on the conservation biology of individual species, the genetic and population levels of biodiversity are of primary concern and the goal of species-based conservation research is therefore to define and preserve the genetic diversity of populations and of the species as a whole. The all-encompassing concept of the conservation of biodiversity replaced the earlier motivation of wildlife ecologists, which was simply to prevent the extinction of a small number of important species, and it is therefore a fundamental concept at the core of modern conservation biology. Biodiversity has unified the different fragments of biology under a common objective (di Castri & Younès, 1996) and has provided a conceptual focus for the science of conservation biology and its practice (Jeffries, 1997).

1.2.2 Population paradigms

Disciplines evolve through the formation of paradigms and the endorsement or refutation of the statements therein. One of the major discussion-provoking hypotheses in conservation biology has been the suggestion that the discipline is divided into two separate bodies guided by different population paradigms (Caughley, 1994). In his 1994 paper, resulting from an overview of the conservation literature over the preceding 20 years, Caughley identified a dichotomy in conservation approaches led by two conflicting ideas that he referred to as the declining-population paradigm and the small-population paradigm. These two paradigms essentially relate to deterministic and stochastic forces respectively. The declining population paradigm focuses on the external causes of population decline and aims to reverse the process through the identification of the causal factors (Caughley, 1994; Caughley & Gunn, 1996). It has operated largely through an empirical approach and on a case-by-case basis without significant theoretical backing. In contrast, the small-population paradigm is concerned with the consequences of rarity and small population size and aims to manage threats to the persistence of small populations (Van Dyke, 2003). It has a wide theoretical base that has arisen through the identification of specific demographic, genetic and environmental threats to small populations (Franklin, 1980; Frankel & Soulé, 1981; Shaffer, 1981, 1987; Lande & Barrowclough, 1987; Lande, 1988;) and the development of stochastic models describing the interaction of these threats (Gilpin & Soulé, 1986). The main criticism of this approach is the lack of applied research and the fact that practitioners may neglect to address the question of why the population is small.

Although Caughley advocates an integration of the two paradigms, he has been widely criticised for constructing a false dichotomy thus polarising conservation biology researchers (Hedrick *et al.* 1996). Specific areas of research within the field of conservation biology can be classified in terms of one or other of the paradigms but in reality individual studies may incorporate aspects of both. Nevertheless, stating the paradigms as conflicting approaches can only put pressure on researchers to subscribe to one or the other and this is therefore an unhelpful exercise that only succeeds in dividing the research community. There are undoubtedly some studies that focus too

keenly on intrinsic aspects of population persistence and, conversely, those that ignore the wider-scale causes of decline. Although the paradigms have been found to be useful in describing the failings of these approaches (Asquith, 2001), the imposition of such a strong dichotomy was unnecessary.

A further paradigm concerned with the level of the population, and highly relevant to conservation biology, is that of metapopulation theory. Levins was the first author to define a metapopulation and described it as '*any real population [that] is a population of local populations which are established by colonists, survive for a while, send out migrants, and eventually disappear*' (Levins, 1970). The concept has been developing since Andrewartha and Birch first stated that '*A natural population occupying any considerable area will be made up of a number of... local populations or colonies*' and emphasised that dispersal ability influences the number of occupied patches at any one time (Andrewartha & Birch, 1954). This formed the basis for the population dynamic theory of metapopulations, the mathematical model of which was developed by Levins (1969, 1970) and extended by Hanski and Gilpin (Hanski, 1991; Hanski & Gilpin, 1991). The equations of Levins' model are structurally equivalent to the equations for the change in frequency of a non-dominant gene in a series of sub-populations developed by Wright (1940) and the genetic theory of metapopulations has been built on this early work (Caughley, 1994). There is now a large body of work on metapopulation theory and its applications and it has become one of the most important paradigms in modern conservation biology.

Inherent within the idea of the metapopulation is the dynamic balance between the survival and extinction of its constituent parts. Extinctions occur in some populations but the metapopulation is sustained by recolonisations of vacant habitat areas. The theory is founded on principles very much related to MacArthur and Wilson's model of island biogeography (1967) with common principles including distance-dependent rates of propagule transfer between island units and area-dependent rates of extinction (Harrison, 1994). The rise in research on the conservation biological applications of the theory can be attributed to its applicability to the fragmented populations of threatened species, and the incorporation of metapopulation concepts into population models has

been widely applied to species conservation. Metapopulation modelling was successfully used in the formulation of a recovery program for the Pitcher's thistle, *Cirsium pitcheri* (Torr. ex Eat.) Torr. & Gray (Asteraceae) (McEachern *et al.*, 1994).

1.3 CONSERVATION GENETICS

1.3.1 Genetics as a basis for conservation

Genetic diversity is the most fundamental unit of biodiversity and is therefore of vital importance to the field of conservation biology (Frankel & Soulé 1981). The genetic variability of plant populations can have both short-term and long-term implications for population persistence and the study of population genetics has become an integral part of research into the conservation of threatened plant species. While the demographic and ecological analyses that form the basis of many plant studies are important in the assessment of short-term threats to populations, ultimately it is the genetic structure of a population that will govern its ability to persist on an evolutionary time-scale (Frankel, 1970; 1974). This is embedded in the Fundamental Theorem of Natural Selection (Fisher, 1930), which states that the rate of evolution at a locus is limited by the degree of genetic variability. Lack of genetic variability can leave a population susceptible to disease epidemics or to future environmental change (Huenneke, 1991). In addition to this long-term consideration, genetic processes can also have more immediate effects on population viability through interaction with other factors, and through processes such as inbreeding depression that can have direct effects on the reproductive fitness of individuals. The relevance of the concepts of population genetics to the field of conservation management was stressed in Berry's paper to the British Ecological Society Symposium of 1970 (Berry, 1971) and the genetic perspective has since become an integral part of conservation biology. Recent developments in DNA extraction and analysis technology, in particular the advent of the Polymerase Chain Reaction (PCR), have greatly facilitated this integration and the techniques used are now readily accessible. This has led to the incorporation of population genetics into a large number of plant conservation studies (eg. Prober *et al.*, 1990; Godt & Hamrick, 1996; Delgado *et al.*, 1999; Hogbin & Peakall, 1999).

The genetic perspective to species conservation is now firmly established as a fundamental consideration in conservation research and this is reflected by its appearance on the international and global political platform through the Convention on Biological Diversity and the EU Habitats Directive (Council Directive 92/43/EEC). The importance of genetic diversity as a component of biological diversity is also recognised by the World Conservation Union (IUCN), which includes genetic diversity as one of the three levels of biodiversity in need of conservation. Through such media the concept of genetic diversity has filtered down to become an integral part of conservation legislation in many countries and has been responsible for schemes such as the establishment of Biogenetic Reserves in Europe.

There have been many critics who have called into question the relative importance of genetic aspects of conservation, stating that ecological factors may be more important (eg Lande, 1988). However, ecological and genetic factors are intimately linked in the evolution and extinction of populations and if numbers of individuals are reduced by deterministic, ecological or demographic factors it is the genetic composition of the population that will dictate its ability to expand and survive.

1.3.2 The genetics of small populations

Many of the genetic processes that occur in plant populations are stochastic in nature and therefore become less stable the smaller the population size. Genetic drift, the random change in allele frequencies from generation to generation, becomes more pronounced in small populations where it is more likely to lead to the loss of alleles (Futuyma, 1986) and to the accumulation and fixation of deleterious mutations (Crow & Kimura, 1970; Ellstrand & Elam, 1993). It also occurs independently of mutation, recombination and selection. The fixation of sub-optimal alleles through genetic drift can occur in any population but the increased sampling error inherent in the reproductive functioning of small populations increases the probability of such events occurring and can lead to mutational meltdown (Lynch *et al.*, 1995). In a review of data from 10 separate studies, Ellstrand and Elam (1993) found that the results were consistent with the hypothesis that the effects of genetic drift vary with population size. In addition to this, population bottlenecks

can trigger extreme cases of genetic drift, as even the initial population is a highly biased sample of the parent population, and bottleneck events can therefore result in dramatic changes in allele frequencies. The division of a population or disruption of gene flow between populations through habitat destruction can result in such bottlenecks and genetic drift can therefore be a dominating force in the genetics of fragmented populations of threatened plant species.

A further contributor to genetic stochasticity is inbreeding, which often occurs as a consequence of reduction in population size due to other factors and causes a loss of heterozygosity. Where inbreeding is widespread, particularly in small populations, the progeny of closely related individuals become subject to the effects of inbreeding depression. This is manifested as a loss of fitness in progeny derived from inbreeding relative to those derived from outcrossing (Husband & Schemske, 1996). The significance of inbreeding depression in plant populations was first noted by Darwin who saw it as a negative selective pressure driving the evolution of outcrossing adaptations in plants (Darwin, 1876; 1877). The evidence for the role of inbreeding depression in the evolution of animals and plants was reviewed by Charlesworth and Charlesworth (1987) and they concluded that it is a sufficiently strong force to have played a significant role in the evolution of breeding systems such as self-incompatibility and dioecy. It is therefore an important factor in plant populations that may be compromised in terms of size and genetic variability.

Two models for the genetic basis of inbreeding depression exist. The first, the partial dominance model, attributes the loss of fitness to the expression of deleterious alleles in the homozygous state. These alleles are recessive, or partly recessive (Fenster & Dudash, 1994). Inbreeding reduces heterozygosity and thus allows fixation of these alleles. The second model is the overdominance model in which the fitness of the heterozygote is greater than that of either homozygote. These two models are compatible with each other and the two processes can occur simultaneously. They may operate at the level of the single locus or exert their effects over many loci (Fenster & Dudash, 1994). However, the two different theories have different implications for the genetic load of a population. If inbreeding depression occurs through partial dominance, selection can reduce the frequency of deleterious alleles but if it results from overdominance the

deleterious alleles will be maintained within the heterozygotes which will continually re-create homozygous recessives (Frankham *et al.*, 2002). Evidence from plant and animal species supports the partial dominance model (eg. Fu & Ritland, 1994; Husband & Schemske, 1997; Roff, 2002) and it is now generally accepted that partial dominance is the main mechanism responsible for the expression of inbreeding depression (Falconer and Mackay, 1996; Lynch and Walsh, 1998; Charlesworth and Charlesworth, 1999).

The occurrence of inbreeding depression has been measured in many plant populations. Levin (1989) documented the phenomenon in self-fertilising *Phlox* (Polemoniaceae) and Dudash (1990) detected a difference in fitness between selfed and outcrossed progeny of *Sabatia angularis* L. (Gentianaceae). The loss in fitness is often detected in reproductive characteristics (Mousseau and Roff, 1987; Falconer and Mackay, 1996; Lynch and Walsh, 1998) and effects of inbreeding include a reduction in fruit set (Schmidt & Jensen, 2000; Severns, 2003), seed production (Weller & Ornduff, 1991; Schmidt & Jensen, 2000) and percentage germination (Buza *et al.*, 2000), and lower seedling survival rates (Newman & Pilson, 1997). Inbreeding depression has also been found to be greater in the field than in controlled greenhouse experiments (Dudash, 1990) although this could be due to the negative effects of ecological factors such as pollen limitation or other factors that may have an effect on reproductive output. This is supported by the finding that inbreeding depression is greater when plants are under stress (Heschel and Paige, 1995) and greater when plants are in harsh environments (Roff, 1997). There is also evidence to suggest that it is a frequency- and density-dependent phenomenon in relation to the frequency and number of outbred versus inbred plants (Cheptou *et al.*, 2001). In general, however, inbreeding depression may be underestimated by controlled studies due to the inability to detect the effects at every stage in the life cycle and across all fitness-related characteristics (Charlesworth & Charlesworth, 1987). Even in populations where inbreeding is not resulting in a direct loss of fitness, inbreeding depression could still be occurring due to the potential gain in fitness if a cross occurred with an unrelated individual from a different population (Charlesworth & Charlesworth, 1987). The

depression may be in stasis, not resulting in further loss of fitness but still restricting the individuals in the inbred population.

The standard measure of the degree of inbreeding is the coefficient of inbreeding, F, as described by Wright (1921). This is the probability that both alleles at a locus are identical by descent (Crow & Kimura, 1970) and its range is therefore 0 to 1, from entirely outbred to entirely inbred respectively. The mean inbreeding coefficient of progeny resulting from self-fertilisation of a parent plant is therefore 0.5. Frankham's model of inbreeding (1995) showed that this inbreeding coefficient is negatively correlated with survivorship and that at low levels of inbreeding, a change in the inbreeding co-efficient does not markedly affect survival but at high levels of inbreeding the same change in the inbreeding co-efficient produces a large negative effect on survivorship. This has implications for small populations with high levels of inbreeding as any further inbreeding could have a significant effect on population viability. A further consideration is that in the absence of gene flow, inbreeding increases with time in all finite populations (Frankham *et al.*, 2002) and the rate of increase is dependent on the population size. The inbreeding coefficient increases more rapidly with time in small populations than in large populations. This has significant consequences for small, fragmented populations with high levels of inbreeding.

An important factor determining how plant species react to genetic pressures such as inbreeding depression is the breeding system. It is widely suggested that selfing can remove deleterious alleles from a population through the purging of the genetic load (Stebbins, 1950) so self-compatible species are expected to display lower levels of inbreeding depression. This is supported by experimental work such as that of Groom (1998) who showed that there was no difference in seed production between selfing and outcrossing plants of *Clarkia concinna* (Fisch. & C.A. Mey.) Greene (Onagraceae), a self-compatible, annual plant species. Wright (1977) has reviewed a large amount of evidence that suggests inbreeding depression is usually reduced in plant species that are habitual inbreeders compared to outbreeding species. Although the supporting evidence comes mainly from domesticated species it still has important implications

for the potential effects of inbreeding depression on natural populations of plant species. An extreme case of inbreeding depression can occur when an obligate outbreeding species has a self-incompatibility system as part of its breeding biology. If population sub-division or a severe bottleneck occurred such that the only individuals remaining were all of the same compatibility type, complete reproductive failure would ensue (eg. Demauro, 1993).

The detrimental genetic effects of drift and inbreeding depression in small populations are enhanced by the degree of fragmentation and isolation of the populations. The severity of fragmentation determines the initial genetic composition and variation of the population sub-units and the degree of isolation determines the relative contribution of gene flow to the population genetics. Gene flow can be vital in ameliorating the effects of drift and inbreeding depression and the genetic input made by even one immigrant per generation can maintain genetic variation and reverse the deleterious genetic trend towards loss of alleles and heterozygosity (Menges, 1991b).

There is a wealth of evidence to suggest that genetic factors play a significant role in the functioning of small populations but they cannot be viewed in isolation from each other. The loss of diversity, heterozygosity and fixation of deleterious mutations resulting from genetic drift can enhance the effects of inbreeding depression, which in turn can reduce population size further thus enhancing the effects of genetic drift. What is clear is that the loss of genetic variability resulting from a combination of these forces can have pronounced impacts on population viability and risk of extinction. A study on *Clarkia pulchella* demonstrated this explicitly, where the probability of population extinction increased as genetic effective population size decreased (Newman & Pilson, 1997). More studies are required to verify whether this effect is widespread in natural populations of plant species but nevertheless, the potential for decreased persistence times as a result of genetic factors endorses the importance of a consideration of genetic factors as part of conservation research.

1.3.3 Molecular tools in conservation biology

The central concern in the area of conservation genetics is the degree of genetic variation held within a species and how this is partitioned within and between populations. Evaluation of this, using molecular methods, can contribute greatly to the assessment of population viability (Haig, 1998). The techniques also allow for the investigation of population structure, effective population size, inbreeding depression and levels of gene flow between populations and are therefore applicable to a range of questions raised by conservation biology.

The first molecular method to be widely used in the field of population genetics was allozyme electrophoresis and involves indirect analysis of the genome via its protein-based gene products. Variation in soluble gene-coded enzymes is revealed using gel electrophoresis and polymorphisms can be detected by the differential migration of enzymes coded for by alternative alleles at a locus (allozymes). An inherent limitation of the process is that the analysis of variation is restricted to only a small subset of the total genome, which is not selectively neutral. The variation quantified may not correspond to selectively important variation and therefore may not be valid to considerations of evolutionary potential. However, in general, allozyme variation seems to be indicative of the overall level of genomic variation as revealed by other methods (Schaal *et al.*, 1998).

Recent advances in molecular technology have provided conservation biologists with the opportunity to analyse the genome directly at the level of nucleic acid sequences. This has largely been made possible by the development of techniques utilising enzymes that allow sampling of the genome at a number of points (Karp *et al.*, 1997). Molecular marker techniques utilise this sampling approach to obtain a genetic fingerprint of individuals and can be subdivided into two major categories based upon the use or otherwise of the Polymerase Chain Reaction (PCR). PCR is a method of amplifying specific fragments of the genome and has been instrumental in the development of DNA-based molecular methods. Techniques that do not employ the PCR method, such as Restriction Fragment Length Polymorphism analysis (RFLP) require large quantities of

DNA and involve lengthy procedures, and for this reason are not widely used where other techniques are available (Karp *et al.*, 1996).

Methods utilising PCR can be further subdivided into arbitrary primed techniques and site-targeted PCR (Karp *et al.*, 1997). A commonly used arbitrary primed technique is RAPD (Random Amplified Polymorphic DNA) analysis, which involves the use of single arbitrary primers in the PCR. The primers comprise an arbitarily chosen nucleic acid sequence, which binds to complementary regions of the DNA. Providing two such regions are located on opposite strands, the DNA between the primers will be copied and amplification then occurs in a PCR reaction. The products will only be detectable if the primers are close enough for amplification to occur. The products of the PCR are generally stained using ethidium bromide separated by electrophoresis on an agarose gel and viewed under ultraviolet light to enable sizing and scoring of fragments (Karp *et al.*, 1997).

There are several limitations to RAPD analysis, the main one being that the expression of bands is dominant and as such no differentiation can be made between the homozygous and heterozygous state (Cruzan, 1998). It is also difficult to maintain the levels of consistency in reaction conditions that are required for reproducibility of results (Karp *et al.*, 1997). However, despite the limitations they can provide an indication of levels of genetic variation, which can form the basis for determining whether further study using higher resolution markers is needed.

A more recent technique that is considered to be more powerful and reproducible than RAPD analysis is Amplified Fragment Length Polymorphism (AFLP) analysis (Vos *et al.*, 1995). This method involves digestion of the DNA using restriction enzymes followed by the addition of adaptors with known sequences to the fragment ends. The amplification primers are designed to incorporate the known sequences plus one or more additional base pairs and in the final PCR are radio-labelled or labelled with a fluorescent dye. PCR amplifies the relevant fragments and the products are separated on polyacrylamide gels by electrophoresis. Where radiolabelled primers are used, banding patterns can be viewed following exposure to X-ray film (Karp *et al.*, 1997),

and fluorescently-labelled products can be run on an automated sequencer. The AFLP technique requires a greater amount of DNA than is needed for RAPD analysis and the procedure is more complex but it is an effective, reproducible method for detecting large numbers of polymorphisms in a single analysis (Karp *et al.*, 1997). However, it still has the limitations associated with dominant markers.

The alternative to using arbitrary primer methods is to target specific sites in the genome. Microsatellites, for example, are simple sequence repeats (SSRs) that occur at many locations along a chromosome and primers can be designed to flank and amplify such regions. The results arising from the use of SSRs are highly informative in population studies as they can yield as many as 30-50 alleles per locus (Parker *et al.*, 1998) and they are co-dominant markers allowing allele frequency to be accurately estimated. However, the development of the primers requires a large investment of time (Parker *et al.*, 1998), and for this reason they are not as accessible as other categories of markers, unless previous researchers have identified variable microsatellite regions in the species or genus of interest. Universal chloroplast markers are available but these are rarely variable enough for use in intra-specific studies (pers. obs.).

Overall, the use of non-coding regions of the genome can provide high resolution of population structure (Amos & Hoelzel, 1992) and the molecular techniques that exploit the information contained in the variation within these regions are valuable tools in conservation biology. However, with the exception of allozymes, all deal with non-selective areas of the genome and the assumption must be made that the markers reflect evolutionary history and potential.

1.4 POPULATION VIABILITY

At the fundamental level, species-based conservation biology is essentially concerned with the unit of the population (Caughley and Gunn, 1996) and it is necessary to quantitatively define certain population parameters before conservation status can be objectively assessed. Populations are spatiotemporal entities (Allee *et al.*, 1949) consisting of groups of co-existing individuals existing separately in space or time from other such groups, and this separation subjects them to

independent biological processes. Extinction probabilities are totally dependent on the effect of these biological processes on population parameters and thus the survival of a species is dependent on the viability of its constituent populations. Population viability has therefore been the subject of much research and debate in the conservation biology literature, resulting in the publication of the volume 'Viable Populations for Conservation' edited by Michael Soulé (1987). One of the theoretical concepts to arise out of the discussion was that of the existence of a threshold number of individuals required to constitute a viable population (Gilpin & Soulé, 1986). This implies that there is a critical population size, termed the Minimum Viable Population (MVP) size (Soulé, 1980; Gilpin & Soulé, 1986), below which extinction is imminent (Stewart & Hutchings, 1996). The MVP size is related to extinction probability due to the fact that large populations have a greater capacity to be buffered against stochastic effects than small populations (Soulé, 1980; Shaffer, 1981; Menges, 1991b).

The first person to address the problem in detail was Shaffer (1981) who proposed the following definition: '*A minimum viable population for any given species in any given habitat is the smallest isolated population having a 99% chance of remaining extant for 100 years despite the foreseeable effects of demographic, environmental and genetic stochasticity, and natural catastrophes*' (Shaffer, 1981). This is clearly a highly specific definition and in reality the probability level and time scales are set at the discretion of the investigator.

Shaffer clearly identifies four random processes that threaten a population's viability, each of which increase in importance as population size decreases (Shaffer, 1981). The first, demographic stochasticity, refers to the random shifts in population number and composition which occur as a result of fluctuations in the gains and losses to the population (Roughgarden,1975; Shaffer,1981). In a uniform environment, persistence time (or alternatively time to extinction), increases as a geometric function of population size (Goodman, 1987). This implies that demographic stochasticity is an important threat only to small populations. Kieding (1975) has suggested that the effects of demographic stochasticity are usually important only in populations of 50 or fewer individuals.

However, very few populations exist in a predictable environment and therefore environmental stochasticity is a second factor affecting population viability. Random fluctuations in the biotic and abiotic environment can have a large effect on survival and reproduction and can increase the vulnerability of the population to other factors, or cause extinction in an already depleted population. When environmental stochasticity is incorporated into a model alongside demographic stochasticity, persistence time increases linearly with population size (Goodman, 1987). This suggests there is no clear critical population size above which a high probability of survival is ensured; any loss to a population significantly reduces the expected survival time and the designation of a minimum viable population size is therefore highly subjective. If natural catastrophes are also considered, their potential to affect all individuals simultaneously amplifies this effect and persistence time increases only with the natural log of population size (Ewens *et al.*, 1987). In determining the MVP the conservation biologist must therefore arbitrarily decide on an extinction risk that is 'acceptably' low and the inherent subjectivity in this decision can lead to dispute over the level of security that should be offered to a population.

Another aspect of viability hinges on the genetic stability of a population. Stochastic processes operating on the genetics of a population can severely impair viability and can be an important factor in population extinctions (Franklin, 1980). Random changes in genetic structure can occur through founder effects, genetic drift and through inbreeding. The founder effect constitutes a bottleneck effect, where a small sample of the population's genes act as the source for a new population. The qualitative result of such a process is the loss of alleles. Quantitatively, the degree of variability of specific traits will be reduced (Frankel & Soulé, 1981). When numbers of individuals are low, a population is effectively experiencing a bottleneck every generation (Frankel & Soulé, 1981) and this constitutes a process known as random genetic drift. This also results in a loss of alleles and can lead to the fixation of deleterious alleles (Lande & Barrowclough, 1987).

The influence of genetical structure on viability analyses has prompted many estimates of minimum viable population sizes to deal with effective population size rather than total numbers

of individuals. The derivation of this parameter is based upon the assumptions that at any one time only a subset of the total population has the capacity to reproduce and that individuals do not contribute equally to reproduction due to individual and environmental variation and a possible skew in the sex ratio (Lande & Barrowclough, 1987). In addition to this, where flowering plants are the subject of study, the occurrence of self-fertilisation must also be considered (Lawrence and Marshall, 1997).

All these factors contribute to the random changes in allele frequencies which constitute random genetic drift and cause populations to act as though smaller than their actual size (Lawrence & Marshall, 1997). Wright (1931) was the first author to name this subset of the population the effective population size (N_e) and defined it as the size of a theoretical population that would experience the same amount of genetic drift as the real population. Franklin (1980) and Soulé (1980) based their respective derivations of theoretical MVP's on effective population size and suggested that a minimum effective population size of 50 would be required to combat inbreeding depression, a further contributor to genetic stochasticity. There is also a variance effective population size, which has the same rate of increase in genetic variance as the actual population (Crow & Kimura, 1970). This contrasts conceptually with the inbreeding effective population size, which has the same rate of decrease in homozygosity as the actual population but the two estimates are often quite similar. However, there are certain situations where the two approaches produce very different figures. For instance, in an expanding population, it is likely that variance N_e will be greater than inbreeding N_e, whereas in a declining population it is likely that the opposite will be true (Simberloff, 1988). In populations where the number of individuals fluctuates rapidly, the equations on which the estimates are based become inaccurate (Lande & Barrowclough, 1987). Another source of inaccuracy is the assumption of non-overlapping generations and caution must be exercised in the derivation of management strategies based on estimates made under these conditions.

In terms of genetic stochasticity, inbreeding has been seen as the principal threat to the short-term survival of populations and genetic drift is viewed as a more serious threat in the long-term

(Shaffer, 1987). The long-term threat comes as a result of the effect of genetic drift in reducing genetic variation, which holds the potential for evolutionary flexibility and adaptation (Frankel & Soulé, 1981). The long-term potential for a population to adapt to changes in its environment is an important consideration and when looked at in conjunction with the other stochastical factors, it has a significant bearing on the derivation of MVP size. Soulé (1980) emphasizes the point that to maintain the evolutionary potential of a population, estimates of viable numbers need to be significantly higher than those derived only from considerations of demographic and environmental stochasticities. Franklin (1980) reiterates this in suggesting that maintaining sufficient genetic variation to allow adaptation to future environmental shifts requires population sizes in the range of 500 individuals.

The interaction of stochastic factors on population survival has been conceptualised by Gilpin and Soulé (1986) as a system of feedback loops which, when triggered by the action of one or more factors, can create an extinction vortex. The action of one stochastic process increases the vulnerability of the population to the others and the effect is a downward spiral to extinction. For example, in a population with low genetic variability, a lack of pollinator activity can increase the degree of selfing, causing inbreeding depression and thus affecting the fitness of the F1 generation. This in turn de-stabilises the demographic structure of the population and the factors enter a synergistic feedback loop where the effects of one factor create the conditions necessary for the full effect of another factor. The integrated study of these feedback loops is considered to be an essential component of viability analysis (Gilpin & Soulé, 1986) as is a consideration of the spatial structure of the population (Gilpin, 1987). Population structure can have a large effect on the dynamics of the feedback loops and its omission from theoretical considerations can invalidate estimates of population viability. Environmental stochasticity and natural catastrophes may not affect all units of a metapopulation nor all parts of a local population in a heterogeneous environment (Gilpin, 1987; Lawrence & Marshall, 1997). Local extinction therefore may be prevented by recolonisation of the affected area and spatial structure may therefore have a significant effect on extinction probability (Gilpin, 1987).

The practical application of population viability theory to the study of plant and animal populations is termed Population Vulnerability (or Viability) Analysis. The ultimate aim of a PVA is to estimate a viability parameter such as time to extinction or persistence time, or to calculate the probability of a population surviving for an arbitrarily chosen period of time. The process embraces the same principles and methods as the MVP question but without defining the lower limit of viability (Boyce, 1992). In theory, such an analysis should incorporate a complete study of all the population effects of the stochastic factors and their feedback loops (Gilpin & Soulé, 1986) but in reality, only one or two parameters can be studied in any detail.

PVA has been carried out on several plant species such as in Menges' (1990) analysis of the endangered *Pedicularis furbishiae* S. Wats. (Scrophulariaceae). Such analyses often involve standard population biology measurements of mortality, survivorship and fecundity, which are arranged in an algebraic matrix. The use of matrices has been a prominent feature of demographic studies since Leslie (1945) first introduced them into population mathematics, and they can be either age-structured (Leslie, 1945) or arranged in terms of life history stage or size (Lefkovitch, 1965; Caswell, 1982). The matrix method can be used to derive several parameters such as λ, the finite growth rate of the population. Sensitivity analysis looks at the response of this parameter to disturbances at various points in the life table and can thus help to identify vulnerable life-history stages.

The predictive capacity of PVA generally relies upon complex modelling of the population incorporating the measured parameters. However, a balance must be found between simulating the natural population and environment as realistically as possible, and keeping the model simple enough so that it is manageable and does not entail the estimation of too many parameters (Eberhardt, 1987). Despite the advantages of predictive modelling, it has many limitations that must be considered before lending too much weight to their predictions. In many cases, important factors are omitted to simplify the model and interactions between factors are overlooked (Boyce, 1992). The statistical reliability of many models is also ambiguous and contrasting results from deterministic and stochastic models often lead to different conclusions regarding viability (Boyce,

1992). A further limitation, which is applicable to all plant population studies, is the omission of seed bank data from projection matrices. The seed is an important life history stage and the need for the inclusion of seed bank data in demographic studies has been emphasised by Bouwmeester & Karssen (1996). The seed bank of a population decreases the likelihood of extinction (Griggs & Jain, 1983), increases the available genetic variation and also confers greater genetic stability on the effective breeding population (Epling *et al.*, 1960; Baskin & Baskin, 1978).

The value of Population Viability Analysis is restricted by the quantity and quality of data available and the accuracy of the models that are used and as a result it is at best an approximate science and at worst a wildly inaccurate estimator of extinction probability. However, although the final outcome of the process may be invalid, the process itself provides useful information that can be used to make informed conservation decisions. A full PVA has not been carried out as part of this research but the concept of population viability and its many contributing factors has directed the method of study and it is therefore a useful approach.

1.5 Plant Conservation in Ireland

1.5.1 Conventions and legislation

There are a number of statutory instruments relating to plant conservation in Ireland and these are listed below. The government agency responsible for the implementation and enforcement of conservation legislation in Ireland is The National Parks and Wildlife Service, formerly Dùchas, the Heritage Service, and this is currently part of the Department of the Environment, Heritage and Local Government.

Flora Protection Order

The primary basis for plant conservation in Ireland is the Flora (Protection) Order, 1999 (S.I. No. 94 of 1999), which is rooted in the Wildlife Act, 1976 (No. 39 of 1976). This order defines components of the Irish flora that are rare or threatened and gives them legal protection from activities that impinge on them or their habitats. The Irish flora contains approximately 1000 flowering plant species (Webb *et al.*, 1996) and of these, 68 are currently protected under this

legislation. Species are included on the Order if they have restricted distributions in the Republic of Ireland or if, whether common or not in the Republic, they are rare in a European context. Licenses, issued by the National Parks and Wildlife Service, are required to disturb, take material from, or interfere with the habitat of any of the plants on this list.

Convention on Biological Diversity

Ireland is one of 188 parties to the Convention on Biological Diversity, which arose from the 1992 Rio Earth Summit. Ireland ratified the Convention in 1996 and the State is therefore committed to promoting the conservation and sustainable use of biodiversity. It has put in place a National Biodiversity Plan to provide a framework for the implementation of this commitment (Department of Arts, Heritage, Gaeltacht and the Islands, 2002).

EU Habitats Directive (92/43/EEC)

Under this legislation Ireland is required to designate Special Areas of Conservation (SAC) for the protection of biodiversity. This system is an effective means of plant species protection as many SACs are designated for the presence of rare and threatened plant species and many more species and populations are protected by default through the designation of their habitats. Appendix II of the Habitats Directive lists species whose conservation requires the designation of Special Areas of Conservation and four of the higher plants listed occur in Ireland. These are: *Trichomanes speciosum* Willd., *Saxifraga hirculus* L., *Najas flexilis* (Willd.) Rostk. & Schmidt, and *Luronium natans* (L.) Raf. Two bryophytes are also listed in Appendix II, the moss *Drepanocladus vernicosus* (Mitt.) Warnst. and the liverwort *Petalophyllum ralfsii* (Wilson) Nees & Gottsche ex Lehm.. The Habitats Directive was integrated into Irish Law by the European Union (Natural Habitats) Regulations, 1997, which specified the obligation of the Minister to propose a list of SACs for designation.

Bern Convention

Ireland is a contracting party to the Bern Convention on the conservation of European wildlife and natural habitats (Bern, 19.IX.1979). The aim of this convention is to protect European flora,

fauna and natural habitats, with particular attention given to species in decline or under threat. Appendix I of the convention lists strictly protected flora and includes 5 Irish higher plant species and two bryophytes. These are *Trichomanes speciosum*, *Luronium natans*, *Najas flexilis*, *Saxifraga hirculus, Zostera marina* L., the moss *Drepanocladus vernicosus* and the liverwort *Petalophyllum ralfsii*. The Bern Convention is implemented through the Wildlife and Countryside Act (1976 and amendments).

Plant Conservation Strategies

The European Plant Conservation Strategy was developed by Planta Europa and the Council of Europe to act as a template for plant conservation throughout Europe. It identifies 42 targets to be met by 2007 and has been accepted as a contribution to the Global Plant Conservation Strategy. The primary objective of the Global Strategy for Plant Conservation is to halt the decline in the world's plant diversity and it was adopted unanimously by the 187 governments present at the 6th Conference of the Parties to the Convention on Biological Diversity at The Hague, Netherlands in April 2002. It aims to achieve its ultimate objective through the implementation of 16 targets to be met by the year 2010. These targets include the cataloguing and conservation assessment of all known plant species, the protection of regions of high plant diversity, successful *ex situ* conservation of 60% of threatened species and the recovery and restoration of 10% of these, the development and implementation of management plans for alien species that threaten native plant diversity, the sustainable use of plants, increasing input into raising public awareness of plant diversity and its importance, increasing numbers of plant conservationists and strengthening national, regional and international networks for plant conservation.

Such strategies are important milestones in the implementation of the Convention on Biological Diversity and the framework that the Global Plant Conservation Strategy sets out allows all bodies involved in plant conservation to focus their activities, to produce similar, time-restricted, outcome-oriented targets and to collaborate in working towards specific contributions to the conservation of global plant diversity.

1.5.2 The Irish Red Data Book of Vascular Plants

The Red Data Book scheme is a globally-accepted method for the identification of species in need of conservation and as a result it is used by governments, conservation groups and scientific institutions alike to prioritise research activities, practical conservation measures and conservation legislation. The first Red Data Book, published by the IUCN (Lucas & Synge, 1978), identified 250 globally threatened plant species and this became the template for subsequent country-specific Red Data publications, aiming to identify and assess the conservation status of plant species threatened on a national level. The Irish Red Data Book I Vascular Plants (Curtis & McGough, 1988) was produced in response to a need for further information on the status of plant species on the 1980 Flora Protection Order and contains a detailed assessment of 142 plant species.

The criteria for inclusion of species in the Red Data book were based primarily on distributional data, and species were included if they occurred in ten, or less than ten, 10 kilometre squares, with respect to the National Grid; if they had declined by more than two thirds since 1970 based on this distribution, or if they were considered threatened in a European context. All species listed on the previous 1980 Flora Protection Order and all Northern Ireland Scheduled species were automatically included and also included were other plant species considered at the time of selection to be rare or threatened. A threat assessment was undertaken for each species and a score was given under each of the following categories: scale of localities, percentage decline since 1970, attractiveness, remoteness, accessibility, and habitat vulnerability. For each category, the higher the score, the greater the threat to the persistence of the species, with a score of zero implying no threat. An overall threat number (with a maximum of 14) was then derived for each species by summing the score in each category.

To aid the interpretation and comparison of these data between countries, a system was developed to translate the threat numbers into the internationally recognised IUCN threat categories. The threat numbers were divided into ranges and their relationship to the five IUCN categories is shown in Table 1.1 (taken from Curtis & McGough, 1988).

Table 1.1 The relationship of Irish Red Data Book threat numbers to IUCN Threat Categories

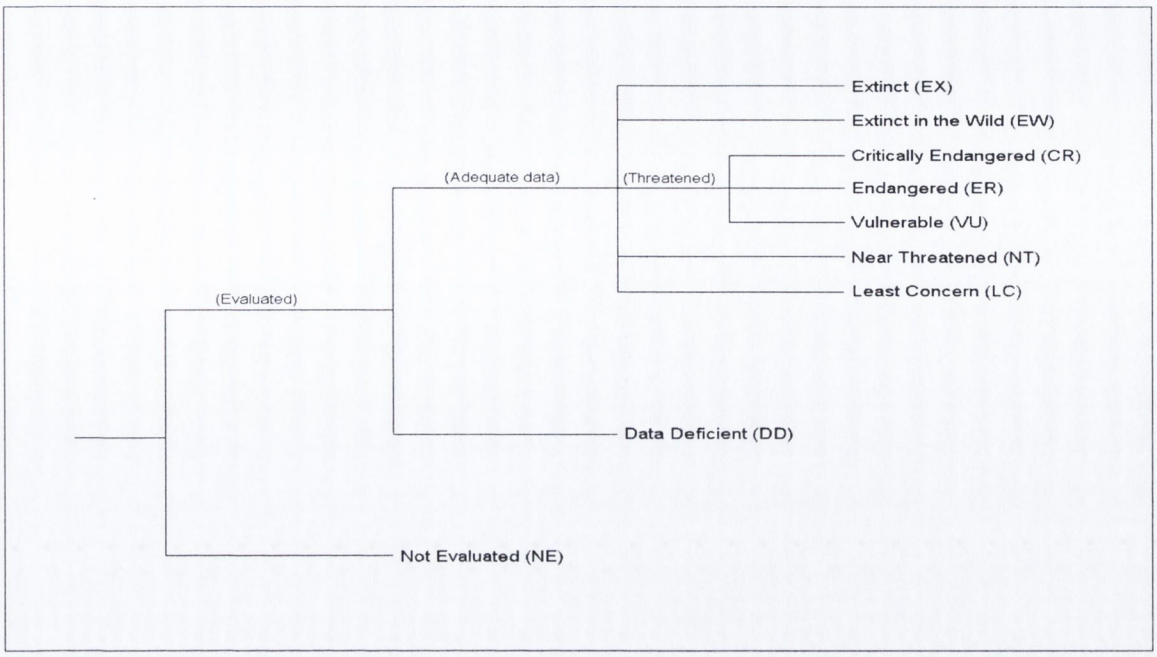
Threat Number Range (Red Data Book)	IUCN Threat Category	Abbreviation
12-14	Endangered	E
9-11	Vulnerable	V
8 or less	Rare	R
Indeterminate	Indeterminate	I
Extinct	Extinct	EX

The threat assessments detailed in the Irish Red Data Book of Vascular Plants formed the basis for the revision of the 1980 Flora Protection Order, leading directly to the publication of the 1987 Flora Protection Order. In addition to this, the specific threat assessments have allowed the funding for research to be focused on priority species and have influenced the proposal of areas for the designation as conservation areas. The Red Data Book has therefore been instrumental in the successful implementation of conservation legislation in Ireland, allowing the state to fulfil its commitment to the protection of biodiversity.

IUCN Threat Categories

Since the publication of the Irish Red Data Book of Vascular Plants in 1988, the IUCN threat categories have been revised and the direct comparison shown in Table 1.1 is therefore no longer applicable. The most recent IUCN Red List Categories and Criteria (2001), prepared by the IUCN Species Survival Commission, were approved by the 51st meeting of the IUCN Council, Gland, Switzerland on 9th February, 2000 and include a larger number of categories summarised in Figure 1.1. Although the categories apply on an international scale, they can also be applied at a local level to determine threat status of species within countries.

Figure 1.1 Structure of the 2001 IUCN Red List categories for threatened species.



Reproduced from IUCN (2001)

All taxa fit into at least one of the categories and for those with adequate data, there is a set of criteria that define which category they should be placed in. Taxa are always placed in the highest priority threat category so, for example, where a species fits the criteria for both Vulnerable and Endangered, it is classed as Endangered. The criteria are based on observed or projected reductions in population size and/or geographic range and also on mathematical predictions of extinction threat. Depending on the level of decline, taxa are assigned to one of the five threat categories in Figure 1.1. Critically Endangered is the highest priority threat category and any taxa in this category are '*considered to be facing an* ***extremely high*** *risk of extinction in the wild*' (IUCN, 2001). Endangered taxa are '*considered to be facing a* ***very high*** *risk of extinction in the wild*' and Vulnerable taxa are '*considered to be facing a* ***high*** *risk of extinction in the wild*' (IUCN, 2001). Taxa are classified as Near Threatened if they do not meet the criteria for these threat categories but are likely to qualify in the near future. Taxa are placed in the Least Concern category if they are not under direct or predicted threat and therefore do not meet the criteria for any of the other categories. Where there are insufficient data to assess and evaluate threat status, taxa are classed as Data Deficient, and taxa that have not yet been assessed are placed in the Not

Evaluated category. Taxa for which there is no reasonable doubt that no living individuals remain are classed as Extinct and taxa that are known only from cultivated specimens, or from captivity, or from naturalised populations well outside their past range are classified as Extinct in The Wild. The study species of this research fit into the 'Least Concern' category on an international scale but in Ireland they have a highly localised distribution centred on the Nore Valley in the southeast.

1.6 THE NORE VALLEY

1.6.1 Location

The valley of the River Nore is located in the south-eastern quarter of Ireland in the Province of Leinster. The river arises in northeast Tipperary, flows north-eastwards into County Laois and then turns southwards to travel the length of County Kilkenny before joining the River Barrow above New Ross in County Wexford. The waters enter the sea through Waterford harbour on the south coast (Figure 1.2).

1.6.2 Physical environment

The geological features of the Nore Valley are highly diverse. According To the Geological Survey of Ireland (1956) the headwaters arise in north-east Tipperary in Old Red Sandstone and are also fed from the Old Red Sandstone, Silurian and Lower Avonian Shales and Sandstones of the Slieve Bloom Mountains as the river travels into County Laois. From there, and for the majority of its course, the River Nore flows through Lower, Middle and Upper Carboniferous limestone, crossing only a small region of Upper Avonian Shales and Sandstones. At Thomastown, County Kilkenny, the river intersects narrow bands of Lower Avonian Shales and Sandstones, a Devonian Kiltorcan Bed (the highest beds of Old Red Sandstone) and a band of the main series of Old Red Sandstone. For the remainder of its course it flows through Ordovician rocks, having cut through a granite ridge immediately south of Inistioge. The river also crosses a region of igneous intrusive rock, poor in silica, before joining with the River Barrow north of New Ross. The combined waters continue to flow through Ordovician bedrock until they reach

Figure 1.2 Map showing the location of the River Nore. County boundaries are shown in black.

small regions of Igneous Volcanic rock rich in silica, and Old Red Sandstone, which occur on either side of the river as it flows down into the estuary emptying into Waterford Harbour on the south coast of Ireland (Geological Survey of Ireland, 1956).

The prevailing geology has influenced both the course of the River Nore and the features of the river valley, and while some sections meander through rich agricultural land, south of Kilkenny the river has cut through a granite ridge and is surrounded by a steep-sided gorge. The present steepness of the gorge is likely to be due to the discharge of glacial meltwater from the later ice sheets of the Midlands. However, the gorge was not initiated by glacial meltwater and the prevailing view is that the ridge of granite was slowly raised up into the path of the Nore, which cut a course through it as it rose (Mitchell & Ryan, 2001).

The climate varies along the course of the river but the rainfall in the East of Ireland is approximately 700mm (Mitchell & Ryan, 2001). The number of rain days per annum is less than 150 in the southern reaches and 150-175 nearer the source (Mitchell & Ryan, 2001). The accumulated temperature in day degrees per annum is in the range 2000-2500 for the majority of the Nore Valley, and only drops below 2000 near the source (Mitchell & Ryan, 2001). Although the general climatic conditions are similar throughout much of the river's course, there are many varied microclimates, related to the height and aspect of the valley sides and other physical features of the landscape.

1.6.3 Study species

The plant species that form the basis of this research are *Colchicum autumnale* L. (Colchicaceae) and *Campanula trachelium* L. (Campanulaceae). Both species have limited distributions in Ireland centred on the Nore Valley, both have suffered a decline in the last century, as revealed by distribution data held by the National Parks and Wildlife Service, and both are listed in the Irish Red Data Book of Vascular Plants (Curtis & McGough, 1988). *Colchicum autumnale* has been classified as 'Endangered' (IUCN Country (Irish) Category) on the basis of a threat assessment detailed in the Red Data Book and it is also protected under the Flora Protection Order (1999). It

is one of the six most threatened species in Ireland and its conservation is therefore of high priority. There has been much debate over whether it is native to the Irish landscape or whether it was introduced through monastic sources (Tom Curtis, pers. comm.), and although it is intrinsic to Irish natural and cultural heritage it is questionable whether scarce conservation resources should be allocated to the protection of a non-native species. The uncertainty over the native status of *Colchicum autumnale* in Ireland needs to be resolved to enable its conservation status to be effectively evaluated.

Campanula trachelium was categorised as Rare under the Red Data Book threat assessment methodology but is no longer listed under the Flora Protection Order due to the discovery of additional sites (Dr. Tom Curtis, pers. comm.). However, its distribution remains fragmented, possibly leaving the smaller populations susceptible to genetic drift and inbreeding depression with subsequent local extinctions.

Colchicum autumnale and *Campanula trachelium* were chosen as the study species as they occur in the same geographic locality in Ireland, allowing the efficient use of research resources. They also both present interesting questions relating to their conservation that would benefit from a biological study of their constituent populations.

1.7 AIMS

The overall aim of this research was to assess the Irish conservation status of *Colchicum autumnale* and *Campanula trachelium* through the collection, analysis and review of population data relevant to the survival of the species in Ireland. The main focus of the project was the conservation genetics of the species but aspects of ecology, reproductive biology and demography were also addressed where they were considered to complement the genetic data or to answer relevant questions outside the realm of population genetics. The philosophy behind the thesis was that the species are rare and/or declining because of one or a combination of factors operating on some aspect of species biology. An attempt was therefore made to identify the factors limiting the distribution of the species and to identify present and future threats to the populations and the

effects they might have on population growth and survival. Thus the objective of the thesis was to collect and consolidate biological information from many aspects of the species' biology and to utilise this information to construct effective conservation objectives for the species. Specific aims were to collect evidence pertaining to the native status of the species in Ireland, to estimate and monitor population size, to investigate the reproductive output of the populations, to assess the level and partitioning of genetic diversity in Irish and other European populations and where possible to investigate biogeographical relationships between populations.

The precise aims for each species will be stated in the relevant chapters.

CHAPTER TWO

METHODS - CONSERVATION GENETICS

2.1 SAMPLING

To investigate the comparative levels of genetic diversity between populations in a taxon, a large number of populations must be sampled (Kay and John, 1997). Populations from across the range of the species should also be included in the analysis to enable the results to be placed in the context of the species as a whole. Samples were therefore collected from as many Irish populations as possible and from populations in at least two other countries in the European range of the study species.

To enable the results to be used in obtaining genetic statistics for the populations, sampling within populations was carried out randomly. Without the random sampling strategy, samples would violate the assumptions of the population genetic analyses. When collecting leaf material, care was taken to avoid diseased leaves, leaves with aphid infestations and leaves covered with organic matter as the collection of material in this state may lead to contamination of the extracted DNA with fungal or invertebrate DNA. Samples were placed into sample bags containing Type III indicating silica gel for rapid desiccation of the leaf material. This preservation method was chosen as the large numbers of samples involved made extraction from fresh material inviable. DNA was extracted from the samples within one year of collection.

2.2 DNA EXTRACTION

List of abbreviations used in this section:

ATP	Adenosine triphosphate	**HCl**	Hydrochloric acid
BSA	Bovine serum albumen	**$MgCl_2$**	Magnesium chloride
CTAB	hexadecyltrimethylammonium bromide	**NaCl**	Sodium chloride
CI	24:1 chloroform:isoamyl alcohol	**PCR**	Polymerase Chain Reaction
DTT	Dithiothreitol	**Tris**	tris(hydroxymethyl)aminomethane
EDTA	Ethylenediaminetetraacetic acid		

Note: The chemical constituents of buffers and solutions referred to in the text are listed at the end of each section.

DNA extraction was carried out using a protocol modified from the CTAB extraction method of Doyle and Doyle (1987). 10ml of 2X CTAB buffer was added to re-usable 30ml centrifuge tubes, which were then preheated to 65°C in a water bath. 40µl of mercaptoethanol was added to each tube immediately prior to use. 0.15g of silica gel-dried plant material was ground using a pre-heated pestle and mortar with a small amount of the 2X CTAB buffer. The remainder of the buffer was added slowly and the mixture ground further until no visible leaf fragments remained. The liquid was swirled to suspend the slurry, poured back into the centrifuge tube and incubated for 10 minutes at 65°C with occasional mixing. Following this, 10ml of CI was added to each tube. The tubes were then inverted, and the caps were subsequently loosened to release the gas. The tubes were placed on a shaker for 30 minutes to gently mix the contents and then centrifuged at 4000g (rcf) on an MSE Harrier 15/80 centrifuge for 10 minutes. The uppermost aqueous layer containing the DNA was transferred to a universal specimen tube and an equal volume of isopropyl alcohol was added. The isopropyl alcohol had been stored in a –20°C freezer and the sample tubes were then placed into the freezer for at least 24 hours to allow the DNA to precipitate. Following this, samples were centrifuged at 2000g (rcf) for 10 minutes in an MSE Harrier 15/80 centrifuge to pellet the DNA. The supernatant was drained off and 3ml of wash buffer (70% ethanol) was added to the tubes and gently mixed. Samples were centrifuged once more at 2000g (rcf) for 5 minutes to re-pellet the DNA and the supernatant was drained off. The tubes were placed upside down on paper towels for 10 minutes to drain off excess ethanol and then placed into a fume cupboard for 30 minutes, or until the ethanol had evaporated. The DNA pellets were then suspended in 0.5ml of TE buffer, transferred into labelled 1.5ml centrifuge tubes and stored at -20 °C.

Reagents

2X CTAB extraction buffer: 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB (w/v). The buffer has a pH of approximately 8 and was pre-heated to 65°C before use so that the optimum conditions for DNAases and other degradative enzymes were avoided. The components of the buffer have various functions: CTAB solubilises the plant membranes and binds with the

DNA, EDTA inhibits the action of metal-dependent enzymes and mercaptoethanol limits the effects of polyphenols, quinones and tannins.

TE buffer: 10mM Tris-HCl pH 8.0 (Tris base brought to pH 8.0 with HCl), 1mM EDTA.

2.3 DNA Purification

The extracted DNA was purified and concentrated using the Gibco BRL ® Concert™ Rapid PCR Purification System. 100µl of the extracted DNA was placed in a 1.5ml tube. 400µl binding solution, containing concentrated guanidine hydrochloride, EDTA, Tris-HCl and isopropanol was added to each sample, these were mixed thoroughly by pipetting and then loaded into a spin cartridge with a 2ml collection tube. The samples were centrifuged at 13,000g (rcf) for 1 minute in an MSE Micro Centaur microcentrifuge, the flow-through was discarded and the spin columns were returned to the collection tube. 700µl wash buffer, containing NaCl, EDTA and Tris-HCl was loaded into the spin cartridges and these were centrifuged once again at 13,000g for 1 minute. The flow-through was discarded and the samples were centrifuged at 13,000g for 1 minute to remove all residual wash buffer. The spin cartridges were placed in individual 1.5ml tubes and 50µl TE buffer pre-heated to 65°C was added directly to the centre of each spin cartridge. The cartridges were incubated at room temperature for one minute and then centrifuged at 13,000g for 2 minutes. The suspended DNA was stored at -20 °C until use.

2.4 DNA Quantification

DNA was quantified using an Eppendorf Biophotometer set at a wavelength of 260nm. The dilution was set at 5µl sample to 95µl diluent and a blank sample containing 5µl TE buffer and 95µl ultra-pure water was used to set a zero reference reading. Samples were quantified by adding 5µl of the stock DNA (suspended in TE) and 95µl ultra-pure water to 220-1600nm Eppendorf tubes, which were placed within the chamber. Light with a wavelength of 260nm was passed through the sample and the readout gave DNA concentration in ng/µl (equivalent to µg/ml).

2.5 AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS

Amplified Fragment Length Polymorphism (AFLP) is a multi-locus genetic fingerprinting technique that combines restriction digestion with two selective PCRs to produce a large number of variable genetic markers. These are bi-parentally inherited and dispersed throughout the genome (Ouborg *et al,* 1999). The technique was developed by Vos and co-workers (Vos *et al.*, 1995) and it has since become an important analytical tool in conservation biology as it does not require *a priori* knowledge of the genome of the target species and requires only a small amount of DNA (typically 250ng). This is important in the study of threatened plant species where the availability of plant material may be limited (Qamaruz-Zaman *et al.*, 1998) and where sampling needs to be carried out without serious adverse affects on the growth and development of the plant.

A schematic diagram of the three-stage AFLP process is shown in Figure 2.1 and the full sequences of the EcoRI and MseI adaptors and the pre-selective and selective primers are given in Appendix I. The first stage of the process involves the digestion of total DNA with two restriction enzymes, a frequent cutter (MseI) and a rare cutter (EcoRI). The ligation of adaptor sequences onto the cut ends of the DNA is carried out in the same reaction and these provide a recognition sequence for the primers in the subsequent selective PCRs. The ligation step does not re-create the restriction enzyme recognition site and therefore does not affect the restriction reaction. It is also advantageous to combine the two steps in the same reaction as addition of the adaptor pairs to the cut ends ensures that artefacts are not produced by the linkage of two cut fragments.

The second stage is a pre-selective PCR designed to reduce the number of fragments for selective amplification. The EcoRI primer recognises fragment ends with the EcoRI adaptor sequence and adjacent guanine then thymine bases, and the MseI primer recognises fragment ends with the complementary MseI adaptor sequence and adjacent thymine then guanine bases. Fragments with sequences matching that of the pre-selective primers at each end are amplified.

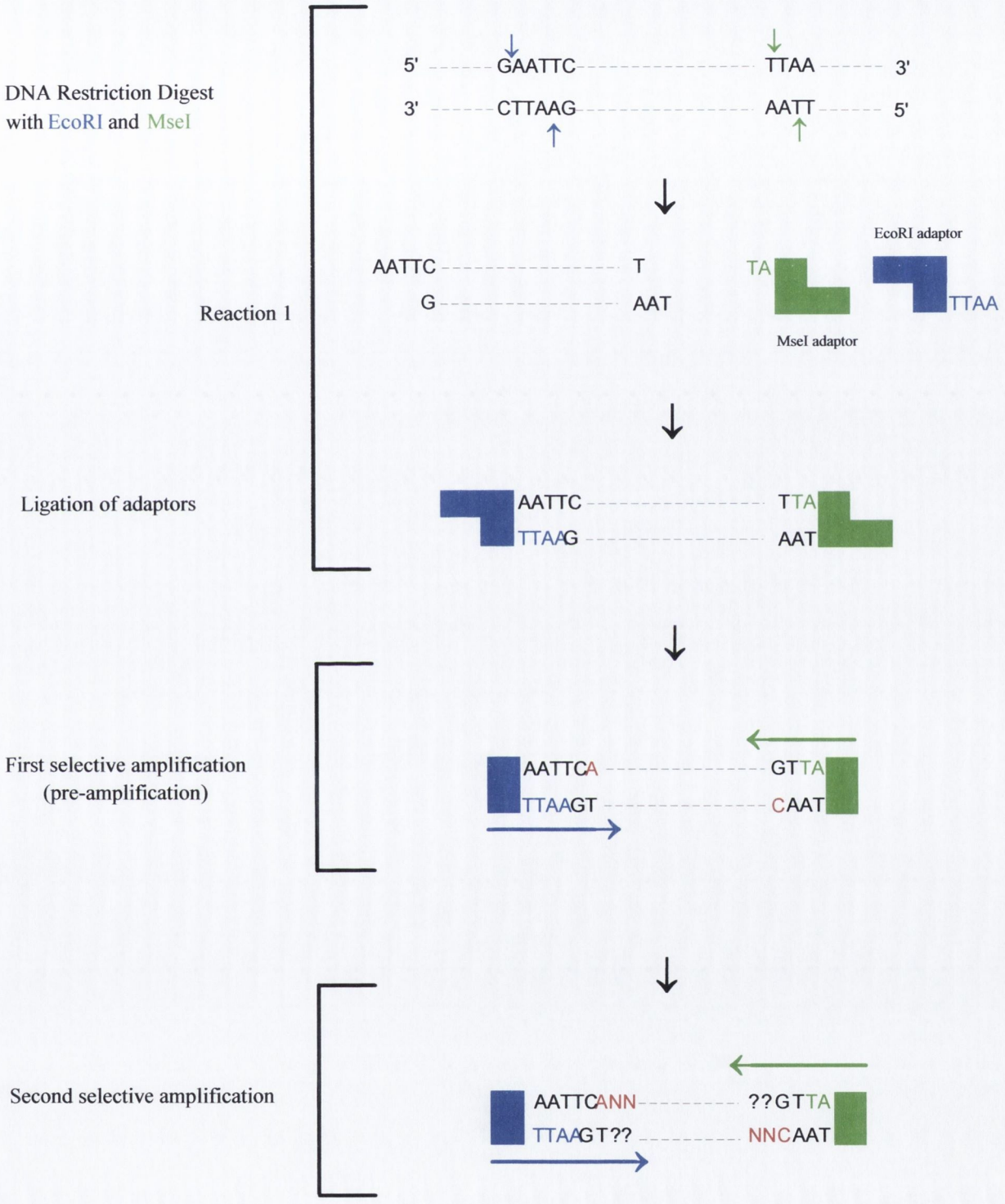


Figure 2.1 Schematic representation of the AFLP genetic fingerprinting technique

Letters in red are the same as the selective base sequence of the pre-selective/selective primers

N represents a base that can be varied by the experimenter during primer trials.

The complementary bases will depend on this choice and are therefore indicated by question marks

Arrows represent the direction of the primers

The final step involves the use of primers with an additional sequence of two or more bases for further reduction of fragments. The primers used in this study contain two additional selective base-pairs. Primer pair combinations are chosen from a set of 16 primers in the Applied Biosystems AFLP I primer kit so there are 64 possible combinations. The available MseI and EcoRI selective sequences are listed below:

Mse I primer (end sequence): CAA, CAC, CAG, CAT, CTA, CTC, CTG, CTT

Eco RI primer (end sequence): AAC, AAG, ACA, ACC, ACG, ACT, AGC, AGG

Due to the nature of the initial restriction enzymes, the final products consist predominantly of fragments with double MseI ends with a smaller number of fragments with one EcoRI end and one MseI end. However, it is the EcoRI primers that are radioactively or fluorescently-labelled, so only fragments with both adaptors present are visualised. The result is a multi-locus fingerprint that can be resolved on a gel or an automated sequencer and scored for the presence or absence of fragments of a particular size.

2.5.1 Reaction protocols

All AFLP reactions were carried out with the use of Applied Biosystems AFLP I kits. When making master mixes, 2 extra volumes were added to allow for pipette error; 'n' is therefore the number of DNA samples to be analysed plus two. In the PCR reactions all ramping temperatures were set at 1°C per second. Negative and positive controls were used in each set of reactions; a negative control, using sterile water in place of DNA, was used to detect contamination and a positive control, consisting of a DNA sample known to produce a clear and consistent fingerprint, was used to account for reaction failure and to allow comparison of fingerprints from different reactions.

Reagents

T4 DNA Ligase buffer: 300 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM DTT and 10 mM ATP.

AFLP Amplification core mix: Applied Biosystems AFLP I amplification core mix containing buffer, nucleotides and Amplitaq® DNA Polymerase.

$TE_{0.1}$ buffer: 2ml 1M Tris-HCl (pH8), 40µl 0.25M EDTA, 97.96ml H_2O.

Restriction-Ligation

0.25µg genomic DNA was added to labelled 0.2ml PCR tubes and the volume was made up to 2.75µl with ultra-pure water. The tubes were placed in racks over ice until required. Annealing of the adaptor pairs was then carried out by adding the total required volume of each of the adaptor pairs (0.5n µl) to two separate 0.2ml tubes. These tubes were heated at 95°C for 5 minutes and allowed to cool over the next 10 minutes. While the adaptor pairs were cooling, master mixes were made up allowing for 2 extra samples. Master mix 1 was prepared by adding the following amounts per reaction to a 0.2ml tube:

0.5 units T4 DNA Ligase
0.05µl 10X T4 DNA ligase buffer with ATP
0.05µl 0.5M NaCl
0.025µl 1mg/ml BSA
0.5 units MseI
2.5 units EcoRI.

The total volume of the master mix was then made up to 0.5n µl with ultra-pure water, mixed thoroughly by tapping, spun down by pulsing to approximately 3000g in an MSE MicroCentaur microcentrifuge and stored on ice. Master mix 2 was then prepared by adding the following amounts per reaction to a 0.5ml tube:

0.5µl 10X T4 DNA ligase buffer with ATP
0.5µl 0.5M NaCl
0.25µl 1mg/ml BSA

The MseI and EcoRI adaptor pairs and Master mix 1 were added to Master mix 2, which was then mixed thoroughly by tapping and spun down by pulsing to approximately 3000g in an MSE Micro Centaur microcentrifuge. 2.75µl of this combined mix was added to each 2.75µl sample of DNA. The tubes were mixed by tapping, spun down in a microcentrifuge and then incubated at 37°C for 2 hours. After this time, the products were diluted by adding 94.5µl $TE_{0.1}$ buffer to each reaction and tapped to mix. The restriction products were stored at 4°C until the next stage or for long-term storage were placed in a -20°C freezer.

Pre-selective amplification

1µl of the diluted restriction-ligation products were added to new labelled 0.2ml PCR tubes and placed in a rack over ice. A master mix was prepared containing 0.25µl per reaction of pre-amplification primers and 3.75µl per reaction of AFLP core mix. This was mixed by tapping and spun down in a microcentrifuge. 4µl of the master mix was added to each tube of restriction products making a total reaction volume of 5µl. The tubes were then placed in an MJ Research PTC200 Peltier Thermal Cycler and run through the following sequence:

HOLD	2 mins at 72°C		
CYCLE x 20	1 sec at 94°C	30 secs at 56°C	2 mins at 72°C
HOLD	30 mins at 60°C		
HOLD	4°C forever		

Pre-selective amplification products were stored at 4°C.

Selective amplification

Three new sets of PCR tubes were labelled per sample to enable the reactions to be carried out with three different fluorescently-labelled primers. In each pair of primers, the primers that are complementary to the Eco RI sequence have been labelled at the 5' end with blue (FAM-labelled), green (JOE-labelled) or Yellow (NED-labelled) fluorescent dyes. This fluorescent tagging enables the fragments to be detected by laser in an automated sequencer.

1.5µl of diluted pre-selective products were added to each set of tubes, which were then placed on ice. The following master mix was prepared for each primer pair with volumes given per sample:

0.5µl MseI primer at 5µM
0.5µl EcoRI primer at 1µM
7.5µl AFLP core mix

The three master mixes for the FAM-labelled, JOE-labelled and NED-labelled primer pairs were mixed thoroughly by tapping and spun down in a microcentrifuge. 8.5µl of each master mix was added to one set of the 2µl diluted pre-selective amplification products and these were mixed by tapping and spun down in a microcentrifuge. The tubes were then placed in an MJ Research PTC200 Peltier Thermal Cycler and run through the following sequence:

No. Cycles			
1	2 mins at 94°C	30 secs at 65°C	2 mins at 72°C
1	1 sec at 94°C	30 secs at 64°C	2 mins at 72°C
1	1 sec at 94°C	30 secs at 63°C	2 mins at 72°C
1	1 sec at 94°C	30 secs at 62°C	2 mins at 72°C
1	1 sec at 94°C	30 secs at 61°C	2 mins at 72°C
1	1 sec at 94°C	30 secs at 60°C	2 mins at 72°C
1	1 sec at 94°C	30 secs at 59°C	2 mins at 72°C
1	1 sec at 94°C	30 secs at 58°C	2 mins at 72°C
1	1 sec at 94°C	30 secs at 57°C	2 mins at 72°C
23	1 sec at 94°C	30 secs at 56°C	2 mins at 72°C
HOLD	30 mins at 60°C		
HOLD	4°C forever		

Selective amplification products were stored at 4°C.

2.5.2 Analysis

The AFLP fingerprints were detected and visualised using an ABI prism 310 Genetic Analyser. The selective amplification products from the 3 primer pairs were combined in a 0.5ml tube in the following amounts:

FAM-labelled – 0.6µl
JOE-labelled – 0.8µl
NED-labelled – 1.3µl

40µl of formamide and 0.3µl of ROX size standard were added to the tubes (ROX size standard contains DNA of a known sequence of sizes, labelled on a single strand with ROX NHS-ester dye to allow laser detection). The samples were incubated at 95°C for 5 minutes to denature the DNA strands and then placed immediately on ice. The samples were spun down, the tube lids were removed and replaced with rubber septae and the tubes were then placed on ice until ready to load onto the ABI 310 Genetic Analyser. The electrical current was applied to each sample for a duration of 26 minutes.

ABI Prism 310 Collection Software

This software controls the ABI Prism genetic analyser, monitors the progress of the run and collects the raw data.

310 Genescan® 3.1.2

The Genescan program processes the raw data from the ABI Prism 310 Collection software by sizing and quantifying the DNA fragments and converting the output from the sequencer into electropherograms of fragment size against band intensity. There are a number of support files associated with the software. There are matrix files, consisting of mathematical matrices that allow any spectral overlap of fluorescent emissions data to be corrected for, a size standard file containing size standard data that enables the sizing of fragments, and an analysis settings file containing information on how the algorithm processes the data. The settings contained in this file are determined by the user.

Genotyper® 2.5.2

Genotyper® 2.5.2 is a genetic data analysis package that provides a user interface to allow lane-by-lane comparison of results and facilitate band scoring, whether by automatic or manual scoring methods. An example of the plot window with labelled peak sizes is shown below in Figure 2.2. The four samples shown are derived from the same individual, but were run in four separate AFLP reactions and were run separately on the ABI Prism Genetic Analyser. The figure therefore illustrates the reproducibility of the technique.

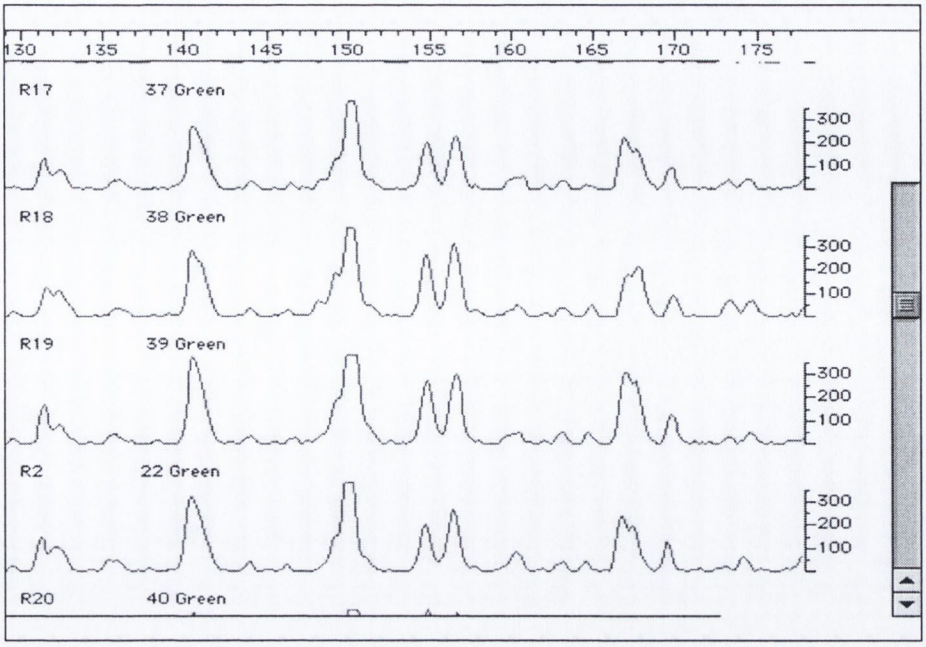


Figure 2.2 Example of the output from the Genotyper™ software package. The length of the fragment in base pairs is given in the scale at the top of the output.

Band scoring

The scoring of bands was carried out by manual inspection of the Genotyper output with peaks labelled by size. Initially, a macro for generating tables of presence and absence of different size fragments was used but this was found to be highly inaccurate and was therefore discarded. The inaccuracies resulted from the incorrect classification of fragments falling close to the demarcation limits of the size classes so that, for example, a fragment with a size of 12.49 was classified as being different from a marker with a size of 12.51. The scoring of bands was therefore done manually.

Genescan sample files were imported into Genotyper and to eliminate subjectivity in the scoring method, samples were referenced only with a number so that their origin was not immediately identifiable. Size standards were checked for consistency and the peaks were then labelled with fragment size to aid scoring. The size of fragments was rounded up or down to the nearest whole number of base pairs and a zero-one matrix was constructed based on the absence or presence of markers in the samples. The default Genotyper setting does not label peaks below a fluorescence intensity threshold of 50 but in this analysis, peaks of low intensity were scored as present if they

could be unambiguously distinguished from the background signal. This is justified if the overall signal is weaker for a particular sample but it can be seen that the banding pattern is the same despite the low intensity. This approach has also been followed in other studies using AFLPs (eg. Tribsch *et al.*, 2002). Positive and negative controls were used in each set of reactions and these were checked for anomalies. Any sets of reactions where the negative controls contained bands were rejected and the samples were re-run. When scoring was complete, the sample numbers were cross-referenced with the run sequence of samples and were replaced with the relevant sample identification codes to allow analysis and interpretation.

2.5.3 Primer selection

The number and quality of bands produced by AFLP analysis varied with the primer combination used. There are 64 possible combinations of primers that can be used in the selective amplification reaction and some preliminary work was carried out to select three primer combinations for the final analysis. A sample of ten individuals from a selection of populations covering a wide geographic spread was used to test 24 primer pairs in eight separate AFLP analyses. Primers were selected on the basis of the quantity and quality of the bands produced, with the optimum primers yielding a large number (over 50) of clearly differentiated bands with a high intensity. The samples used in the primer trials were labelled with arbitrary numbers and not run in any significant sequence to avoid the selection of primers being influenced by preconceptions of the structuring of populations. Different primers were selected for each species and these are described in the relevant chapters.

2.6 Analytical Theory and Techniques

2.6.1 General assumptions

There are a number of fundamental assumptions associated with the use of dominant molecular markers for the genetic description of populations. These are described by Stewart and Excoffier (1996) and are listed below:

- the banding pattern is interpreted without error.
- each band represents a two-allele locus where the present band is dominant over its absent counterpart.
- only one allele at a locus is amplifiable so that each band represents a different locus.
- there is no co-migration of bands.
- loci are independent.
- genotypic frequencies are in Hardy-Weinberg equilibrium at each locus.

The final assumption has the potential to greatly influence the outcome of all analyses of population genetic structure and is therefore of fundamental importance. Populations are in Hardy-Weinberg equilibrium if random mating maintains allele frequencies at constant levels from one generation to the next and this is often the case in large outbreeding populations (Frankham *et al.*, 2002). However, small inbreeding populations, or populations with a large degree of immigration, may exhibit a departure from the Hardy-Weinberg equilibrium and there is no way of evaluating this in the AFLP analysis of a single generation (Lynch & Milligan, 1994). Various corrections exist to account for the occurrence of inbreeding but this can only be assessed over multiple generations and unless prior research has been carried out on inbreeding levels within a species, it must be assumed that the populations are in Hardy-Weinberg equilibrium. This is therefore a limitation of the use of dominant genetic markers to describe population genetic structure, but Bayesian approaches are being developed that do not rely on this assumption and incorporate uncertainty about the magnitude of inbreeding (eg. Holsinger *et al.*, 2002)

2.6.2 Estimation of allele frequencies

The presence/absence matrices derived from the banding patterns of the genetic fingerprint prevent a distinction from being made between dominant homozygotes and heterozygotes at individual loci, as both genotypes will result in the amplification of DNA. The use of dominant genetic markers in the description of population genetic structure therefore involves an initial estimation of allele frequencies. Various estimation techniques are in use to extract allele frequencies from dominant data and the accuracy of the genetic parameters and relationships

inferred from the data can vary depending on which approach is used. All approaches are based on statistical distributions and the accuracy of the resulting frequency estimates is entirely dependent on the accuracy with which the estimated distribution fits the real population genetic structure.

The first solution to the problem was to use the square root procedure whereby the frequency of the null allele at an individual locus is taken to be the square root of the frequency of the null AFLP marker (Stewart & Excoffier, 1996). This was the most widely-used method among RAPD studies but is statistically biased, leading to a downwardly biased estimate of the null allele frequency and highly inaccurate estimates of genetic parameters (Zhivotovsky, 1999). This was recognised by Lynch and Milligan (1994) who proposed an estimation procedure for the analysis of RAPD data based on the transformation

$$\hat{q} = \hat{x}^{1/2}\left(1 - \frac{Var(\hat{x})}{8\hat{x}^2}\right)^{-1}$$

where q is the estimated frequency of the null allele, and x is the observed frequency of null homozygotes. The sampling variance of the frequency of null homozygotes is given by

$$Var(\hat{x}) = \frac{\hat{x}(1-\hat{x})}{N}$$

and the sampling variance of the allele frequency is approximated by

$$Var(\hat{q}) = \frac{1-\hat{x}}{4N}$$

The statistical basis of the transformation led Lynch and Milligan to suggest that loci where the band frequency is greater than 1-(3/N) should be removed from the analysis. This reduces the statistical bias of the estimation procedure but as it selects against loci with low frequencies of null alleles it still leads to biased estimates of genetic variation and genetic distance. For N=10,

this involves the removal of markers whose incidence is less than 0.7. The results are also sensitive to sample size (Zhivotovsky, 1999).

A third estimation technique was introduced by Zhivotovsky (1999), based on a Bayesian approach. This method gives almost unbiased estimates of allele frequency and is therefore the preferred method for the derivation of genetic diversity statistics. The Bayesian approach does not select against loci with low frequencies of null alleles and is therefore a more accurate estimator of the true population genetic parameters. It is also a more robust method, as illustrated by the case where there are no null homozygotes in the sample. In this case, the square root estimator gives zero as the null allele frequency even though null alleles are present in heterozygote individuals. The Lynch and Milligan approach is also inadequate as the estimating equation cannot be resolved due to the presence of a zero denominator (see above). The Bayesian method yields estimates that are intermediate between those of the square root transformation and the Lynch and Milligan transformation, and is a more balanced approach with less inherent bias (Zhivotovsky, 1999).

The accuracy of the three estimation procedures for null allele frequencies was assessed by Krauss (2000). Using data from the analysis of 116 polymorphic AFLP loci in 21 individuals of the outcrossing angiosperm, *Persoonia mollis* R.Br. (Proteaceae), he showed that mean heterozygosity, as estimated by the square root transformation, the Lynch and Milligan procedure and the Bayesian estimate were very similar, producing accurate estimates of observed average heterozygosity. He therefore concluded that even when the number of individuals sampled is small, the biases involved in the estimation of null allele frequencies are negligible if a large number of polymorphic loci are used in the analysis.

2.6.3 Measures of genetic diversity

Population genetic variation can only be quantified exactly by the analysis of all structural loci present within all individuals (Nei, 1987). As the number of individuals within a population and also the number of loci per individual can be in the order of tens of thousands, the genetic

variability of a population must be estimated by sampling a sub-set of individuals and a sub-set of loci. The genetic data obtained from such analyses can be processed in a number of different ways and the genetic variability of a population can therefore be expressed using a variety of measures. The simplest measure of genetic variability is the percentage of polymorphic loci in the population. This is a useful statistic but should not be considered in isolation, as it is highly susceptible to sampling error with respect to the number of individuals and the number of loci sampled. It is also not considered to be a robust measure due to the assignment of an arbitrary cut-off point in the criterion for a polymorphic locus, which is usually defined as a locus where the frequency of the most common allele is equal to or less than 0.99. As a result of these sources of error and ambiguity, the percentage of polymorphic loci is only used in conjunction with other population genetic statistics.

Heterozygosity, another measure of genetic diversity is defined in terms of gene frequencies and is therefore a more robust measure. The heterozygosity for a single locus is given as

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

where x_i^2 is summed over i=1 to i=m, m is the number of alleles and x_i is the frequency of the ith allele at a locus. Average heterozygosity, H, is the average of h over all loci and therefore describes the average proportion of heterozygotes per locus for a randomly mating diploid population in Hardy-Weinberg equilibrium. It also describes the expected proportion of heterozygous loci in a randomly chosen individual from a population.

The most widely-used genetic diversity measures are Nei's (1972, 1973 and 1978) gene diversity statistics. Gene diversity is also a measure of heterozygosity but it is derived from the probability that two randomly chosen alleles from a population are different (Nei, 1973) and can therefore be applied to haploid, diploid or polyploid species.

For gene diversity, allele frequency, x_i, is estimated by:

$$x_i = X_{ii} + \sum X_{ij}/2$$

where $j \neq i$. The sample gene diversity is then calculated in the same way as heterozygosity above using the estimate of allele frequency.

It should be noted that the equation $\mathbf{h = 1-\sum x_i^2}$ is a biased estimator of heterozygosity and was therefore modified to give an unbiased estimate of heterozygosity / gene diversity (Nei, 1973), which is the measure used throughout this thesis. It is given by

$$\hat{h} = 2n(1-\sum x_i^2) / (2n-1)$$

The average gene diversity (H) is estimated by the sampling of loci and is therefore given by

$$\hat{H} = \sum_{j=1}^{r} \frac{\hat{h}j}{r}$$

where r is the number of loci sampled and $\hat{h}_j$ is the value of $\hat{h}$ for the jth locus.

Total gene diversity can be partitioned into within and between population diversity by expressing the total population diversity estimate H_T in terms of its components, within-population diversity, H_S and between population diversity, D_{ST}.

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

Gene diversity between populations can then be expressed as a proportion of the total population diversity to give G_{ST}, a measure of population differentiation referred to as the co-efficient of gene differentiation.

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

Finally, the proportion of gene diversity within populations can be calculated as $1 - G_{ST}$. Nei also describes the derivation of gene diversity statistics for the analysis of subdivided populations (Nei, 1973;1987).

There are a number of variations in the estimation of heterozygosity that attempt to reduce the bias that is inherent in the use of dominant markers. In particular, the model developed by Lynch and Milligan (1994) for the estimation of null allele frequency was extended to the estimation of heterozygosity and is widely used in population genetics. It is based on Nei's conventional measure of gene diversity (Nei, 1972) and therefore describes the probability that a random pair of alleles will consist of a marker and a null allele. The asymptotically unbiased estimator of gene diversity is described by Lynch and Milligan (1994) as:

$$\hat{H}_j(i) = 2\hat{q}_j(i)\left[1 - \hat{q}_j(i)\right] + 2Var[\hat{q}(i)]$$

where Var[q(i)] is the sampling variance of the null allele frequency at the ith locus. $\hat{H}_j(i)$ is the gene diversity of population j at the ith locus, or the probability that two genes drawn at random from population j will differ at the ith locus. Its sampling variance is approximately:

$$Var\left[\hat{H}_j(i)\right] = 4\left[1 - 2\hat{q}_j(i)\right]^2 Var\left[\hat{q}_j(i)\right]$$

The mean observed gene diversity in the jth population is given by averaging over all loci, where L is the total number of loci:

$$\hat{H}_j = \frac{1}{L}\sum_{i=1}^{L}\hat{H}_j(i)$$

Where n populations have been sampled an estimation of mean within-population diversity is given by:

$$\hat{H}_w = \frac{1}{n}\sum_{j=1}^{n}\hat{H}_j$$

The sampling variance of this quantity is a function of the variance associated with the sampling of populations, individuals and loci. Lynch and Milligan (1994) extend this model to describe gene diversity between populations, the probability that a gene drawn randomly from population j

differs from a gene drawn randomly from population k at the ith locus. An asymptotically unbiased estimator of this quantity is given by:

$$\hat{H}_{jk}(i) = \hat{q}_j(i) + \hat{q}_k(i) - 2\hat{q}_j(i)\hat{q}_k(i)$$

However, where there is no population subdivision the gene frequencies are the same in all populations and Lynch and Milligan (1994) therefore defined gene diversity between populations in terms of the heterozygosity in excess of that observed within populations, given by:

$$\hat{H}_{jk}(i) = \hat{H}_{jk}(i) - \frac{\hat{H}_j(i) + \hat{H}_k(i)}{2}$$

The mean gene diversity between populations j and k is then given by averaging this quantity over all L loci.

$$\hat{H}_{jk} = \frac{1}{L}\sum_{i=1}^{L} \hat{H}_{jk}(i)$$

The mean between-population gene diversity averaged over all distinct pairs of populations is then given by:

$$\hat{H}_B = \frac{2}{n(n-1)}\sum_{j<k} \hat{H}_{jk}$$

The sampling variance of $\hat{H}_B$ accounts for the sampling of populations, individuals and loci and the full treatment of all these estimates and their variances can be found in the original paper (Lynch and Milligan, 1994).

Other measures of diversity are based on statistics that have been widely used to describe phenotypic variation, such as Shannon's diversity index, H, calculated by

$$H = - \sum p_i \ln p_i,$$

where p_i is the frequency of the ith polymorphic marker (Shannon and Weaver, 1949).

2.6.4 Distance measures

The most commonly used measure of genetic distance is that of Nei (1972). It is derived from his expression of gene identity, I (equivalent to 1-gene diversity) and is given by

$$D = -\ln I$$

Genetic identity is estimated by

$$I = \frac{\sum x_i y_i}{\sqrt{\sum x_i^2 \sum y_i^2}}$$

where x_i and y_i are the frequency of the ith allele at a locus in individual x and y respectively. This parameter is summed over all loci. There is also an unbiased version for use when sample size is small and this subtracts unity from each of the estimates in both the numerator and denominator (Nei, 1978).

A further method for deriving genetic distances between individuals is based on the estimation of the number of nucleotide substitutions that have occurred since the individuals have diverged. The similarity of the individuals is given by:

$$S = 2n_{xy} / (n_x + n_y)$$

where n_{xy} is the number of identical sites shared by the two individuals and n_x and n_y are the total number of restriction sites in individuals x and y respectively (Nei & Li, 1979). This method can be used to analyse data from genetic fingerprinting using dominant markers if it is assumed that the presence or absence of bands is a result of a difference in the restriction site.

In traditional distance measures, shared absence is given equal status to shared presence and this obscures the determination of true genetic distance (Legendre & Legendre, 1998). The Sørenson-Dice measure of similarity accounts for this by giving more weight to shared presence. It is calculated by:

$$\text{Similarity} = 2a/\ 2a+b+c$$

where a is the number of shared fragments and b and c are the numbers of fragments that are unique to either of the two individuals (Legendre & Legendre, 1998).

Finally, the construction of phylogenetic trees is often carried out using Euclidean distance or mean character difference to construct a distance matrix.

2.6.5 Population differentiation

The degree of genetic differentiation between populations can be described using F-Statistics, which describe the variation in estimated gene frequencies between populations. The calculations were first developed by Wright (1921) to quantify the effect of reduced heterozygosity arising from the subdivision of populations and they involve the comparison of heterozygosity in different levels of population hierarchy with respect to each other. The statistics obtained from these comparisons have since been called fixation indices and are used to assess levels of differentiation and gene flow between populations and sub-populations. The fixation index is described by the general formula:

$$F = (H_1-H_2) / H_1$$

where H_1 denotes heterozygosity in the larger of the two hierarchical levels and H_2 denotes heterozygosity in the smaller of the two. For example, F_{ST} measures overall divergence and describes the difference in heterozygosity between the most and least inclusive levels of population hierarchy, expressed as a proportion of the heterozygosity in the total population.

$$F_{ST} = (H_T-H_S) / H_T$$

Similar comparisons can be made between regional aggregates and the total combined population or between sub-populations and regional aggregates (Hartl & Clark, 1997).

F_{ST}, the most inclusive form of F-statistic, measures overall genetic divergence between sub-populations and has a theoretical range of 0 to 1, from no genetic divergence to fixation of

different alleles in different subpopulations (Hartl & Clark, 1997). Wright (1978) divided this range into a number of categories to aid interpretation of fixation indices and suggested that 0-0.05 indicates little genetic differentiation, 0.05-0.15 indicates moderate differentiation, 0.15-0.25 indicates a great level of differentiation and values above 0.25 indicate very great levels of differentiation (in Hartl and Clark, 1997). Although these categories act as a guide to the interpretation of the magnitude of F-statistics, they may not apply equally to all levels of population hierarchy and an F_{ST} value of 0.05 among sub-populations cannot be considered to indicate a negligible degree of differentiation (Wright, 1978).

Sources of error in the calculation of F statistics to estimate genetic differentiation arise from the effects of the sampling of sub-populations and the sampling of individuals within sub-populations. Various attempts have been made to correct for these sampling errors and these are discussed by Weir and Cockerham (1984), who suggest an approach that includes the number of populations and individuals sampled. The suggested variance-based calculation is shown below and does not require information about heterozygote frequencies.

$$F_{ST} = \frac{s^2 - \frac{1}{2\bar{n}-1}\left[\bar{p}(1-\bar{p}) - \frac{r-1}{r}s^2\right]}{\left[1 - \frac{2\bar{n}C^2}{(2\bar{n}-1)r}\right]\bar{p}(1-\bar{p}) + \left[1 + \frac{2\bar{n}(r-1)C^2}{(2\bar{n}-1)r}\right]\frac{s^2}{r}}$$

s^2 is the sample variance of allele frequencies over populations, r is the number of populations, $\bar{p}$ is the mean allele frequency, $\bar{n}$ is the mean sample size and C^2 is the squared coefficient of variation of sample sizes.

Another variant of F_{ST} is given by Lynch and Milligan (1994) who have derived an asymptotically unbiased estimate of the quantity, given by:

$$F_{ST} = \frac{\hat{H}_B}{\hat{H}_T}\left(1 + \frac{\hat{H}_B Var(\hat{H}_W) - \hat{H}_W Var(\hat{H}_B) + (\hat{H}_B - \hat{H}_W) Cov(\hat{H}_B, \hat{H}_W)}{\hat{H}_B \hat{H}_T{}^2}\right)$$

where $H_T = H_w + H_B$ and H_w and H_B are within and between –population gene diversity as described by Lynch and Milligan (1994) and given in the equations in section 2.5.3 and Cov and Var represent covariance and variance respectively.

All these methods of calculating F-statistics are reliant upon the assumption of Hardy-Weinberg equilibrium or on knowledge of the inbreeding co-efficient, and yet populations often deviate from an equilibrium state and information on levels of inbreeding is rarely available. A Bayesian approach was therefore developed by Holsinger *et al.* (2002) that incorporates uncertainty about the magnitude of inbreeding, thereby obtaining estimates of F-statistics that are not dependent on the assumption of Hardy-Weinberg equilibrium nor on a prior knowledge of the level of inbreeding. This approach was shown to provide accurate estimates of population parameters (Holsinger *et al.*, 2002) and it is likely that it will be increasingly used in future studies in conservation genetics where populations are frequently small and/or inbred and unlikely to be in Hardy-Weinberg equilibrium.

Analysis of Molecular Variance

The statistical approach of Analysis of Molecular Variance (AMOVA) is based on the derivation of F-statistics and tests the hypothesis that the molecular variance among sub-populations is greater than the molecular variance within sub-populations. It can utilise molecular marker data, sequence data, or phylogenetic trees that are based on these data, to analyse the level of sub-population differentiation. The analysis of variance framework for use with molecular data was first described by Cockerham (1969) and was later developed further by Weir and Cockerham (1984) and others. The most recent approach is that of Excoffier *et al.* (1992) and although this is also based on the analysis of gene frequencies, it differs from previous approaches by incorporating a consideration of the number of mutations between molecular haplotypes. When AMOVA is used with dominant markers, the assumption is made that the mating pattern is the

same in all subpopulations and this can be a limitation for its use within species that are prone to inbreeding under certain conditions. In addition to this, F-statistics provide important information about the structuring of populations but do not indicate the factors involved in creating this structure. Allele frequencies in different populations diverge as a result of a variety of random processes, including migration, genetic drift and natural selection and it is therefore difficult to ascertain the cause of the observed differentiation (Hartl & Clark, 1997). F-statistics are nevertheless important estimators of population differentiation and AMOVA can therefore be highly informative with respect to the conservation of populations, regardless of the causal factors of the divergence.

2.6.6 Tree construction

Trees are mathematical structures used to infer and display evolutionary relationships between taxa and can be constructed using many different methods (Page & Holmes, 1998). In the analysis of population genetic data, distance matrix methods are used for tree construction, and these include unweighted pair-group method with arithmetic mean (UPGMA), neighbour-joining, neighbours relation and minimum evolution. Neighbour-joining and neighbours relation are similar approaches but neighbour-joining is more commonly used and more widely available in the analytical software. The minimum evolution method is computationally demanding and as neighbour-joining provides a fast approximation of the minimum evolution tree this is the preferred method (Wen Hsuing, 1997). Both UPGMA and neighbour-joining are used in the analysis of data in this thesis and these are discussed below.

UPGMA (Unweighted Pair Group Method with Arithmetic Mean)

This is a form of cluster analysis, where similarity or distance data are displayed in the form of an ultrametric tree where each of the branches arising from a node is the same length (Swofford *et al.*, 1996). It is the most basic method of tree construction and proceeds in a stepwise manner, combining the most similar taxonomic units first, thereby constructing the tree in the order of

decreasing similarity. It provides a good estimation of phylogenetic structure for taxa that have a constant rate of evolution but errors can be introduced if this assumption is not met.

Neighbour-joining

Neighbour-joining (described by Saitou and Nei, 1987) constructs trees using a star decomposition method and although it is related to cluster analysis, it is not based on the assumption that the data are ultrametric (Swofford *et al.*, 1996). It also tracks the nodes on a tree rather than clusters and the separation between nodes is adjusted based on their divergence from all other nodes (Swofford *et al.*, 1996). The overall objective of the process is to minimise the overall length of the tree and the method starts with a star-like cluster from which taxa are separated sequentially, based on the outcome that gives the smallest sum of branch lengths (Wen Hsuing, 1997). The ability of this technique to process large datasets has led to its widespread use in molecular systematics.

Bootstrap analysis

Boostrap analysis is a standard statistical method that can be used for testing the reliability of phylogenetic trees (Efron, 1982; Dodds, 1986, both cited in Hillis *et al.*, 1996). It operates through re-sampling the character matrix at random and creating new matrices of the same size that are treated as replicate datasets. Each original sample has an equal probability of occurring in the replicate datasets and some may therefore appear many times while others will not be represented at all. The bootstrap value for each branch is the percentage of replicate data sets that are in agreement with the sample data in the construction of that particular branch and this gives an indication of the stability of the tree structure and the confidence that can be placed in particular sample groupings.

2.6.7 Analytical Programs

There are many programs in circulation for the analysis of population genetic data, most of which are freely available for download from the World Wide Web. The programs vary in their capacity to perform different analytical tasks and also in the methods on which the calculation of genetic

parameters is based. It is therefore advisable to use a number of different analytical software packages to allow an accurate assessment to be made and to allow greater confidence to be placed in conclusions drawn from the data. The packages used in the completion of this thesis are described below.

POPGENE Version 1.32

This is Windows-based freeware for the analysis of population genetic data (Yeh *et al.*, 1999). It can be used for the analysis of diploid and haploid individuals and for co-dominant or dominant markers. It allows the calculation of gene diversity statistics (Nei, 1973), F-statistics (G_{ST} - Nei, 1973), genetic distance (Nei, 1972; 1978) and estimates gene flow. It carries out these calculations on populations, sub-populations or groups of populations and produces UPGMA dendrograms displaying the relationships between these population units.

PAUP v.4.0b10

PAUP is an acronym for **P**hylogenetic **A**nalysis **U**sing **P**arsimony and it is software that enables the construction and analysis of phylogenetic trees. Methods of tree construction for population genetic data include UPGMA and neighbour-joining and support for the data is provided by bootstrap analysis. Mean or total character difference or Nei-Li's restriction site difference (Nei & Li, 1979) can be used to construct trees from a presence-absence matrix of molecular markers or genetic distance matrices derived from other analyses can be inserted into the program and used in the same way.

AFLP-SURV 1.0

This is free genetic analysis software that gives estimates of genetic diversity and population genetic structure and computes genetic distance matrices between populations. It is distributed by the author (Vekemans, 2002). The computations are designed for samples that have been analysed using dominant genetic markers such as RAPDs or AFLPs. Hardy Weinberg equilibrium is assumed unless prior information has been obtained on any deviations from this state. Three alternative methods of allele frequency estimation are provided, fragment frequency

(for haploid organisms or fixed homozygosity), the square root method or a Bayesian method with uniform prior distribution of allele frequencies. The approach of Lynch and Milligan (1994) is then used to derive diversity statistics from the estimated allele frequencies.

Arlequin Ver 2.000

Arlequin is free downloadable software for population genetic data analysis (Sneider *et al.*, 2000). It has the capacity to form an extensive range of statistical tests and can utilise genotypic or haplotypic data. When molecular marker data are used, each distinct fingerprint is treated as a haplotype and a matrix of squared Euclidean distance is computed. This is an evaluation of the number of mutational steps between haplotypes. Arlequin can perform intra and inter-population analyses based on this matrix. Intra-population analyses include the calculation of standard gene diversity indices, estimators of fixation indices and haplotype frequency estimations. Inter-population analyses include the calculation of pairwise genetic distances based on F_{ST} for short divergence time and hierarchical Analysis of Molecular Variance to evaluate the structuring of populations. It also performs an exact test of population differentiation and can compute the correlation between matrices of genetic and geographic distances between populations.

PART I : *Colchicum autumnale*

Colchiques dans les prés
Fleurissent, fleurissent
Colchiques dans les prés
C'est le fin d'Été

CHAPTER THREE

INTRODUCTION TO *COLCHICUM AUTUMNALE*

3.1 TAXONOMY AND MORPHOLOGY

Colchicum autumnale L. belongs to the Liliales order of flowering plants with recent taxonomic treatments placing it in the family Colchicaceae, for which it is the type genus (Angiosperm Phylogeny Group, 2003). There are 23 European species in the genus and *Colchicum autumnale* defines the *Colchicum autumnale* group, which also contains *C. rhodopaeum, C. neapolitanum, C. lusitanum, C. lingulatum* and *C. parnassicum* (Tutin, 1980). Members of the genus *Colchicum* are perennial herbs with an underground corm enclosed by tunics that often extend above the corm to form a neck. The corm has a basal ring from which adventitious roots develop and it replaces itself fully each year with the new corm developing on top of the old. The flowers arise from a bud on the corm and elongate along a groove running up one side. They are purple, pink or white in colour and can be solitary or in groups of two or more. Each flower is subtended by a small bract and is very shortly pedicillate. The perianth segments occur in two whorls that are fused below into a long narrow tube. The six stamens are inserted at the base of the perianth segments above this tube. There are three free styles and the superior ovary is subterranean. The fruit is a septicidal capsule that forms underground and matures at, or slightly above, ground level. Seeds are spherical and numerous (Butcher, 1954; Tutin, 1980a).

Characteristics of the *C. autumnale* group include narrowly campanulate flowers, an ovoid to subglobose corm, a dark brown leathery or membranous tunic, glabrous leaves that develop after anthesis, 1-6 flowers, styles curved at apex, long-decurrent stigmas, filaments 10-16mm in length, anthers 5-8mm and fruit an oblong capsule 20-60mm. *Colchicum autumnale* itself is distinguished from other members of the group by possessing yellow anthers and broadly lanceolate leaves which are erect or erecto-patent and approximately five times as long as wide. An albino form is present in one of the Irish populations (Plate 3.1) and this has also previously been recorded from other populations in the British Isles (Foord-Kelcey, 1910). Diploid chromosome numbers of 38 and 42 have been recorded (Butcher, 1954; Tutin *et al.*, 1980a; Clapham *et al.*, 1987).

3.2 DISTRIBUTION

Colchicum autumnale is a widespread species but is limited in the number of localities in which it occurs. It is distributed throughout central Europe and occurs northwards to Schleswig and the Baltic, east to Lithuania and Romania and south to north Italy and Spain (Butcher, 1954). Its occurrence in Spain and other Mediterranean countries is restricted to the montane regions in the north and these define the limits of its Euro-Siberian phytogeographic range. In the United Kingdom, there are three centres of abundance focused on Suffolk in the east, the Yorkshire river valleys in the north and the Wye, Severn and Thames river valleys in the south (Butcher, 1954; Preston *et al.*, 2002). In Ireland it has a highly localised distribution (Figure 3.1) occurring predominantly along the valley of the River Nore in the southeast, with only one population occurring outside this area in County Wexford. It was formerly more widespread in Ireland and Figure 3.1 also shows its past distribution as indicated by records held by the National Parks and Wildlife Service of the Department of the Environment, Heritage and Local Government. The reason for its decline in Ireland and elsewhere throughout its range is likely to be agricultural intensification (Curtis & McGough, 1988). The base-rich alluvial soil on which the majority of its Irish populations occur forms prime agricultural land and many of its sites may have been destroyed by ploughing or by excessive fertiliser application. Further decline may have been caused by deliberate eradication of the species as it is known to be poisonous to cattle.

3.3 ECOLOGY

Colchicum autumnale is a corm geophyte with the corm occurring up to 30cm below the soil surface. It is a lowland species in Britain and Ireland but the altitudinal limit of continental European populations extends to 2200m in the Alps (Butcher, 1954). Its habitats are base-rich riverside meadows and base-rich woodland, due to its requirement for basic soils where the water table is high, but where there is good drainage. These conditions are usually found in riverside meadows but similar conditions are often found in open woodland in calcareous regions such as in the Cotswolds (Butcher, 1954). Throughout Europe it occurs predominantly in the riverside meadow habitat but in some areas, for example in Wiltshire in the United Kingdom, its localities

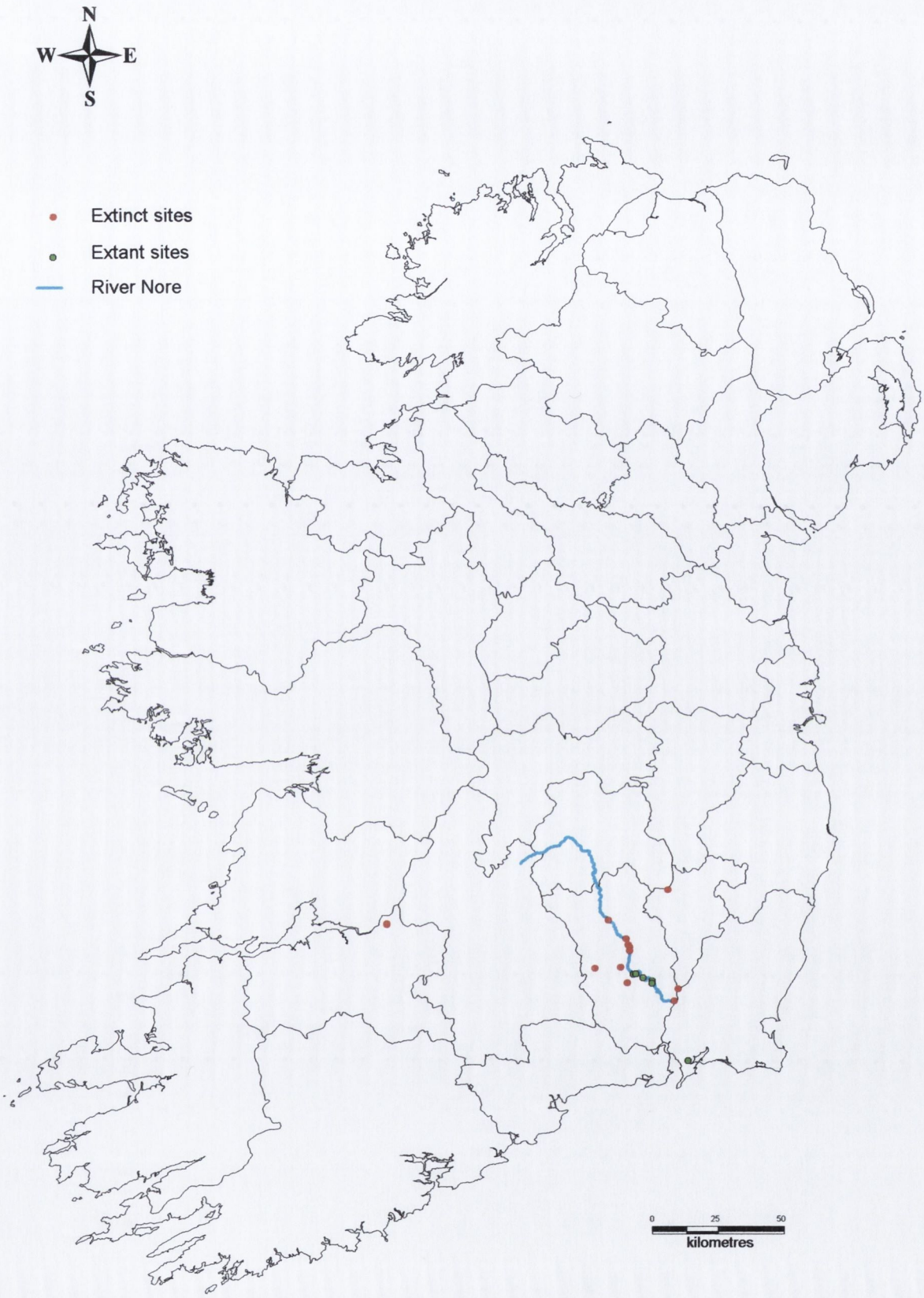


Figure 3.1 Map showing the locations of extant and extinct populations of Colchicum autumnale in Ireland

are almost all woodland sites. It is found on a wide variety of base-rich soil types; in Britain alone it occurs on the chalky boulder clay of Suffolk, on the Magnesian and Mountain limestones of Yorkshire and Nottingham, on Lias clay in Worcestershire and Warwickshire and on Oolite limestone in the Cotswolds. In Ireland it occurs predominantly on the base-rich alluvial soil of the Nore floodplain (Butcher, 1954).

The plant communities in which it occurs are mainly seasonally wet meadows that have been improved in the past containing low overall diversity. All its localities in Ireland are dominated by grass species and most are open meadows, the exception being Fourpenny Rock, a sparsely wooded site with light shading. In open meadow sites, grazing of the surrounding vegetation is an integral part of the ecology of the species, creating conditions that are favourable to growth, anthesis and reproduction. This is evident by the sparsity of individuals and occasional reproductive failure in sites where the vegetation is dominated by long ungrazed grasses and aggressive ruderal species.

Although the leaves and seeds are toxic to grazing animals and other mammals, leaf herbivory by slugs is common and seed predation often occurs (Butcher, 1954).

3.4 Reproduction

Colchicum autumnale undergoes both vegetative and sexual reproduction. Vegetative reproduction is likely to predominate and occurs through the production of cormels, buds that arise on the upper portion of mature corms and eventually become separated. Vegetative reproduction can be extensive but the process is slow as only fully mature corms reproduce in this way and they rarely produce more than one cormel in any one season (Butcher, 1954). Sexual reproduction is also slow with plants flowering only in their third year or later, germination occurring two years after seed set and seedling development taking place over two seasons (Butcher, 1954).

Flowering occurs in late summer, around the third week of August in Ireland, and continues until the second or third week in September. The flowers are entomophilous and secrete nectar into the

base of furrows that run longitudinally along the perianth segments. The furrows are lined with hairs and the mature anthers deposit the majority of their pollen content onto these hairs. Invertebrate pollinators crawl down the furrows to the nectar source and are dusted with pollen from the hairs or directly from the anthers. They then deposit their load onto the styles of subsequent flowers. Pollinators include representatives from several insect orders including the Diptera, Hymenoptera, Lepidoptera and Thysanoptera (Butcher, 1954). Flowers are protogynous but the styles become recurved towards the furrows as the flower ages and autogamy can then occur in the absence of cross-pollination. Cleistogamy and apomixis have not been observed (Butcher, 1954).

The flowers are subtended by a white spathe and this grows to cover the 3-celled ovary, which develops underground over the winter months and is pushed to the surface by the developing foliage in early spring. The fruit matures above ground level and dehisces to shed its seed when the leaves have died back. The mode of seed dispersal is not known but the seeds become gelatinous when wet and it is possible that they adhere to the hooves of herbivores and are transported to new meadows by the movement of livestock (pers. obs.).

3.5 HISTORICAL CONTEXT

Plants in the genus *Colchicum* contain colchicine, a water-soluble alkaloid that is toxic and has the capacity to cause permanent chromosomal changes in dividing cells through its action as a mitotic inhibitor (Dustin, 1984). In small amounts however, it can be useful in the treatment of rheumatism and gout and was first noted and used for these properties by the ancient Egyptians. It was also documented by the ancient Greeks who referred to the plant as 'the soul of the joints' because of its ability to cure these painful diseases of the joints. The name *Colchicum* comes from the classical Greek name Kolchikon, named after Kolchis, an ancient province located on the Black Sea where the mythical witch Medea was said to brew poisons. The genus was first described for medicinal use in *De Materia Medica* written by Padanius Dioscorides, a Greek surgeon in the Roman army in the first century A.D. (Singer, 1996). *Colchicum autumnale*

features widely in herbals and medical texts such as Bentley & Trimen's volume of medicinal plants (Bentley & Trimen, 1880), which lists *Colchicum autumnale* as a medicinal plant, particularly useful for the treatment of gout and rheumatism but also for other inflammatory infections, and for its use as a diuretic, as a cholagogue on the liver and as a cathartic and emetic on the intestinal canal. It also features in Gerard's Herbal (Woodward, 1994) where the 7th century Alexandrian physician Paulus Aegineta is quoted as recommending 'meade saffron' as a treatment for gout whilst also warning of death through improper use.

The medicinal use of *Colchicum autumnale* in medieval times is difficult to trace due to the indiscriminate use of latin terms to describe a number of different plant species. Hunt (1989) mentions that the Linnean *Colchicum autumnale* is equivalent to the old latin name 'Hermodactila' but this has also been used to describe *Allium vineale* and *Allium ursinum.* Latham (1965) also refers to 'hermodactylus' and 'hermodactilus' as *Colchicum* but Gerard states that it is not the true Hermodactyl (Woodward, 1994). The Irish equivalent for Hermodactuli in medieval medical literature is Teine talmhan (A. Nic Dhonnchadha, pers. comm.) but 'tine talún' currently refers to *Ranunculus bulbosus.* Despite the indiscriminate use of the term 'hermodactilus', it is clear that the medicinal properties of *Colchicum autumnale* were widely known from ancient times through to the modern day and it is therefore possible that this species was moved around and cultivated on account of its useful properties. Orders of monks such as the Cistercians and Augustinians were known for the creation of herb gardens and the cultivation of medicinal plants and this, coupled with the limited distribution of *Colchicum autumnale* in Ireland, has led to the suggestion that the species may have been introduced with the spread of monasteries into Ireland. The main concentration of sites in the Nore Valley occurs in an area rich in monasteries and abbeys and the extant populations may be derived from previously cultivated plants. However, the habitat in which the Irish populations occur is typical of the native habitat in which it occurs throughout its range, and where the plant occurs in close proximity to abbeys, as at Jerpoint and Tintern, the monks may have been using a native resource, moving some plants to the abbey grounds for cultivation.

The earliest botanical record for *Colchicum autumnale* in Ireland is listed in *Cybele Hibernica* (Colgan and Scully, 1898) where J. Blashford recorded it as occurring on the banks of the River Nore between Inistioge and Thomastown in 1799. This volume gives numerous other entries for the species, mainly in County Kilkenny but also near Carlow and on the banks of the River Shannon in County Limerick where it was described as 'plentiful'. It is also recorded as occurring in County Dublin at Finglas and Scribblestown, and at Grey Abbey, County Down but it was stated that the species had not been found recently at these sites and therefore probably occurred as a garden escape. It was therefore considered native at the other sites. An entry in the Irish Naturalists' Journal (Phillips, 1925) lists *Colchicum autumnale* among the surrounding flora of *Serratula tinctoria* L., which was stated to have consisted of 'purely native species'. It was therefore regarded by many of the early naturalists as a native species.

3.6 CONSERVATION STATUS

In the Republic of Ireland, *Colchicum autumnale* has been classified as 'Endangered' (IUCN Country (Irish) Category) on the basis of a threat assessment detailed in the Irish Red Data Book of Vascular Plants (Curtis and McGough, 1988). It is therefore listed under the Flora (Protection) Order, 1999 (S.I. No. 94 of 1999) and is fully protected under the Wildlife Act, 1976 (No. 39 of 1976). All of its Nore Valley sites will also be protected under the Habitats Directive through their occurrence within the Nore and Barrow candidate Special Area of Conservation (cSAC 2162). This legislation will have a full legal basis when all landowners have been notified of the designation of their land and when the SAC designation has been published with no official objections within three months of publication date. Reasons for the decline of the species in Ireland were identified as being drainage and intensification of agricultural practices but these threats should be eliminated through the inclusion of its extant Nore Valley sites in the area to be covered by the SAC designation. Any alteration in land-use will then be considered as a notifiable action, and must be declared to the National Parks and Wildlife Service.

3.7 SITE DESCRIPTIONS

In Ireland, there are five sites for *Colchicum autumnale* within the Nore river valley and one in County Wexford. This sixth site was discovered after sampling for genetic diversity was carried out, and when the demographic monitoring was in its third year, and was therefore not included in this study. Four of the five study sites are wet meadow habitats on the River Nore floodplain and of these, three are grazed while the fourth is subjected to rolling twice a year to flatten the vegetation. The fifth site is a small sparsely-wooded area adjacent to the river Nore and is the only site with shading. The locations of the study sites are shown in Figure 3.2 and are numbered below in accordance with this figure.

In addition to the Irish study sites, a number of other European sites were sampled for genetic analysis. A description of each is given in Section 1.3.3 and the locations of the sites are shown in Figure 3.3. They are numbered in accordance with this figure.

The sole objective of the majority of site visits was to collect leaf material for population genetic analysis and as a result of time constraints at certain sites, descriptions of the plant communities present vary in detail. This was an unavoidable consequence of undertaking intensive sampling in a short period of time but enough detail is given to allow an adequate assessment of the habitat to be made. Nomenclature follows Stace (1997).

3.7.1 Irish sites

All Irish study sites are located within County Kilkenny in the south-east of Ireland.

1. Jerpoint

Location: County Kilkenny.
Grid reference: S567408

This site defines the northern limit of *Colchicum autumnale* in Ireland and is located on the East bank of the Nore, north of Thomastown. The vegetation at this site has a high component of ruderal species such as *Urtica dioica*, and *Pteridium aquilinum* becomes dominant during late summer. The site is therefore subjected to a rolling regime in early February and in late July. In

Figure 3.2 Map showing the locations of the five Irish study populations of *Colchicum autumnale*

1. Jerpoint, Co. Kilkenny, Ireland
2. Thomastown, Co. Kilkenny, Ireland
3. Fourpenny Rock, Co. Kilkenny, Ireland
4. Inistioge, Co. Kilkenny, Ireland
5. Inistioge Lower, Co. Kilkenny, Ireland
6. Llanmerewig Glebe, Powys, Wales
7. Cwm Bwchel, Monmouthshire, Wales
8. West Wood, Gloucestershire, England
9. Asham Wood, Somerset, England
10. Fonds l'Olivier, Meuse, Lorraine, France
11. Val des Nonnes, Meurthe-et-Moselle, Lorraine, France
12. Moncley, Doubs, Franche-Comte, France
13. Avanne, Doubs, Franche-Comte, France
14. Valleraugue, Hérault, Languedoc Roussillon, France
15. la Tindière, Loire Atlantique, Pays de la Loire, France
16. Campo-Esera, Huesca, Aragón, Spain
17. Campo-Rialbo, Huesca, Aragón, Spain
18. Lapizea, Navarre, Spain
19. Gasteiz-Vitoria, Alava, Basque Country (Euskadi), Spain
20. Sendadiano, Alava, Basque Country (Euskadi), Spain

Figure 3.3 Approximate location of all European study populations of *Colchicum autumnale.*
For Ireland, the location and County are given, for France, location, Region and Département are given and for Spain, location, Province and Autonomous Community are given.

early spring and summer, the vegetation is dominated by the grass species *Alopecurus pratensis* and *Festuca arundinacea* with *Anthoxanthum odoratum* and *Dactylis glomerata* also present. Other associated species include *Allium vineale*, *Cardamine pratensis, Cirsium palustre, Eupatorium cannabinum, Filipendula ulmaria, Heracleum sphondylium, Ranunculus ficaria, R. flammula, R. repens* and *Vicia sativa.* By late summer, *Filipendula ulmaria, Pteridium aquilinum,* and *Urtica dioica* dominate the vegetation (plates 3.2 and 3.3) and the July rolling allows *Colchicum autumnale* flowers to emerge in late August. This site is located on the northeast bank of the River Nore.

2. Thomastown

Location: County Kilkenny.
Grid reference: S578410

This is the site of the largest Irish population of *Colchicum autumnale* and is a sheep-grazed damp meadow (plate 3.4). It has been re-seeded and fertilised in the past but has regained a high diversity of species. The vegetation is dominated by *Agrostis stolonifera, Arrhenatherum elatius, Dactylis glomerata, Holcus lanatus* and *Poa trivialis.* Other species include *Alopecurus pratensis*, *Bromus hordeaceus, Festuca arundinacea, Festuca pratensis, Helictotrichon pubescens, Phleum pratensis, Poa trivialis, Carex hirta, Juncus articulatus, J. inflexus, Cardamine pratensis, Crepis biennis, Filipendula ulmaria, Lathyrus pratensis, Lotus corniculatus, Rorippa nasturtium-aquaticum, Rumex acetosa, Rumex crispus, Ranunculus acris, R. repens, Stellaria media, Tragopogon pratensis agg., Trifolium pratense* and *T. repens.*

3. Fourpenny Rock

Location: County Kilkenny.
Grid reference: S605395

This is the only wooded *Colchicum* site in Ireland and occurs on the East bank of the Nore below Inistioge. The site is lightly shaded by a low density of *Fraxinus excelsior, Quercus robur* and *Ulmus glabra.* The ground flora is grassy and dominated by *Agrostis capillaris, Brachypodium sylvaticum,* and *Dactylis glomerata* with some *Arrhenatherum elatius, Anthoxanthum odoratum,*

Cynosurus cristatus, Festuca pratensis and *Lolium perenne.* Other species include *Anthriscus sylvestris, Arum maculatum, Filipendula ulmaria, Galium aparine, Geranium robertianum, Geum urbanum, Glechoma hederacea, Hedera helix, Heracleum sphondylium, Hyacinthoides non-scripta, Lathyris pratensis, Lonicera periclymenum, Potentilla sterilis, Ranunculus ficaria, Taraxacum agg.* and *Veronica chamaedrys, Vicia sativa* and *Viola riviniana.*

4. Inistioge, Dyer's Field

Location: County Kilkenny.
Grid reference: S639383

This is an improved sheep-grazed meadow located on the east bank of the Nore at Inistioge. The vegetation is dominated by *Lolium perenne, Festuca rubra, Poa trivialis, Holcus lanatus* and *Agrostis stolonifera* with *Cynosurus cristatus* and *Anthoxanthum odoratum* also occurring. Other species include *Achillea millefolium, Cardamine pratensis, Cerastium fontanum, Ranunculus acris, R. bulbosus, R. repens, Rumex acetosa, Trifolium pratense, T. repens* and *Urtica dioica.*

5. Inistioge Lower

Location: County Kilkenny.
Grid reference: S638373

This is a horse-grazed meadow occurring at a bend in the River Nore immediately south of Inistioge. It is separated from the river by an access road and carpark. The site has been improved and the dominant grass species is therefore *Lolium perenne* although *Holcus lanatus* and *Arrhenatherum elatius* are locally dominant. Other species include *Equisetum arvense, Allium vineale, Cardamine pratensis, Filipendula ulmaria, Heracleum sphondylium, Ranunculus repens, R. acris, Solanum dulcamara* and *Urtica dioica.*

3.7.2 Other European sites

6. Llanmerewig Glebe

Location:Montgomeryshire, Wales, UK.
Grid reference: SO160930

Llanmerewig Glebe is a 1.5 hectare nature reserve purchased by the Montgomeryshire Wildlife Trust in 1987 to protect the only remaining colony of *Colchicum autumnale* in Montgomeryshire (Montgomeryshire Wildlife Trust, 1997). The land formerly belonged to the Church in Wales and is adjacent to the old rectory. The damp meadow habitat in which *Colchicum autumnale* is found at this location is relatively species-rich in comparison to the fertilised and re-seeded meadow habitats in Ireland, and grass species include *Agrostis capillaris, Alopecurus pratensis, Anthoxanthum odoratum, Arrhenatherum elatius, Bromus hordeaceus, Cynosurus cristatus, Dactylis glomerata, Festuca pratensis, Holcus lanatus, H. mollis, Phleum pratense* and *Trisetum flavescens.* Broad-leaved herbaceous plants include *Achillea millefolium, Centaurea nigra, Crepis capillaris, Knautia arvensis, Lathyrus pratensis, Ranunculus acris, R. repens, Rumex acetosa, Succisa pratensis,* and *Trifolium dubium.*

7. Cwm Bwchel

Location: Llanthony, Monmouthshire, Wales.
Grid reference: SO160930

This is sheep-grazed, improved grassland occurring on a hillside opposite Llanthony Abbey. The site occurs on the sloping valley side of the Afon Honddu (river) with a north-western aspect. *Lolium perenne* is the dominant grass species with *Dactylis glomerata* and *Holcus lanatus* also present. The diversity of broadleaved herbaceous plants is low and includes *Ranunculus repens, Rumex obtusifolius, Trifolium repens* and other species associated with re-seeded and heavily fertilised grasslands.

8. West Wood

Location: Nr. Cheltenham, Gloucestershire, England.
Grid reference: SP023237

This is a woodland site, with *Colchicum autumnale* occurring as part of a rich semi-natural woodland ground flora on the sides of a small valley. The woodland is dominated locally by *Quercus robur* and *Fraxinus excelsior* or by *Fagus sylvatica*. There is a rich understorey of *Corylus avellana, Crataegus monogyna, Ilex aquifolium* and *Prunus spinosa* and a highly diverse ground flora comprising *Agrostis capillaris*, *Brachypodium sylvaticum*, *Luzula sylvatica, Anenome nemorosa, Arctium lappa, Arum maculatum, Campanula trachelium, Euphorbia amygdaloides, Galeobdolon luteum, Geranium robertianum, Geum urbanum, Hyacinthoides non-scripta, Mercurialis perennis, Orchis mascula, Paris quadrifolia, Primula veris, P. vulgaris* and *Tamus communis*

9. Asham Wood

Location: Nr. Shepton Mallet, Somerset, England.
Grid reference: ST703459

Colchicum autumnale is common throughout the woodland of the Mendips and the Asham Wood site is an ancient oak woodland with a rich ground flora, of which *Colchicum autumnale* is a significant component. Associated species include *Allium ursinum, Anenome nemorosa, Arum maculatum, Euphorbia amygdaloides, Fragaria vesca, Hyacinthoides non-scripta, Mercurialis perennis, Orchis mascula, Primula vulgaris* and *Viola riviniana.*

10. Fond l'Olivier

Location: Nr. Vigneulles, Meuse, Lorraine, France.
Long/Lat: 5°37.889′E 48°58.603′N

This is a base-rich grassland site adjacent to a small stream and a ruined abbey. The vegetation was dominated by grass species and also included *Allium sp., Bellis perennis, Geum urbanum, Ranunculus repens* and *Trifolium spp.* Part of the site was shaded and in these areas *Colchicum autumnale* was found growing with *Anenome nemorosa, Arum maculatum* and *Viola sp..*

11. Val des Nonnes

Location: Nr. Ecrouves, Meurthe-et-Moselle, Lorraine, France.
Long/Lat: 5°49.611′E 48°42.241′N

The population of *Colchicum autumnale* at this site occurs in grassland adjacent to a small stream. Associated species include *Carex spp., Juncus spp., Allium sp., Bellis perennis, Ranunculus acris, R. ficaria* and *Pulmonaria angustifolia.*

12. Moncley

Location: Doubs, Franche-Comté, France.
Long/Lat: 5°54.276′E 47°18.430′N

This is a sloping base-rich grassland site, with the main population of *Colchicum autumnale* located on a bank approximately 300 metres from a small stream. Associated species include *Heracleum sphondylium, Plantago lanceolata, Primula elatior, Ranunculus repens, R. ficaria, Rumex acetosella, Sanguisorba minor* and *Trifolium pratense.*

13. Avanne

Location: Doubs, Franche-Comté, France.
Long/Lat: 5°57.730′E 47°12.442′N

The *Colchicum autumnale* population at this site spans several large fields of improved grassland and is located approximately two hundred metres from a river. Associated species include *Lolium perenne, Cardamine pratense, Plantago lanceolata, Taraxacum agg.,* and *Trifolium spp.*

14. Valleraugue - Les Cevennes, France

Location: Hérault, Languedoc-Roussillon, France.
Long/Lat: 3°37.800′E 44°4.969′N

This population of *Colchicum autumnale* occurs in an improved grassland habitat on a narrow floodplain, elevated above the River Hérault. Associated species include *Dactylis glomerata, Lolium perenne, Achillea millefolium, Galium mollugo, Glechoma hederacea, Heracleum sphondylium, Ranunculus repens, Plantago lanceolata, Prunella vulgaris, Trifolium pratense* and *Urtica dioica.*

15. La Tindière - le Pont Bérangér, nr. Nantes, France

Location: le Pont Bérangér, nr. Nantes, Loire Atlantique, Pays de la Loire, France.
Long/Lat: 1°50′E 47°10′N

This site occurs along a tributary of the River Acheneau in the Loire Valley south-west of Nantes. It is a former meso-hygrophilous meadow but has recently been planted with *Populus nigra* and is now occupied by a megaforb community with a vegetation height of 25cm-110cm. There are approximately 25 individuals of *Colchicum autumnale* and associated species include *Bromus sterilis, Poa trivialis, Ajuga reptans, Galium aparine, G. verum, Geum urbanum, Oenanthe crocata, Orchis laxiflora, Phalaris arundinacea, Pulmonaria angustifolia, Ranunculus acris, Rumex acetosa, Stellaria graminea* and *Valeriana officinalis*.

16. Campo - Esera

Location: Campo, Huesca, Aragón, Spain.
Long/Lat: 0°23.674′E 42°23.875′N

This is a grassland habitat adjacent to the River Esera. No information is available regarding associated species.

17. Campo - Rialbo

Location: Campo, Huesca, Aragón, Spain.
Long/Lat: 0°23.749′E 42°23.481′N

This is a grassland site, adjacent to the River Rialbo, a tributary of the River Esera. Associated species include *Bromus erectus, Cynosurus cristatus, Dactylis glomerata, Festuca rubra, Poa pratensis, Carex caryophyllea, Bellis perennis, Carum carvi, Centaurea nigra, Crepis biennis, Daucus carota, Galium mollugo, G. verum, Leontodon hispidus, Leucanthemum vulgare, Lotus corniculatus, Muscari neglectum, Plantago media, Primula veris, Potentilla verna, Trifolium pratense, Taraxacum officinale, Ranunculus acris, R. bulbosa, Salvia pratensis* and *Viola riviniana.*

18. Lapizea

Location: Navarre, Navarre Province, Spain.
Long/Lat: 1°19.368′W 42°58.339′N

This is a sloping woodland site on alluvial soil with a small stream running down through the population. Tree species include *Acer campestris, Fagus sylvatica* and *Quercus petraea,* with an understorey of *Corylus avellana, Crataegus monogyna, Daphne laureola, Euonymus europaeus, Ilex aquifolium* and *Prunus spinosa.* The ground flora is diverse and includes *Alliaria petiolata, Cardamine pratensis, Crocus nudiflorus, Geranium robertianum, Geum sylvaticum, Helleborus viridis ssp. occidentalis, Lonicera periclymenun, Potentilla sterilis, Primula veris, Rosa arvensis* and *Sanicula europaea.*

19. Gasteiz-Vitoria

Location: Alava, Euskadi (Basque Country), Spain.
Long/Lat: 2°38.727′W 42°51.381′N

This is a marshy habitat that was formerly grazed but was subsequently abandoned, allowing grasses and ruderal species to dominate. A recent fire has allowed *Colchicum autumnale* to become prevalent in the vegetation. Associated species include *Dactylis glomerata, Poa pratensis, Agrostis sp., Carex spp., Juncus sp., Cardamine pratensis, Filipendula ulmaria, Galium mollugo, Heracleum sphondylium, Iris pseudacorus, Lathyrus pratensis, Mentha sp.,* and *Ranunculus acris.*

20. Sendadiano

Location: Alava, Euskadi (Basque Country), Spain.
Long/Lat: 2°54.222′W 42°53.407′N

Only ten individuals of *Colchicum autumnale* were present at this site and were located in grassland adjacent to a riverbank. No information is available on associated species.

3.8 AIMS

The overall aim of this research into the population biology of *Colchicum autumnale* was to determine the conservation status of the species in Ireland and thus establish a scientific basis for the future conservation of the species and prioritisation of conservation resources. One of the specific aims was therefore to collect genetic evidence to indicate whether the species is native to the Irish flora, through assessment of comparative levels of gene diversity in western European populations and evaluation of biogeographical patterns revealed through genetic similarities between populations. If the species was not afforded native status, it would be considered as a lower conservation priority than other threatened species that are native, and this would duly influence decisions regarding conservation resource management. If introduced, *Colchicum autumnale* would still be worthy of conservation, as it forms an integral part of Ireland's cultural and, to a lesser degree, natural heritage, but in an environment of limited conservation resources it would not take priority over the protection of native components of the Irish flora. Further objectives of the genetic study of *Colchicum* autumnale were to evaluate the diversity and evolutionary potential of the populations through comparison of the gene diversity of Irish populations with those in other parts of western Europe, to investigate possible correlations between population size and gene diversity, to assess the differentiation of populations through the analysis of the partitioning of genetic variation within and between populations and to provide an important source of information in the consideration of population management schemes such as translocation or the establishment of new populations.

The specific aims of the study of the species' demography were to provide estimates of population size, to determine the rate of flux of individuals within the populations, to establish whether sexual recruitment of individuals is taking place and thus determine the relative importance of sexual and vegetative modes of reproduction, and to obtain information on reproductive activity and output in the Irish populations.

The projected outcome of this was that the conservation status of *Colchicum autumnale* in Ireland could be fully evaluated to provide a strong scientific basis for decision-making.

Plate 3.1 The albino form of *Colchicum autumnale.*

Plate 3.2 *Colchicum autumnale* growing amongst *Filipendula ulmaria* at Jerpoint.

Plate 3.3 Vegetation at Jerpoint. The Jerpoint population becomes overgrown with grasses, *Filipendula ulmaria, Pteridium aquilinum* and *Urtica dioica* towards the end of the summer. Unless the site is rolled, the inflorescences of *Colchicum autumnale* cannot penetrate the thick mat of vegetation.

Plate 3.4 Vegetation at Thomastown. In contrast to the Jerpoint population, the grazing management of the meadow at Thomastown has allowed *Colchicum autumnale* to spread prolifically and flowering is not hindered by tall vegetation.

CHAPTER FOUR

POPULATION BIOLOGY OF *COLCHICUM AUTUMNALE*

4.1 INTRODUCTION

The monitoring of population size and stability should be a requirement of any conservation policy (Bradshaw & Doody, 1978) and demographic study is therefore considered to be an essential component of conservation research and practice (Bradshaw, 1981; Davy & Jeffries, 1981; Harvey & Meredith, 1981; Pavlik & Manning, 1993; Iriondo, 1996). The potential for the application of the demographic study of plant populations to their management for conservation was first emphasised by John Harper (Harper, 1971), although conservation bodies had been monitoring rare plant populations prior to this (Wells, 1967). Harper stressed the importance of demographic data in making informed management decisions (Harper, 1971) and since then there have been many population studies focusing on rare and endangered plant species (Griggs & Jain, 1983; Charron & Gagnon, 1991; Pavlik & Manning, 1993; Waite, 1998). Simple census data can provide an indication of population size but can be misleading if considered in isolation from other population statistics (Bradshaw, 1981). Where demographic stochasticity is an important factor in the survival of a species, a detailed population study is required to place census data in context, to allow the identification of vulnerable stages in the life cycle and to provide insights into the factors limiting population survival and growth. However, an understanding of demographic fluxes in populations of long-lived perennials requires survey over a long time period (eg. Tamm, 1956; Ernst, 1979; Hutchings, 1987; Waite, 1998) and is therefore not feasible in all cases.

Many long-lived species, particularly those with underground storage organs, also present difficulties in that they may spend some growing seasons underground, in a dormant state. Orchids, for example, possess this ability and can survive several years without producing any leaves (McKendrick, 1995). This apparent disappearance of plants may cause an incorrect assumption of mortality and when the total survey time is restricted to less than five years, these inaccuracies could result in large errors in the calculation of parameters such as the population growth rate. Clonal species also complicate demographic analysis through the uncertainty of what constitutes a genetic individual, or genet, and many studies focus instead on ramet

demography (eg. Newell *et al.*, 1981). The monitoring of individual ramets removes the need to define or determine the extent of individual genets but even in this situation, the separation of individuals into age classes can be difficult and many genera cannot be aged accurately by size (eg *Calochortus* (Liliaceae), Fiedler, 1987). The solution to this is the classification of plants into size classes and although this is used in many studies (eg. Fiedler, 1987; Ehrlén, 1995), it can still be inappropriate for some long-lived plant species where long survey periods are required to detect transitions between classes. In terms of long term conservation the study of ramets may have little relevance if only a few genets are present in the population.

For many long-lived clonal species, populations can number in the thousands and in species that occur in such large numbers, demographic stochasticity is unlikely to influence population survival (Kieding, 1975; Shaffer, 1981) and other factors may be more important. However, *Colchicum autumnale* occurs in a range of population sizes in Ireland, and a knowledge of the demography of the species can contribute to the assessment of these populations and to the way in which the populations are managed for conservation. One particular question is whether recruitment from seed is occurring in the populations, or whether the spread is purely vegetative. The monitoring of individuals can help to answer this question and also complement the collection of data on genetic aspects of population structure, as the proportion of clonal to sexual reproduction has important genetic consequences. The interplay of demographic and genetic factors and the benefits of following both lines of research was recognised by Lande (1988) and this thesis aims to maximise the available information by using both demographic and genetic techniques as complementary approaches.

This chapter describes the collection of data on the population dynamics and structure of *Colchicum autumnale,* but a full demographic analysis was not possible as the transition between stages in the life cycle could not be accurately determined due to the longevity and slow growth of the species, the large degree of vegetative reproduction and the difficulty in locating seedlings among the dense grass in the meadow habitat. However, by population census and the monitoring of individual ramets and genets, the aim of the demographic study was to monitor survivorship

and fecundity in three of the Irish populations, to learn more about patterns of growth and reproduction within the species and to obtain estimates of the number of individuals in each of the populations sampled for genetic analysis.

4.2 METHODS

All research activities were carried out under licence from the National Parks and Wildlife Service.

4.2.1 Population census

One of the main problems encountered in the census of plant populations is how to determine what constitutes an individual. Over long periods of time, clonal individuals can spread vegetatively to cover wide areas and what appear to be separate plants can be genetically identical. For the purposes of this study, an arbitrary decision had to be made over how to define a genet and is was therefore decided that groups of plants were part of the same genet if they were positioned not more than 5cm away from each other. This was based on observations of the distance between clumps of plants in the field and was also the distance used to delimit clumps in a demographic study of *Narcissus pseudonarcissus* L. (Liliaceae) by Barkham (1980).

A population census at all sites (described in section 3.7) was undertaken during May 2000. At Inistioge and Thomastown, the number of individuals was too large to count and an estimation technique was therefore employed using a system of stratified random sampling. At each site, the population was delimited into regions on the basis of plant density so that within regions, plant density was consistent. The regions were delimited by placing poles at their extreme corners and the length of the lines connecting these poles was measured using tape measures. The regions were thus delimited by geometric shapes and their area was calculated using simple geometry. A random sample of ten $1m^2$ quadrats was taken within each region and the number of ramets and genets within each quadrat was recorded. An estimation of population size was then obtained by multiplying the average number of genets in each quadrat by the area of the region and these were summed over all regions. At Jerpoint, the number of genets and ramets was recorded but due to

the height and density of the vegetation, seedlings would not have been visible and the count therefore represents only well-established genets with a large number of ramets. At Fourpenny Rock and Inistioge Lower, the total numbers of ramets and genets were estimated through counting by eye during May, 2000. The size of other European populations that were sampled for genetic analysis were counted directly or estimated by eye due to the time constraints of the field schedule.

4.2.2 Demographic monitoring

Demographic monitoring of the Thomastown and Inistioge populations was carried out by establishing permanent, unfenced 1m x 1m quadrats and mapping the position of every ramet within this area every year over four years. The Jerpoint population was not included in a full demographic study due to the difficulty in monitoring each individual ramet. This population showed significant differences in growth habit from that exhibited at the other four sites in that the distribution of ramets was highly clumped with large numbers of stems forming widely separated groups. This localised high density of stems made it impossible to relocate individual ramets. However, their wide separation allowed accurate mapping and monitoring of clumps, which were assumed to be genets. Each clump was mapped and an aluminium tag sunk to ground level adjacent to each, to ensure accurate relocation with a metal detector. The number of stems in the clump was recorded along with the number of fruiting ramets and the leaf width of the largest individual. The height of the surrounding vegetation was also measured. Inistioge Lower was not included, as permission to access the site and establish permanant quadrats was not received until the second field season had passed. The height of the vegetation in this field was also a limiting factor as the location of young plants would have been difficult due to their similarity to long grass blades.

At Inistioge and at Thomastown, quadrats were located at set intervals along a transect spanning the length of the population and along two perpendicular transects. The exact position of each quadrat was marked by sinking aluminium tent pegs into the ground on the corners of one side of the quadrat, along the transect line. The line of the transect was identified by lining up physical

features at the site with the start point and by taking a bearing. Fifteen permanent quadrats were set up in each of the two populations along fixed transects. The transects were run along the length of the populations parallel to the river and also transversely across their width to take account of any possible clinal change from the riverbank to the inner limit of growth. For ease of relocation, the transects were lined up with prominent landmarks but where the working line missed significant areas of *Colchicum autumnale* or veered away from the population, the angle of the line was altered and re-aligned with alternative landmarks, or measurements were simply taken out from the fixed line at a 90° angle. Quadrats were placed at regular intervals along the transects but where an area of significantly different density or species composition would be missed in this way, the quadrats were placed at irregular intervals. In this way, the full range of plant densities and community types were encompassed within the quadrats whilst also allowing ease of relocation. Four quadrats were established at Fourpenny Rock but due to re-location difficulties and the height of the vegetation in subsequent years, only two of the quadrats were monitored for two seasons.

At Thomastown, demographic survey was carried out annually for four years, 1999-2002. Inistioge was monitored in 1999, 2000 and 2002 but access to the field was denied in 2001 as a consequence of the foot and mouth disease outbreak in Ireland and the UK. Fourpenny Rock (population 3) was monitored for two years. In the three populations, the position of each ramet within the $1m^2$ quadrats was mapped using Cartesian coordinates. Plants were identified on the basis of the previous year's coordinates and data so that death and recruitment could be noted each year. The disappearance of a plant was recorded as a death unless a ramet with a similar leaf width was recorded in the same position in subsequent years. A distinction was made between recruitment from seed, distinguished by the appearance of small one-leaved plants with a width of less than 10mm and occurring more than 5cm from another plant, and the recruitment of clonal ramets, indicated by the new appearance of a small plant in the vicinity of another ramet. However, in view of the similarity of seedlings to blades of grass, it is likely that many would be overlooked. Any ramets with 2 leaves, a leaf width of less than 15mm, and not occurring within

5cm of an existing plant were assumed to have been recruited from seed in the previous year. The following measurements were taken for each ramet: number of leaves; leaf width (taken ¾ way to base); number of fruits. During the first year, the height of the surrounding vegetation was also recorded. All demographic surveys were carried out during the first two weeks of May.

Although the leaves and fruit of *Colchicum autumnale* are present above ground from late February until June in Ireland, the flowers do not emerge until late August and September. The above-ground reproductive and vegetative stages of development are therefore temporally separated. The flowering period lasts for approximately one month, from the end of August until the end of September, but flowers are relatively short-lived and there is therefore a continuous wave of flower emergence and senescence. The measurement of flowering demographics are therefore subject to error if measured at one or even two occasions during the flowering season, and flowering was therefore not recorded. Reproductive success was measured by the production of fruits but the proportion of flowers producing fruits could not be ascertained.

4.2.3 Fecundity

Fruit production was monitored for Jerpoint, Thomastown, Fourpenny Rock and Inistioge as part of the demographic study. The number of fruits produced by each ramet contained within the $1m^2$ quadrats was recorded along with the other demographic data. Initially, permission was not obtained for conducting a similar analysis at Inistioge Lower and this population was not part of the demographic monitoring study. However, when access permission was granted, the percentage of fruiting ramets was determined by randomly selecting 100 ramets and recording whether fruit was produced.

An estimate of seed production was carried out in June 2001 for the Thomastown (2), Inistioge (4) and Inistioge Lower (5) populations. Plants at Jerpoint (1) had failed to flower during the preceding year and therefore produced no fruits, and the vegetation surrounding Fourpenny Rock (3) had been cut back immediately prior to the site visit and the fruits were therefore damaged. Individual plants were selected randomly and the width at the widest point of the widest leaf was

measured. The number of fruits was recorded and these were collected and stored in paper envelopes. The fruits were later dissected and the number of seeds contained within were counted. Fifty fruiting ramets were selected randomly within each of Thomastown and Inistioge. However, due to the low population size at Inistioge Lower, 50 ramets were selected randomly, regardless of their fruiting status and any fruits produced from these ramets were collected. This would ensure that approximately four fifths of the fruits were left intact.

4.2.4 Seed germination

Various attempts to germinate *Colchicum autumnale* seeds are reported in Butcher (1954). Methods included sowing seeds from green capsules in July, sowing mature seed in November and in March, freezing, scarification and soaking the seed in a hormone solution. All these methods failed to induce germination but in 1951, three seedlings appeared from seed that had been sown two years earlier. Another previous study on the germination of seeds from Irish threatened plant species failed to induce germination in *Colchicum autumnale* (Martin, 1998). The four dormancy-breaking treatments of chilling, scarification, addition of Gibberellic acid and dry storage had no effect on seeds from either of the two sampled populations. It is possible that a chemical in the seed coat (possibly colchicine) prevents germination and in this study various pre-treatments were performed on seeds of *Colchicum autumnale* with the aim of removing this influence. The following treatments were tested:

1/ Scarification – seeds were surface-sterilised and then rubbed lightly with sand paper.

2/ Testa removal – the seed coat was removed entirely.

3/ Soaking – seeds were soaked in water for 24 hours

4/ Control – no treatment

In the germination trials, seeds were placed on moist filter paper in glass petri dishes and were kept moist throughout the experiment. Each treatment was repeated for three temperature regimes giving a total of nine treatments and three controls. The first temperature treatment was stratification where the seeds were placed in a cold store at 5°C for a month and then moved to a

growth room with a 13/11 hour alternating light/dark regime and a 13/11 hour alternating temperature regime of 25°C/20°C. The second replicated natural conditions in the field and the seeds were placed in the growth room for three months and then moved to the cold store. In the third temperature treatment, Petri dishes were placed straight into the growth room. Each of the crossed treatments was represented by two Petri dishes containing twenty-five seeds each.

4.3 RESULTS

4.3.1 Demography

Estimates of population size based on estimates of the number of ramets and the number of genets are shown in Table 4.1. The number of genets is based on the assumption that any plants within 5cm of each other are vegetative clones and part of the same genet. The number of ramets is simply defined as the number of separate stems.

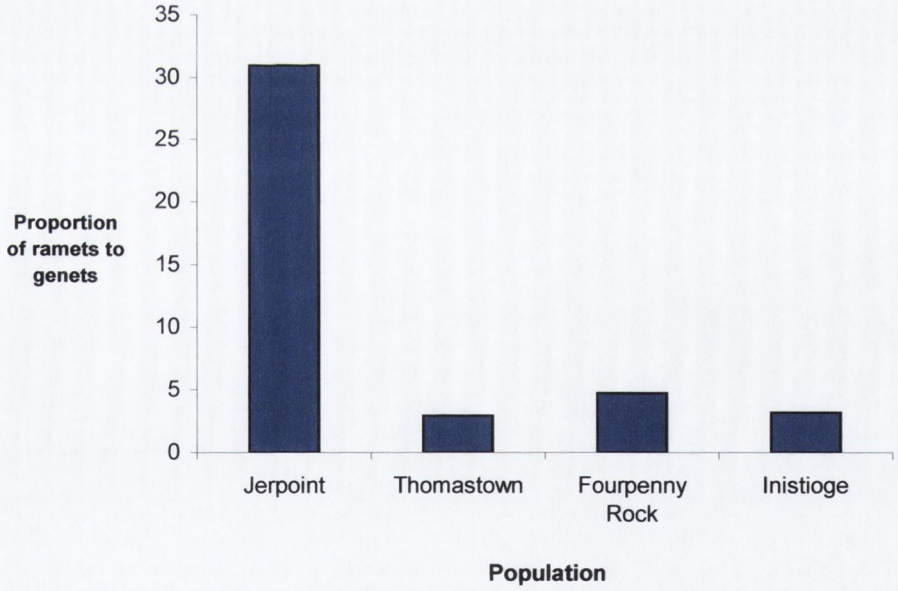
Table 4.1. Estimates of the number of genets and ramets in twenty populations of *Colchicum autumnale*.

Population number	Population	No. genets/clumps	No. ramets
1	Jerpoint	68	2,106
2	Thomastown	15,618	45,741
3	Fourpenny Rock	74	270
4	Inistioge	7,196	22,875
5	Inistioge Lower	250	no data
6	Llanmerewig Glebe	716	no data
7	Cwm Bwchel	2,000	no data
8	West Wood	7,000	no data
9	Asham Wood	4,500	no data
10	Fond l'Olivier	755	no data
11	Val des nonnes	200	no data
12	Moncley	800	no data
13	Avanne	20,000	no data
14	Valleraugue	175	no data
15	la Tindiere	25	no data
16	Campo-Esera	4,000	no data
17	Campo-Rialbo	1,500	no data
18	Lapizea	400	no data
19	Gasteiz-Vitoria	250	no data
20	Sendadiano	10	no data

Numbers of genets in the twenty populations of *Colchicum autumnale* range from 25 to 15,618. The largest population occured in France (Avanne, population 13) but Ireland contained two of the three largest sampled populations: Thomastown and Inistioge. The smallest population

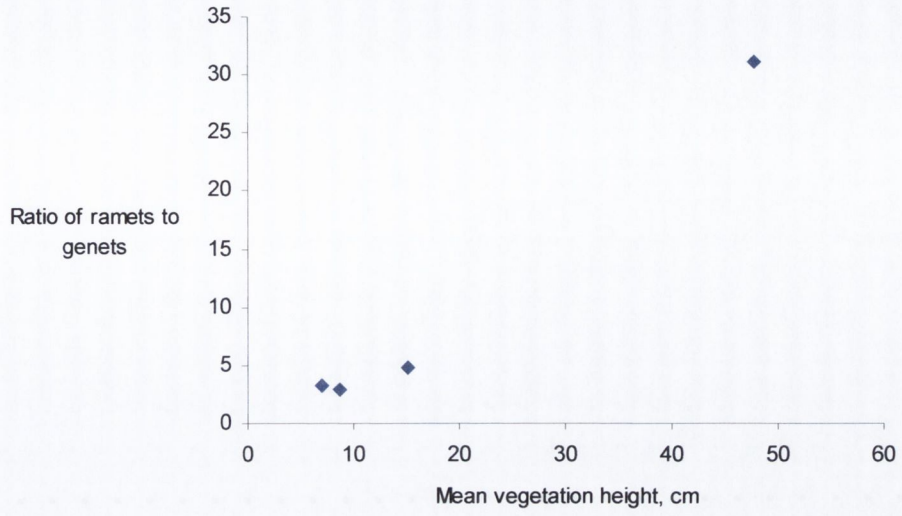
occurred in the Basque country and contained only 10 individuals, occurring at the edge of an improved grassland adjacent to a small stream. The high number of clumped individuals/ genets (over 5000 in four of the populations), shows that in a suitable habitat, *Colchicum autumnale* has the capacity to spread prolifically. Population size was found to be significantly negatively correlated with the mean vegetation height at Jerpoint, Thomastown, Fourpenny Rock and Inistioge (Spearman Rank Correlation = -1, n=4). The nature of the spread of genets appears to be determined by the environment, as in the ungrazed environment of Jerpoint the distribution of genets is highly localised with significant clumping of ramets, whereas the distribution of plants is more even at the grazed sites. The degree of clumping can be described by the ratio of ramets to genets at each of the Irish sites (Figure 4.1). Populations with a high ratio of ramets to genets have a clumped distribution whereas populations with a low ratio are more diffuse.

Figure 4.1 Ratio of ramets to genets in four Irish populations of *Colchicum autumnale*



The ratio of ramets to genets shows a positive relationship with the height of the surrounding vegetation (Figure 4.2). The Spearman Rank Correlation co-efficient between the variables is 0.8 but this is not statistically significant at the 95% level. However, more data are needed to fully evaluate this correlation.

Figure 4.2 Relationship between the ratio of ramets to genets and the mean vegetation height in four populations of *Colchicum autumnale*



The density of *Colchicum autumnale* ramets per metre squared also indicates the degree of clumping. The mean density of ramets was calculated for all quadrats included in the demographic study and these are shown in Table 4.2.

Table 4.2 Mean density of *Colchicum autumnale* ramets in four Irish populations. Figures in brackets are standard deviation

	Mean density of ramets (m^{-2})				
Population	**1999**	**2000**	**2001**	**2002**	**Overall**
1 Jerpoint	30.16 (±20.61)	30.82 (±22.18)	No data	No data	30.49 (±21.40)
2 Thomastown	14.58 (±9.10)	15.83 (±10.36)	15.25 (±9.56)	15.83 (±9.37)	15.38 (±9.60)
3 Fourpenny Rock	14.00 (±5.66)	14.00 (±4.24)	No data	No data	14.00 (±4.95)
4 Inistioge	10.08 (±4.55)	10.83 (±4.37)	No data	10.42 (±4.38)	10.44 (±4.43)

Mean density of ramets ranged from 10 to 31 ramets per metre squared and was highest at Jerpoint and lowest at Inistioge. The order of increasing density corresponds to that of increasing vegetation height at the sites as described earlier. Although the density of ramets was high within quadrats at Jerpoint, the distribution of *Colchicum autumnale* genets was highly localised and the majority of the site does not contain any ramets. The high density represents extremely localised vegetative spread.

Survival, mortality and recruitment

The monitoring of individual ramets at Thomastown (population 2), Fourpenny Rock (population 3) and Inistioge (population 4) allowed survival, mortality and recruitment to be assessed. Due to the inability for exact population size to be determined, the subpopulation of plants represented by those contained within the permanent quadrats was assumed to reflect survival and recruitment in the population as a whole. The population growth rate, λ, was therefore calculated based on changes in the number of individuals within the quadrats. The growth rate is given by:

$$\lambda = N_{t+1} / N_t$$

where N is the number of genets or ramets in the population and t is the year of survey. Where the re-identification of ramets was ambiguous, quadrats were omitted from the study. The Thomastown data are therefore based only on 12 quadrats and the Inistioge study is based on 13 quadrats.

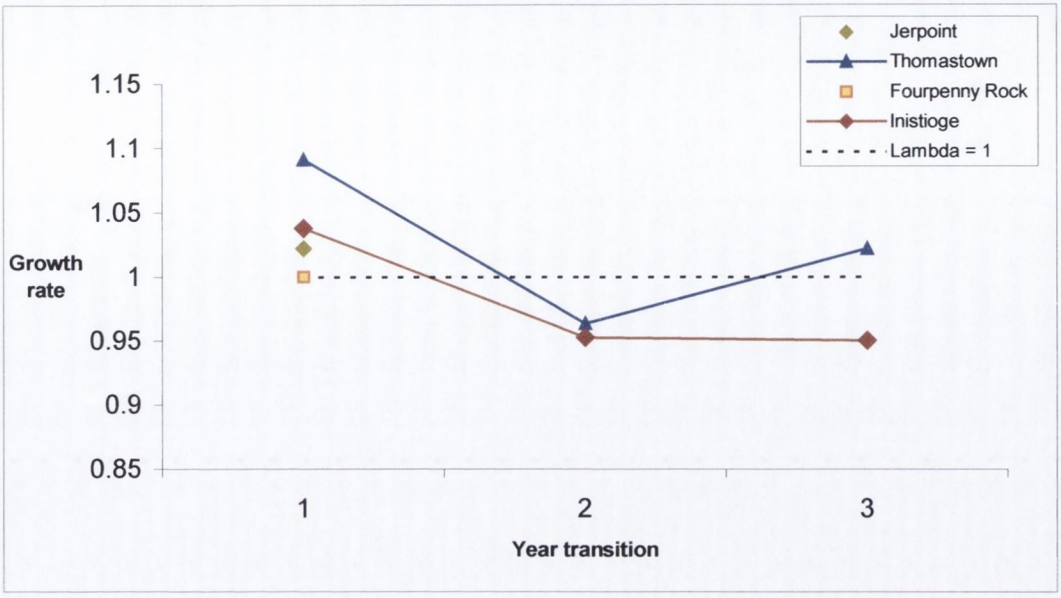
Ramet demography

The ramet population growth rates are given in Table 4.3 over the three yearly transitions for Thomastown and Inistioge, and for the single transition monitored at Fourpenny Rock. As Inistioge could not be monitored in 2001 due to foot and mouth disease restrictions, the mortality and recruitment over the two-year span were divided by two to give average changes per year, and the growth rate was calculated accordingly for the transitions. Although individual ramets were not monitored at Jerpoint, the total number of ramets was counted for two years and the growth rate over this period is also shown for this population. The results are displayed graphically in Figure 4.3.

Table 4.3 Ramet population growth rates for four Irish populations of *Colchicum autumnale*

Population	1999-2000	2000-2001	2001-2002
1 Jerpoint	1.0219	No data	No data
2 Thomastown	1.0914	0.9634	1.0217
3 Fourpenny Rock	1	No data	No data
4 Inistioge	1.0301	0.9526	0.9502

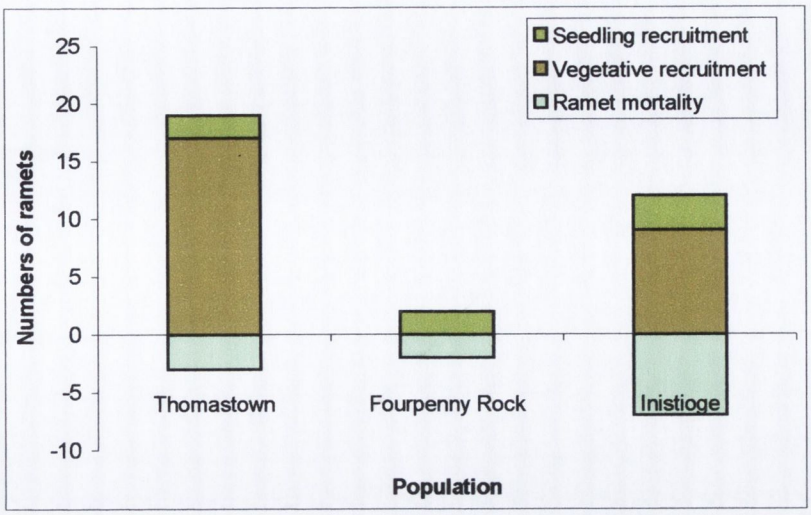
Figure 4.3 Variation in ramet growth rates in four Irish populations of *Colchicum autumnale* over three yearly transitions



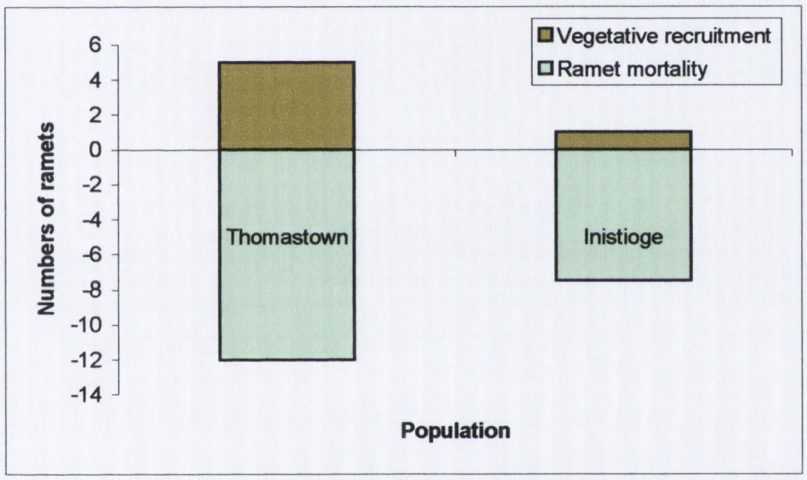
A growth rate of one indicates a steady state, where recruitment to the population balances mortality. The growth rate for Fourpenny Rock was equal to one and the population size remained the same for the two years of monitoring. Growth rates for the other three populations were close to one and variations in population size were therefore relatively small. The numbers of individual ramets at all three populations increased over the period from 1999 to 2000; However, Inistioge showed a decline in the last two years of monitoring. The number of ramets at Thomastown increased between 1999 and 2000, decreased between 2000 and 2001 but showed an increase again in 2002. It therefore appears to be fluctuating about the steady state.

The change in ramet population size can be attributed to ramet and genet mortality, vegetative recruitment and recruitment from seed. The relative proportions of these factors are shown in Figure 4.4 with mortality below the x axis and recruitment above. These plots show that the greater proportion of recruitment is through vegetative spread with only a small amount of recruitment from seed. A number of the ramets classified as recruitment to the population from the vegetative spread of existing ramets may have been surviving ramets that had been dormant in the previous year(s) of survey and this parameter may therefore have been overestimated.

1999-2000



2000-2001



2001-2002

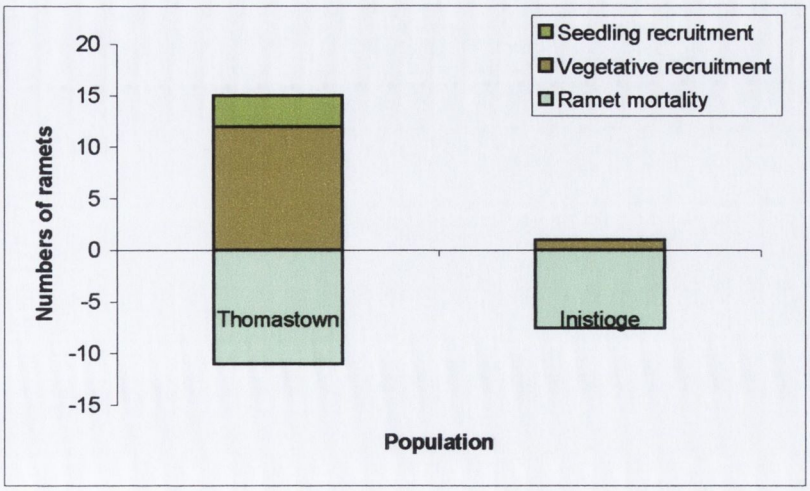


Figure 4.4 Relative proportions of ramet mortality and recruitment in Irish populations of *Colchicum autumnale* over three yearly transitions

Seedling recruitment, vegetative recruitment and mortality expressed as percentages of the total number of ramets in the previous year, are very low (Table 4.4) and as the growth rates do not vary greatly this indicates that the ramets are long-lived.

Table 4.4 Percentage mortality and recruitment in three populations of *Colchicum autumnale*

Population	Period	% Vegetative recruitment	% Ramet mortality	% Recruitment from seed
2 Thomastown	99-00	9.7	1.7	1.1
	00-01	2.6	6.3	0.0
	01-02	6.5	6.0	1.6
3 Fourpenny Rock	99-00	0.0	7.1	7.1
4 Inistioge	99-00	6.8	5.3	2.3
	00-01	0.7	5.5	0.0
	01-02	0.8	5.8	0.0

Recruitment from seed ranged from 0% to 7.1% of the preceding year's ramet population size. There was no observed seedling recruitment at Thomastown over the second transition period (2000-2001) or at Inistioge in the final two years of survey. There was no vegetative recruitment at Fourpenny Rock but percentage vegetative recruitment ranged from 0.7 to 9.7% of the sample size in the other two populations. Ramet mortality ranged from 1.7% to 7.1%, the latter occurring at Fourpenny Rock. Percentage ramet mortality was stable at Inistioge, remaining between 5 and 6% but was more variable at Thomastown ranging from 1.7% to 6.3%.

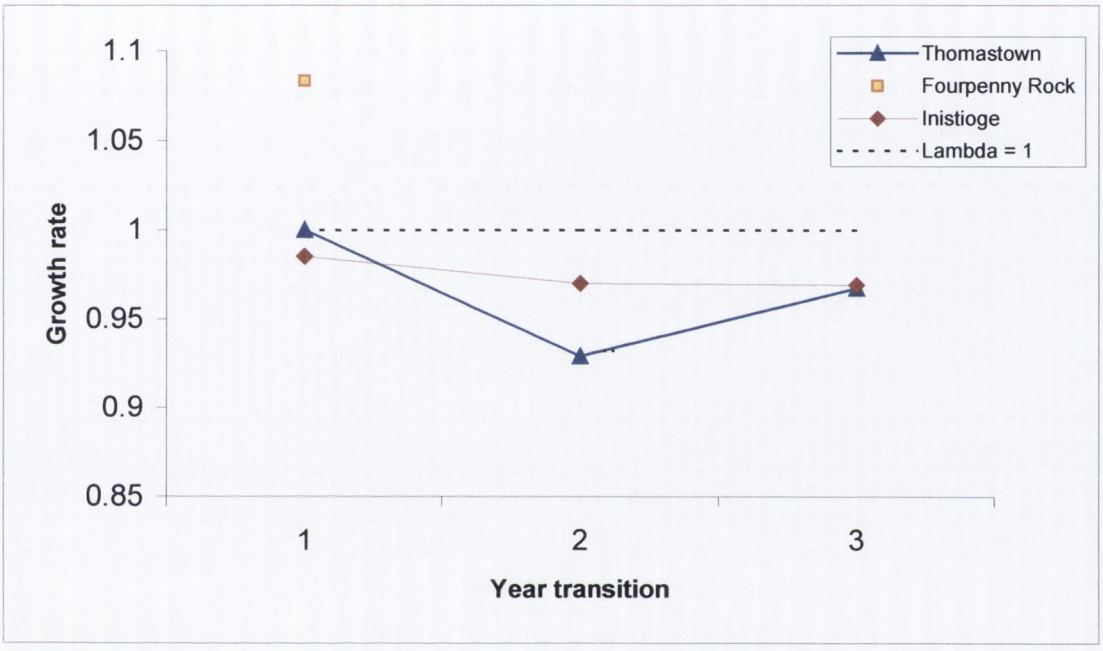
Genet demography

The genet demography of a population can behave differently to ramet demography and although the description of genets was based on a subjective definition, the population parameters were re-calculated as an expression of genet demography, where possible. The population growth rates are shown in Table 4.5 and Figure 4.5.

Table 4.5 Genet population growth rates for four Irish populations of *Colchicum autumnale*

Population	1999-2000	2000-2001	2001-2002
2 Thomastown	1	0.9292	0.9674
3 Fourpenny Rock	1.0833	No data	No data
4 Inistioge	0.9853	0.9701	0.9692

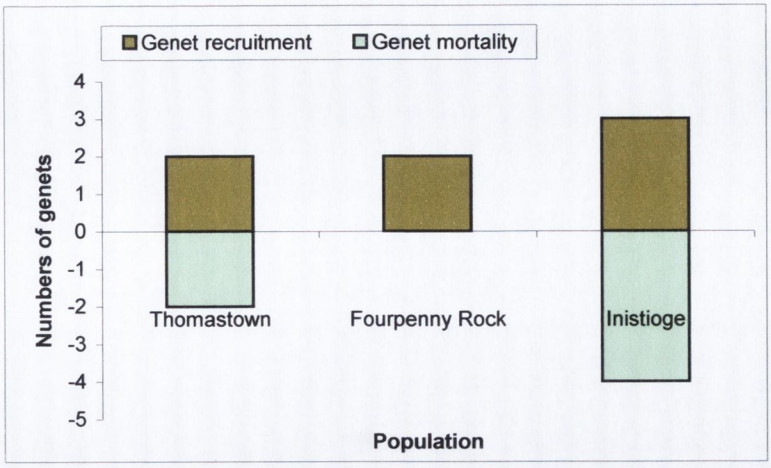
Figure 4.5 Variation in genet growth rates in three Irish populations of *Colchicum autumnale* over three year transitions



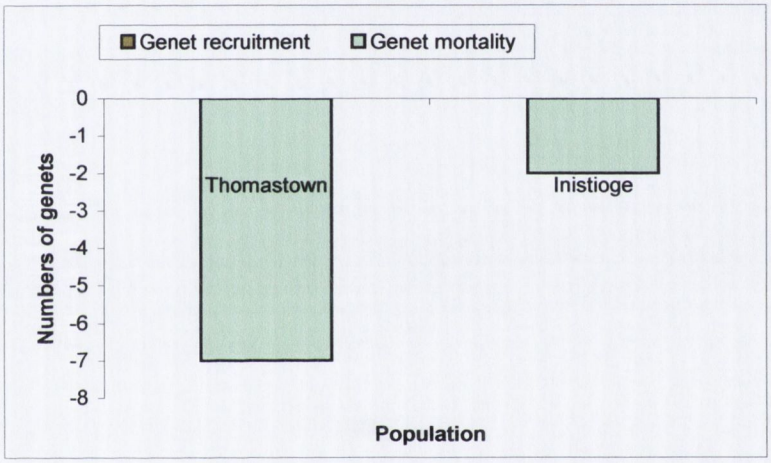
The genet population growth rates ranged from 0.9292 to 1.0833. In contrast to the trends shown in the analysis of ramet demography, Inistioge shows a consistent decrease in numbers over the yearly transitions and while Thomastown remains stable between the first two years, the number of genets also decreases over the second and third transitions. The number of genets showed an increase in Fourpenny Rock, whereas the ramet number had remained stable as indicated by the growth rate of 1 in the previous analysis.

Changes in genet number can be attributed to recruitment from seed and genet mortality and these were therefore calculated as a percentage of the total number of genets. These percentages are shown in Table 4.6. The actual values are shown in Figure 4.6.

1999-2000



2000-2001



2001-2002

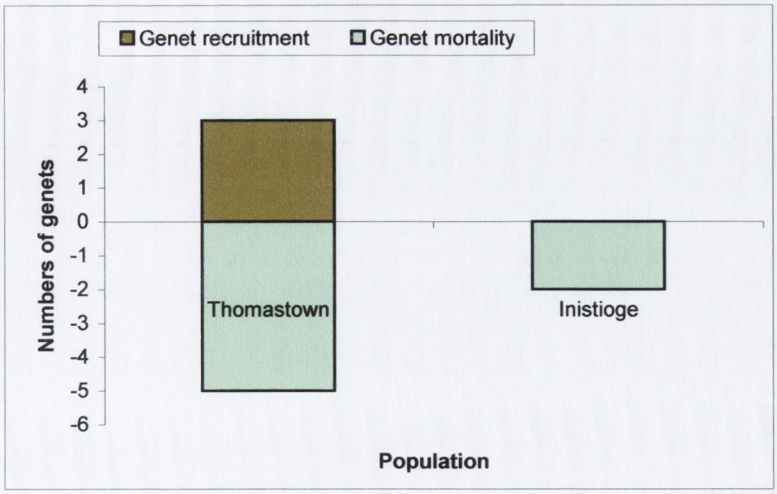


Figure 4.6 Relative proportions of genet mortality and recruitment in Irish populations of *Colchicum autumnale* over three yearly transitions

Table 4.6 Percentage genet mortality and recruitment in three populations of *Colchicum autumnale.* Values are expressed as a percentage of the total number of genets in the population in the first year of the time period.

Population	Period	% Recruitment from seed	% Genet mortality
2 Thomastown	99-00	2.0	2.0
	00-01	0	7.1
	01-02	2.2	5.4
3 Fourpenny Rock	99-00	8.3	0
4 Inistioge	99-00	4.4	5.9
	00-01	0	3.0
	01-02	0	3.1

The percentage recruitment from seed was higher in all populations over all yearly transitions when expressed as a percentage of genet rather than ramet numbers, and ranged from 0 to 8.3. Although this was expected as the number of ramets is much higher than the number of genets, it illustrates the importance of recruitment from seed with respect to the establishment of individuals rather than clonal ramets. As recruitment from seed and genet mortality are the only factors affecting the number of genets, these two figures can be compared directly to assess their relative importance. At Thomastown and Inistioge, the percentage recruitment from seed does not exceed the percentage genet mortality and this is therefore causing the steady state or negative growth rates previously illustrated. Genet mortality occurred over all transitions at Thomastown and Inistioge but did not occur at Fourpenny Rock. In the majority of cases, percentage genet mortality was higher than percentage ramet mortality, ranging from 0 to 7.1.

Seedling demography

The number of seedlings emerging each year, and an estimate of total seedling emergence in each population is shown in Table 4.7. Total seedling emergence was estimated by multiplying the mean number of seedlings per metre squared by the total area of the populations obtained during the population estimates.

Table 4.7 The number of seedlings recorded in three populations of *Colchicum autumnale* over the study period

Population	Year	No. seedlings recorded	Estimated no. seedlings in population
2 Thomastown	1999	5	2609
	2000	2	1044
	2001	0	0
	2002	3	1565
3 Fourpenny Rock	1999	3	27
	2000	0	0
4 Inistioge	1999	2	615
	2000	3	923
	2002	0	0

The numbers of seedlings recorded in the permanent quadrats were very low. However, the proportion of new seedlings surviving until the following year was 100% in all populations for all yearly transitions. Estimated numbers of seedlings ranged from 0 to 2609 at Thomastown, from 0 to 27 at Fourpenny Rock and from 0 to 923 at Inistioge. The longevity of seedlings was assessed by following the fate of a cohort of seedlings recorded during the first year, over the entire study period. The percentage survival is shown in Table 4.8.

Table 4.8 Percentage survival of a cohort of seedlings

Population	% survival after 1 year	% survival after 2 years	% survival after 3 years
2 Thomastown	100	60	40
3 Fourpenny Rock	100	No data	No data
4 Inistioge	100	No data	50

As described previously, all seedlings survived for at least one year after emergence but none reached reproductive maturity during the survey. The majority of seedlings (60%) also survived into the second year at Thomastown although no data was available for Inistioge for that transition. A high percentage of seedlings survived for three years after emergence with 40% survival at Thomastown and 50% survival at Inistioge.

Longevity and dormancy

The longevity of *Colchicum autumnale* is apparent by the large number of ramets surviving throughout the four years of the survey period. At Inistioge, 112 of the 155 ramets recorded in the

first year (85.5%), were still alive at the end of the study. Similarly, at Thomastown, 88.6% (155 of the original 175 ramets) survived over the four years of monitoring. The figures were similar for the genet survival with 88.2% of the genets surviving throughout the study period at Inistioge and 85.9% at Thomastown. These data indicate that the species is a long-lived perennial with a life-span of at least four years, and probably considerably longer than this, as some of the ramets may already have been a number of years old at the start of the study. Dormancy, where a corm survives but does not produce leaves, was observed at Thomastown and Inistioge and lasted one year in all instances. However, as only two transition periods were observed at Inistioge, dormancy periods over 1 year could not be distinguished from mortality. In both populations, new recruits could not be distinguished from re-appearing individuals that had been dormant at the start of the study. At Inistioge, dormancy in 2000 was 4.4% with 6 of the 137 ramets failing to appear above-ground but re-appearing in 2002. At Thomastown, none of the corms were dormant during 2000 but 7 of the 185 corms were dormant in 2001 representing 3.8% of the sampled population.

4.3.2 Reproductive Biology

Fecundity

The fecundity of the populations was measured firstly in terms of the percentage of ramets producing fruit and the mean number of fruits per fruiting ramet. The median number of fruits per fruiting ramet was 1 in all cases. These data are summarised in Table 4.9 and Table 4.10. Figures in brackets in Table 4.10 are the upper and lower limits of the variable range.

Table 4.9 Percentage of ramets producing fruit in five populations of *Colchicum autumnale*

	Population	1999	2000	2001	2002	Mean
1	Jerpoint	4.4	30.2	0	0	8.7
2	Thomastown	15.3	27.9	38.8	54.6	34.2
3	Fourpenny Rock	7.1	28.6	No data	No data	17.9
4	Inistioge	6.7	44.9	No data	62.0	37.9
5	Inistioge Lower	No data	No data	6.0	No data	6.0

Table 4.10 Mean number of fruits produced per fruiting ramet. The range is given in brackets.

Population	1999	2000	2001	2002
1 Jerpoint	1.12 (1-2)	1.24 (1-3)	NA	NA
2 Thomastown	1.11 (1-2)	1.17 (1-2)	1.23 (1-2)	1.35 (1-4)
3 Fourpenny Rock	1.00 (1)	1.00 (1)	No data	No data
4 Inistioge	1.08 (1-2)	1.33 (1-2)	No data	1.37 (1-3)
5 Inistioge Lower	No data	No data	1.00 (1)	No data

No data: no information is available on the fruits produced
NA: Not applicable because no fruits were produced

The percentage of ramets producing fruit ranges from 0 to 62.0 and is highest overall in Thomastown and Inistioge. The largest range occurs in Inistioge, with a maximum of 62% in 2002 and a minimum of 6.7% in 1999. The percentage of ramets producing fruit also has a large range in Jerpoint, with a maximum of 30.2% in 2000 and a minimum of no fruits produced in 2001 and 2002. The mean number of fruits per fruiting ramet is highest in Thomastown and Inistioge and ranges from 1 to 1.37 in all populations. The number of fruits ranged from 1 to 4 per ramet but in all populations the majority of fruiting ramets produce only one fruit. The percentage of ramets producing fruit and the mean number of fruits per fruiting ramet were lowest in 1999 for most populations and highest in 2002 for all populations.

Fecundity was also measured in a separate study on the numbers of seeds produced per fruit and the number of fruits and seeds produced per ramet. The results are summarised in Table 4.11.

Table 4.11 Summary of data collected on reproductive output in three populations of *Colchicum autumnale*. Standard deviation of the mean is given in brackets except for the mean no. fruits where the range of values is given.

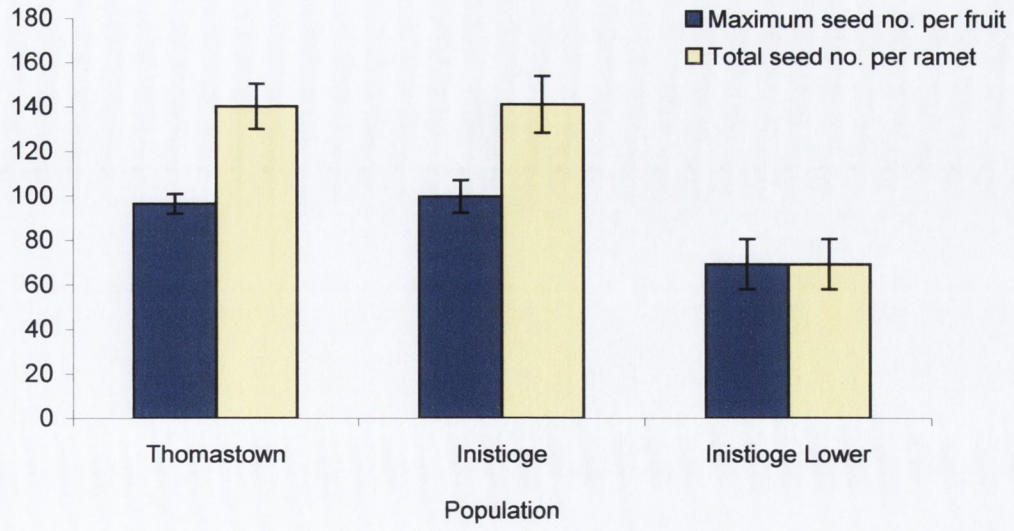
Pop. no.	Pop. size	Sample size	Mean leaf width, mm	Mean no fruits per ramet	Mean no. seeds per fruit	Mean maximum no. seeds per fruit	Mean total no. seeds per ramet
2	15,618	50	31.54 (±4.64)	1.64 (1-3)	84.78 (±25.31)	96.4 (±31.72)	140.18 (±71.95)
4	7,196	48	32.17 (±4.44)	1.58 (1-3)	85.82 (±41.49)	99.6 (±49.16)	140.93 (±85.86)
5	250	6	28.00 (±4.10)	1.00 (0)	69.17 (±27.53)	69.17(±27.53)	69.17 (±27.53)

Mean no. seeds per fruit was calculated for each ramet and then averaged over the population
Mean maximum no. seeds per fruit is the maximum per ramet, averaged over the population
Pop 2 = Thomastown, Pop 4 = Inistioge, Pop 5 = Inistioge Lower

Over the three populations, the mean number of fruits ranged from 1 to 1.64, the mean number of seeds per fruit ranged from 69.17 to 85.82, the mean maximum number of seeds per fruit ranged from 69.17 to 99.6 and the total number of seeds produced ranged from 69.17-140.93. The range of fruit number per ramet was one to three fruits at both Thomastown and Inistioge but all ramets at Inistioge Lower produced only one fruit. The mean leaf width, mean maximum number of seed produced per fruit and the mean total number of seeds produced were higher at Thomastown and Inistioge than at Inistioge Lower.

The variables that represent reproductive vigour are the maximum number of seeds per fruit and the total number of seeds per fruit, averaged over all ramets in the respective populations. The maximum seed number per fruit was compared rather than the mean value to account for the possibility that if a ramet produces more than one fruit, additional fruits may be allocated less seed due to the strain on resources. This would bring down the mean number per fruit and so the maximum number of seed per fruit was compared between populations. These two variables are displayed in Figure 4.7.

Figure 4.7 Mean values of seed output per fruit and ramet in three populations of *Colchicum autumnale* Error bars are standard error of the mean



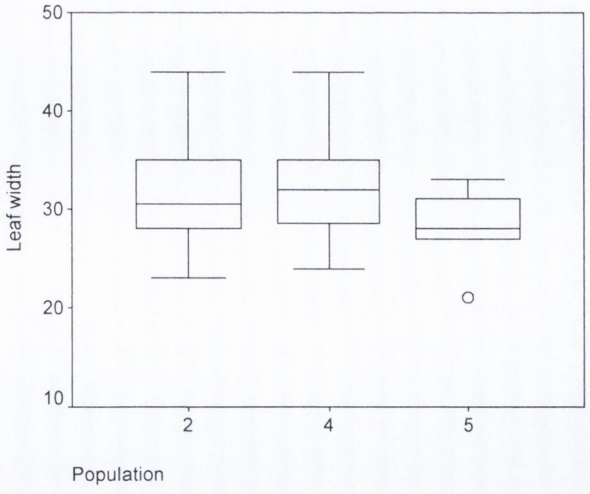
Due to the large differences in sample size, Analysis of Variance was not an appropriate statistical test to perform to identify whether the differences between the populations are significant. Boxplots of the variables were therefore examined (Figure 4.8) and these show that overall there is little difference between the Thomastown and Inistioge populations. However, the Inistioge Lower population has generally lower values of leaf width, mean maximum seed per fruit and mean total seed number per fruit and a much narrower range of all values than in Thomastown and Inistioge. The extremely large range in the Thomastown and Inistoge boxplots encompasses the majority of the range in Inistioge Lower, making differences difficult to detect. As a result of this, and the large differences in sample size, Kruskal-Wallis tests (Table 4.12) were carried out on leaf width and on the reproductive variables, as a non-parametric alternative to Analysis of Variance to look for significant differences between populations.

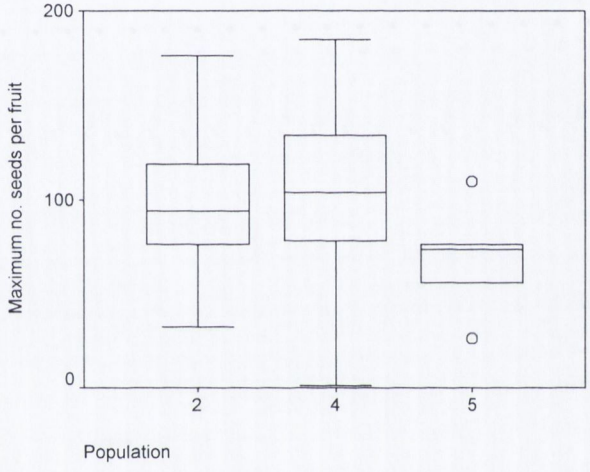
Table 4.12 Results for Kruskal-Wallis analysis of leaf width and reproductive variables in different populations

Variable	Test statistic $_{df}$	Probability
Leaf width	3.796 $_2$	ns
No. fruits	6.028 $_2$	0.049
Maximum no. seeds per fruit per ramet	4.185 $_2$	ns
Total no. seeds per ramet	6.626 $_2$	0.036

ns = not significant and indicates that the probability level is greater than 0.05

Significant differences occur between populations with respect to the number of fruits per ramet and also with respect to the total number of seeds per ramet. These two variables are linked and therefore significantly correlated ($r=0.769$, $p<0.005$, Spearman Rank correlation) as expected. A regression analysis showed that total number of seeds per ramet showed a significant positive linear dependence on leaf width (F ratio = 43.8, df =1,99, $p\leq0.0001$). This relationship is shown in Figure 4.9.





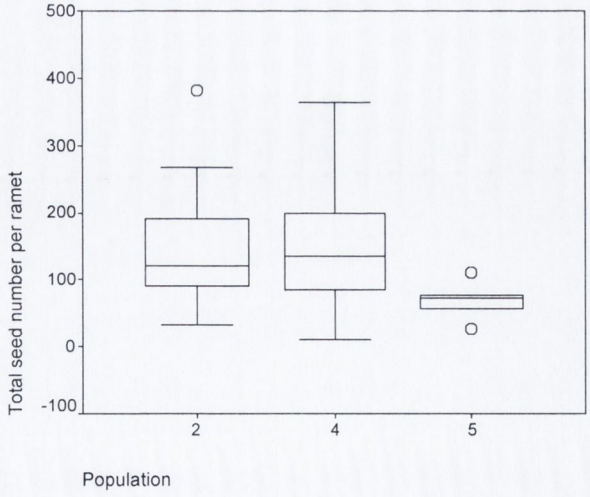
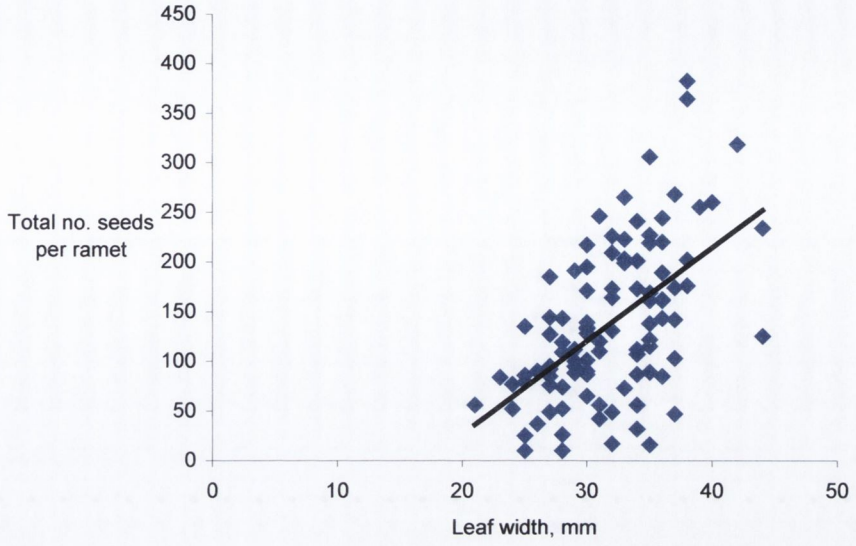


Figure 4.8 Boxplots of leaf width and reproductive output in three populations of *Colchicum autumnale* Population 2 = Thomastown, population 4 = Inistioge, population 5 = Inistioge Lower

Figure 4.9 Relationship between leaf width and the total number of seeds per ramet for all sampled individuals at Thomastown, Inistioge and Inistioge Lower.



The co-efficient of determination was 30.7% and the regression therefore does not explain a very high proportion of the variation. No significant differences were detected between populations with regard to leaf width.

Additional observations on reproductive biology

Although the demography of flowering ramets was not monitored during this study due to the large margin of error, all sites were visited during the flowering season and general notes were taken. The emergence of flowers occurred in the latter half of August in all years and flowering continued until the third week in September. The emergence of flowers was staggered and flowers at various stages of development were present throughout the flowering period. Potential pollinators were in abundance in the meadow habitats at Inistioge and Thomastown and those observed inside the flowers of *Colchicum autumnale* included species from a variety of insect families including Lepidoptera, Coleoptera, Thysanoptera, Diptera and Hymenoptera. Only Lepidopterans and Hymenopterans were observed moving between flowers. Potential pollinators were also present in the other sites but were not as abundant. At the time of flowering in 2001 and 2002, the vegetation at Jerpoint was dense and greater than 50cm and no flowers were observed.

It was not possible to determine whether *Colchicum autumnale* is self-compatible due to the temporal separation of flowering and fruiting phases and the close proximity of individuals, making re-identification between flowering and fruiting ambiguous. However, at Jerpoint where pollen limitation is likely to occur due to the greater height and density of the vegetation, the shrivelled remains of flowers that had failed to develop into fruits were discovered within the emerging leaves in the following spring. This suggests that self-pollination may not occur.

4.3.3 Seed germination

The different treatments had no effect on germination in *Colchicum autumnale* and none of the seeds germinated after one year. However, four out of twenty five untreated seeds that were unintentionally left in the cold store germinated after two years. These seeds had been in the growth room for 3 months prior to placement in the cold store. An observation made during the preparation of the treatments was that the seed coat becomes gelatinous and sticky when exposed to water. This may aid dispersal if the seeds stuck into the hooves of grazing livestock that move around and between fields.

4.4 Discussion

Populations are subject to two factors that have an immediate effect on population size, demographic stochasticity, which affects the vital rate of one or more age classes in a population, and environmental stochasticity, which does not discriminate between demographic groups in its effects on population size (Lande, 1988). Keiding (1975) has suggested that the effects of demographic stochasticity are only important in populations of 50 or fewer individuals but the interaction of demographic and genetic factors can leave populations more susceptible to environmental stochasticity and both are therefore important in threatened species. Irish populations of *Colchicum autumnale* range from 68 to 15,618 estimated genets, but if the assumptions on which the counts of genets were based do not reflect the true distribution of genets in the population, these population sizes could be overestimates and the smaller populations could be more vulnerable than they first appear. However, the Irish populations are

within the range of population sizes represented by the other sampled European populations and the two largest Irish populations, Thomastown and Inistioge, are among the three largest of this sample. These Irish populations occur in extensive riverside meadows and the large area of habitat coupled with a short grass sward maintained by grazing may account for the prolific spread of ramets. At the other sites, grazing is either sporadic, as at Inistioge Lower, or absent, as at Jerpoint, and at Fourpenny Rock the habitat is shaded grassland of a limited area, thus preventing the spread of the population.

The significant negative correlation between population size and mean vegetation height, measured during the fruiting phase, suggests that this may be the primary factor preventing the spread of genets at Jerpoint and Inistioge Lower. The Jerpoint site is part of REPS (the Rural Environment Protection Scheme) and grazing is not permitted at the site as a stipulation of this scheme. As a consequence, there are no animals to aid in seed dispersal by the postulated mechanism and as the vegetation is closed for much of the year between rolling in February and July, seed dispersal is limited and seedling establishment is difficult due to competition for light and resources. There are therefore relatively few genets and those that have established have spread vegetatively in the available space to form large multi-stemmed clumps. The ratio of ramets to genets indicates that the degree of clumping at Jerpoint is approximately six times higher at this site than at the other Irish sites. The mean height of the vegetation at this site was also greater than at all other sites, and the relationship between this environmental factor and the ratio of ramets to genets suggests that it may possibly be responsible for the different growth form. The large degree of clumping may be triggered by repeated failure to reproduce sexually, resulting in a re-allocation of resources to vegetative reproduction. Alternatively, the trait may be genetically determined and may be independent of vegetation height. There may also be a number of other biotic and abiotic factors having a causal effect on population size.

As a result of the clumping, the density of ramets per sampled metre square at Jerpoint was higher than in any other population, but as the site is extensive, the overall density of plants is much lower than in the other Irish populations. It would seem that the spread of *Colchicum autumnale*

at this site requires a grazing regime to be put in place to open the vegetation and stimulate recruitment from seed. Between 1999 and 2000, the growth rate in this population was only 1.0219 whereas the potential for the population to expand is large due to the large area of uncolonised grassland.

Growth rates of both ramets and genets in all populations were close to 1 and the majority of Irish populations of *Colchicum autumnale* therefore appear to be in a steady state. Similar growth rates, with values fluctuating about one with only a small amount of year-to-year variation, have been recorded in other perennial herbs eg. *Panax cinquefolium* L. (Araliaceae) (Charron & Gagnon, 1991), *Chamaelirium luteum* (L.) Gray (Liliaceae) (Meagher, 1982) and *Lathyrus vernus* (L.)Bernh. (Fabaceae) (Ehrlén, 1995) and this is characteristic of long-lived clonal species. Some perennial species have highly variable growth rates, eg *Pedicularis furbishiae* S. Wats. (Scrophulariaceae) (Menges, 1990), which exhibited population growth rates in the range of 0.58-1.81, but the life history and transient habitat of this plant are very different from that of *Colchicum autumnale*. Of the Irish populations, Inistioge was the only population with a negative growth rate at the end of the survey period but this was largely due to the disappearance of all individuals in one particular quadrat and may therefore have been just a localised stochastic effect. This would also account for the decrease in the number of genets in that particular year but, in contrast to the results of the ramet survey, the analysis of genet demography showed that the number of individuals decreased consistently throughout the study period. The number of genets also declined at Thomastown over the second and third yearly transitions. This indicates that the populations are failing to recruit enough individuals from seed to compensate for losses of genets but that they are compensating in most cases by vegetative spread. This may have future genetic effects on the health of the populations and illustrates the importance of considering both ramet and genet demography to obtain a true picture of population fluxes. However, genet death may be part of the normal population turnover and seedling recruitment may have been underestimated due to the difficulty in detecting them amongst the grassland habitat. Overall, mortality and recruitment were less than 10% in all populations over all years and as the

proportions of these components of population change were similar, there were no great fluctuations in population size or growth rates. Seedling recruitment may have been underestimated but conversely, vegetative recruitment may have been overestimated due to the possibility of the classification of returning dormant ramets as new recruits to the population.

The dormancy exhibited by the species has the potential to cause large errors in estimates of survival, as plants that did not re-emerge were assumed to be dead but may re-appear in subsequent years beyond the scope of the survey. Dormancy has been recorded in other groups such as orchids (Hutchings, 1987) and plants can be dormant for significant lengths of time. Plants of *Orchis mascula* (L.) L. have been recorded as dormant for up to twelve years (Tamm, 1972). All confirmed instances of dormancy in *Colchicum autumnale* were for only one year, as in many other species (eg *Orchis militaris* L., Waite & Farrell, 1998) but the possibility that some of the newly emerging ramets were plants that had been dormant for a longer period cannot be ruled out. As a result of this, the estimates of the percentage of the population lying dormant could have been inaccurate. The estimates obtained were between 0 and 5% but could be closer to the percentages observed in other species such as *Orchis militaris* where 14% of the population was dormant (Waite & Farrell, 1998). All these factors highlight the fact that population dynamic trends require survey over a much greater time period than the four years represented by this study, particularly for long-lived species. The high percentage of ramets and genets surviving the length of the study indicate that *Colchicum autumnale* is such a species and as many of the original plants were reproductively mature, it is likely that the life span of the species is considerably in excess of 7 years. In view of this, the change in numbers of ramets and genets over the study period are short-term fluctuations and cannot be interpreted as indications of long-term populations trends.

Another source of inaccuracy in the data is the identification of seedlings. In view of the slow development of seedlings (Butcher, 1954) and the possibility of overlooking seedlings in the first stages of development, it is likely that the individuals identified as seedlings were second or third year plants. If the lag in seedling detection is consistent and the number of seedlings is not

subject to large variation, the conclusions drawn from the examination of recruitment from seed and from the survival of seedlings should still be relevant. Assuming this to be the case, recruitment was low, as expected in established populations of clonal species (eg. Barrett & Silander, 1992). However, even a small amount of seedling recruitment can contribute to genetic diversity in the populations and may therefore limit the effects of genetic drift (Watkinson & Powell, 1993). The reason for low recruitment from seed may be that the seeds require gaps in the vegetation to germinate and become established. However, the survival of seedlings detected in the first year was high, even after 3 years and may also be higher than estimated due to the possibility of dormancy in a proportion of the cohort. If a time lag does occur before detection of seedlings, a significant proportion of seedling mortality may be missed but the important statistic in terms of the population structure is the number of seedlings surviving to adulthood. Longer-term monitoring would be required to determine this but the high percentage survival after three years is encouraging. Although it was difficult to establish without doubt whether new plants had been established from seed or by vegetative reproduction (as is the case in many clonal species, eg. Hutchings *et al.*,1998), seedling recruitment is more likely to have been underestimated rather than overestimated due to the condition that new ramets had to be at least 5cm away from another ramet to be classed as a seedling. In order to verify whether new ramets represented a new genotype or whether they resulted from vegetative spread, the seedlings and all nearest ramets would need to have been fingerprinted to compare genotypes. However, due to the importance of seedlings they could not be sampled at a small size and due to their slow development it was not possible to carry out this analysis as part of this research.

Despite the limitations of this study, it has been shown that recruitment from seed is likely to be occurring in Irish populations of *Colchicum autumnale*, and the populations are not therefore entirely clonal. However, the recruitment of seedlings through to reproductive maturity could not be verified and it is therefore not known whether sexual reproduction contributes to the population.

The establishment of seedlings and the generation of genetic diversity within populations is first dependent on the successful, sexual reproduction of individuals. The study of fecundity in Irish populations of *Colchicum autumnale* indicates that a large amount of seed is potentially entering a seed bank each year but the percentage of ramets producing fruits is very variable. This variation is most pronounced at Jerpoint where the percentage of fruiting ramets dropped from 30.2% to 0 between 2000 and 2001 and remained at 0 for 2002. During the two flowering seasons preceding 2001 and 2002 no flowers were observed at Jerpoint and this accounts for the absence of fruit in these years. The management regime had been changed in these two years and the vegetation was rolled earlier, at the start of July rather than August. This allowed *Pteridium aquilinum*, *Urtica dioica* and *Filipendula ulmaria* to grow back and dominate the vegetation by the end of August. The thick vegetation prevented *Colchicum autumnale* flowers from emerging and any that did would have been unable to attract pollinators. Some flowers may have emerged and may have escaped observation due to the density of the vegetation. However, it is unlikely that these flowers were able to attract pollinators and no fruits were therefore produced. It is clear that the management of this site needs to be carefully considered and this will be discussed further in Chapter 9. The percentage of ramets producing fruit is also low at Inistioge Lower and this could also be a consequence of the habitat. This site is occasionally horse-grazed, but this is sporadic and the vegetation height is often over 60cm at this site (pers. obs.) and could interfere with pollination.

The number of fruits per ramet ranged from 1 to 4, with an average of just above 1 in all populations. This indicates that the majority of ramets produce only one fruit and this contrasts with the results reported by Butcher (1954) who reports that plants produce 1 to 5 fruits with an average number of 2.5. He also reports that the number of seeds per capsule is approximately 30-35, whereas the mean number of seeds per capsule ranged from 69.17 to 85.82 in Irish populations. However, the variation within Irish populations in the seed number per fruit did encompass this range and it may be that the number reported by Butcher was based on a limited number of personal observations. From his own direct observations he also concluded that the

percentage of plants flowering was between 5 and 10 percent. This study reports significantly higher percentages than this in the larger populations. Overall, the two largest populations are more fecund than Jerpoint, Fourpenny Rock and Inistioge Lower with respect to the percentage of fruiting ramets and mean number of fruits per ramet. The number of fruits per ramet showed significant differences between populations. Seed output per capsule was also greater at Inistioge and Thomastown than at Inistioge Lower and as a result of the higher number of fruits per ramet, the total number of seeds per ramet was also higher. The seed output showed a large amount of variation within populations and the differences between populations were only statistically significant for total seed output per ramet.

The total number of seeds per ramet showed a significant linear dependence on leaf width, suggesting that reproductive output may be dependent on the resources available to the plant. However, leaf width is only one indicator of plant vigour and an invasive survey below-ground biomass would need to be undertaken to further investigate the relationship. There were no significant differences in leaf width between populations and this variable does not therefore explain the significant differences in seed output. An alternative explanation for the lower reproductive vigour is that inbreeding depression is being expressed in the population, due to the small population size. Other possible factors include environmental differences between populations resulting in resource limitation. A more detailed study with a greater sample size for Inistioge Lower would be required to ascertain the degree of difference and likely causal factor or factors.

This study of reproductive output in *Colchicum autumnale* was limited in its scope due to the inability to collect data from Jerpoint and Fourpenny Rock. The low sample size at Inistioge Lower also made any differences between populations harder to detect and a full reproductive study would be required for a detailed analysis to be undertaken. Despite the limitations of the study, it suggests that the main threats to the reproductive success of the smaller populations are connected with the management of the sites and are therefore deterministic in nature. Grazing is likely to be an important management tool and alteration of the management regimes at Inistioge

and Jerpoint may greatly increase reproductive vigour and also result in increased recruitment to the populations. This is particularly evident at Jerpoint where although the longevity of individuals has meant that the lack of reproduction has not had a significant impact on the population size, continued reliance on vegetative reproduction will have lasting impacts on the genetic structure of the population. The generation of genetic diversity will be hindered by the lack of sexual recruitment. The hypothesis that the management regime influences the reproductive viability of *Colchicum autumnale* populations could be tested by further monitoring of populations following an agreed change in management.

Both demographic and genetic factors are important in the long-term viability of populations and the genetic aspect to the conservation of the Irish populations of *Colchicum autumnale* will be addressed in the next chapter.

CHAPTER FIVE

GENETIC VARIATION IN IRISH POPULATIONS OF *COLCHICUM AUTUMNALE*: A EUROPEAN PERSPECTIVE

5.1 INTRODUCTION

The recent introduction in 2000 of a scientific journal, *Conservation Genetics*, dedicated entirely to the field of conservation genetics is indicative of the wide acceptance of the importance of population genetics in the theory and practice of plant conservation. The potential applications of genetic research to conservation are wide-ranging and the analysis of population genetic structure can answer many different questions relating to the survival of a species, whilst also providing information on which future conservation strategies can be based. Molecular markers have been used to elucidate phylogeographical relationships among populations (eg. Evans *et al.*, 2001; Loeffler & Morden, 2003), to determine the taxonomic status of threatened groups (eg Vasquez *et al.*, 1999; Coleman et al., 2000), to describe the genetic structure and differentiation of populations of threatened plant species (e.g. Ayres *et al.*, 1997; Cardoso *et al.*, 1998; Palacios *et al.*, 1999; Archibald *et al.*, 2001; Bonnin *et al.* 2002; England *et al.*, 2002), to identify clonal diversity in rare plant species (Bushakra, 1999; Brzosko, 2002), to monitor genetic variation and identity in translocated populations of an endangered plant (Krauss et al., 2002) and to determine the causes and effects of low genetic variation in many threatened plant species (eg. Lammi *et al.*, 1999; Buza *et al.*, 2000; Lutz *et al.*, 2000; Schmidt & Jensen, 2000).

Low genetic diversity is a major concern in small populations of threatened species due to the potentially harmful effects of genetic drift and inbreeding depression, and many studies have therefore focused on inbreeding risks and possible manifestations of inbreeding depression through the reduction of reproductive fitness (eg. Heschel & Paige, 1995; Severns, 2003). However, the breeding system and mode of reproduction greatly affect the response to low levels of genetic variation and plants that have high levels of inbreeding through self-compatibility and selfing, or through a predominantly vegetative mode of reproduction, may have purged their genetic load in the past (Lacy, 1992). A genetic load is incurred through the presence of sub-optimal or detrimental alleles in a population and although inbreeding can increase this load in the short-term, repeated inbreeding can result in a long-term decline in the frequencies of detrimental

alleles as the expression of the alleles results in increased selection against them (Charlesworth & Charlesworth, 1987).

Despite the potential of clonal reproduction to limit levels of genetic variation in populations of plant species, the levels of genetic variation in clonal plants and non-clonal perennials have been found to be similar (Ellstrand & Roose, 1987), suggesting that seedling recruitment may be maintaining diversity; 40% of the species reviewed by Eriksson (1989) exhibited continued seedling recruitment after colonisation of a new site. Where gene flow between populations is limited, mutation and recruitment through sexual reproduction are the sole factors maintaining genetic variation and the input of seedlings to a population will therefore greatly affect genetic diversity. However, although this affects the overall genetic variability of a population, there is still likely to be localised inbreeding among clones, although very few studies have investigated genetic diversity at a small spatial level (eg. Kreher *et al.*, 2000). In view of this probable structure of localised inbreeding, even where genetic variation is moderately high at a population level, plants within clonal patches may have a long history of inbreeding and thus may not be susceptible to inbreeding depression. Inbreeding depression may therefore not be a primary concern in clonal plant species and in such cases longer-term effects such as the ability of the species to adapt to changing environments are more important. Even where asexual reproduction is pronounced and populations can expand rapidly through the production of clones, higher levels of genetic diversity would confer a greater degree of evolutionary flexibility on a species (Huenneke, 1991) and the level of genetic variation is still of concern in the long-term conservation of clonal plant species.

Colchicum autumnale has the potential to reproduce extensively through the vegetative production of cormels and as a result it may be naturally low in genetic diversity and adapted to inbreeding, despite the predominantly outbreeding mode of sexual reproduction enforced by herkogamy and protogyny. This study aims to compare levels of genetic variability in populations from throughout the western European range of the species in an attempt to discover if the Irish populations contain lower gene diversity than expected, and to determine whether the variation

present is a unique component of the total diversity or a subset of the whole. The partitioning of genetic variation among and within populations will also provide information that will be valuable to the assessment of the conservation status of the species in that one of the primary issues is the ambiguity over its native status in Ireland. The allocation of conservation resources to a species that is not a part of the native flora of Ireland is controversial and is an issue that needs to be resolved to enable the prioritisation of species with regard to research and management inputs. Introduced populations are typically established from a few individuals and may be expected to contain lower genetic diversity than natural populations of similar sizes. Genetic similarity between populations will also provide evidence to support or refute the suggestion that *Colchicum autumnale* is introduced. Morphologically there is very little variation in the species throughout its range, other than the elongation of leaves relative to the height of the surrounding vegetation (pers. obs.) but genetic techniques allow the possibility for relationships between populations to be quantified.

To evaluate the level of genetic variation within Irish populations of *Colchicum autumnale*, which are populations occurring at the north-westerly limit of the species' range, a European perspective was required. In view of the conservation aspect to the population genetic study, it is advantageous that the species is not threatened in all areas of its range and comparisons are therefore possible between populations in areas of widespread and restricted distribution to make inferences about the origin and genetic health of the endangered Irish populations. This broad approach was not only a requirement for the assessment of the evolutionary capacity of the populations, it was chosen to allow the investigation of the hypothesis that the species is an introduction to the Irish flora, an archaeophyte linked to the settlement of monastic communities within the Nore Valley. The information obtained from genetic fingerprinting allows a number of different but complementary analyses to be performed, and the objectives of the molecular analyses of populations were to assess the comparative level of gene diversity in populations from different regions, to elucidate relationships between populations through the investigation of genetic distance, to use this information to evaluate the native status of *Colchicum autumnale* in

the Irish flora, to assess its genetic potential for adaptation to future changes in its environment and to provide information pertinent to the suitability of management strategies for the species in Ireland.

5.2 METHODS

5.2.1 Sampling

The sampling of populations for genetic analysis was carried out using the methodology detailed in Chapter two. Twenty populations were sampled in total and their locations are shown in Figure 5.1. Five of the six Irish populations were sampled in April 2000; the population adjacent to Tintern Abbey in County Wexford was not recorded until 2001 and was not accessible during the following year. In addition to the sampling of Irish populations, a European sampling trip was undertaken in April 2000 and this was part-funded by grants from the Botanical Society of the British Isles and Dùchas, the Heritage Service. Fourteen populations were sampled, two from Wales, two from England, five from France and five from Spain. A further French population was sampled during June, 2003 by Frédéric Bioret of l'Université de Bretagne Occidentale. The leaf material was subsequently sent to Trinity College where it was processed and added to the analysis. Twenty-five samples were collected randomly from each population, with the exception of population 20 where the population size was small and only three samples were taken. This represents an excess of samples but this number was collected to allow for difficulties in the extraction of DNA and the possibility of reaction failures. Vegetative and flowering voucher specimens were collected from Inistioge in April 2000 and in September 2000 and were lodged in the Trinity College Herbarium housed in the Department of Botany, Trinity College Dublin.

5.2.2 Genetic Analysis

DNA was extracted from the three samples collected from population 19 and from ten samples from all other populations using the CTAB extraction method detailed in Chapter 2. Quantification of DNA was carried out using an Eppendorf Biophotometer and primer trials were then conducted using a sample of ten individuals from across the range of populations. 8 primer

1. Jerpoint, Co. Kilkenny, Ireland
2. Thomastown, Co. Kilkenny, Ireland
3. Fourpenny Rock, Co. Kilkenny, Ireland
4. Inistioge, Co. Kilkenny, Ireland
5. Inistioge Lower, Co. Kilkenny, Ireland
6. Llanmerewig Glebe, Powys, Wales
7. Cwm Bwchel, Monmouthshire, Wales
8. West Wood, Gloucestershire, England
9. Asham Wood, Somerset, England
10. Fonds l'Olivier, Meuse, Lorraine, France
11. Val des Nonnes, Meurthe-et-Moselle, Lorraine, France
12. Moncley, Doubs, Franche-Comte, France
13. Avanne, Doubs, Franche-Comte, France
14. Valleraugue, Hérault, Languedoc Roussillon, France
15. la Tindière, Pays de la Loire, Loire Atlantique, France
16. Campo-Esera, Huesca, Aragón, Spain
17. Campo-Rialbo, Huesca, Aragón, Spain
18. Lapizea, Navarre, Spain
19. Gasteiz-Vitoria, Alava, Basque Country (Euskadi), Spain
20. Sendadiano, Alava, Basque Country (Euskadi), Spain

Figure 5.1 Approximate location of all *Colchicum autumnale* populations sampled for genetic analysis. For Ireland, the location and County are given, for France, location, Province and Département are given and for Spain, location, Province and Autonomous Community are given.

pairs were tested. Table 5.1 lists the three primers selected on the basis of the number and intensity of bands produced.

Table 5.1. The sequence of selective nucleotides selected for the analysis of *Colchicum autumnale*

Eco RI primer (end sequence)	Mse I primer (end sequence)
AGG	CAA
AAG	CAT
AGC	CTT

Following primer selection, samples were processed using the AFLP genetic fingerprinting technique, following the protocol outlined in section 2.4.1. Failed and weak reactions were repeated; if no improvement occurred, the samples were discarded from the analysis and DNA was extracted from other samples in the relevant populations. Where possible, this was repeated until results were obtained for a total number of ten individuals per population. The numbers of amplifiable AFLP reactions used in the analysis of fingerprinting patterns are shown in Table 5.2.

Table 5.2 The number of samples used in the genetic analysis of *Colchicum autumnale* populations

Population no.	Population name	No. samples
1	Jerpoint	10
2	Thomastown	10
3	Fourpenny Rock	9
4	Inistioge	10
5	Inistioge Lower	10
6	Llanmerewig Glebe	10
7	Cwm Bwchel	10
8	West Wood	10
9	Asham Wood	10
10	Fond l'Olivier	10
11	Val des nonnes	10
12	Moncley	10
13	Avanne	10
14	Valleraugue	10
15	la Tindiere	10
16	Campo-Esera	10
17	Campo-Rialbo	10
18	Lapizea	10
19	Gasteiz-Vitoria	10
20	Sendadiano	3

All samples were run on an ABI Prism Genetic Analyser and the fragments sized by Genescan® 3.1.2 software. The Genescan files were imported into Genotyper® 2.5.2 where the

electropherograms were viewed and the presence and absence of bands were scored by eye. A macro for the automatic scoring of bands was available but was not used due to significant errors inherent within the band size classification method. A presence/absence matrix was obtained from the scoring of bands and this was analysed using a number of different software packages to allow comparison of the results derived from different methods of analysis. Details of the analyses performed are presented below. Due to the significant error in the calculation of diversity statistics for small sample sizes, population 20 (Sendadiano) was left out of the analyses of gene diversity performed by POPGENE and AFLPSURV; these calculations were therefore based on 19 populations.

PAUP v.4.0b10

Neighbour-joining analyses were performed on the presence-absence matrix for all 20 populations using mean character difference and Nei-Li's restriction site difference (Nei and Li, 1979) for comparison. A genetic distance matrix computed between 19 populations was also used as the basis for a neighbour-joining analysis. Bootstrap analyses were performed on all the datasets using 10,000 replicates, to obtain confidence estimates for the tree groupings. Trees were drawn using midpoint rooting so as not to assume relationships between samples. This method roots the tree based on the maximum divergence in the dataset

POPGENE version 1.32

UPGMA dendrograms of populations based on Nei's (1978) genetic distance estimates were plotted. This estimate of genetic distance accounts for small sample sizes. The multi-population genic variation statistics were calculated as described in Nei (1987) and total gene diversity and within population diversity were based on Nei's analysis of gene diversity in sub-divided populations (Nei, 1987). The population statistics obtained are Ht, the total genetic diversity, which is partitioned into Hs the within population diversity and D_{ST} the between-population diversity. D_{ST} is the average gene diversity observed between populations in excess of that observed within populations. G_{ST} is the between-population diversity expressed as proportion of total diversity and therefore gives an indication of differentiation; it is obtained from the

following calculation: $G_{ST} = D_{ST} / H_T$. From the partitioning of diversity, POPGENE calculates an estimate of gene flow using the following equation: $Nm = 0.5(1 - G_{ST})/G_{ST}$ (see McDermott and McDonald, 1993).

AFLP-SURV 1.0

AFLP-SURV was used to calculate population statistics and to generate a genetic distance matrix derived from a Bayesian estimation of allele frequency with non-uniform prior distribution (Zhivotovsky, 1999). The distance matrix was used as the basis for a UPGMA dendrogram using PAUP. The gene diversity statistics obtained are Ht, the total genetic diversity, which is partitioned into Hw the within population diversity and Hb, the between-population diversity. An overall F-statistic for the differentiation of populations is provided by AFLP-SURV following the methodology of Lynch and Milligan (1994).

Arlequin Ver 2.000

Analysis of Molecular Variance (AMOVA) was carried out on the samples to obtain estimates of population structure. Analyses were carried out on all populations, and on all populations with a sub-structure of four groups; Irish populations, British populations, and French populations. Separate analyses were also carried out on each individual country. A further analysis was carried out on biogeographic regions as indicated by the trees obtained in the previous analyses. Mantel tests with 1000 permutations were carried out for all populations and separately for all Irish populations to determine the correlation between genetic and geographic distance matrices.

Le Progiciel R v4·0d

Principal Coordinates Analysis was performed on the data using le Progiciel (Casgrain, 1999), in order to show spatial relationships between samples. The analysis was carried out on a distance matrix derived from the original presence-absence matrix using the Dice similarity measure (Dice, 1945) and subtracting all values in the matrix from 1.

5.3 RESULTS

The AFLP analysis of *Colchicum autumnale* populations yielded 155 markers that could be scored unambiguously for all samples. Marker size ranged from 38 to 370 base pairs in length.

5.3.1 Gene diversity

The three diversity measures derived from the POPGENE analysis, the proportion of polymorphic loci, Shannon's index and gene diversity, are plotted for all populations in Figure 5.2, with populations arranged in geographic regions as labelled in Figure 5.1. Total gene diversity (Ht), mean gene diversity averaged over all loci (H), mean within population gene diversity (Hs) and the mean Shannon's Index (I) were calculated for two levels: all populations and populations within geographic regions. The diversity measures are summarised in Tables 5.3 and 5.4.

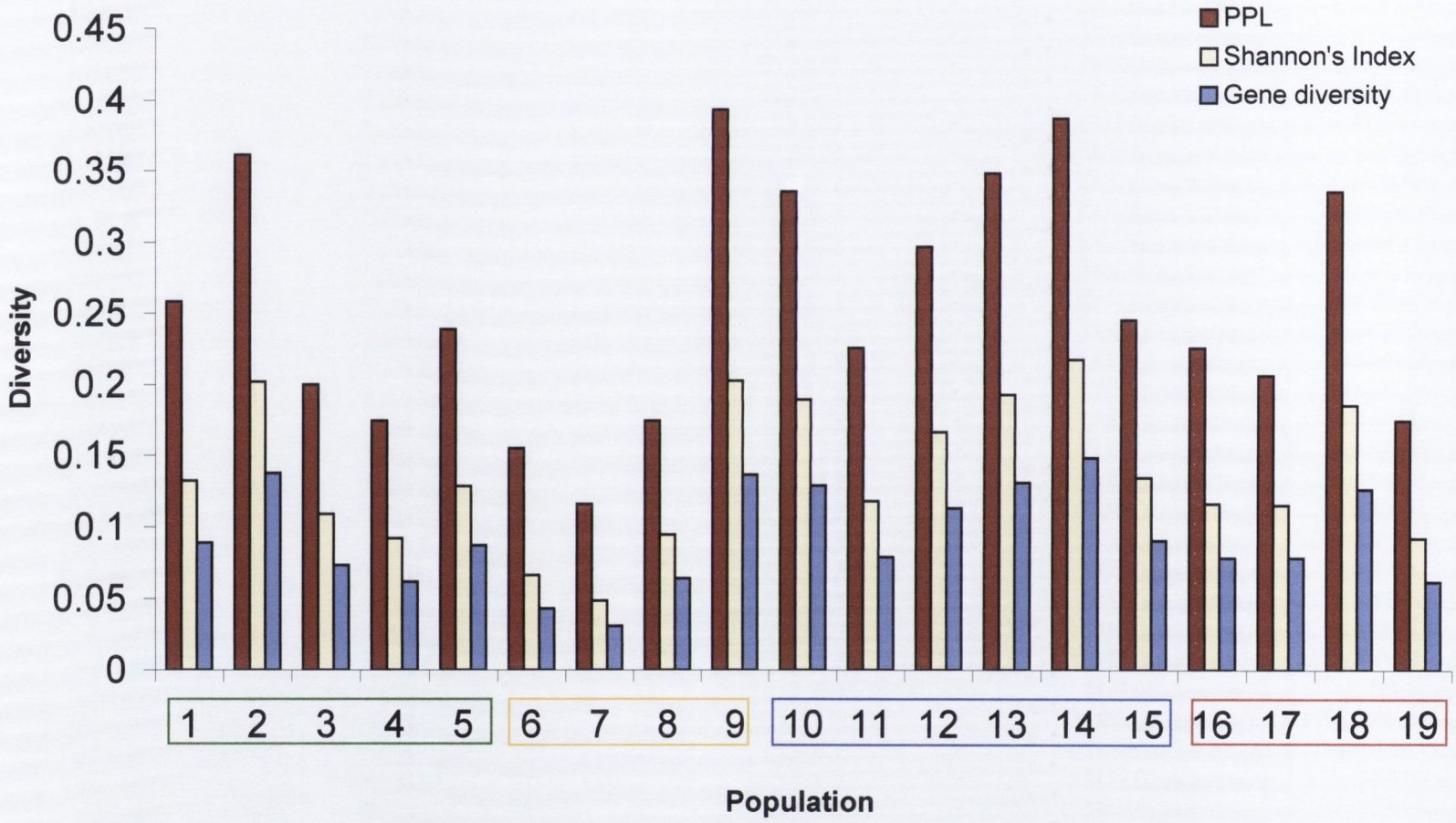


Figure 5.2 Measures of genetic diversity in 19 populations of *Colchicum autumnale,* showing the proportion of polymorphic loci, Shannon's Information Index and Nei's (1973) gene diversity.

PPL = Proportion of polymorphic loci
Irish populations are outlined in Green, British populations in Yellow, French in Blue and Spanish in Red

Geographic Region	Population	Population name	Population size (genets)	No. samples	PPL	Shannon's Index	Gene diversity
Ireland	1	Jerpoint	68	10	0.2581	0.1325	0.0892
	2	Thomastown	15,618	10	0.3613	0.2019	0.1378
	3	Fourpenny Rock	74	9	0.2000	0.1093	0.0738
	4	Inistioge	7,196	10	0.1742	0.0920	0.0622
	5	Inistioge Lower	250	10	0.2387	0.1287	0.0874
Britain	6	Llanmerewig Glebe	716	10	0.1548	0.0669	0.0434
	7	Cwm Bwchel	2,000	10	0.1161	0.0487	0.0310
	8	West Wood	7,000	10	0.1742	0.0947	0.0644
	9	Asham Wood	4,500	10	0.3935	0.2026	0.1363
France	10	Fond l'Olivier	755	10	0.3355	0.1893	0.1291
	11	Val des nonnes	200	10	0.2258	0.1180	0.0795
	12	Moncley	800	10	0.2968	0.1666	0.1132
	13	Avanne	20,000	10	0.3484	0.1927	0.1310
	14	Valleraugue	175	10	0.3871	0.2176	0.1485
	15	la Tindiere	25	10	0.2452	0.1344	0.0909
Iberian Peninsula	16	Campo-Esera	4,000	10	0.2258	0.1165	0.0787
	17	Campo-Rialbo	1,500	10	0.2065	0.1152	0.0787
	18	Lapizea	400	10	0.3355	0.1852	0.1261
	19	Gasteiz-Vitoria	250	10	0.1742	0.0926	0.0621

PPL = Proportion of polymorphic loci
Gene diversity (Nei, 1973)

Table 5.3 Genetic diversity estimates within 19 European populations of *Colchicum autumnale*

Table 5.4 Summary of molecular diversity within populations of *Colchicum autumnale*

Population level	No. samples	PPL	Shannon's Index	Ht	Hs
All populations	189	0.9032	0.4150	0.2741	0.0928
Irish populations	49	0.5161	0.1923	0.1162	0.0901
British populations	40	0.4903	0.1637	0.0979	0.0688
French populations	60	0.6387	0.2609	0.1659	0.1154
Spanish populations	40	0.7032	0.3704	0.2497	0.0864

PPL = Proportion of polymorphic loci Ht = Total gene diversity Hs = Within-population diversity

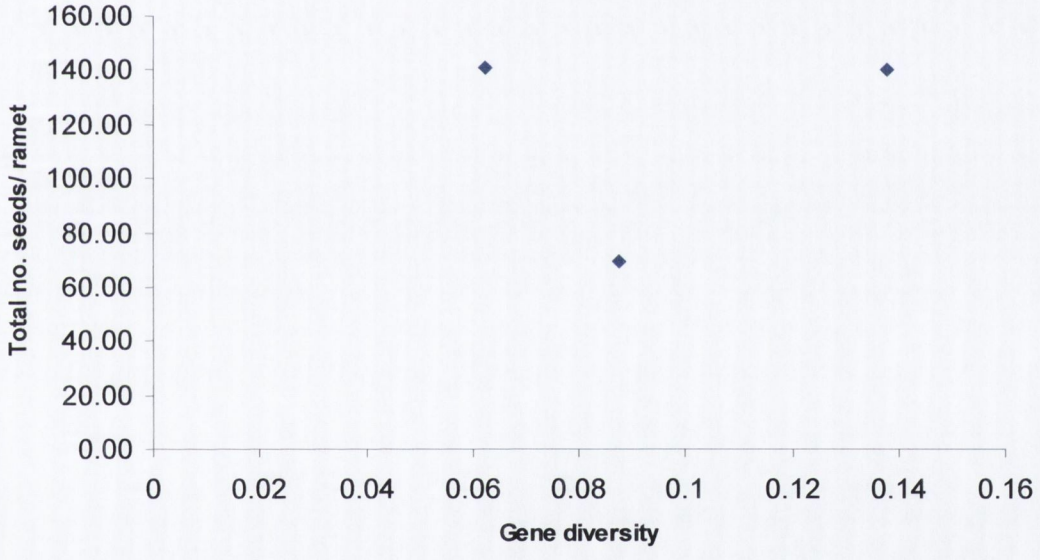
Genetic diversity varied greatly between the nineteen populations of *Colchicum autumnale.* The three diversity measures give different but comparable estimates and the relative pattern of variation is therefore the same in each. The proportion of polymorphic loci is 0.9032 in the sample as a whole but ranges from 0.1161 to 0.3935 in individual populations. The Shannon Index ranges from 0.0487 to 0.2176 and Nei's gene diversity estimate ranges from 0.0310 to 0.1485. The lowest levels of gene diversity occur in the two Welsh populations (Llanmerewig Glebe and Cwm Bwchel) and the highest are found in populations 9 (Asham Wood) and 14 (Valleraugue). Overall, Irish and British populations contain the lowest total diversity, while the Iberian populations contain the highest overall diversity but low within-population diversity. This indicates significant differentiation between Iberian populations and this will be investigated in further analyses detailed in section 5.3.3. Within-population diversity is highest in the French populations and lowest in the British populations. Among the Irish populations, although the average diversity values are low, the largest (population 2, Thomastown) contains the second highest level of gene diversity of all the European populations. In the five Irish populations of *Colchicum autumnale*, the proportion of polymorphic loci ranges from 0.17 to 0.36, Shannon's Index ranges from 0.09 to 0.20, and gene diversity ranges from 0.0622 to 0.1378.

Gene diversity was not significantly correlated with population size (Spearman Rank coefficient = 0.034, n=19) or the log of population size (Pearson Product Moment coefficient = 0.111, n=19). Gene diversity was also not significantly correlated with any of the measures of reproductive output obtained from the studies described in Chapter 4. The Spearman Rank correlation coefficient was 0.5 when gene diversity was tested against the number of fruits and was –0.5

when tested against the maximum number of seeds per fruit per ramet and the total number of fruits per ramet. As there were only three pairings in all these analyses, this value was not significant and the relationship would need to be evaluated fully with further study on all five populations.

The relationship between total seed output per ramet and gene diversity shown in Figure 5.3, shows that the population with the lowest gene diversity had the highest reproductive output. The same relationship is displayed between all pairings of all the gene diversity estimates and all reproductive output estimates.

Figure 5.3 Relationship between gene diversity (Nei, 1973) and total seed number per ramet



Bayesian estimation

Gene diversity statistics were also calculated using the AFLP-SURV analytical program, which employs a Bayesian estimate of allele frequency estimation. Estimates of Nei's genetic diversity were then calculated using the estimated gene frequencies and following the treatment of Lynch and Milligan (1994). The population values obtained for gene diversity are shown in Table 5.5 alongside the estimates of gene diversity obtained from POPGENE, and both are plotted in Figure 5.4.

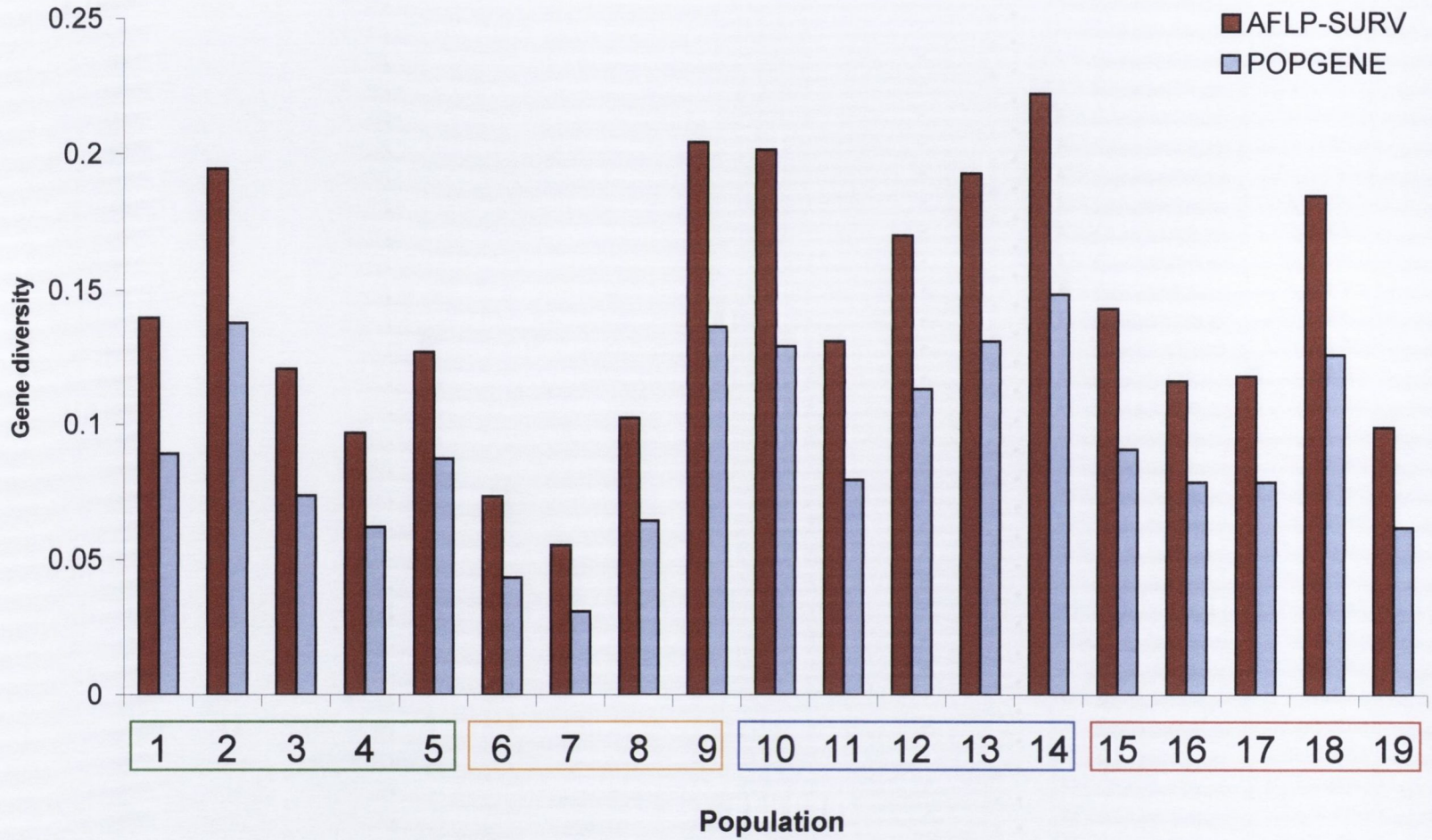


Figure 5.4 Comparison of genetic diversity estimates derived from square root (POPGENE) and Bayesian (AFLP-SURV) estimates of gene frequency

Irish populations are outlined in Green, British populations in Yellow, French in Blue and Spanish in Red

Table 5.5 Comparison of diversity estimates obtained from POPGENE and AFLP-SURV programs

Country	Population	No. samples	Gene diversity	Hj (using Bayesian estimate)
Ireland	1	10	0.0892	0.13986
Ireland	2	10	0.1378	0.19455
Ireland	3	9	0.0738	0.12081
Ireland	4	10	0.0622	0.09679
Ireland	5	10	0.0874	0.12697
Britain	6	10	0.0434	0.07344
Britain	7	10	0.0310	0.05539
Britain	8	10	0.0644	0.10244
Britain	9	10	0.1363	0.20424
France	10	10	0.1291	0.20137
France	11	10	0.0795	0.13106
France	12	10	0.1132	0.17028
France	13	10	0.1310	0.19281
France	14	10	0.1485	0.22242
France	15	10	0.0909	0.14322
Spain	16	10	0.0787	0.11611
Spain	17	10	0.0787	0.11795
Spain	18	10	0.1261	0.18481
Spain	19	10	0.0621	0.09903

The Bayesian estimate of allele frequency calculated by AFLP-SURV gives significantly higher values of gene diversity than the square root estimation method employed by POPGENE. Using the Bayesian estimation, total genetic diversity was 0.3049 (0.2741 with POPGENE) and mean within population diversity was 0.1418 (0.0928 with POPGENE). Gene diversity values for individual populations, as calculated by AFLP-SURV, range from 0.05539 to 0.22242 in comparison with 0.0310 to 0.1485. However, the relative values between populations are similar and both methods of estimation give the highest value of gene diversity for Valleraugue (population 14) and the lowest for Cwm Bwchel (population 7). Using this method of estimation, gene diversity in Irish populations of *Colchicum autumnale* ranges from 0.10 to 0.19.

A significant positive correlation occurs between the two estimates of gene diversity (Pearson Product Moment co-efficient = 0.995, $n=19$, $p<0.05$). The overall conclusions drawn from the previous analysis of diversity in the 19 European populations of *Colchicum autumnale* are therefore maintained in the Bayesian analysis.

5.3.2 Genetic distance

The presence-absence matrix of AFLP markers was used to elucidate relationships between populations through the calculation of pairwise genetic distance. Gene diversity estimates for the 19 populations were used to construct a matrix of genetic distance between populations (Nei,

1978) using POPGENE and a UPGMA dendrogram of populations was produced (Figure 5.5). As a further comparison, a genetic distance matrix was obtained from a Bayesian method of gene frequency estimation (Zhivotovsky, 1999) using the AFLP-SURV analytical freeware (Vekemans, 2002) and this was also used to construct a UPGMA dendrogram, which was found to show the same grouping of populations and is therefore not shown. The genetic distance matrices from which the dendrograms are derived are shown in Tables 5.6 and 5.7. Distances between populations are lower in magnitude when the Bayesian method of allele frequency estimation is used due to the higher estimates of within-population diversity described earlier. However, as before, the relative values are similar and both UPGMA dendrograms show exactly the same relationships between populations, which are organised into four sub-groups within two major groups. The major division in the dendrogram splits French populations from British and Irish populations, with the four Spanish populations divided evenly between the two groups. Within the bottom division, all French populations occupy an exclusive sub-group that is linked to the two Spanish populations from the eastern Pyrenees. Irish and British populations are un-segregated within the top division and an interesting feature of this group is the presence of a sister group containing populations Lapizea and Gasteiz-Vitoria. Figure 5.1, showing the distribution of sampled populations, reveals that these populations are found to the west of the Pyrenees.

The relationships between samples from different populations were investigated more closely using a method of cluster analysis known as neighbour-joining, performed in PAUP version 4.0b10. The original presence-absence matrix was used in the neighbour-joining analysis and individual samples were therefore grouped based on the similarity of the banding patterns. As this does not involve estimation of allele frequencies, all individuals were included in this analysis. Figure 5.6 shows a neighbour-joining phylogram of samples, based on mean character difference and plotted using mid-point rooting. The branch lengths of this phylogram correspond to genetic distance as determined by mean character difference and each character difference between samples corresponds to a distance of 0.006452. The scale of 0.01 corresponds to 1.55

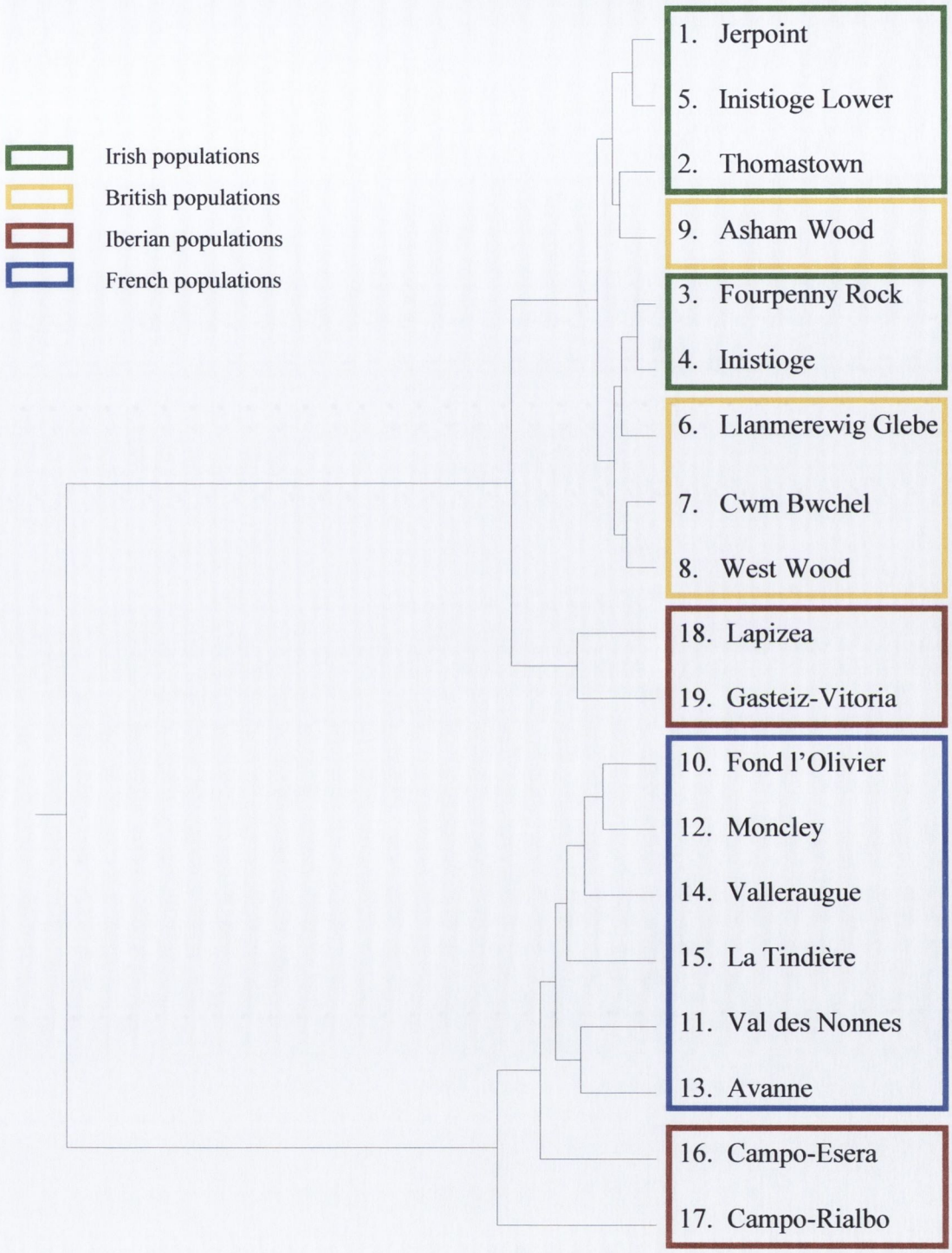


Figure 5.5 UPGMA dendrogram of 19 European populations of *Colchicum autumnale* based on Nei's (1978) genetic distance

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	0.0000																		
2	0.0271	0.0000																	
3	0.0308	0.0475	0.0000																
4	0.0345	0.0559	0.0132	0.0000															
5	0.0163	0.0264	0.0272	0.0330	0.0000														
6	0.0475	0.0441	0.0233	0.0252	0.0335	0.0000													
7	0.0330	0.0526	0.0306	0.0290	0.0357	0.0327	0.0000												
8	0.0339	0.0437	0.0293	0.0324	0.0272	0.0240	0.0209	0.0000											
9	0.0390	0.0256	0.0445	0.0548	0.0303	0.0506	0.0583	0.0437	0.0000										
10	0.4310	0.4257	0.4233	0.4423	0.4221	0.4425	0.4253	0.4197	0.4188	0.0000									
11	0.4363	0.4271	0.4339	0.4551	0.4413	0.4669	0.4496	0.4409	0.4341	0.0763	0.0000								
12	0.4362	0.4358	0.4388	0.4547	0.4289	0.4748	0.4423	0.4424	0.4283	0.0381	0.0648	0.0000							
13	0.3850	0.3865	0.3937	0.3892	0.3879	0.4187	0.3885	0.3824	0.3946	0.0786	0.0539	0.0643	0.0000						
14	0.3858	0.3856	0.4052	0.3952	0.3885	0.4317	0.3929	0.3934	0.3885	0.0594	0.0698	0.0431	0.0564	0.0000					
15	0.4321	0.4370	0.4467	0.4456	0.4312	0.4659	0.4315	0.4354	0.4329	0.0730	0.0652	0.0582	0.0984	0.0601	0.0000				
16	0.4548	0.4566	0.4734	0.4697	0.4595	0.4981	0.4741	0.4731	0.4606	0.1183	0.0786	0.0729	0.0827	0.0664	0.0764	0.0000			
17	0.4667	0.4662	0.4855	0.4982	0.4573	0.4871	0.4799	0.4709	0.4543	0.1095	0.1148	0.0903	0.1432	0.1316	0.1041	0.0983	0.0000		
18	0.1163	0.1070	0.1202	0.1176	0.1083	0.1224	0.1100	0.1075	0.1161	0.3172	0.3304	0.3256	0.3096	0.2887	0.3383	0.3473	0.3570	0.0000	
19	0.0908	0.1057	0.0840	0.0840	0.0947	0.0965	0.0719	0.0807	0.1129	0.3701	0.3820	0.3696	0.3402	0.3508	0.3799	0.4038	0.4009	0.0561	0.0000

Table 5.6 Genetic distance between 19 European populations of *Colchicum autumnale*
(Nei's genetic distance (Nei, 1978) derived from square root estimation of null allele frequency)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	0.0000																		
2	0.0114	0.0000																	
3	0.0153	0.0283	0.0000																
4	0.0209	0.0381	0.0030	0.0000															
5	0.0048	0.0112	0.0129	0.0195	0.0000														
6	0.0352	0.0296	0.0138	0.0172	0.0223	0.0000													
7	0.0238	0.0393	0.0225	0.0222	0.0262	0.0273	0.0000												
8	0.0214	0.0267	0.0174	0.0220	0.0154	0.0163	0.0140	0.0000											
9	0.0213	0.0064	0.0263	0.0369	0.0136	0.0352	0.0442	0.0269	0.0000										
10	0.3917	0.3775	0.3889	0.4137	0.3879	0.4152	0.4020	0.3896	0.3685	0.0000									
11	0.4058	0.3940	0.4046	0.4303	0.4144	0.4430	0.4294	0.4154	0.3958	0.0558	0.0000								
12	0.4051	0.3969	0.4090	0.4294	0.4022	0.4490	0.4224	0.4160	0.3867	0.0183	0.0482	0.0000							
13	0.3509	0.3464	0.3609	0.3624	0.3565	0.3920	0.3657	0.3546	0.3480	0.0548	0.0384	0.0438	0.0000						
14	0.3487	0.3400	0.3676	0.3666	0.3542	0.3996	0.3671	0.3598	0.3374	0.0335	0.0516	0.0237	0.0343	0.0000					
15	0.4016	0.3997	0.4153	0.4214	0.4048	0.4413	0.4115	0.4080	0.3925	0.0543	0.0511	0.0432	0.0787	0.0436	0.0000				
16	0.4271	0.4236	0.4433	0.4453	0.4336	0.4752	0.4543	0.4457	0.4211	0.0970	0.0664	0.0578	0.0643	0.0509	0.0631	0.0000			
17	0.4397	0.4334	0.4572	0.4753	0.4333	0.4672	0.4613	0.4464	0.4174	0.0901	0.0989	0.0741	0.1205	0.1079	0.0892	0.0843	0.0000		
18	0.0951	0.0836	0.0971	0.0982	0.0881	0.1055	0.0944	0.0897	0.0896	0.2811	0.3026	0.2949	0.2736	0.2531	0.3087	0.3223	0.3304	0.0000	
19	0.0784	0.0891	0.0719	0.0735	0.0817	0.0872	0.0642	0.0707	0.0958	0.3423	0.3587	0.3457	0.3142	0.3197	0.3562	0.3796	0.3785	0.0405	0.0000

Table 5.7 Genetic distance between 19 European populations of *Colchicum autumnale*
(Nei's genetic distance after Lynch and Milligan (1994) and based on Bayesian estimate of null allele frequency)

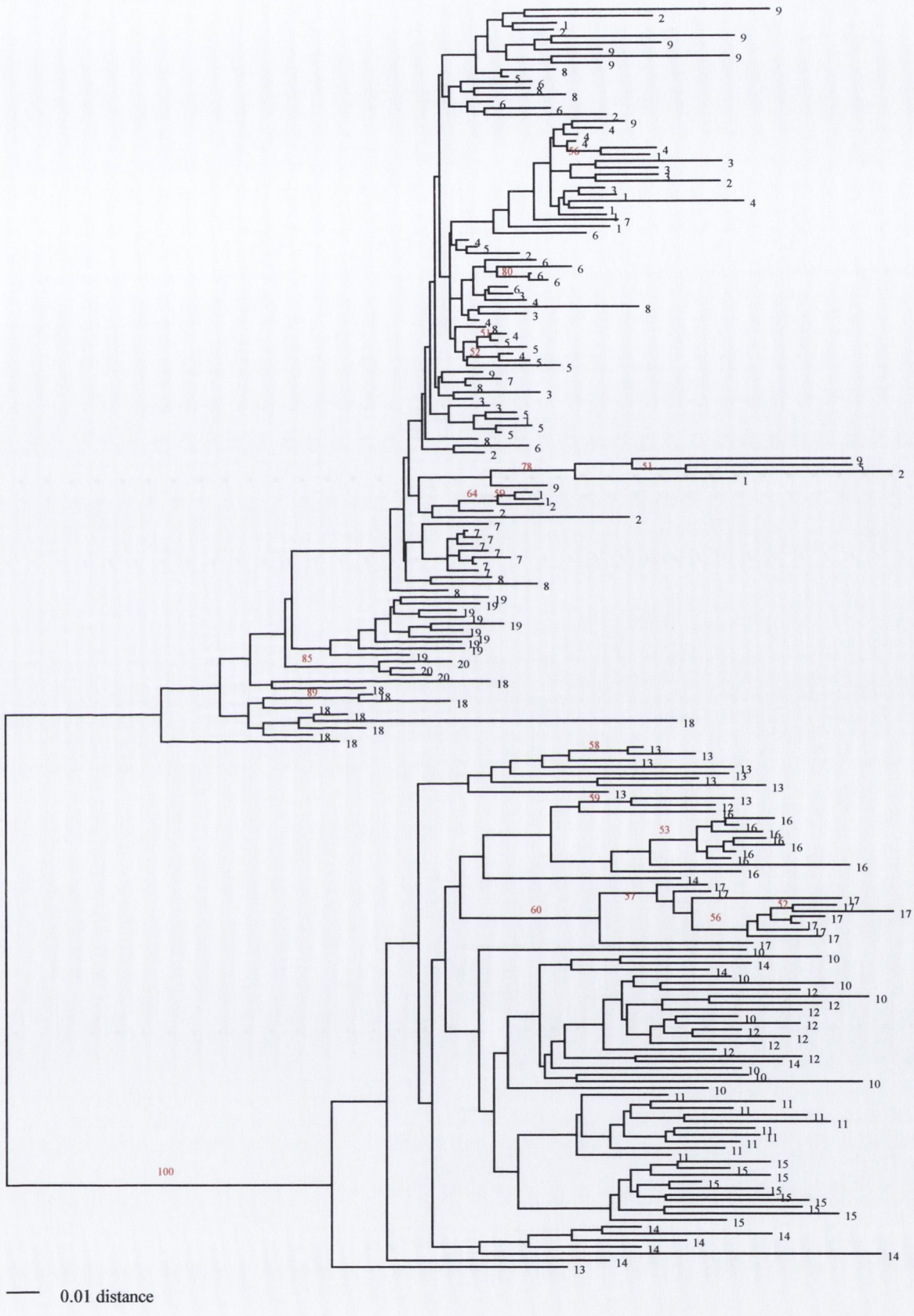


Figure 5.6 Neighbour-joining phylogram of individuals from 20 European populations of *Colchicum autumnale,* based on mean character difference. Bootstrap support values over 50%, from an analysis of 10,000 replicates, are shown in red on the relevant branches. Population numbers correspond to the populations as mapped in Figure 5.1.

character differences. The maximum character difference between two samples of *Colchicum autumnale* was 76 differences and this occurred between a sample from the Spanish Campo-Rialbo population (17) of the eastern Pyrenees and a sample from the English Asham Wood population (9). The minimum character difference between samples was 1 and this occurred between two pairs of individuals from population 7, Cwm Bwchel in Wales.

Figure 5.6 supports the findings of the population dendrograms through the separation of all French populations from the British and Irish samples. The Iberian samples are also split between the two major groups in the same way as shown in Figure 5.5 and Sendadiano, which is now included in the analysis, groups with the other western Pyrenean populations, reinforcing their relationship with the British and Irish populations. The major group containing the French populations and the Iberian populations 16 and 17 is supported by 100% of the bootstrap replications and the significant separation of this group from all other populations is therefore a prominent feature of the molecular dataset. To highlight the grouping of samples from different countries, Figure 5.7 shows the same neighbour-joining phylogram with the population numbers colour-coded by country.

Many of the French and Iberian populations form population-specific sub-groups in Figure 5.6 (populations 11, 15, 16, 17, 18, 19 and 20) indicating very high levels of differentiation, whereas the Irish and British populations are interspersed with each other with a smaller degree of same-population grouping. The branch lengths are also generally longer for the French and Iberian populations. Other than the 100% bootstrap support for the major group containing the French populations, only a small number of sample pairs and small groups were well supported by the bootstrap analysis (>50%). The only population-specific clusters with good support are Sendadiano (population 20) with 85% bootstrap support and Campo-Rialbo (population 17) with 60% bootstrap support.

The neighbour-joining analysis was repeated under the assumption that each difference in the amplification of an AFLP marker results from a restriction or amplification site difference in the

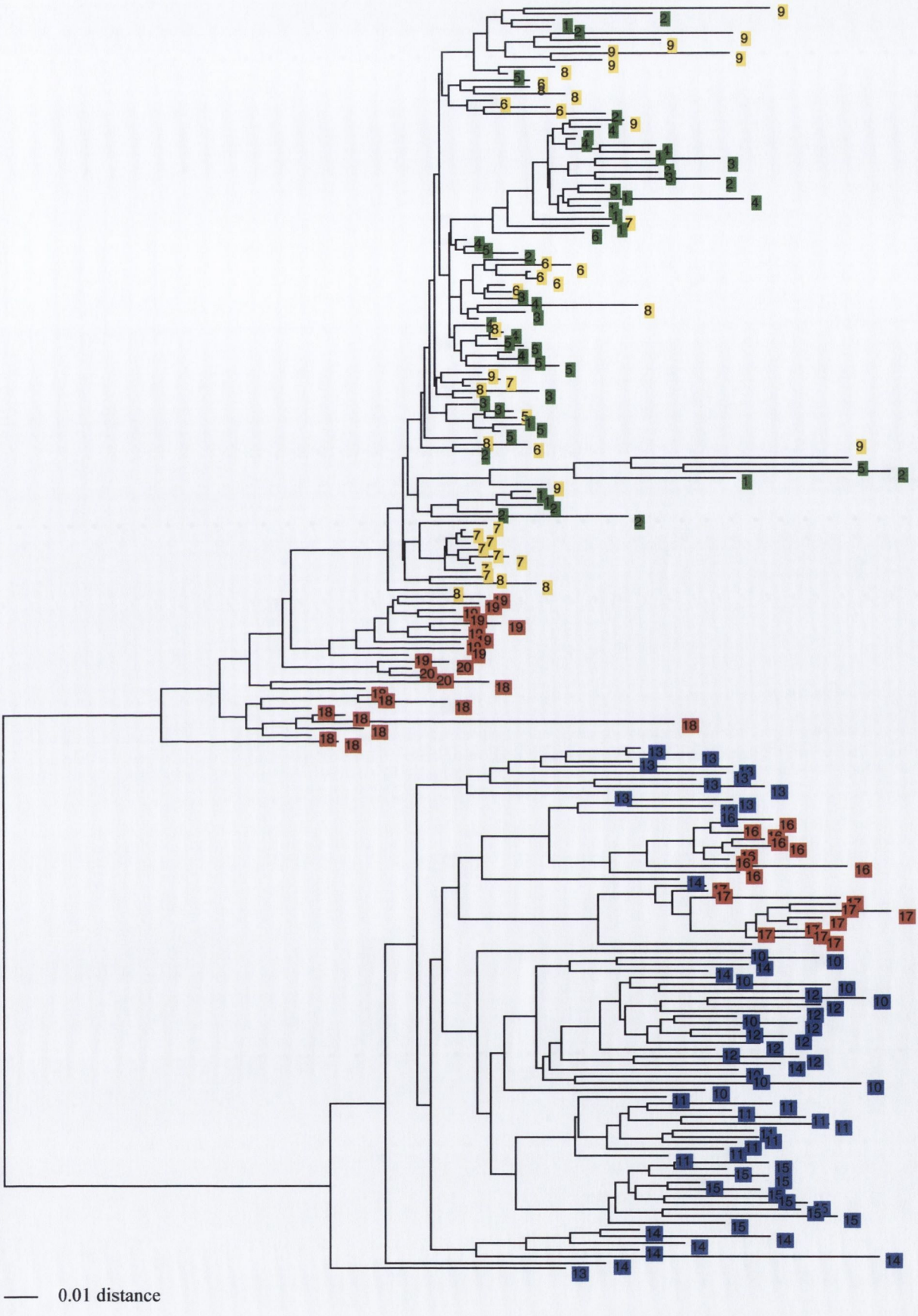


Figure 5.7 Neighbour-joining phylogram of 20 European populations of *Colchicum autumnale* based on mean character difference and colour-coded by region.
Population numbers correspond to the populations as mapped in figure 5.1 and are colour-coded by region – Irish populations are shown in green, British populations in yellow, French populations in blue and Iberian populations in red.

DNA at that locus (Nei and Li, 1979). The neighbour-joining tree based on this calculation of distance using Nei-Li's restriction site distance (Figure 5.8) displays the same general structure as the tree derived from the calculation of mean character difference but the distances between samples are a factor of 10 smaller. Once again, the major group containing the French populations is supported by 100% of the bootstrap replicates and the biogeographical separation of the two groups of Iberian populations is reinforced by the exclusion of Lapizea, Gasteiz-Vitoria and Sendadiano (populations 18,19 and 20) from this group. Samples from Val des Nonnes, La Tindière, Campo-Rialbo and Sendadiano (populations 11, 15, 17 and 20), form exclusive population groups but samples from Irish and British populations are interspersed, with only a small degree of population-specific groupings. Once again the population groups for Sendadiano and Campo-Rialbo are well supported with 83% and 57% bootstrap support respectively.

The genetic relationships between populations were also subjected to a multivariate ordination analysis. Principal coordinates analysis was carried out using Le Progiciel. The first two axes accounted for 23.02% of the variation in the dataset and their eigenvectors are plotted in Figure 5.9. The first axis accounts for 21.49% of the variation in the whole dataset, in comparison with 1.53% for axis two and this is seen by the significant separation of samples along axis 1. The analysis divides the samples into two main groups, consistent with the separation of samples into two major groups in the neighbour-joining and UPGMA analyses. The first axis also separates the western Pyrenean populations from the British and Irish cluster and the second axis separates Campo-Rialbo (17) represented by the group at the bottom right, from Campo-Esera (16) and the French populations. This population also formed a well-supported, exclusive group in the neighbour-joining analysis using mean character difference (Figure 5.6). Despite the low proportion of variation accounted for overall, the Principal Coordinates analysis supports the conclusions drawn from the other analyses that two major groups occur among the population samples, that the two north-western Pyrenean populations show affinities to the Irish and British populations but form a distinct group and that apart from this, the clustering at lower levels within the Irish and British groups is largely unsupported.

Figure 5.8 Neighbour-joining phylogram of individuals from 20 European populations of *Colchicum autumnale,* based on Nei-Li's restriction site difference. Bootstrap support values over 50%, from an analysis of 10,000 replicates, are shown in red on the relevant branches. Population numbers correspond to the populations as mapped in figure 5.1.

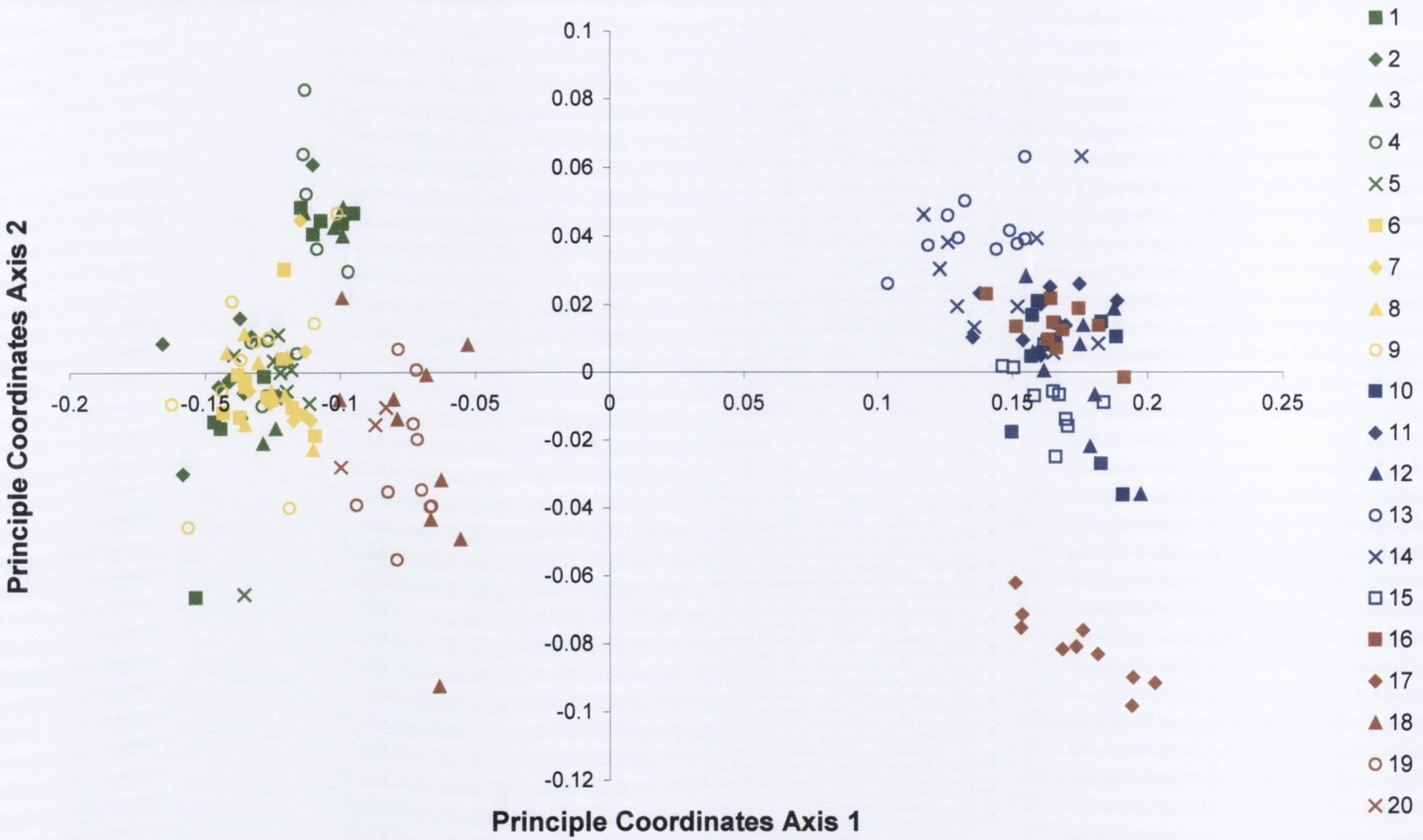


Figure 5.9 Plot of first two axes from a Principal Coordinates Analysis of a 1- Dice similarity matrix for 20 European populations of *Colchicum autumnale.*

Axis 1 accounts for 21.49% of the variation in the dataset. Axis 2 accounts for 1.53%. The symbols correspond to different populations as shown on the right and are colour coded by region. Irish populations are shown in green, British in yellow, French in blue and Iberian in red.

5.3.3 Population differentiation

None of the AFLP markers was diagnostic of any individual population. The level of population differentiation in all the sampled European populations of *Colchicum autumnale* was assessed with both POPGENE and AFLP-SURV analytical programs at two different levels: the overall level of differentiation between populations and the levels of differentiation between populations in each of the four major geographic regions. The results are shown in Table 5.8.

Table 5.8 Population differentiation estimates obtained from POPGENE and AFLP-SURV programs

	POPGENE analysis				AFLP-SURV analysis			
Population level	**Ht**	**Hs**	**G_{ST}**	**Nm**	**Ht**	**Hw**	**Hb**	**F_{ST}**
All populations	0.2741	0.0928	0.6614	0.2559	0.3049	0.1418	0.1631	0.5331
Irish populations	0.1162	0.0901	0.2252	1.7198	0.1503	0.1358	0.0145	0.0954
British populations	0.0979	0.0688	0.2976	1.18	0.1339	0.1089	0.0250	0.1770
French populations	0.1659	0.1154	0.3047	1.1408	0.2135	0.1769	0.0366	0.1704
Iberian populations	0.2497	0.0864	0.6540	0.2646	0.3205	0.1295	0.1910	0.5836

Ht Total gene diversity
Hs / Hw Within-population diversity
Hb Between-population diversity
G_{ST} Proportion of between-population diversity
F_{ST} F-statistic for differentiation of populations (Lynch & Milligan, 1994)
Nm Gene flow

Estimates of total and within-population gene diversity are consistently higher in the AFLP-SURV analysis based on the estimation of allele frequencies using a Bayesian method with non-uniform prior distribution than in the analysis based on the square root estimation. Total diversity in European populations of *Colchicum autumnale* is high but the within-population diversity is surprisingly low indicating significant differentiation as confirmed by the high G_{ST} and F_{ST} statistics and low estimate of gene flow. The level of between-population diversity is highest in Iberian populations and lowest in Irish populations for both G_{ST} and F_{ST} estimates of differentiation. Permutation tests for genetic differentiation among populations (F_{ST}) were carried out in AFLP-Surv, based on resampling statistics with 10,000 random permutations of individuals among populations. The level of population differentiation is highly significant in all groups ($p<0.01$) and the Irish populations are therefore significantly differentiated despite containing low between-population diversity.

A hierarchical analysis of diversity was also carried out using POPGENE, to partition the level of differentiation among countries and among populations within countries. The total diversity of groups was 0.1562. The proportion of between group diversity was 0.4302 and the proportion of among-population diversity was 0.4058 (Table 5.9). There is therefore a high level of differentiation between groups and between populations within groups, and low levels of gene flow.

Table 5.9 Population differentiation estimate for populations grouped by country.

Ht	Hc	Hs	Gst	Gcs
0.2741	0.0928	0.1562	0.4302	0.4058

Ht = Total gene diversity, **Hc** = Within-population diversity, **Hs** = Total diversity within countries
Gst = Proportion of between-country diversity, **Gcs** = Proportion of between-population diversity

This hierarchical analysis was repeated with the populations structured into two biogeographic groups based on the structure shown by the sample and population trees obtained from neighbour-joining and UPGMA analyses. The Irish, British and western Pyrenean populations were placed in one group and the eastern Pyrenean and French populations in another. The results are shown in Table 5.10.

Table 5.10 Population differentiation estimates for populations grouped by biogeographic regions.
Region 1 includes all Irish and British populations and the two western Pyrenean populations, Lapizea and Gasteiz-Vitoria. Region 2 includes all French populations, Campo-Rialbo and Campo-Esera.

	Ht	Hc	Hs	Gst	Gcs
All populations	0.2741	0.0928	0.1491	0.4561	0.3776
Region 1	0.1331	0.0831			0.3759
Region 2	0.1711	0.1962			0.3794

Ht = Total gene diversity, **Hc** = Within-population diversity, **Hs** = Total diversity within countries
Gst = Proportion of between-country diversity, **Gcs** = Proportion of between-population diversity

The grouping of populations into biogeographic groups has increased the differentiation between groups and decreased the differentiation among populations within groups. Total gene diversity and within-population gene diversity is highest for the French and eastern Pyrenean

biogeographic group. The populations in this group also show a marginally higher level of differentiation. These estimates of differentiation are lower than the previous estimates and it is clear that the previous analyses were influenced by the grouping of all the Pyrenean populations together in an artificial group. The biogeographic grouping of populations provides a more meaningful analysis.

The partitioning of genetic variation was also investigated with Analysis of Molecular Variance (AMOVA) at two different levels. Firstly, the overall partitioning of molecular variance was calculated among and within populations. 68.25% of the variation was distributed among populations and 31.75% within populations. The level of differentiation (Fst = 0.6825) was highly significant ($p < 0.00001$). Secondly, the division of variance among countries, among populations within countries and within populations was investigated. 51.03% of the variation was distributed among countries, 10.73% among populations and 28.24% within populations. Fixation indices were significant at all levels indicating that countries, and populations within countries, are significantly differentiated from each other. The results of the AMOVA are shown in Table 5.11.

Table 5.11 Partitioning of molecular variance among and within countries

Component of variation		df	SS	Variance component	% Variation	Fixation index ϕ	Significance
All populations	Among populations	18	2603.649	13.89205	68.25	0.68253	p<0.00001
	Within populations	170	1098.489	6.46170	31.75		
Groups	Among countries	3	1799.135	11.67426	51.03	0.51026	p<0.00001
	Among pops within countries	15	804.514	4.74307	10.73	0.42331	p<0.00001
	Within populations	170	1098.489	6.46170	28.24	0.71757	p<0.00001

df = degrees of freedom
SS = sum of squares

AMOVA was also carried out on a country basis to provide a further insight into the partitioning of variance indicated above. The results are presented in Table 5.12.

Table 5.12 Partitioning of molecular variance among and within populations, calculated for individual countries

Component of variation		df	SS	Variance component	% Variation	Fixation index ϕ	Significance
Ireland	Among populations	4	51.089	0.70806	10.82	0.10820	p<0.00001
	Within populations	44	256.789	5.83611	89.18		
Britain	Among populations	3	59.550	1.47889	22.61	0.22613	p<0.00001
	Within populations	36	182.200	5.06111	77.39		
France	Among populations	5	185.500	2.85852	25.13	0.25134	p<0.00001
	Within populations	54	459.800	8.51481	74.87		
Spain	Among populations	3	508.375	16.39111	74.71	0.74714	p<0.00001
	Within populations	36	199.7	5.54722	25.29		

df = degrees of freedom, SS = sum of squares

The fixation indices are low for the Irish, British and French groups, with the larger component of variation occurring within populations. However, the Spanish populations show a much greater degree of differentiation and it is evident that this high level of differentiation would have greatly influenced the overall analysis of populations. In view of the large genetic differences previously detected between the two groups of Spanish populations, a further hierarchical analysis was carried out based on the two biogeographic groups suggested by the previous UPGMA dendrograms and neighbour-joining phylograms. Campo-Esera and Campo-Rialbo were included with the French populations and Lapizea and Gasteiz-Vitoria were included in a group with the Irish and British populations. AMOVA was then carried out as a comparison to the country analysis to detect whether the high divergence in Spanish populations was responsible for the high fixation index among populations within countries obtained in Table 5.11. The results are shown in Table 5.13.

Table 5.13 Partitioning of molecular variance among and within groups, based on genetic similarity of populations

Component of variation	df	SS	Variance component	% Variation	Fixation index ϕ	Significance
Among biogeographic regions	1	1951.159	20.72841	68.19	0.68187	p<0.00001
Among pops within regions	17	652.49	3.20932	10.56	0.33185	p<0.00001
Within populations	170	1098.489	6.46170	21.26	0.78744	p<0.00001

df = degrees of freedom
SS = sum of squares

As in the previous analysis, the smallest proportion of variation occurs among populations within regions. However, the fixation index among populations within regions is lower without the influence of the highly differentiated Spanish populations (0.33 compared to 0.42). Both analyses show that there is significant differentiation between regions and between populations overall, but that the genetic distinction between populations that occur in the same region is comparatively much lower.

An overall Mantel test found the matrix of F_{ST} values between populations (Table 5.14) to be highly significantly correlated with the matrix of geographic distances between populations (Table 5.15) with a probability value of $p<0.000001$. No significant correlation was found between genetic and geographic distance for Irish populations ($p=0.219$), British populations ($p=0.254$), French populations ($p=0.442$) or Spanish populations ($p=0.259$). The test was repeated for each of the two major biogeographic groups; the first containing Irish, British and western Pyrenean populations and the second containing French and eastern Pyrenean populations, and both were found to show significant genetic structuring with geographic distance ($p<0.000001$ and $p=0.006$ respectively).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2	0.25000																	
3	0.28421	0.65741																
4	0.32292	0.38710	0.35052															
5	0.21978	0.37143	0.40777	0.36634														
6	0.27368	0.34000	0.51000	0.53608	0.11000													
7	0.30526	0.55072	0.70667	0.62667	0.51807	0.53846												
8	0.40206	0.20792	0.27723	0.28283	0.34375	0.21978	0.39175											
9	0.49020	0.43678	0.32941	0.36145	0.43182	0.36782	0.49038	0.39130										
10	0.97273	1.00000	1.00000	1.00000	1.00000	0.98165	0.95370	0.99074	0.98148									
11	0.98182	1.00000	0.96330	0.99091	1.00000	1.00000	0.95370	0.99091	0.97248	0.55238								
12	0.99091	1.00000	1.00000	0.99091	1.00000	1.00000	0.99091	1.00000	0.99083	0.56731	0.33663							
13	1.00000	0.98165	1.00000	1.00000	1.00000	1.00000	0.99083	0.99091	1.00000	0.35714	0.25234	0.63208						
14	0.97248	0.95327	0.97248	0.96330	0.99083	0.98182	0.97248	0.98165	0.98182	0.37255	0.57009	0.35000	0.34615					
15	0.99091	0.98182	0.99091	1.00000	1.00000	1.00000	0.98182	1.00000	0.99091	0.71429	0.24038	0.63551	0.74074	0.27885				
16	0.99091	1.00000	1.00000	1.00000	0.99091	1.00000	1.00000	0.98182	1.00000	0.30476	0.26733	0.29907	0.76147	0.24762	0.65421			
17	0.66000	0.34951	0.50962	0.53465	0.56436	0.49485	0.66019	0.67000	0.74257	0.99091	0.93578	0.99083	0.99091	0.93458	1.00000	0.96296		
18	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.74312	0.23077	0.66038	0.85981	0.39796	0.76364	0.66038	1.00000	
19	0.43137	0.71818	0.95413	0.91589	0.90000	0.80189	0.45055	0.68224	0.45918	1.00000	0.99091	0.98165	0.99091	0.97273	0.99091	1.00000	0.33654	0.99091

Table 5.14 Matrix of pairwise Fst values for 19 European populations of *Colchicum autumnale*. Population numbers correspond to those displayed and listed in figure 5.1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	0.00																		
2	1.07	0.00																	
3	3.92	3.11	0.00																
4	7.55	6.67	3.63	0.00															
5	7.78	7.02	3.91	0.94	0.00														
6	265.40	264.50	261.90	258.50	258.70	0.00													
7	288.60	287.50	284.60	280.90	280.90	66.54	0.00												
8	360.20	359.30	356.60	352.90	352.90	110.40	73.20	0.00											
9	356.50	355.80	352.70	349.10	348.80	156.80	91.70	83.84	0.00										
10	980.80	980.80	980.80	980.80	980.80	737.80	695.80	629.00	625.10	0.00									
11	1008.00	1008.00	1008.00	1008.00	1008.00	767.80	723.80	657.70	651.60	32.55	0.00								
12	1098.00	1098.00	1098.00	1098.00	1098.00	873.80	823.60	763.30	743.10	185.60	155.40	0.00							
13	1108.00	1108.00	1108.00	1108.00	1108.00	885.00	834.50	774.50	753.60	198.10	167.80	12.73	0.00						
14	1229.00	1229.00	1229.00	1229.00	1229.00	1068.00	1005.00	965.60	913.60	566.20	542.90	402.10	393.00	0.00					
15	876.20	876.20	872.20	868.70	868.70	697.80	636.10	594.30	545.40	345.20	342.60	307.60	311.30	371.80	0.00				
16	1259.00	1259.00	1259.00	1259.00	1259.00	1159.00	1092.00	1073.00	1004.00	836.40	819.50	698.70	691.70	321.20	543.40	0.00			
17	1259.00	1259.00	1259.00	1259.00	1259.00	1159.00	1092.00	1073.00	1004.00	836.40	819.50	698.70	691.70	321.20	543.40	0.43	0.00		
18	1147.00	1147.00	1147.00	1147.00	1147.00	1073.00	1006.00	995.70	920.40	854.70	843.20	743.30	738.70	415.40	528.20	152.50	152.90	0.00	
19	1126.00	1126.00	1126.00	1126.00	1126.00	1079.00	1013.00	1010.00	931.30	933.00	924.00	834.00	830.30	522.40	595.90	251.40	252.20	108.80	0.00

Table 5.15 Matrix of pairwise geographic distance (km) for 19 European populations of *Colchicum autumnale*. Population numbers correspond to those displayed and listed in figure 5.1.

5.4 DISCUSSION: GENETIC VARIATION IN EUROPEAN POPULATIONS OF *COLCHICUM AUTUMNALE*

The use of molecular markers to investigate genetic diversity in threatened plant species has contributed greatly to the assessment of population viability (Haig, 1998). Ultimately, the diversity present at this most fundamental level determines the capacity of a population to survive and reproduce effectively and is therefore a prime factor in determining its viability. In order to assess diversity and evaluate viability, information is required on the level of genetic variation that is expected to occur within populations, and the most widely used comparisons are based on the breeding system and life history of the study species. The breeding systems of plant species can have a large influence on levels of within-population genetic variation (Loveless & Hamrick, 1984) and comparisons should only be made between plants in the same reproductive category (Barrett and Kohn, 1991). However, even in species with similar life histories and modes of reproduction, levels of diversity can vary greatly. A review of diversity within clonal plant species undertaken by Ellstrand and Roose (1987) concluded that species reproducing mainly by a vegetative mode of reproduction generally display lower levels of genetic diversity than species reproducing sexually. However, this was not true in all cases and many clonal species contain high levels of genetic variability (eg. in American *Cyprepidium* (Orchidaceae), Case, 1994 and *Vaccinium vitis-idaea* L. (Ericaceae), Persson & Gustavsson, 2001), indicating that other ecological and historical factors contribute to the determination of population genetic structure. This highlights the dangers of making assumptions based on comparisons with other species but even within species, there are genetic factors that must be taken into account. There is an expectation of lower levels of genetic diversity at the periphery of a species' range due to the genetic consequences of spread and divergence from a centre of diversity (Godt *et al.*, 1995) and there is also a possibility of divergence, with significant differentiation of peripheral and central populations (Lesica & Allendorf, 1995). To evaluate the level of genetic variation within Irish populations of *Colchicum autumnale*, which are populations occurring at the northwesterly limit of the species' range, a European perspective was therefore taken.

5.4.1 Genetic diversity and population differentiation

Levels of genetic diversity within populations of *Colchicum autumnale* were similar to those observed in clonal species, despite its capacity for outcrossing. The overall value for within-population gene diversity of 0.0928 was close to the value of 0.099 observed in allozyme diversity of *Calystegia japonica* (Choisy.) (Convolvulaceae) (Kim & Chung, 1995) and that of 0.091 in *Ophiopogon xylorrhizus* (Liliaceae *s.l.*) (He *et al.*, 2000). RAPD variation in *Astelia australiana* (J.H. Willis) L. B. Moore (Liliaceae), a clonal plant that reproduces almost entirely vegetatively, also produced a similar gene diversity estimate of 0.0926 (James & Ashburner, 1997). However, as previously discussed, in view of the differences between species, an evaluation of the evolutionary potential and conservation status of the Irish populations is dependent on a comparison with other European populations rather than with other species. From this perspective, it can be concluded that the Irish populations contain a moderate level of genetic diversity (0.0901 compared with the overall mean within-population diversity of 0.0928). In the five Irish populations of *Colchicum autumnale*, the proportion of polymorphic loci ranges from 0.17 to 0.36, Shannon's Index ranges from 0.09 to 0.20, and gene diversity ranges from 0.0622 to 0.1378 or from 0.10 to 0.19, using a Bayesian estimate. All these values are within the range of diversity estimates from the other sampled populations and the Irish populations therefore have similar levels of genetic diversity to native populations throughout the western-European range of the species.

The pattern of genetic diversity in the sampled populations indicates that France is the centre of diversity for the species. Diversity is lower in British and Irish populations, which represent the northwestern periphery of the species' European distribution. The Iberian Pyrenean populations are at the southwestern periphery, defining the limit of the Euro-Siberian phytogeographic range of the species and with the exception of population 18 (Lapizea), these also contain low within-population diversity in comparison to the French populations. However, as a region, Iberia contains the highest total gene diversity and this is a result of two clearly differentiated groups of populations existing within this region.

None of the measures of genetic diversity was correlated with population size and it is therefore unlikely that the small populations are more susceptible to genetic threats than the larger ones. Genetic variation within populations is influenced by a number of different variables and historical factors can also confuse the expected relationship between population size and genetic diversity (Ouborg & Van Treuren, 1995). For example, a genetic bottleneck followed by the large expansion of a population through vegetative spread will result in a large population with a low level of genetic diversity. The relative contribution of vegetative spread to a population size disrupts the genetic expectations based on assumptions of genetic drift in sexual populations and in this study of *Colchicum autumnale*, it has been shown that assumptions cannot be made about the evolutionary potential of populations based on the numbers of individuals present. The population with the lowest level of genetic diversity is Inistioge and yet this has an estimated population size of just over seven thousand genets. The other Irish population with low genetic diversity is Fourpenny Rock but variation in this population is still higher than three of the four native Welsh and English populations. An immediate concern with this population is that the low genetic diversity may cause inbreeding depression and result in impaired sexual reproduction, thus further reducing the genetic diversity in the population in a positive feedback loop. Although this population was not included in the reproductive study, none of the measures of genetic diversity were correlated with reproductive vigour in those that were. Seed production was only measured in three populations so the investigation of correlation between variables was based only on three pairs of observations and this is a severe limitation of the analysis. However, evidence suggests that species with a high degree of inbreeding are not susceptible to the dominance effects of inbreeding depression (Lacy, 1992) and genetic theory in conjunction with the results of this study suggest that inbreeding depression is probably not affecting Irish populations of *Colchicum autumnale* although there were too few populations to fully evaluate the relationship.

The partitioning of genetic variation shows that within biogeographic regions, the greatest proportion of variation occurs within populations. This is consistent with the expectation for

outbreeding plant species and *Colchicum autumnale* is therefore not exclusively clonal. However, overall, the greater proportion of variation occurs among populations and this can be attributed to significant differences between regions, as shown by the distribution of variation when populations are grouped according to country and when grouped according to biogeographic regions. The genetic distances between samples and populations suggest that a large degree of differentiation has occurred between two major groups of populations in Western Europe. This is reflected in the high overall values of Gst and Fst and is clearly visible in the population dendrograms, the neighbour-joining phylograms and the Principal Co-ordinates plot. The correlation between the different methods of analysis indicates that this division is inherent within the dataset and not an artefact of the analytical techniques. A clear split occurs between all Irish and British populations, which form one group, and all French populations, which form the basis of the other, but the Iberian populations are divided between these two groups. There is a geographical pattern to this divide and samples that group with the British and Irish samples are from the three populations that occur nearest the northern coast of Spain. These populations are separated geographically and genetically from the other two Pyrenean populations, which show more affinity to the French samples.

This biogeographic connection between northern Iberia, Ireland and Britain is reflected in a number of plant groups, such as in the Ericaceae and among *Saxifraga*, and suggests a colonisation route that progressed up the Atlantic seaboard of western Europe into southwest Britain and Ireland. The high genetic distances between the western Pyrenean populations and population 15, La Tindière, in the lower Loire valley appear to suggest against an migration route passing along the Atlantic side of France but it is possible that post-glacial recolonisation of Europe by *Colchicum autumnale* originated from two separate glacial refugia, and that following a migration northwards from Iberia, a second wave of colonisation from the Alps, Italy or the Balkans into France proceeded far enough to engulf the populations on the western coast and also progressed along the south coast of France into the Eastern Pyrenees. The post-glacial colonisation of Britain and Ireland from an Iberian refugium has been suggested by many studies

and there is strong evidence this occurred in *Hedera* (Grivet & Petit, 2002), in *Ilex aquifolium* (Rendell & Ennos, 2003) and in the genus *Quercus* (Cottrell *et al.*, 2002; Petit *et al.*, 2002; Kelleher *et al.*, 2004).

Although there is a link between the populations from the western Pyrenees and populations from Britain and Ireland, the latter are not nested within the former and are therefore unlikely to have been introduced from this source. The evidence suggests a significant period of isolation between these two groups in comparison to British and Irish populations. The genetic data showed that they have not diverged to a sufficient degree to result in complete separation of British and Irish populations in either the neighbour-joining or UPGMA analyses. This suggests that these populations have not been separated for a sufficient period of time for total divergence of their genetic characteristics. As the genetic distance between populations cannot be dated, there is a possibility that this is due to a very recent separation and that Irish populations are derived from British populations and were introduced as monasteries spread into Ireland from Britain. However, if this were the case, the genetic diversity in Irish populations would form a sub-set of the British diversity, due to founder effects. This would also be expected if the populations had been introduced from Spain or if an introduction had occurred from Ireland to Britain, as may be inferred from the low levels of variation in the British populations, but was not found to be the case. In addition to this, the large degree of diversity within populations would indicate multiple introductions, with extensive sexual reproduction as in *Pueraria lobata* (Fabaceae), an introduced clonal plant of the south-eastern US (Pappert *et al.*, 2000). However, evidence from the study of population dynamics in Irish populations has suggested that sexual recruitment is responsible for only a small proportion of total recruitment and the ability of populations founded from even multiple introduced corms to generate such a large amount of variation with such a small degree of sexual reproduction is unlikely.

A Mantel test for correlation between pairwise geographic and genetic distance between populations showed a significant positive correlation between the two matrices and this indicates significant genetic structuring with respect to geographical location. The structuring was shown

to occur within the two major biogeographic groups, indicating historical gene flow between the populations within biogeographic groups, but not within individual countries. The Irish populations are therefore not differentiated on the basis of their geographic separation.

The Irish populations are less differentiated than populations in all other regions and this reflects the close proximity of the populations and the possibility of gene flow through seed transfer between sites along angler's paths, through dispersal directly by river water or by seed adherence to mats of vegetation that may be carried to new sites by floodwater.

5.4.2 Conservation status of *Colchicum autumnale* in Ireland

The moderately high levels and partitioning of genetic diversity in Irish populations has indicated that the species is not lacking in evolutionary potential and is unlikely to have been introduced into Ireland. Introduced populations are not always less variable than native populations and this was shown in a comparison of genetic diversity in domesticated and native populations of Turk's-cap lily (*Lilium martagon* L. (Liliaceae)) (Persson *et al.*,1998). However, this diversity was conjectured to have been generated by high levels of sexual reproduction following the introduction of multiple clones. As sexual recruitment is limited in Irish populations of *Colchicum autumnale,* the high levels of diversity are more likely to reflect a long period of occupation in Ireland, suggesting that introduction is unlikely to have occurred. The habitats in which the species is found are very similar to habitats in which it occurs in across its European range and this supports the genetic evidence presented here. The evidence supporting the native status of *Colchicum autumnale* in Ireland justifies its position on the Flora Protection Order (1999) and also justifies the allocation of resources to the effective conservation management of the sites.

Genetic differentiation between groups and within groups is significant and to effectively preserve the total genetic diversity present in European populations of *Colchicum autumnale,* populations in all groups must be maintained. Irish populations follow the overall trend and show significant differentiation despite exhibiting the lowest levels of within group differentiation (Gst and Fst in

Table 5.6). A small amount of gene flow is suggested among Irish populations (Nm= 1.72) and although there are limitations in the calculation of this parameter based on genetic models (Bossart & Prowell, 1998) it does reflect the degree of shared genetic characteristics between populations. The absolute value may not be accurate but this parameter does indicate that some genetic transfer between populations is occurring or has occurred in the recent past. The riverside meadow sites of *Colchicum autumnale* all have anglers paths at their boundary with the River Nore and people moving between sites could act to disperse the seed, which becomes gelatinous when wet and may stick to shoes. A small isolated clump of *Colchicum autumnale* occurs approximately 200m up-river from the most-northerly population, along the anglers path and this could be a possible explanation for its appearance at this location. Another isolated clump observed at Mount Juliet, another 200 metres up-river could also have been founded in this way. The movement of anglers along the riverbank has also been postulated as a dispersal mechanism in riverbank populations of *Silene tatarica* Pers. (Caryophyllaceae) (Tero *et al.*, 2003). Other possible mechanisms of dispersal include flotation or the attachment of seed to mats of vegetation that may be carried away by the river and lodged further downstream.

In conclusion, *Colchicum autumnale* is a valuable component of the Irish flora and its populations contain moderate levels of genetic diversity, giving them the capacity to adapt to future environmental changes. All populations have undergone genetic differentiation and are therefore valuable from the perspective of the preservation of genetic diversity. In a European context, the Irish and British populations are significantly differentiated from all other European populations and therefore represent a unique sub-section of the overall genetic diversity. Although the Irish and British populations were not sufficiently differentiated to form distinct population-specific groups in the analyses presented here, together with populations from the Basque Country and Navarre, they form a biogeographic and genetic group that is significantly differentiated from other European populations and should therefore be recognised as a unique genetic unit in the conservation genetics of *Colchicum autumnale* in Europe.

PART II : *Campanula trachelium*

CHAPTER SIX

INTRODUCTION TO *CAMPANULA TRACHELIUM*

6.1 TAXONOMY AND MORPHOLOGY

Campanula trachelium L. is a member of the Campanulaceae family of flowering plants in the Order Asterales (Angiosperm Phylogeny Group, 2003). Its vernacular names include nettle-leaved bellflower and bats-in-the-belfry. Members of the genus *Campanula* are biennial or perennial plants with undivided, alternate leaves without stipules; actinomorphic flowers arranged in racemes or panicles; blue, 5-lobed corollas; 5 free stamens; 3-5 stigmas and 3-5-celled inferior ovaries that develop into capsules, dehiscing by lateral or basal pores (Stace, 1997; Rose, 1981). White-flowered morphs occur occasionally. *Campanula trachelium* is a perennial species, 50-100cm in height with hispid, angular stems; coarsely-toothed leaves; cordate, long-stalked basal leaves; short-stalked stem-leaves; flowers arranged in groups of 1 to 4 in a paniculate inflorescence; blue (occasionally white) corollas, 30-40mm in length with internal and external hairs; erect calyx teeth and globular fruits that disperse seed through basal pores. *Campanula trachelium* has well-developed rhizomes and survives the winter as a basal rosette (Rose, 1981; Stace, 1997). The diploid chromosome number is 34 (Tutin *et al.*, 1980b; Clapham *et al.*, 1987).

6.2 DISTRIBUTION

Campanula trachelium is present throughout most of Europe where it is common in the south and locally common elsewhere, occurring north to Denmark, east to Siberia and south to North Africa. In Britain it is absent from Scotland, with its northern limits in the north of England and north Wales. Its occurrence is occasional to frequent in the north but it is locally common in the south and is also known to have been widely naturalised from gardens (Stace, 1997). Although widespread throughout Britain, its distribution in Ireland is highly localised and it is almost entirely restricted to the Nore and Barrow river catchments in the southeast. Outside this region, populations occur in only four isolated sites, one in each of counties Carlow, Clare, Laois and Offaly (Figure 6.1). A recent survey of the native woodlands of Counties Kilkenny and Carlow, undertaken by BEC Consultants Limited for the National Parks and Wildlife Service, did not record any new locations for *Campanula trachelium* (J. Martin, pers. comm.).

Figure 6.1 Map showing the locations of extant and extinct populations of *Campanula trachelium* in Ireland County boundaries are shown

Botanical records held by the National Parks and Wildlife Service show that the species was formerly more widespread and has therefore undergone a significant decline, as illustrated in Figure 6.1. Even within the Nore valley, which encompasses the majority of its sites, it exists only as a series of mainly small, isolated subpopulations varying in size from ten to four hundred individuals. It is possible that the distribution of the plant along the river valleys was once continuous and that its decline has paralleled the fragmentation of the river valley woodland, its primary habitat.

6.3 HABITAT

Campanula trachelium is a semi-rosette hemicryptophyte that favours base-rich soils. Its primary habitat is base-rich woodland on the sides of river valleys but it also occurs in hedgebanks and on riverbanks (Stace, 1997 and pers. obs.). The plant communities in which it occurs are diverse due to its tolerance of a large range in light intensity and it can be part of a typical woodland ground flora or can occur with a diversity of hedgerow and riverbank species. In Ireland and Britain, it occurs predominantly at the edges of base-rich woodland and in hedgerows and is therefore found mainly in woodland-edge plant communities.

6.4 REPRODUCTION

The inflorescence of *Campanula trachelium* is a leafy panicle with the bell-shaped, hispid flowers borne on short branches in groups of one to four. In Ireland, flowering occurs from July through to the end of September and is highly staggered with some plants flowering while others are shedding seed. The timing of flowering within individuals is also staggered. A particular feature of the reproduction of *Campanula trachelium,* in common with many of the Campanulaceae, is the mechanism of pollen presentation whereby the short-stalked anthers deposit their pollen grains on the style before the flowers open. They are held in this region by pollen-collecting hairs, outgrowths of the stylar tissue, and are thus isolated from the stigma and at the same time 'presented' to incoming pollinators (see Plate 6.1). In addition to this, the floral development is strongly protandrous and the species is therefore expected to be predominantly outbreeding. The flowers are entomophilous and pollinators include insects from the Hymenoptera, Diptera and

Lepidoptera orders. The flowers secrete a nectar reward at their base but many pollinators also feed on the nutrient-rich pollen. The mature capsules are 5-celled and dehisce to form basal pores. Seeds are small and numerous and are released through the pores and dispersed by autochory, through the movement of the flowering stem. Once released, some of the seeds are also likely to be dispersed by wind.

6.5 Historical Context

The earliest botanical records for *Campanula trachelium* are listed in *Cybele Hibernica* (Colgan & Scully, 1898). Records are given for the occurrence of the species at Abbeyleix, Ballyraggett, Thomastown and from Woodstock to New Ross where it is described as abundant by Hart (1885d). He also recorded the plant on the left bank of the Barrow above Portarlington (Hart, 1885). In County Kilkenny, it was also recorded at Archer's Grove, Sheestown and Laggbush by the Rev. S. Madden (Colgan & Scully, 1898), on the River Barrow above New Ross by Mackay (1825) and at Woodstock by Templeton (1793-1820). There is also a record of the occurrence of *Campanula trachelium* from a hedgebank between Glasnevin and Finglas in County Dublin (Colgan and Scully, 1898) but the authors reported that it had not been seen recently and was undoubtedly an introduction. More recently, the plant was recorded in 1940 near Longford but these plants were considered by the recorder to be an introduction (Lamb, 1940). Other more recent records are held by the National Parks and Wildlife Service and are incorporated into Figure 6.1 showing the extinct and extant Irish sites. The suggestion has been made that *Campanula trachelium* was introduced into the Irish flora but as is the case with *Colchicum autumnale*, the localities in which it occurs are identical to the habitats in which it occurs in its supposed native sites in England and Wales. The horticultural value of the species opens the possibility that the plant has become naturalised from garden populations but this is unlikely with regard to the present distribution of the species. Nevertheless, the question over its native status needs to be resolved in order to establish its priority status for conservation.

6.6 CONSERVATION STATUS

Campanula trachelium is listed in the Irish Red Data Book of Vascular Plants (Curtis and McGough, 1988) where it was assigned to the 'Vulnerable' IUCN Country (Irish) threat category. It was also protected under the 1987 Flora Protection Order (S.I. No. 274/1987), but was not included under the revised FPO (S.I. No. 94 of 1999) due to an increase in the number of its localities. However, its populations remain under threat from recreational use of its riverside locations, erosion of the riverbanks, trampling by people and livestock and from the collection of flowering stems. As a result of these factors and due to its highly localised distribution, it is still considered to be a rare plant species in an Irish context and in need of close monitoring.

6.7 SITE DESCRIPTIONS

In Ireland, the distribution of *Campanula trachelium* is centred around the Nore River Valley and the majority of sites therefore occur within the Nore catchment. The remaining sites are distributed widely and fall within the catchments of the Rivers Barrow, Shannon and Slaney, with the exception of Tulla in County Clare where the population occurs at the edge of a turlough. Turloughs are temporary lakes that fill and empty through swallowholes in the limestone bedrock; this is the only known population of *Campanula trachelium* to occur in this habitat and was recently discovered by Goodwillie (1999).

Information on the species present at the sites was collected during sampling for genetic analysis and the species lists were enhanced during subsequent visits for populations that were studied in more detail. *Campanula trachelium* was rediscovered at the Abbeyleix site during 2002 and due to the small number of mature plants, was not sampled for genetic analysis. The locations of the Irish sites are shown in Figure 6.2 and are described below in accordance with this numbering.

In addition to the Irish sites, plants were sampled from two sites in Monmouthshire, Wales and seed obtained from the Musée National d'Histoire Naturelle, Paris was germinated to obtain individuals from a French population. Nomenclature follows Stace (1997).

1 Tulla, Co. Clare
2 Eagle Hill, Co.Offaly
3 Knocknagroagh, Co. Laois
4 Dunmore, Co. Kilkenny
5 Archer's Grove, Co. Kilkenny
6 Mount Juliet, Co. Kilkenny
7 Boherduff, Co. Kilkenny
8 Thomastown, Co. Kilkenny
9 Brownsbarn, Co. Kilkenny
10 Ballyduff, Co. Kilkenny
11 Ballygallon, Co. Kilkenny
12 Island at Oldcourt, Co. Kilkenny
13 Woodstock, Co. Kilkenny
14 Clonamery, Co. Kilkenny
15 Murphys of the River, Co. Kilkenny
16 Rathsnagadan, Co. Kilkenny
17 Pollmonty, Co.Carlow
18 Tintine, Co. Kilkenny
19 Tankardstown Cross, Co. Carlow
20 Clonlea, Co. Dublin (garden population)
24 Abbeyleix, Co. Kilkenny
25 Inistioge, Co. Kilkenny
26 Coolnamuck, Co. Kilkenny

Figure 6.2 Location of Irish study populations of *Campanula trachelium*
Sites are numbered with the population number
County boundaries are displayed and the River Nore is shown in blue.

6.7.1 Irish sites

The main populations of *Campanula trachelium* in Ireland are described below, but it should be noted that in addition to the well-established populations, smaller numbers of individuals are often recorded in hedgerows and along riverbanks and appear to be transient.

Of the populations sampled for genetic analysis, the following group all consisted of less than 5 individuals occurring along the riverbanks of the Rivers Nore or Barrow. The vegetation at these sites was not surveyed due to time constraints and the nature of the sampling, which was done by boat. However, some of the plants occurred at the edges of reed swamp habitat with adjacent beds of *Phragmites australis*, plants at Rathsnagadan were growing on *Salix* trunks and at other sites, the plants were growing with *Phalaris arundinacea*, *Epilobium hirsutum, Impatiens glandulifera, Lythrum salicaria, Oenanthe crocata* and *Valeriana officinalis.*

12. Island at Oldcourt

Location: River Nore, County Kilkenny.
Grid reference: S648368

14. Clonamery

Location: River Nore, County Kilkenny.
Grid reference: S651355

15. Murphy's of the River

Location: River Nore, County Kilkenny.
Grid reference: S660349

16. Rathsnagadan

Location: River Nore, County Kilkenny.
Grid reference: S670335

17. Pollmonty River

Location: Tributary of R. Barrow, County Carlow.
Grid reference: S737353

18. Tintine

Location: River Barrow, County Kilkenny.
Grid reference: S730329

26. Coolnamuck

Location: River Nore, County Kilkenny.
Grid reference: S668341

The remainder of the sites contained populations of more than 10 individuals and are described below.

1. Tulla

Location: Co. Clare, Ireland.
Grid reference: M367015

This is an important site for *Campanula trachelium* due to its status as the largest Irish population and as the only population in Europe to occur in *Corylus avellana* woodland, at the edge of a turlough basin. The population is concentrated in the woodland but also spreads out into the adjacent open calcareous grassland habitat. The woodland canopy is dominated by *Corylus avellana* with an understorey of *Rhamnus catharticus* and *Crataegus monogyna.* Associated species in the woodland habitat include *Agrostis capillaris, Brachypodium sylvaticum, Angelica sylvestris, Arctium minus, Filipendula ulmaria, Geranium robertianum, Hedera helix, Mentha aquatica, Oxalis acetosella, Plantago major, Prunella vulgaris, Rubus fruticosus, Rumex sanguineus, Taraxacum* agg., *Urtica dioica, Vaccinium myrtilis, Vicia cracca, V. sepium* and *Viola riviniana.* The open grassland habitat contained a different community of species including *Pteridium aquilinum, Anthoxanthum odoratum, Arrhenatherum elatius, Dactylis glomerata, Holcus lanatus, Centaurea nigra, Cirsium arvense, Galium aparine, Lathyrus pratensis, Leucanthemum vulgare, Odontites verna, Rhinanthus minor* and *Trifolium pratense.*

2. Eagle Hill, Roscrea

Location: Roscrea, County Offaly, Ireland.
Grid reference: S105925

This site is on the north slope of an esker ridge with the Little Brosna River, part of the River Shannon catchment, occurring to the south-west of the site. The population of mature flowering *Campanula trachelium* individuals is concentrated along a *Crataegus monogyna/Fraxinus*

excelsior-dominated hedgerow but the open calcareous grassland of the esker slope supports a large population of seedlings and non flowering rosettes. This may be due to the trampling or grazing of flowering stems by the livestock that graze the esker or may be a consequence of the high light intensity and low nutrients on the esker slope, preventing plants reaching maturity or sending up flowering stems. The esker had recently been cleared of scrub and the plants on the open slope may be relicts of a woodland population. Associated species include *Carex flacca, Agrostis capillaris, Arrhenatherum elatius, Brachypodium sylvaticum, Cynosurus cristatus, Holcus lanatus, Sesleria albicans, Dactylis glomerata, Achillea millefolium, Alchemilla sp., Arenaria serpyllifolia, Arum maculatum, Bellis perennis, Centaurium erythraea, Cerastium fontanum, Crepis capillaris, Cirsium arvense, C. palustre, Digitalis purpurea, Euphrasia sp., Geranium robertianum, Glechoma hederacea, Hedera helix, Hypericum perforatum, Leontodon saxatilis, Leucanthemum vulgare, Linum catharticum, Medicago lupulina, Plantago lanceolata, Potentilla sterilis, Prunella vulgaris, Ranunculus repens, Rubus fruticosus, Senecio jacobea, Stachys palustris, Taraxacum* agg., *Trifolium pratense, Ulex europaeus, Urtica dioica, Veronica chamaedrys, Viola riviniana* and *V. sativa* subsp. *nigra.*

3. Knocknagroagh

Location: County Laois, Ireland.
Grid reference: N473036

This population stretches over 2 miles of an esker ridge and the plants occur on roadside and trackside *Corylus avellana*-dominated hedgebanks and along a *Corylus avellana*-dominated field boundary hedgerow. The Triogue River, part of the River Barrow catchment, flows at the base of the ridge. Associated species include, *Arrhenatherum elatius, Brachypodium sylvaticum, Dactylis glomerata, Centaurea nigra, Galium aparine, G. verum, Geranium robertianum, Geum urbanum, Glechoma hederacea, Hedera helix, Heracleum sphondyllium, Knautia arvensis, Lonicera periclymenum, Potentilla reptans, Rosa canina* agg., *Rubus fruticosus, Sanicula europaea, Scrophularia nodosa, Taraxacum* agg., *Torilis japonica, Veronica chamaedrys* and *Vicia sepium,* with *Ilex aquifolium* and *Fraxinus excelsior* saplings.

4. Dunmore

Location: N. of Kilkenny City, County Kilkenny, Ireland.
Grid reference: S495598

This is an area of woodland to the east of the River Nore north of Kilkenny city and is protected under the designation of Natural Heritage Area as part of the Dunmore complex of woodland. It is dominated by *Fagus sylvatica* with *Acer pseudoplatanus, Crataegus monogyna, Fraxinus excelsior* and *Quercus petraea. Hedera helix* dominates the ground flora but other species present include *Phyllitis scolopendrium, Carex nigra, Carex remota, Angelica sylvestris, Arum maculatum, Geranium robertianum, Geum urbanum, Hedera helix, Lonicera periclymenum, Rosa canina, Scrophularia nodosa, Vaccinium myrtilis, Veronica chamaedrys* and *Viola riviniana.*

5. Archer's Grove

Location: S. of Kilkenny City, County Kilkenny, Ireland.
Grid reference: S532544

This is a strip of deciduous woodland on the sloping valley side on the southern bank of the River Nore, to the south-east of Kilkenny city. The woodland is dominated by *Fagus sylvatica, Quercus robur* and *Fraxinus excelsior* with an understorey of *Corylus avellana, Crataegus monogyna, Ilex aquifolium* and *Prunus spinosa.* Associated species in the ground flora include *Dryopteris dilitata, Phyllitis scolopendrium, Carex remota, Brachypodium sylvaticum, Arctium minus, Chrysosplenium oppositifolium, Circaea lutetiana, Epilobium montanum, Geranium robertianum, Geum urbanum, Glechoma hederacea, Hedera helix, Hyacinthoides non-scripta, Prunella vulgaris, Ranunculus repens, Rumex sanguineus, Solanum dulcamara, Urtica dioica, Veronica chamaedrys and Viola riviniana.*

6. Mount Juliet

Location: Nr Thomastown, County Kilkenny, Ireland.
Grid reference: S550425

This population occurs in a wooded area of the Mount Juliet Country Estate on the west bank of the Nore west of Thomastown. The woodland is dominated by *Quercus* spp. and the ground flora includes *Phyllitis scolopendrium, Brachypodium sylvaticum, Angelica sylvestris, Chelidonium*

majus, Epilobium hirsutum, Geranium robertianum, Geum urbanum, Glechoma hederacea, Hedera helix, Heracleum sphondyllium, Lapsana communis, Ranunculus repens, and *Rubus fruticosus.*

7. Boherduff

Location: Nr Thomastown, County Kilkenny, Ireland.
Grid reference: S561418

This is a broadleaved woodland site on the sloping east bank of the River Nore, west of Thomastown. The canopy is dominated by *Quercus robur* and *Fagus sylvatica* with an understorey of *Crataegus monogyna* and *Ilex aquifolium.* The population of *Campanula trachelium* is associated with a typical woodland flora including *Phyllitis scolopendrium, Brachypodium sylvaticum, Alliaria petiolata, Arctium minus, Circaea lutetiana, Geranium robertianum, Geum urbanum, Glechoma hederacea, Heracleum sphondyllium, Lapsana communis, Ranunculus repens, Rosa canina* agg., *Rubus caesius, Stachys sylvatica, Urtica dioica, Valeriana officinalis, Veronica chamaedrys, Vicia sepium* and *Viola riviniana.*

8. Thomastown

Location: County Kilkenny, Ireland.
Grid reference: S580410

This is a band of broadleaved woodland on the steeply sloping southern side of the Nore valley to the south-west of Thomastown. The tree species are dominated by *Fraxinus excelsior, Acer pseudoplatanus* and *Fagus sylvatica* with occasional *Quercus robur* and *Ulmus procera.* The understorey consists of *Crataegus monogyna, Corylus avellana, Prunus spinosa* and *Ilex aquifolium.* The woodland stretches down to the riverside meadow below, where it meets an area of marsh and *Salix cinerea* occurs at this margin. The ground flora is sparse but includes *Dryopteris filix-mas, Phyllitis scolopendrium, Polystichum setiferum, Luzula sylvatica, Galium odoratum, Geum urbanum, Geranium robertianum, Glechoma hederacea, Hedera helix, Lapsana communis, Lonicera periclymenum, Rubus fruticosus,* and *Viola riviniana.*

9. Brownsbarn and 10. Ballyduff

Location: N of Inistioge, County Kilkenny, Ireland.
Grid reference: S616385

Both these populations occur in the same area but on opposite banks of the River Nore. They are long linear populations occurring over a distance of approximately 2.5 kilometres. *Campanula trachelium* shows a disjunct distribution along this stretch but the distance between plants is never more than approximately 30 metres on either bank. The populations are therefore assumed to be continuous along each individual bank but the populations on opposing banks were separated due to the barrier of the River Nore, which may prevent gene flow. The plants occur in woodland, riverbank hedgerows and open riverbanks along this stretch of the Nore and are therefore associated with a range of species. The banks are mainly wooded or with light shading from hedgerows or sparse trees and the species present are therefore typical woodland edge species as described for other woodland sites. However, in the open habitats seedlings on the banks were found growing with species such as *Agrostis capillaris, Brachypodium sylvaticum, Deschampsia caespitosa, Phalaris arundinacea, Calystegia sepium, Filipendula ulmaria, Hedera helix, Hypochaeris radicata, Oenanthe crocata, Origanum vulgare, Rumex hydrolapathum* and *Solanum dulcamara.*

11. Ballygallon

Location: Inistioge, County Kilkenny, Ireland.
Grid reference: S641384

This is a west-facing hedgebank population occurring on a roadside on the steeply sloping valley side above the River Nore at Inistioge. It is located at the edge of a mixed coniferous and deciduous woodland and light shading is given by *Fraxinus excelsior* and *Crataegus monogyna.* Associated ground flora species include *Dryopteris dilitata, Phyllitis scolopendrium, Pteridium aquilinum, Arrhenatherum elatius, Brachypodium sylvaticum, Holcus lanatus, Lolium perenne, Centaurea nigra, Galium aparine, Geranium robertianum, Geum urbanum, Glechoma hederacea, Hedera helix, Heracleum sphondylium, Lonicera periclymenum, Potentilla reptans, P. sterilis,*

Rosa canina agg., *Rubus fruticosus, Rumex acetosa, R. sanguineus, Taraxacum* agg., *Torilis japonica, Vicia sepium* and *Viola riviniana.*

13. Woodstock

Location: Inistioge, County Kilkenny, Ireland.
Grid reference: S647356

Woodstock is an estate planted with a range of coniferous and broad-leaved tree and shrub species and is located on the west bank of the Nore on the steeply-sloping valley sides above the village of Inistioge. There are a number of trackways through the estate and the population of *Campanula trachelium* occurs in the banks bordering the tracks. Some of the plants are shaded by tree species including *Castanea sativa, Fagus sylvatica* and *Quercus robur*, some are shaded by natural rock faces that border the tracks and others are in more open vegetation. Associated species include *Blechnum spicant, Phyllitis scolopendrium, Pteridium aquilinum, Luzula sylvatica, Carex pendula, Agrostis stolonifera, Arrhenatherum elatius, Brachypodium sylvaticum, Cynosurus cristatus, Odontites verna, Dactylis glomerata, Phleum pratense, Angelica sylvestris, Calluna vulgaris, Chamaenerion angustifolium, Cirsium palustre, Filipendula ulmaria, Fragaria vesca, Hypercium calycinum, Juncus subnodulosus, Lotus uliginosa, Plantago lanceolata, Potentilla erecta, Pulicaria dysenterica, Rosa canina* agg., *Rubus fruticosus, Salix aurita, Senecio jaobea, Solidago virgaurea, Stachys officinalis, Teucrium scorodonium, Tragopogon pratensis, Valeriana officinalis* and *Vicia sepium.* The flora includes many introduced species such as *Hypericum calycinum*, *Acaena* sp. and *Leycesteria formosa*, suggesting that *Campanula trachelium* may be planted at this site.

19. Tankardstown Cross

Location: Nr. Tullow, County Carlow, Ireland.
Grid reference: S893761

This is a roadside population occurring along a *Fraxinus excelsior* hedgerow near the Dereen River, a tributary of the River Slaney. Associated species include *Dryopteris filix-mas, Phyllitis scolopendrium*, *Arrhenatherum elatius, Dactylis glomerata, Holcus lanatus, Anthriscus sylvestris, Digitalis purpurea, Epilobium montanum, Filipendula ulmaria, Galium aparine, Geranium*

robertianum, Geum urbanum, Hedera helix, Heracleum sphondyllium, Rosa canina agg., *Rubus fruticosus, Rumex sanguineus, Urtica dioica* and *Vicia sepium.*

24. Abbeyleix

Location: County Kilkenny, Ireland.
Grid reference: S417820

This site held a record for *Campanula trachelium* in the late 1980s but the plant has not been seen at this location between then and 2002 when it appeared again following clearance of the banks of a drainage channel leading into the River Nore. The site is a woodland site, part of the Abbeyleix Estate and the population is located on the western side of the River Nore along a narrow drainage channel. The canopy directly above the population is dominated by either *Corylus avellana* and *Crataegus monogyna,* or *Acer pseudoplatanus, Fraxinus excelsior* and *Quercus robur.* The ground flora associated with *Campanula trachelium* is sparse as most of the plants are growing on the muddy banks of the drainage channel but species include *Phyllitis scolopendrium, Brachypodium sylvaticum, Ajuga reptans, Angelica sylvestris, Geum urbanum, Glechoma hederacea, Viola riviniana* and *Urtica dioica.* The bryophyte flora is also rich.

25. Inistioge

Location: County Kilkenny, Ireland.
Grid reference: S637379

The population at Inistioge is located on the east bank of the River Nore. It occurs in lightly shaded riverbank vegetation and numbered approximately thirty-five individuals during the first year of survey, 1999. However, when genetic sampling was carried out, there were only eight plants, possibly a result of the erosion of the riverbank. Associated species include *Asplenium trichomanes, Brachypodium sylvaticum, Phalaris arundinacea, Luzula sylvatica, Epilobium hirsutum, Eupatorium cannabinum, Geranium robertianum, Geum urbanum, Hedera helix, Lythrum salicaria, Orobanche hederae, Rumex sanguineus, Solanum dulcamara* and *Viola riviniana.*

6.7.2 Other sites

20. Clonlea

Location: Blackrock, County Dublin, Ireland.
Grid reference: O208294

This is a garden population located in the suburbs of Dublin.

21. Henllan

Location: Henllan, Monmouthshire, Wales.
Grid reference: SO2925

Two isolated plants were discovered in a hedgerow near Henllan.

22. Strawberry Wood

Location: Monmouthshire, Wales.
Grid reference: SO 313 216

This is a woodland site under the ownership of Gwent Wildlife Trust. The dominant tree species are *Fraxinus exclesior* and *Quercus robur* with an understorey of *Corylus avellana, Crataegus monogyna, Ilex aquifolium* and *Sambucus nigra.* Associated species include *Carex remota, Dryopteris dilitata, D. filix-mas, Brachypodium sylvaticum, Dactylis glomerata, Chrysosplenium oppositifolium, Circaea lutetiana, Epilobium montana, Galium aparine, Geranium robertianum, Geum urbanum, Hedera helix, Lapsana communis, Melica uniflora, Mercurialis perennis, Rubus fruticosus, Silene dioica,* and *Viola riviniana.*

23. Forêt de Chatillon

Location: Cote d'Or, Bourgogne, France
No grid reference available

Samples for the genetic analysis of this population were taken from seedlings grown from seed obtained from the Musée National d'Histoire Naturelle, Paris (accession number 20010128). No further information is available regarding the site of the population.

6.8 AIMS

The objective of this research was to investigate the biological functioning of populations of *Campanula trachelium* and to utilise this information to identify the conservation requirements of the species in Ireland. As a predominantly outbreeding species that has been subjected to fragmentation of its habitat, it is particularly susceptible to the detrimental effects of population decline such as inbreeding depression. A major aim of this investigation was therefore to establish whether the populations displayed any signs of reproductive or genetic deterioration. The study focussed on the Nore Valley populations for the investigation of population and reproductive biology but the conservation genetic status of the species was assessed through the study of the majority of Irish populations and two other populations from the European range of the species.

Campanula trachelium has shown signs that it operates on a metapopulational level, with the larger populations acting as a source of seed for the establishment of smaller, transient populations. If this were the case, it would have important implications for the conservation of the species and was therefore investigated through the study of the population genetics of the species. The genetic study also aimed to assess the evolutionary potential of the Irish populations and aimed to provide evidence regarding the native status of the species in Ireland through the comparison of levels of diversity and genetic relationships between Irish populations and a limited sample of two other European populations.

The aims of this study of *Campanula trachelium* were to monitor fluctuations in population size, to compare the reproductive output of populations, to estimate genetic diversity within populations and to investigate correlations between these variables. Further aims of the population genetic study were to describe the genetic structure of populations in terms of the partitioning of genetic variation within and between populations and the genetic relationships between populations. The overall objective was to consolidate all available information to assess the conservation status of the species in Ireland and to prioritise future conservation efforts.

Plate 6.1. Inflorescence of *Campanula trachelium* showing the pollen presentation mechanism. The style of the top flower is loaded with pollen that was deposited by the ripened anthers onto stylar hairs prior to the flower opening.

CHAPTER 7

POPULATION AND REPRODUCTIVE BIOLOGY OF *CAMPANULA TRACHELIUM*

7.1 Population Census

7.1.1 Introduction

The survival probability of a population is entirely a function of population size and the factors that influence it. It is therefore a standard measure that is fundamental to the interpretation of all other information and is a common denominator in the assessment of demographic, genetic and environmental stochasticity. Populations of *Campanula trachelium* are highly variable with respect to population size and frequently contain less than 50 individuals, often occurring in isolated clumps along riverbanks and hedgerows. As a result, the monitoring of populations is particularly important in order to establish the degree of variation in population size from year to year. Information on relative population size is also required to interpret patterns in genetic and reproductive variation and estimates of population size were obtained for all populations.

7.1.2 Method

The habitat and accessibility of *Campanula trachelium* populations prevented a full demographic analysis from being undertaken. However, a yearly population census was carried out for a sub-set of the Nore valley populations and the population size of all other populations sampled for genetic analysis was estimated at the time of sampling. It was decided that only mature flowering plants would be counted due to time restrictions and the difficulty in locating immature plants amongst the vegetation. The counting of only a sub-set of the demographic structure of the populations provides an effective census technique providing that the same class is censused each year (Morris and Doak, 2002) and the same technique is used for all populations.

Population sizes of *Campanula trachelium* are relatively small and mature plants can therefore be censused by walking through the site and counting the number of groups of flowering stems. An assumption had to be made regarding what constituted an individual plant and this had to be applied consistently throughout the census of all populations. It was decided that stems arising in groups, within 5cm of another stem in the same group, could be considered as one individual with multiple flowering stems. Although neighbouring plants could be the same genetic individual due

to extensive rhizomatous growth, this cannot be determined in the field and separate groups of flowering stems were therefore counted as separate individuals. As a result of this, it must be noted that the population census overestimates the true genetic effective population size of flowering plants.

7.1.3 Results

The number of flowering individuals present in each of the populations at the time of collection of material for genetic analysis is shown in Table 7.1. Where no material was collected, the estimates given were made on the first visit to the population. These counts will be referred to as population size from here forward.

Table 7.1. Estimates of the number of mature *Campanula trachelium* plants in 25 populations.

Population number	Population	Population size	Year of census
1	Tulla turlough	600	2000
2	Eagle Hill	79	2000
3	Knocknagroagh	84	2000
4	Dunmore	29	2000
5	Archer's Grove	347	2000
6	Mount Juliet	53	2001
7	Boherduff	60	2000
8	Thomastown	69	2000
9	Brownsbarn	175	2000
10	Ballyduff	412	2000
11	Ballygallon	82	2000
12	Oldcourt Island	1	2000
13	Woodstock	281	2000
14	Clonamery	10	2000
15	Murphy's of the River	2	2000
16	Rathsnagadan	3	2000
17	Pollmonty	2	2000
18	Tintine	1	2000
19	Tankardstown Cross	54	2000
20	Clonlea	35	2000
21	Henllan	2	2001
22	Strawberry Wood	20	2001
24	Abbeyleix	16	2002
25	Inistioge	8	2000

Three of the smaller populations were surveyed in detail and total population counts were made of flowering plants and rosettes. The relative numbers of rosettes and flowering plants are shown in Table 7.2

Table 7.2 The relative numbers of flowering plants and rosettes in four populations of *Campanula trachelium.*

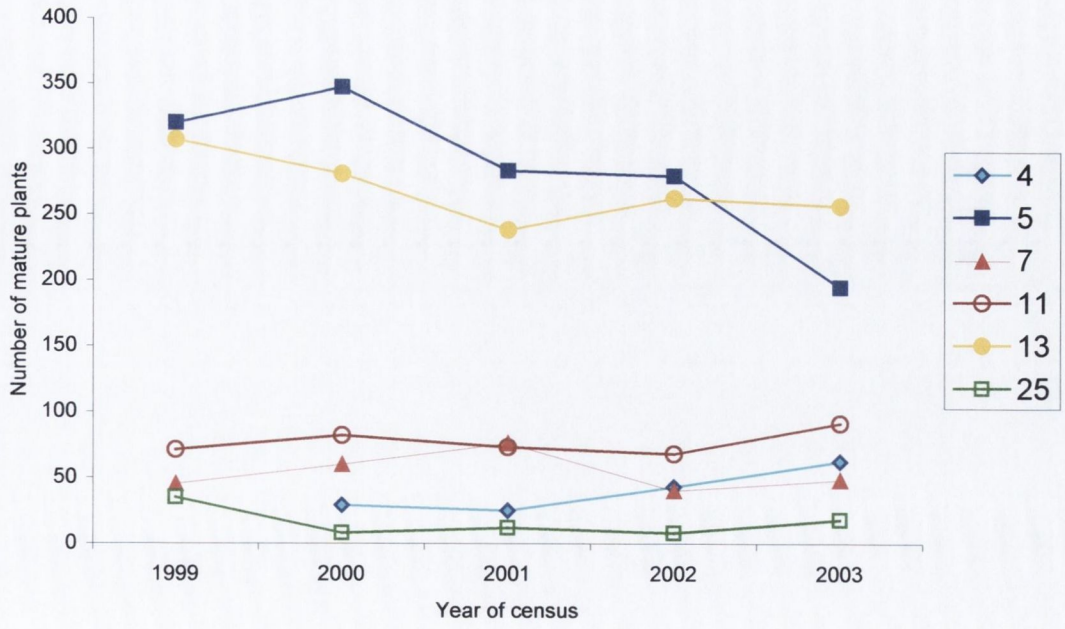
Population number	Population name	Year of survey	No. flowering plants	No. rosettes
4	Dunmore	2003	62	10
24	Abbeyleix	2002	16	28
25	Inistioge	2003	18	10

Five populations were surveyed for five consecutive years to monitor variation in population size. A further population, located in the second year of study was surveyed for four consecutive years. The population estimates are displayed in Table 7.3 and Figure 7.1.

Table 7.3 Estimates of the number of flowering stems in six populations of *Campanula trachelium* over five years of census

Population no.	Population name	1999	2000	2001	2002	2003
4	Dunmore	*	29	25	43	62
5	Archer's Grove	320	347	283	279	194
7	Boherduff	45	61	76	40	48
11	Ballygallon	71	82	73	68	91
13	Woodstock	307	280	238	262	256
25	Inistioge	35	8	12	8	18

Figure 7.1 Variation in the number of mature plants in six populations of *Campanula trachelium* over five years of census. The legend shows the population number.

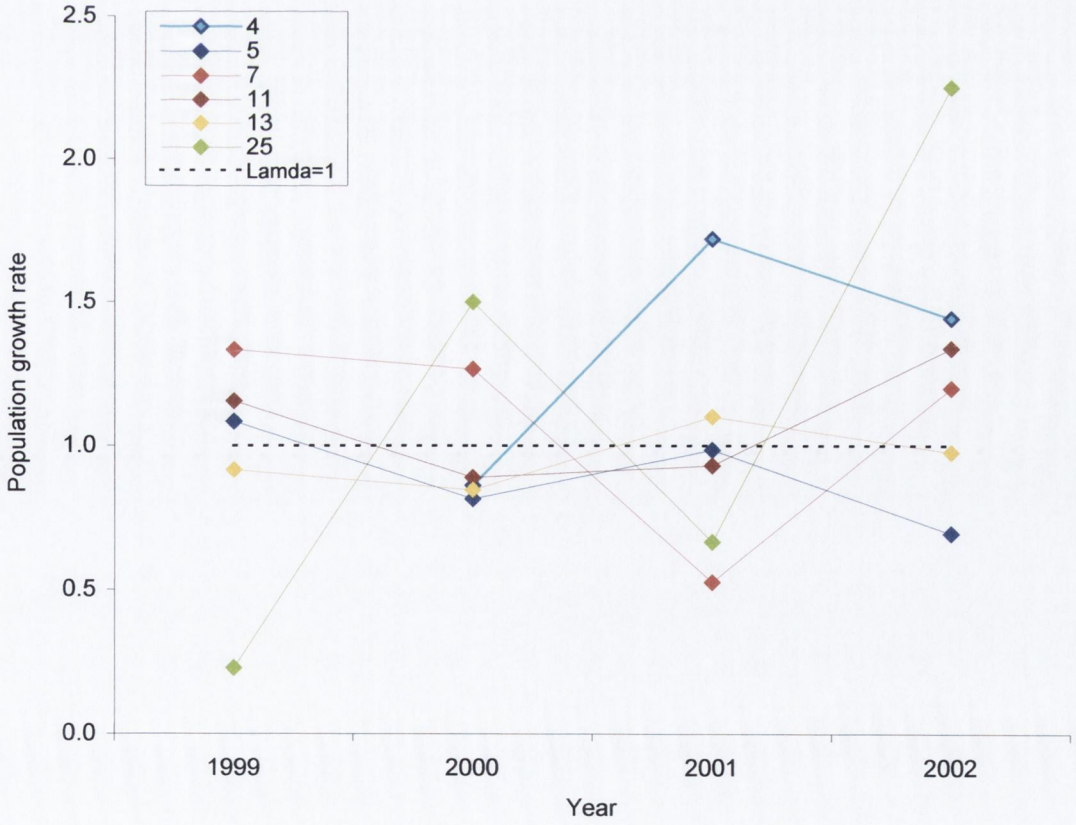


The larger populations have decreased in size since the start of the study but the majority of smaller populations show a net increase in population size over the five years. The exception is Inistioge, which decreased from 35 to 18 flowering individuals during the study period. The changes in population size can be expressed as the growth rate, λ, which is defined by

$$\lambda = \frac{N_{t+1}}{N_t}$$

where N_t is the population size at year t and N_{t+1} is the population size in the following year. The variation in growth rate is shown in Figure 7.2.

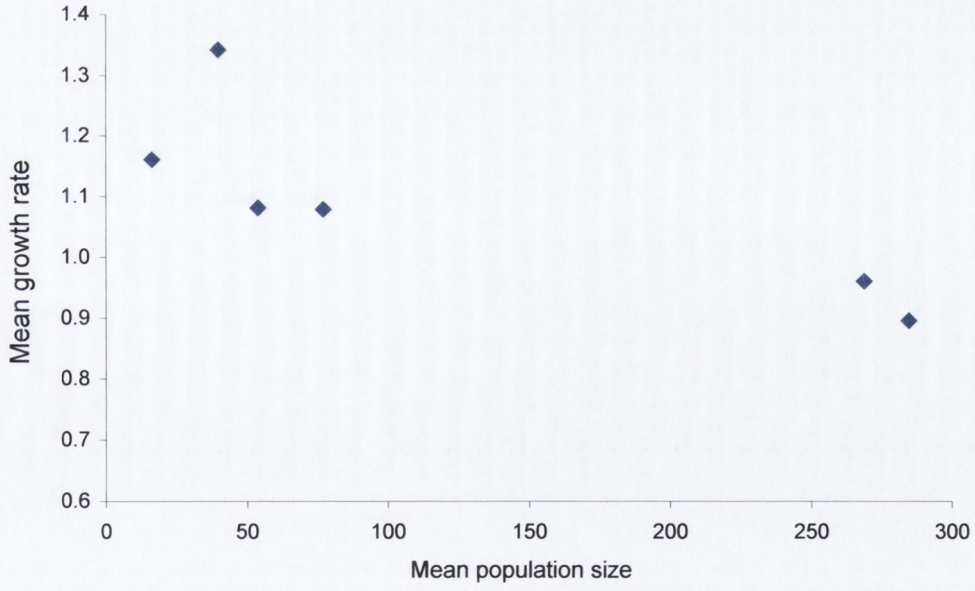
Figure 7.2 Variation in the growth rate, λ, in six populations of *Campanula trachelium* over four years. The legend shows the population number. 4=Dunmore, 5=Archer's Grove, 7= Boherduff, 11=Ballygallon, 13= Woodstock, 25= Inistioge.



A growth rate of 1 indicates a steady state in which the population size does not increase or decrease. Values above this therefore represent population growth and values below 1 indicate population decline. The growth rate ranged from 0.229 to 2.250. It can be seen that population 25, Inistioge, has the most variable growth rate and is the smallest Irish population. Any changes in population size are therefore relatively large.

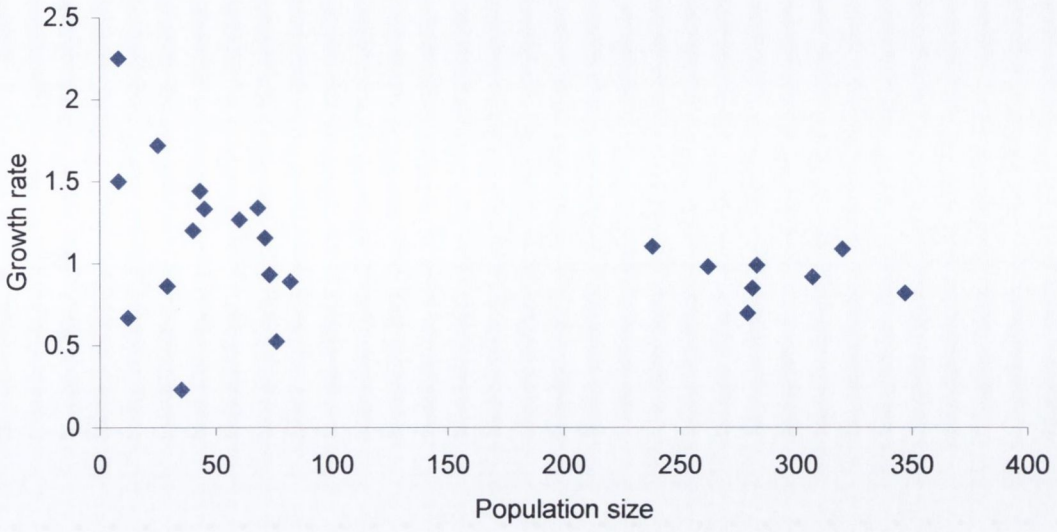
Mean growth rate over all years was found to be significantly negatively correlated with mean population size (Figure 7.3), with a Spearman Rank correlation of –0.943 ($n = 6$, $p < 0.01$). This indicates that the growth rate may be density dependent with large populations stabilising or decreasing due to competition between individuals for available resources and less available habitat for recruitment.

Figure 7.3 Relationship between mean growth rate and mean population size over the study period in six populations of *Campanula trachelium*.



When each individual population size is plotted against its growth rate for the year following measurement (Figure 7.4), it can be seen that at small population size, the variation in the growth rate is large in comparison to the situation at large population sizes. Small populations are therefore subject to large stochastic increases or decreases in population size whereas larger populations are more stable.

Figure 7.4 The relationship between population size and growth rate, λ, in *Campanula trachelium*. Pairs of observations in all populations over all years are shown.



7.2 PRELIMINARY STUDY OF POSITION EFFECTS ON SEED OUTPUT

Prior to any comparisons of reproductive output within populations of *Campanula trachelium*, a preliminary study was carried out to determine whether the reproductive output of individual flowers varied with the vertical positioning of the flower on the inflorescence. This was required to ensure that position effects were taken into account during future population comparisons of reproductive output.

7.2.1 Method

During September, 1999, four populations were visited (Archer's Grove, Boherduff, Ballygallon and Woodstock) and seed capsules were collected randomly from between 5 and 10% of the total population before capsule dehiscence. One capsule was collected from both the top and bottom of one flowering stem for each individual. Following drying, the collected capsules were broken apart and the capsule debris separated from the seed therein and from any seed already released. The seed from each capsule was photographed at a fixed distance with a Nikon digital camera and the resulting images were analysed using the software 'NIH Imaging 1.62' to obtain total seed number for each capsule collected.

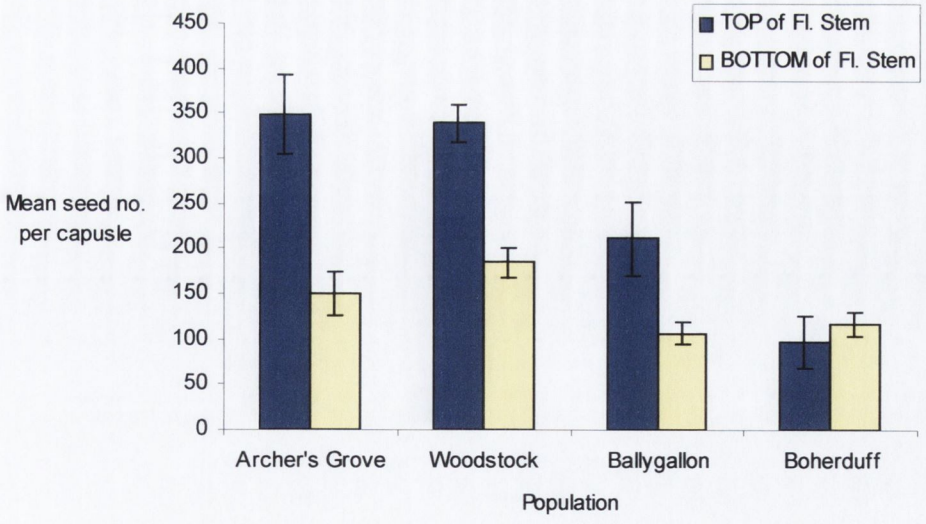
7.2.2 Results

Flowers were found to open from the top of the flowering stem. The results of the analysis of seed output are summarised in Table 7.4 and displayed graphically in Figure 7.5.

Table 7.4 Mean seed output per capsule, with respect to the position of the capsule on the inflorescence, in four populations of *Campanula trachelium*. Standard deviation of the mean is given in brackets.

Population	Pop. size (1999)	Sample size	Position	Mean no. seeds per capsule
5 Archer's Grove	320	30	Top	348.03 (240.56)
		30	Base	150.46 (130.30)
13 Woodstock	307	30	Top	338.86 (111.65)
		30	Base	184.27 (95.62)
11 Ballygallon	71	9	Top	210.7 (86.08)
		9	Base	106.89 (38.69)
7 Boherduff	45	5	Top	97.29 (89.50)
		5	Base	117.00 (28.02)

Figure 7.5 Comparison of mean seed output per capsule, with respect to the position of the capsule on the inflorescence, in four populations of *Campanula trachelium*. Error bars show standard error of the mean. Population size decreases from left to right.



The results show that in three of the four populations the mean number of seeds per capsule is greater in capsules at the top of the flowering stem than in those positioned at the base of the inflorescence. Overall, the seed output appears to be greater in the larger populations (Archer's

Grove and Woodstock) than in the two smaller populations (Ballygallon and Boherduff). To investigate whether the effects of position and population on seed output are significant, a crossed ANOVA was carried out. The results are summarised in Table 7.5.

Table 7.5 Results of a crossed ANOVA and Bonferroni post-hoc tests on the effects of capsule position and population on seed output in four populations of *Campanula trachelium*. For the comparison of pairs, the position or population with the highest mean is listed first.

	ANOVA results		Bonferroni post-hoc test results	
Factor	**F-Ratio** $_{df}$	**Significance**	**Comparison**	**Significance**
Position	13.604 $_{1, 140}$	p=0.0003	Top - Bottom	p=0.0003
Population	5.8064 $_{3, 140}$	p=0.0009	5-7 13-7 13-11	p=0.0136 p=0.0054 p=0.0409
Position*Population	2.0844 $_{3, 140}$	ns		

df = degrees of freedom (factor, error)
ns = not significant (p>0.05).
Populations: 5=Archer's Grove, 7=Boherduff, 13=Woodstock

The ANOVA shows that there are significant differences in seed output with respect to the position of the capsule on the stem and significant differences between populations. Seed output is significantly higher in capsules positioned at the top of the flowering stem and is also significantly higher in larger populations. The number of seeds per capsule is significantly higher in Woodstock than in Boherduff and Ballygallon and is also significantly higher in Archer's Grove than in Ballygallon. The interaction between the factors of population and position of the capsule on the stem is not significant. As a result of these preliminary findings, further investigations of reproductive output were designed to eliminate position effects and a more detailed study was carried out to investigate differences between populations of different sizes. These are described in subsequent sections.

7.3 GYNODIOECY

7.3.1 Introduction

Gynodioecy is the occurrence of two floral sex states within a population, hermaphrodite and female (Richards, 1997). It may form an evolutionary progression towards dioecy (Charlesworth, 1991), but is an evolutionary stable strategy in itself and is approximately twice as common as dioecy in temperate floras (Richards, 1997). It can occur as a result of unstable gender expression in a dioecious species, but more commonly occurs through the existence of a male sterile polymorphism in predominantly hermaphroditic species (Richards, 1997). The determination of sex state in gynodioecious species has been traced in many incidences to both cytoplasmic and nuclear genetic factors that interact to determine the sex phenotype of individual plants (Saumitou-Laprade *et al.*, 1994). Cytoplasmic CMS alleles cause male sterility by inhibiting the development of male function but their expression can be altered by nuclear alleles that restore this male function. The frequencies of females and hermaphrodites in a population are therefore determined by the epistatic interaction of mitochondrial and nuclear loci and the degree of polymorphism displayed at either or both types of loci (McCauley & Taylor, 1997). This type of interaction produces what is described by Ross (1978) and Bawa (1980) as stable gynodioecy, as distinct from unstable gynodioecy where gender is determined by a single genetic factor. Stable gynodioecy can still occur when under the control of a single nuclear locus, but only if females are better vegetative reproducers than hermaphrodites (Stevens & van Damme, 1988).

Gynodioecy develops when females have a fitness advantage over hermaphrodites. The advantage can occur as a result of the re-allocation of resources from male to female function and was first recognised by Darwin (1877) as a possible mechanism for the increase in females or males in hermaphrodite populations. This resource compensation can be expressed through increased fruit and seed production (eg. Asikainen & Mutikainen, 2003) or through an increase in seed mass, which has been found to confer a survival advantage on the resulting seedlings of many plant species (Delph *et al.*, 1999). However, in some species, there is no detectable difference in reproductive vigour or seed provisioning between hermaphrodites and male sterile

plants and the difference is found instead in vegetative biomass. This was found to be the case in *Chamaecrista fasciculata* Rydb. (Fabaceae) where females were a third more massive than hermaphrodites, suggesting the re-direction of resources to vegetative rather than reproductive development (Williams & Fenster, 1998). It is debatable whether this use of resources can lead to increased fitness but it may be important for provisioning for increased reproductive effort in subsequent years (Williams & Fenster, 1998).

In addition to resource compensation, another theoretical advantage of the female sex state is the avoidance of inbreeding. The progeny of females may be more outcrossed and therefore have higher heterozygosity with associated hybrid vigour. These effects are therefore manifested post-zygotically and have been documented in a number of species including *Saxifraga granulata* L. (Stevens, 1988). In *Silene acaulis* (L.) Jacq. (Caryophyllaceae), Shykoff (1988) compared the relative performance of outcrossed progeny from females and hermaphrodites and found that the fitness of female offspring was higher than that of hermaphrodite offspring, despite there being no difference in relative seed size. Delph and co-workers (1999) extended this study to test whether female seeds were higher in nutrients than those of hermaphrodites in *Silene acaulis*, but found that this was not the case. The difference in the performance of progeny may therefore be explained by heterotic effects.

The theoretical fitness advantages of the female state cannot be considered in isolation from population structure. As gynodioecy is a heritable trait, female individuals often form a clumped distribution, particularly when pollen and seed dispersal ability is limited. This increases the frequency of females in the immediate population and density effects come into play, where female advantages from resource compensation may be counteracted by the fact that the reduction in available pollen limits seed output. This was shown by Graff (1999), where both sex morphs in a gynodioecious population were pollen limited. The population sex structure was shown to favour hermaphrodites under these conditions. Alternatively, the scarcity of hermaphroditic pollen donors may force self-compatible species to produce a high proportion of selfed seed, which may have lower survival potential due to the effects of inbreeding depression.

In August 1999 an individual of *Campanula trachelium* was discovered with under-developed anthers. The anthers were later dissected under a microscope and no pollen grains were contained within (Plate 7.1). The aims of this study were therefore to establish whether *Campanula trachelium* is gynodioecious in Ireland, to determine whether the male sterile state is environmentally-induced or heritable, to determine the frequency of male sterile individuals in a number of populations and to compare the reproductive output of male sterile and hermaphrodite plants. This information will be important in the consideration of conservation strategies for *Campanula trachelium*.

7.3.2 Methods

Character state of gynodioecy

An initial survey of the Woodstock population was carried out to determine whether the variation in the amount of pollen in the anthers is a discrete or continuous character in *Campanula trachelium*. One flower was collected from each of fifty plants in the population and these were taken back to the laboratory and dissected. An arbitrary five-point scale was used to quantify the amount of pollen present, with 1 indicating a small amount of pollen and 5 indicating abundant pollen. The scale is even with each class representing the same increment of pollen increase and scoring was carried out after first examining all the flowers to determine the extent of variation.

Constancy of gynodioecy

The constancy of the female condition was assessed by marking fifteen female plants in the Woodstock population and determining their sex each year over a period of three years. Plants were marked by sinking an aluminium peg into the ground at the base of the stem and were re-located in subsequent years using a metal detector.

Frequency of gynodioecy

A total of eight populations were surveyed during July 2000, to determine the frequency of female plants. Plants were chosen at random on a walk through the population and the sex determined.

This could only be carried out on unopened or recently opened flowers. The limitations of this method are that it only gives the relative frequencies of females and hermaphrodites at one point in time. This does not take account of the fact that there may be phenological differences between females and hermaphrodites such that the frequency of flowering females may change as the season progresses. However, from personal observation, this is not the case and this method should therefore give an accurate estimate of the true frequency of females in the populations.

Assessment of female advantage

To determine whether female plants are more fecund than hermaphrodites, reproductive success was measured in the Woodstock population. Capsules were collected from 20 male sterile and 20 hermaphroditic plants for which the sex had been determined earlier in the season. Plants were marked during the flowering season in 2000 with black tags to indicate females and green tags to indicate hermaphrodites and were then re-visited before dehiscence. The top three capsules were collected from each plant and stored in sealed envelopes. Stem height, total number of capsules per stem and total number of stems per plant was recorded for each individual sampled. Following drying, the collected capsules were broken apart and the capsule debris separated from the seed therein and from any seed already released. The seed from each capsule was counted and weighed as described previously, and the total mass was divided by seed number to obtain mean seed mass per capsule.

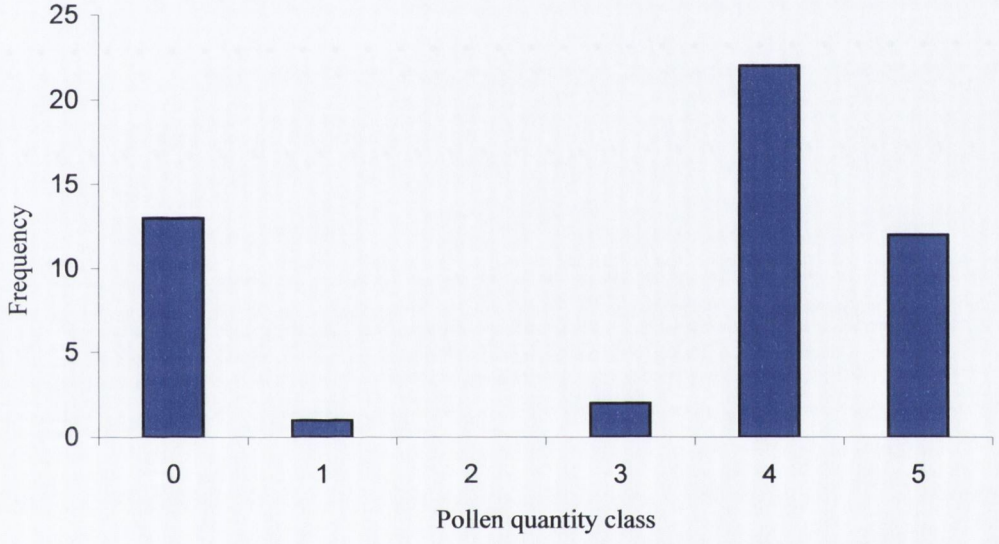
Percentage germination was not assessed as the capsules were collected before dehiscence and may therefore have not fully completed their development. This may have affected germination success.

7.3.3 Results

Character state of Gynodioecy

The frequency distribution of the pollen classes is shown below in Figure 7.6. It shows that the distribution of size classes is bimodal with no continuous expression of pollen quantity. A normal distribution would be expected if anthers with no pollen were part of a continuous variation in pollen quantity with very few individuals lacking pollen altogether.

Figure 7.6 Frequency distribution of pollen quantity classes in a population of *Campanula trachelium* Graded scale. 0 = no pollen and 5=maximum pollen quantity.



Constancy of gynodioecy

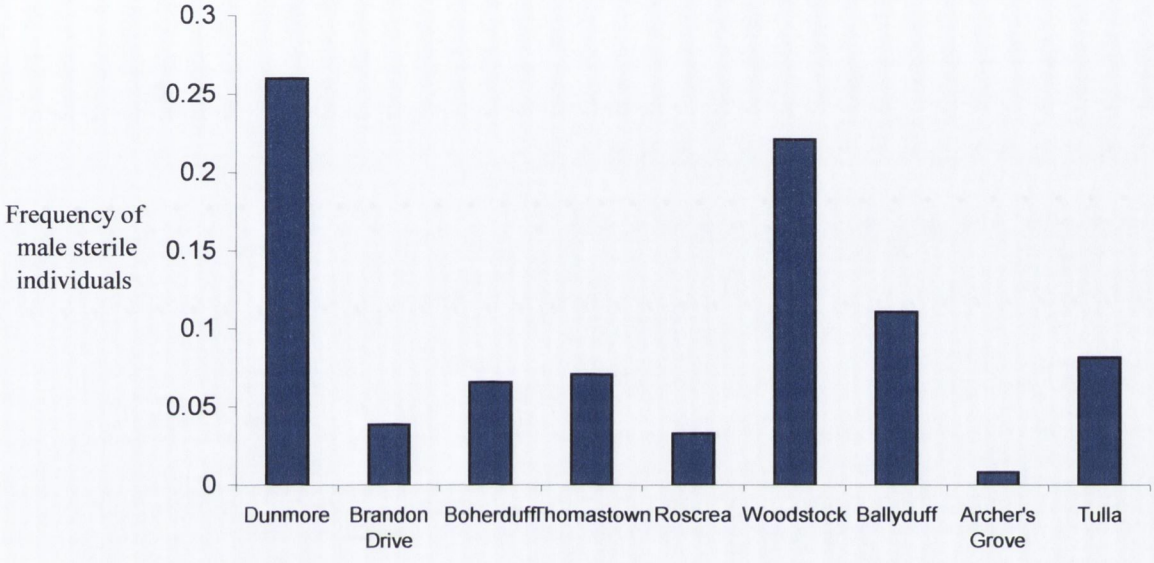
The fifteen plants retained their female floral sex state over three years of observation indicating that the gynodioecy is not likely to exhibit morphological plasticity. The male sterile plants were observed to exhibit a clumped distribution but were scattered throughout the population, indicating that the male sterility is unlikely to be environmentally-induced.

Frequency of gynodioecy

The frequency of female plants observed in nine populations of *Campanula trachelium* is shown in Figure 7.7. Populations are arranged in increasing order of population size. The frequency of female individuals ranges from 0.08 to 0.26. The mean frequency of females in Irish populations

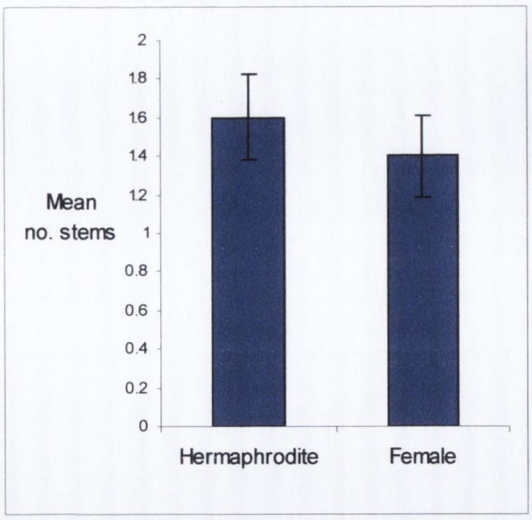
is 0.099 with a standard error of 0.0029. The frequency of females was not significantly correlated with population size during the year of survey (Spearman Rank correlation co-efficient = -0.092, n=9, p>0.2). Isolated clumps along the River Nore were also examined for the expression of male sterility and at locations such as Clonamery, two thirds of the plants were found to be female.

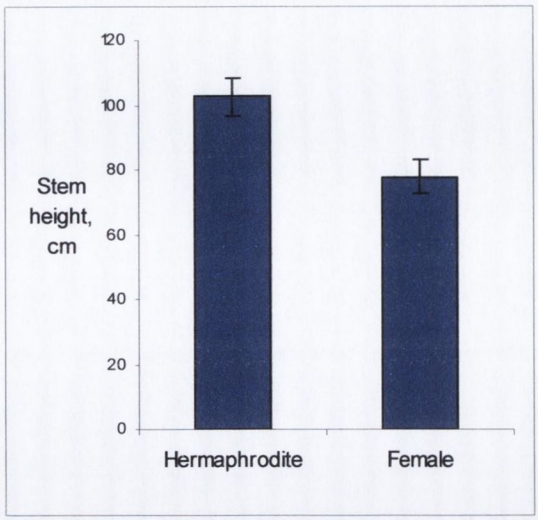
Figure 7.7 Frequency of male sterility in nine Irish populations of *Campanula trachelium*

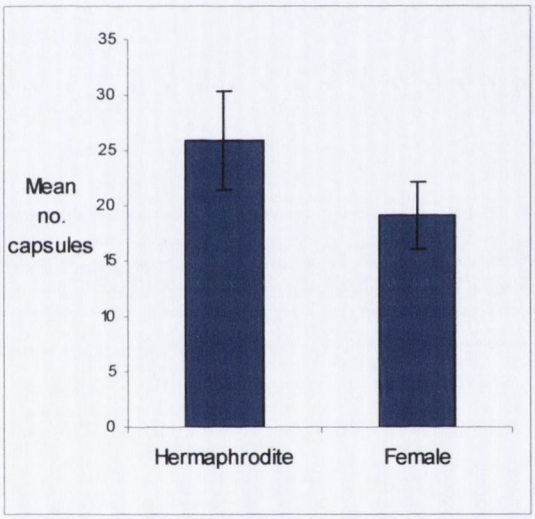


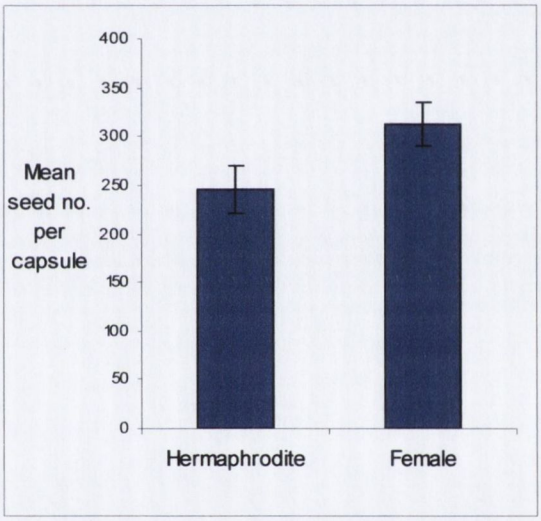
Assessment of female advantage

The characteristics measured at the time of capsule collection were analysed to investigate whether the floral sex state of individuals is a significant factor in vegetative and reproductive success. Total seed production per plant was estimated by multiplying the average seed number per capsule by the number of capsules per plant. However, this variable should not be given too much weight, as it is likely to be an inaccurate measure due to the variation in seed number with position on the flowering stem. The results of a one-way analysis of variance (ANOVA) for each of the variables are shown in Table 7.6 and graphs of the data are shown in Figure 7.8. The number of stems per individual was not normally distributed and a non-parametric test was therefore carried out. A Wilcoxon signed ranks test gave a probability value of 0.9731 (n=20,









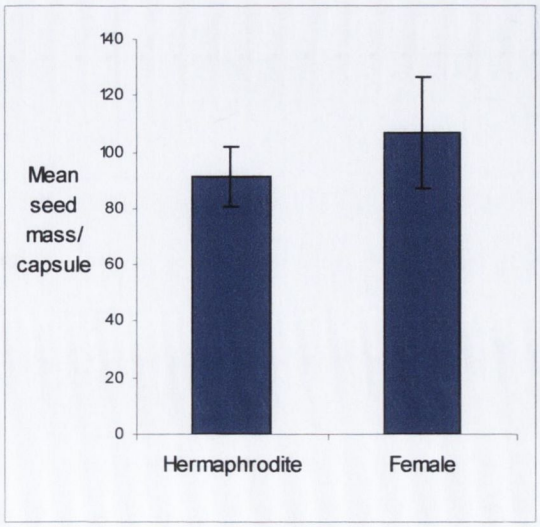


Figure 7.8 Comparison of mean fitness traits between hermaphrodite and female plants of *Campanula trachelium*

W=-0.034) and the null hypothesis was therefore accepted that there is no significant difference between the number of stems in individuals with different floral sex states.

Table 7.6 ANOVA results for reproductive variables in different populations

Dependent Variable	F Ratio	df sex, error	Significance	Variable higher in:
Height of stem	10.622	1, 38	p=0.0024	Hermaphrodites
Number of capsules	1.620	1, 38	ns	
Number of seeds per capsule	4.110	1, 80	p=0.0497	Females
Mean seed mass, µg	0.478	1, 37	ns	
Total seed no.per plant	0.0639	1, 38	ns	

The seed number per capsule was measured for three capsules per plant and a nested ANOVA was therefore carried out for this variable. The F-Ratio for the plant factor (nested within sex) was 2.66, with 38 degrees of freedom and significant at p=0.0001. There is therefore significant variation between plants within the sex states with regard to seed output per capsule.

The number of stems and capsules per plant were found to be greater in hermaphrodite than female plants but these differences were not statistically significant. Hermaphrodite flowering stems were also found to be significantly taller than female stems. Female plants were found to produce significantly more seed per capsule than hermaphrodites. The average seed mass per capsule was also greater in females although this result was not significant.

7.4 FECUNDITY

7.4.1 Introduction

The fecundity of threatened plant species is of primary concern with respect to their conservation, particularly where population size is variable. Small, isolated populations are dependent on successful reproduction but they are also the most susceptible to factors that impact on this success. Low reproductive success in such populations can lead to extinction in relatively short periods of time (Groom, 1998). It is therefore important to collect information on aspects of the reproductive output of populations in order to evaluate their capacity to survive and expand. This importance is reflected in the large number of conservation-based studies that focus on

reproductive traits in the survival of populations of rare and threatened plant species (eg. Sipes & Tepedino, 1994; Bernardello *et al.*, 1999; Kaye, 1999) and those that use such information in conjunction with genetic and demographic information to provide an indication of the reproductive response to changes in other stochastic factors in population biology (eg. Bosch *et al.*, 1998; Wong & Sun, 1999; Burne *et al.*, 2003).

The reproductive biology of small or fragmented plant populations can be diminished in a variety of ways, many of which are attributable to Allee effects. This phenomenon was first described in animal populations (Allee, 1951) but can also occur in plant populations and describes the situation where fitness increases as a result of the benefits of the aggregation of conspecific individuals. For example, larger plant populations may have a greater ability to attract pollinators, reducing pollen limitation and increasing seed production and this has been demonstrated in *Clarkia concinna* (Fisch. & C.A. Mey.) Greene (Onagraceae) (Groom, 1998). There are many components that can contribute to the overall Allee effect and its cause can be rooted in ecology, genetics, demography or any combination of these factors. However, different causes have different implications for conservation and as the overall decline in fitness with decreasing population size can have severe consequences for threatened plant species one of the functions of conservation biology is to separate out the causal factors and take appropriate action.

The deterioration of small populations is not always a result of Allee effects such as pollen limitation, and of particular concern is the role that inbreeding depression may be playing in reducing the reproductive success of populations with low genetic variability. This phenomenon is often manifested through the loss of reproductive fitness and can therefore have a significant impact on the breeding population (Demauro, 1993). There are many studies that document the detrimental effects of low genetic variability on the reproductive success of plant populations (Newman & Pilson, 1997) and the effects include lower reproductive output, lower germination rates, lower seedling survival rates and lower estimated mean fitness (Demauro, 1993; Buza *et al.*, 2000; Severns, 2003). These effects are more pronounced in small, isolated populations where there is little gene flow into the population and where genetic drift and inbreeding reduce genetic

variation further. The link between small population size and fitness traits has been investigated by many researchers. Fischer and Matthies (1998) studied the effects of population size on reproductive traits in *Gentianella germanica* (Willd.) E.F. Warb, a rare biennial plant in central Europe. They reported significantly fewer seeds per fruit and per plant in small, compared to large populations. The same trends were observed by Heschel and Paige (1995) who found that seed size and percentage germination were significantly reduced in populations with less than or equal to 100 flowering plants. In Fisher and Matthies' study, the effects were also manifested in the progeny and after 17 months cohorts grown from seed of large populations had a significantly higher proportion of flowering plants and more flowers per planted seed than those grown from the seed from small populations.

Campanula trachelium is a strongly outbreeding species that has undergone significant decline in Ireland and now occurs in a series of fragmented populations, many of which contain less than 100 individuals. This breeding system, encouraged by herkogamy and protandry leaves the species particularly susceptible to inbreeding depression (Wright, 1977). During the first year of survey it was noticed that some populations of *Campanula trachelium* produced noticeably larger capsules than others and measurements of capsule diameter confirmed a significant difference. It was conjectured that this difference in capsule size was related to the numbers of seeds produced within and it was therefore decided that a survey of seed number per capsule should be undertaken for a number of populations of varying size. The aim of this study was to investigate the link between small population size and the possibility of reproductive deterioration as a result of inbreeding depression.

7.4.2 Methods

Five populations, exhibiting a range of population sizes were chosen to investigate differences in reproductive fitness between populations. These were Dunmore, Archer's Grove, Boherduff, Thomastown and Woodstock (populations 4, 5, 7, 8 and 13 respectively). During July 2001, plants were chosen randomly from each population and the number of stems was recorded. If a

plant contained more than one flowering stem, one stem was chosen at random and the stem height and number of capsules per stem was recorded. The terminal capsule was then collected, providing that dehiscence had not occurred, and was placed in a paper envelope with the edges folded over to prevent seed loss through the joins. The terminal capsule was chosen due to earlier observations that number of seeds per capsule differed significantly from the top to the bottom of the flowering stem. In addition to this, in any group of three capsules on a branch, the terminal capsule was also observed to be the largest. This was consistent throughout populations and between populations so it was decided that for an unbiased comparison to be made, the position on the stem from which the capsule was collected should remain constant. To give an accurate indication of total seed produced, it would be necessary to collect capsules from the top, middle and bottom of the infructescence but this may have significantly affected the seed output of the smaller populations and would also have required many more days in the field due to the temporal separation of flowering and capsule production. Due to availability of suitable test plants and the difference in population size, the sample size varied between populations.

The capsules were left in the envelopes, allowed to dry and stored until analysis. Following drying, the collected capsules were broken apart and the capsule debris separated from the seed therein and from any seed already released. The seed number per capsule and the mean mass of seeds was determined as previously described.

To account for the possibility that measurements of seed mass may be influenced by the developmental stage of the capsule when collected, the populations were re-visited a number of times during September and seed was collected from the dehisced capsules of thirty plants per population. The seed was collected in five envelopes, so that each envelope contained seed from six individuals. Ten samples of 100 seeds were then taken, two samples per envelope, and weighed on a Sartorius 1801 electronic weighing balance. The total mass was divided by 100 to give mean seed mass in each sample. This variable is referred to as mean seed mass on dehiscence to distinguish it from mean seed mass as measured from the capsules collected prior to dehiscence.

Pollen limitation

Pollen limitation is regarded as one of the principal factors limiting plant reproduction (Kull, 1998) and this may be a possible cause of the differences between seed numbers in the different populations. Variation may occur in the abundance of pollinators between the sites and this may account for the differences in seed set.

To investigate this hypothesis, pollen supplementation experiments were carried out in two of the populations during July 2001. Populations 4 and 5 (Dunmore and Archer's Grove) were chosen for the experiment as they have contrasting population sizes. From previous surveys Dunmore (population 4), had appeared to be producing fewer and smaller capsules than other populations while population Archer's Grove (population 5), produced abundant, large capsules. The two populations were visited at the beginning of August 2001 and flowering stems were selected randomly. The stigma was then checked for receptivity and the stem was rejected if the three stigma lobes had not yet separated or if they were yellowed or folded back towards the anthers. When the pollen-receiving test flowers had been chosen, unopened flowers from other plants, located at least 20 metres away from the test plants, were picked and the petals were peeled back to expose the pollen-loaded stylar hairs. The styles were then used to gently brush pollen onto the receptive stigmas of the test flowers until the surface contained abundant pollen grains. The pollen-supplemented flowers were then marked with a lightweight plastic tag secured around the pedicel. Stem height and total number of stems were recorded.

The plants were re-visited at the end of August and the beginning of September and capsules that had swollen but not dehisced were collected in paper envelopes with the sides folded over to prevent seed loss. One other free-pollinated capsule was collected from each plant to provide a comparison. Due to possible flower position effects, the supplemented and free-pollinated flowers were alternated between terminal and peripheral positions. On larger plants, the terminal flower from side branches was used rather than a peripheral flower in the same group of flowers. Some plants had been destroyed by livestock or by horses using the woodland tracks and some tags were missing therefore the sample size was small. Eight capsules were retrieved from

Dunmore (population 4) and ten from Archer's Grove (population 5). Seed number and average seed mass were determined in the same way as described previously.

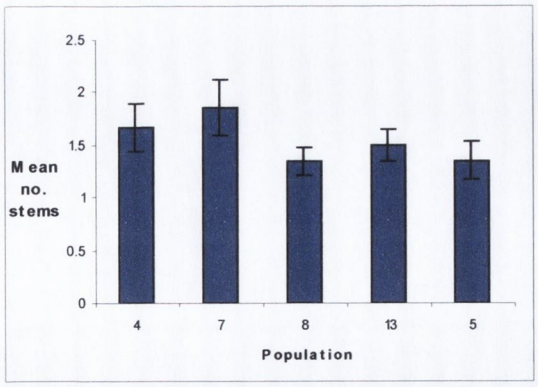
7.4.3 Results

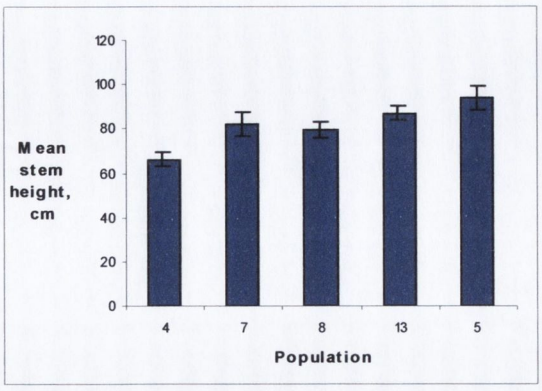
The mean values for the reproductive traits compared in the fecundity study are given in Table 7.7 and displayed graphically in Figure 7.9. Population size refers to the number of flowering stems recorded during August, 2001 and the populations are listed in increasing order of population size.

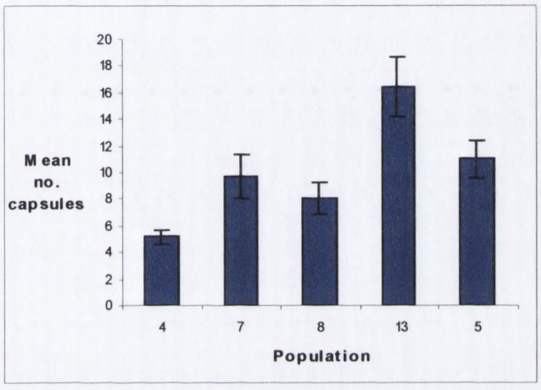
Table 7.7 Summary of data collected on reproductive traits in five populations of *Campanula trachelium*. The standard deviation of the mean is given in brackets.

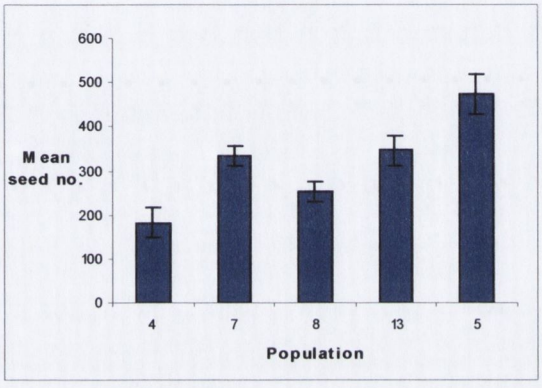
Pop. no.	Pop. size	Sample size	Mean no. stems	Mean stem height, cm	Mean no. capsules	Mean no. seeds per capsule	Mean seed mass per capsule, µg	Mean seed mass after dehiscence, µg
4	25	12	1.67 (±0.78)	66.25 (±10.89)	5.17 (±1.75)	181.75 (± 114.44)	87.56 (±47.88)	109.69 (±8.17)
7	76	20	1.85 (±1.18)	84.11 (±25.09)	9.70 (±7.06)	334.50 (±100.28)	129.05 (±25.41)	162.02 (±8.13)
8	78	20	1.35 (± 0.59)	79.21 (±15.35)	8.05 (±5.19)	252.20 (±100.11)	134.87 (±24.84)	140.00 (±11.69)
13	238	20	1.50 (± 0.69)	86.65 (±14.14)	16.35 (±10)	345.60 (±147.82)	144.45 (±32.08)	195.07 (±38.61)
5	283	20	1.35 (±0.79)	93.64 (±23.31)	11 (±6.17)	473.65 (±193.08)	63.33 (±24.44)	132.95 (±22.42)

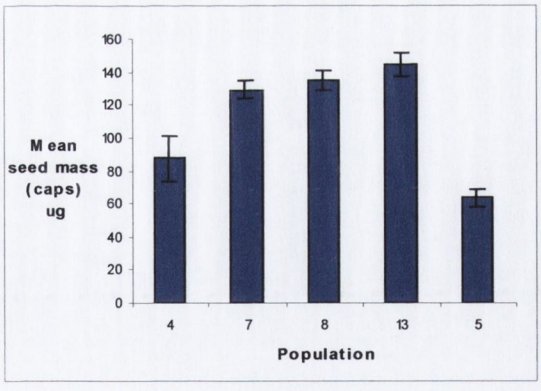
To determine whether significant differences occurred between populations with respect to the reproductive traits measured, ANOVA was carried out on the raw data for the five populations. Bonferroni post-hoc tests were performed on significant variables to determine which populations exhibited significant differences. The Bonferroni approach divides the error rate among the tests performed, and is the most conservative of the post-hoc tests. Total seed number per plant was estimated for population comparison by obtaining the product of mean seed number per capsule, number of capsules per plant and number of stems. The absolute magnitudes of these estimates are likely to be inaccurate and overestimates due to variation in capsule size and seed number per capsule with position on the flowering stem and differences between flowering stems of the same plant. However, they can be compared between populations on the assumption that the trends involving capsule size and seed number at different stem positions are consistent between populations. Although the same trend for increasing capsule size with increasing capsule height above the ground was observed in all populations, the limitations of this variable must be taken into account. The results of the ANOVAs are summarised in Table 7.8. Significant Bonferroni











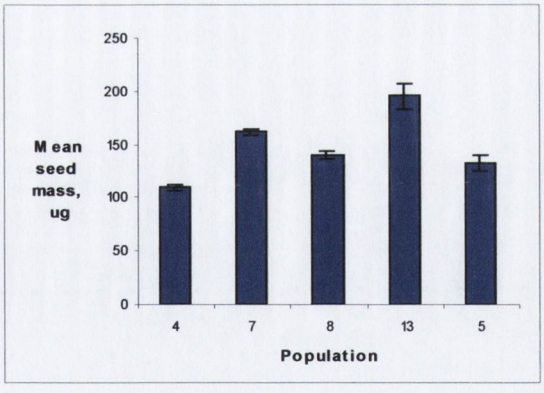


Figure 7.9 Comparison of mean fitness traits between five populations of *Campanula trachelium* displayed in increasing order of population size. Error bars are standard error of the mean. Mean seed mass (caps) was calculated for capsules collected from plants sampled randomly as part of the comparison of reproductive traits between populations. Mean seed mass was calculated from seed collected separately by randomly sampling plants throughout the populations and calculating the mean of a sub-sample of these seeds – see methods section for more detail. Population 4=Dunmore, 7=Boherduff, 8=Thomastown, 13=Woodstock and 5=Archer's Grove.

test results are displayed and where population pairs are given, the population with the highest mean is listed first.

Table 7.8 Results of ANOVA and Bonferroni post-hoc tests to compare stem height, number of capsules, mean number of seeds per capsule, total seed number per plant, mean seed mass per capsule and mean overall seed mass in five populations of *Campanula trachelium*.
Population 4=Dunmore, 5=Archer's Grove, 7=Boherduff, 8=Thomastown and 13=Woodstock

	ANOVA results		Bonferroni post-hoc test results	
Variable	**F-Ratio$_{df}$**	**Significance**	**Population pair**	**Significance**
Stem height	4.498 $_{4,87}$	$p = 0.0024$	13 - 4 5 - 4	$p = 0.0337$ $p = 0.0011$
Number of capsules	6.147 $_{4,87}$	$p = 0.0002$	13 - 4 13 - 7 13 - 8	$p = 0.0003$ $p = 0.0293$ $p = 0.0025$
Number of seeds per capsule	10.588 $_{4,87}$	$p \leq 0.0001$	7 - 4 13 - 4 5 - 4 5 - 7 5 - 8 5 - 13	$p = 0.0309$ $p = 0.0158$ $p < 0.0001$ $p = 0.0192$ $p < 0.0001$ $p = 0.0411$
Total seed number per plant	153.700 $_{4,87}$	$p < 0.0001$	13 - 4 13 - 8 5 - 4 5 - 8	$p = 0.0007$ $p = 0.0102$ $p = 0.0016$ $p = 0.0224$
Mean seed mass	24.850 $_{4,87}$	$p \leq 0.0001$	7 - 4 8 - 4 13 - 4 7 - 5 8 - 5 13 - 5	$p = 0.0032$ $p = 0.0005$ $p < 0.0001$ $p < 0.0001$ $p < 0.0001$ $p < 0.0001$
Mean seed mass after dehiscence	23.041 $_{4,45}$	$p \leq 0.0001$	7 - 4 8 - 4 13 - 4 13 - 7 13 - 8 7 - 5 13 - 5	$p < 0.0001$ $p = 0.0259$ $p < 0.0001$ $p = 0.0114$ $p < 0.0001$ $p = 0.0370$ $p < 0.0001$

All the measured traits, with the exception of mean seed mass after dehiscence, were highly variable within populations, indicated by the large standard deviations of the means. As displayed in Figure 7.9, the general trend with respect to stem height, number of capsules, seed number per capsule and total seed number per plant, was for the large populations (>100 individuals) to have higher means than the three smaller populations. This trend was reversed for the number of flowering stems, with the highest mean values occurring in the two smallest populations, but

differences between populations were not significant for this variable. Mean seed mass, as compared using mean seed mass calculated by seed within capsules, and mean seed mass calculated using seed collected after dehiscence, exhibited significant differences between populations but with no obvious trends displayed. Mean seed mass appears to increase as population size increases but the very low mean value for Archer's Grove does not support this trend.

Results of the Bonferroni post-hoc tests reveal where the significant differences between populations lie with respect to the measured variables. Stem height and total number of seeds per plant were found to be significantly different between the two largest populations (Archer's Grove and Woodstock) and the smallest (Dunmore). The number of capsules was significantly greater in Woodstock than in the three smallest populations and this was also the case for mean seed mass. The number of seeds per capsule was significantly greater in the two largest populations and Boherduff than Dunmore. The largest population (Archer's Grove) had significantly higher numbers of seeds per capsule than all other populations but significantly less mean seed mass than all populations except the smallest. In the same way, Dunmore was found to have significantly lower mean seed mass than all other populations except the largest. This was also true for mean seed mass after dehiscence.

The number of flowering stems per plant did not meet the criteria for Analysis of Variance and a non-parametric Kruskal-Wallis test was carried out to investigate whether significant differences occurred between populations with respect to this variable. The chi-square value of 4.272 was not significant ($df=4$, $p=0.371$), and it can therefore be concluded that this variable does not exhibit significant differences between populations.

Correlation tests (Spearman Rank and Pearson Product Moment) and regression analyses were performed on the mean values in Table 7.7 to determine whether any of the reproductive variables show a correlation with, or are linearly dependent on population size. The results are summarised

in Table 7.9 and graphs showing the significant relationships are displayed in Figure 7.10. Standard error was used to construct error bars.

Table 7.9 Results of correlation and regression analyses between each of five variables and the factor 'population size'. n = 5 for all correlations. A Spearman Rank correlation test was carried out on the number of stems but all other variables were tested against population size using Pearson Product Moment tests.

	Correlation		Regression Analysis		
Dependent variable	**Correlation co-efficient**	**Significance**	**R-Squared (%)**	**F ratio** $_{df}$	**Significance**
No. stems	-0.718	ns	NA	NA	NA
Stem height	0.892	$p<0.05$	79.6	$11.7_{1,3}$	$p = 0.0420$
Number of capsules	0.780	ns	60.8	$4.65_{1,3}$	ns
Number of seeds per capsule	0.879	$p<0.05$	77.3	$10.2_{1,3}$	$p = 0.0496$
Total seed number per plant	0.922	$p<0.05$	85.1	$17.1_{1,3}$	$p = 0.0257$
Seed mass (capsules)	-0.221	ns	4.9	$0.154_{1,3}$	ns
Seed mass (after dehiscence)	0.442	ns	19.6	$0.73_{1,3}$	ns

NA = not applicable due to violation of assumptions for parametric tests.
ns = not significant at the 95% level.

There is a significant positive correlation between population size and three of the variables, stem height, number of seeds per capsule and the total seed output per plant. From the results of the regression analysis stem height, number of seeds per capsule and total seed produced per plant were found to show a significant linear dependence on population size with a high proportion of the variation accounted for by the regression.

In summary, significant differences between populations occurred with respect to stem height, number of capsules, number of seed per capsule, total seed per plant, mean seed mass (calculated by capsule) and mean seed mass after dehiscence. There is a consistent difference between Dunmore and Woodstock in all traits, with the smallest population (Dunmore), displaying the lowest means. Dunmore has the lowest mean values for all traits, with the exception of mean seed mass where only Archer's Grove has a lower mean, and shows significant differences between populations of higher population size for all measured traits. Two of the measured traits, stem height and number of seeds per capsule showed a significant linear dependence on population size.

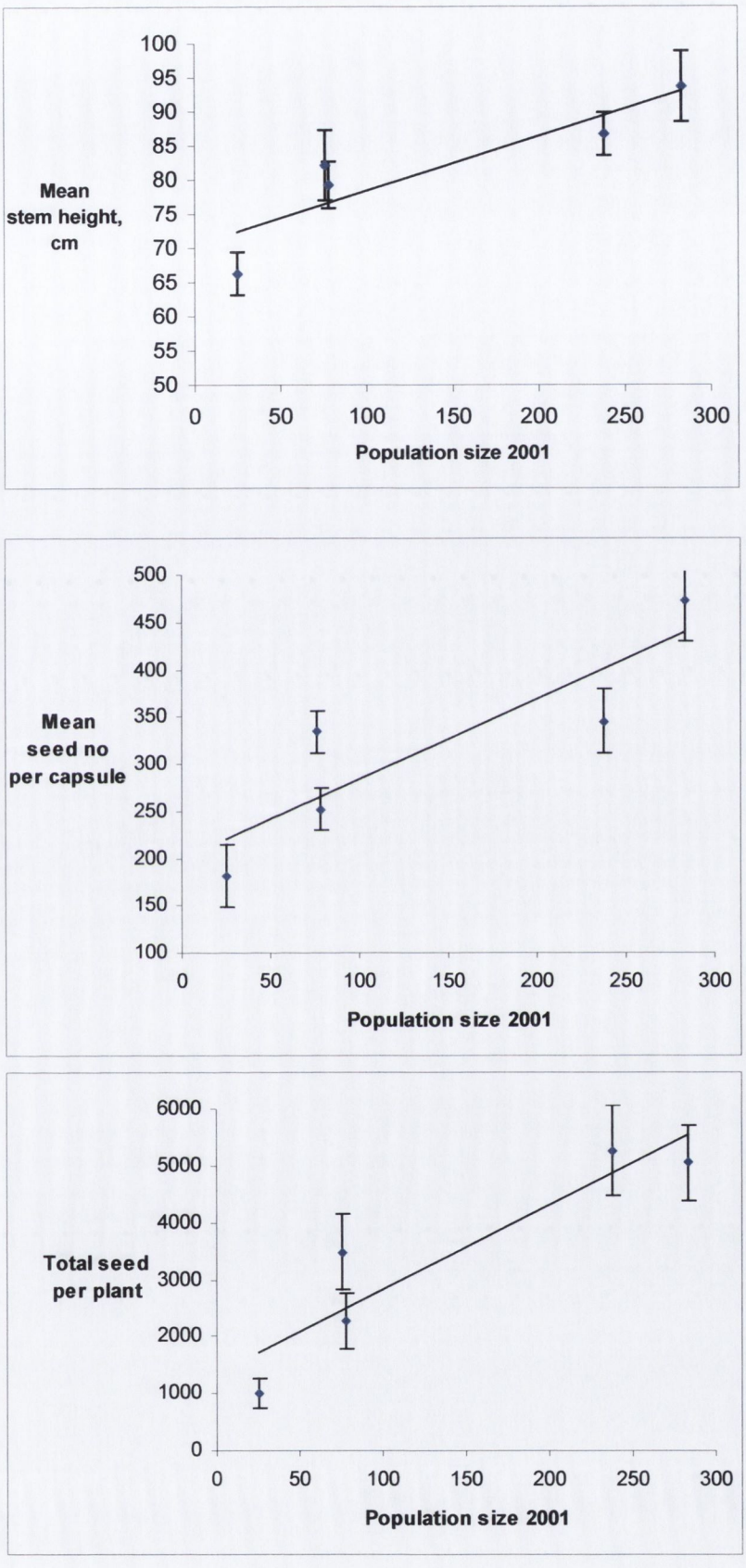


Figure 7.10 Graphs showing the significant linear relationship between population size and two fitness traits in five populations of *Campanula trachelium.* Error bars are standard error of the mean.

Pollen limitation

Due to damage to plants and tags and the limited availability of pollen donors and receptive stigmas in the Dunmore population, the sample size for this investigation was very small. The data contained four treatment pairs for Dunmore (population 4) and five for Archer's Grove (population 5). The mean data for the two treatments in the two populations along with graphical representation is displayed in Figure 7.11. The mean number of seeds per capsule was lower at Dunmore than Archer's Grove whereas the converse was true for mean seed mass per capsule. In the Dunmore population, seed number per capsule and seed mass per capsule increased slightly following pollen supplementation while at Archer's Grove they decreased.

The effect of supplemental pollen application to plants in the two populations was tested using a crossed ANOVA model with population and treatment as factors. The results are summarised in Table 7.10.

Table 7.10 Results of crossed ANOVA for pollen supplementation data.

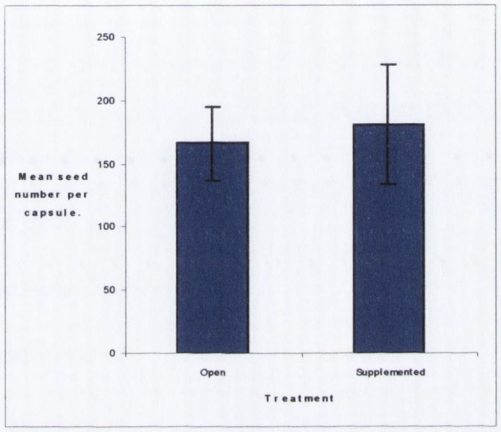
Dependent Variable	Factor	F Ratio	df (factor, error)	Significance
Number of seeds per capsule	Population	23.85	1, 14	p=0.0002
	Treatment	0.7492	1, 14	ns
	Treatment*Population	1.4763	1, 14	ns
Seed mass	Population	1.0720	1, 14	ns
	Treatment	0.0014	1, 14	ns
	Treatment*Population	0.0123	1, 14	ns

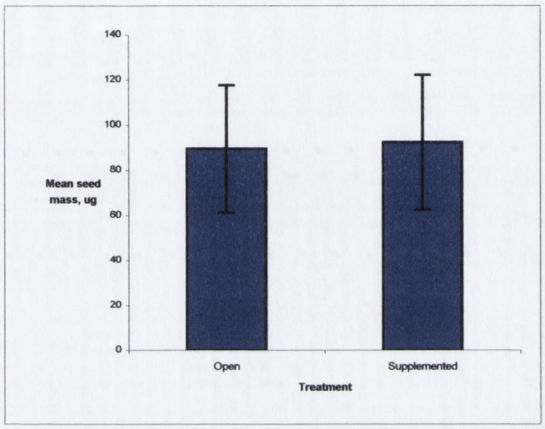
df = degrees of freedom
ns=not significant ($p<0.05$)

The number of seeds per capsule was significantly different between populations and this reinforces the earlier ANOVA comparison of populations and the relationship between seed number and population size. However, there were no significant differences between pollination treatments in either of the two variables and it can be concluded that the number of seeds per capsule and seed mass are not restricted by a deficit of pollen and pollen limitation is not responsible for the differences between populations with respect to these variables. The interaction between factors was not significant for either variable.

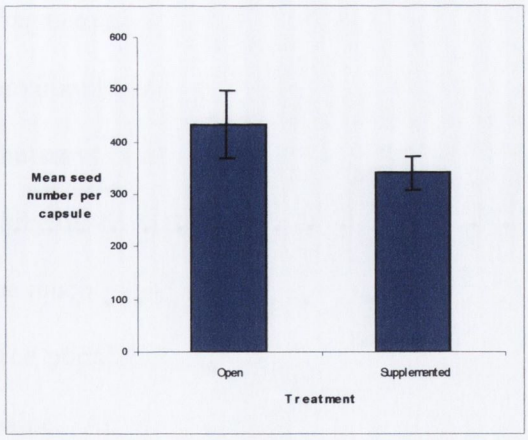
Population	Population size	Treatment	Mean no. seeds per capsule, µg (+/- standard error)	Mean seed mass per capsule, µg (+/- standard error)
4	25	Open pollination	166 (±57.10)	89.48 (±56.40)
		Supplemental pollination	181.25 (±93.79)	92.36 (±59.59)
5	283	Open pollination	432.2 (±127.82)	71.59 (±23.14)
		Supplemental pollination	341.4 (±65.15)	70.18 (±15.47)

Population 4 - Dunmore





Population 5 – Archer's Grove



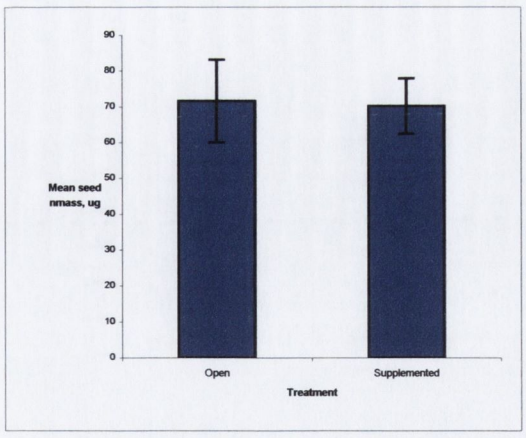


Figure 7.11 Comparison of mean seed number per capsule and mean seed mass (µg) between open and pollen-supplemented flowers in two populations. Error bars on the graph show standard error

7.5 DISCUSSION

The search to understand the intrinsic problems of small population size, and the way in which they interact to affect the survival of plant populations, is prominent in the conservation biology literature. The feedback loops and extinction vortices described by Gilpin and Soulé (1986) are triggered by a decrease in population size, and stochastic events affecting even stable small populations can lead to the same downward spiral to extinction. However the process begins, the outcome is dependent on population size and, in relative terms, small populations are more likely to face extinction than large populations (Shaffer, 1981, Lynch *et al.*, 1995a). Whether due to Allee effects such as the inability of small numbers of plants to attract pollinators or to intrinsic effects of population genetic structure, persistence is explicitly related to population size and there is a growing body of research into the causes and consequences of small population size in both threatened and widespread species. Many studies have focused on the reproductive health of a population, as it is an important measure of the capacity of a population to expand, but it can also be an indicator of underlying genetic effects.

Campanula trachelium has a high proportion of small populations, with the majority containing less than 100 flowering individuals. Coupled with isolation effects, possibly due to the fragmentation of its woodland habitat, these populations are vulnerable to population decline from environmental, demographic and genetic effects. However, the population sizes obtained during the surveys are underestimates and the proportion of non-flowering to flowering plants within three of the smallest populations, as high as 1.75, suggests that the actual numbers of plants could be much greater than estimated. Despite this, effective population size is always lower than the true population size and an underestimation of population size may be closer to this theoretical value. As the investigation of the population biology of *Campanula trachelium* was concerned with comparisons and relative effects of population size, the estimates are sufficient to draw accurate conclusions on the relative performance of the populations.

The six populations monitored over the five years of study showed fluctuations in growth and this was expressed in the variable growth rates. Other studies of herbaceous perennial plants have

shown an even greater variability in growth rate, such as a study on *Ipomopsis aggregata* (Pursh) V. Grant (Polemoniaceae) where growth rate ranged from 0.55 to 12.88 in different populations over 2 years (Heschel and Paige, 1995). Although the larger populations of *Campanula trachelium* showed a net decline over the five years, the growth rates of the smaller populations were more variable and this highlights their vulnerability to demographic stochasticity. The population at Inistioge declined from 35 to 18 flowering individuals over the five-year study period and this has serious implications for the survival of the population. Anglers use the riverbank habitat of this population heavily and the bank is prone to erosion and collapse. The loss of more plants from the population could seriously impact the survival probability of this population.

7.5.1 Gynodioecy

The occurrence of male sterility in the Irish populations of *Campanula trachelium* was noted during 1999 and the gynodioecious state of the species was subsequently verified by the investigation of a large sample of flowers. The bimodal distribution showed that the male sterility was not a continuously-varying trait and the monitoring of male sterile individuals and observations on their distribution also suggested that the male sterility is likely to be a constant, heritable sex state. Due to local seed dispersal, the non-random clumping of genders is expected if the sterility is heritable (Graff, 1999) and this was observed in several populations. It can therefore be inferred that the species exhibits heritable gynodioecy and although this hypothesis requires further testing, the implications of the occurrence of male sterility should be considered as part of the conservation of the species.

The frequency of female plants in the population varied greatly but marked sex ratio variation is characteristic of many gynodioecious species (eg. McCauley & Brock, 1998). Female frequency ranged from 0.11 to 0.8 in populations of *Thymus vulgaris* L. (Lamiaceae) (Thompson & Tarayre, 2000) and such variation is not unusual, although populations exhibiting stable gynodioecy show little change in the frequency of females over time (Richards, 1997). However, due to the

potential for female density to act as a selective pressure, the frequency of females becomes an important consideration in small populations of *Campanula trachelium*. A large frequency of females may force hermaphrodites to self due to lack of available pollen and leave females unable to set seed. This situation could lead to population decline and increased inbreeding, and stochastic events would then threaten such a population. Female advantage through possible resource compensation, as indicated by significantly higher amounts of seed produced per capsule, may encourage the spread of females through a population and in small populations a significant shift in female frequency in one generation may be enough to initiate these detrimental effects. However, the situation is complex and the increased diversity of even a small amount of outcrossed seed from females may compensate for inbreeding effects among hermaphrodites.

The results of the assessment of female advantage indicated that stem height and the number of seeds per capsule are significantly greater in females than in hermaphrodites. The advantage in greater flowering stem height may be to attract pollinators, an important trait for plants that have no capacity for self-pollination. Although the females produced greater numbers of seed per capsule, the mean capsule number was greater in hermaphrodites than in females, although not significantly so. The estimated total seed production was therefore not significantly different, although this could not be accurately assessed as only seed from the terminal capsules was counted and seed number varies with position on the flowering stem. Even accounting for this, there may be a female advantage in producing taller stems and more seed at the terminal position on the stem in that dispersal ability would be enhanced. Mean seed mass per capsule was also greater in the female capsules although this difference was not significant. Increased seed mass can provide seedlings with a better chance of survival (Jackson *et al.*, 1992) and this may outweigh the benefits of seed number. The complex interactions between the measured reproductive traits could not be fully assessed in this study and a larger sample with more thorough consideration of stem position would be required.

7.5.2 Fecundity

The fecundity of plant populations is the key to their survival and high fecundity secures the perpetuation of a population through the creation of a large input into a new generation. The deterioration of genetic variation, through genetic drift and inbreeding can result in a reduction in fecundity through inbreeding depression and this may be occurring in smaller populations of *Campanula trachelium.* The analysis of fecundity and reproductive fitness showed that significant differences occurred in five out of six reproductive characters; number of stems, stem height, number of capsules, number of seed per capsule and mean seed mass (per capsule and overall in the population). The smallest of the populations displayed the lowest mean values for four of the traits and was significantly different to Woodstock (population 13), one of the largest populations, for all measured traits. The largest population (5 - Archer's Grove) showed a surprisingly low value for mean seed mass per capsule. This may have been caused by the collection of capsules at an earlier stage of development than in the other populations but the overall mean seed mass, calculated from seed collected after dehiscence, although increased, also showed this population to have a low mean value for this trait. The geographic isolation of this population may have resulted in differentiation with respect to seed mass, and this may have allowed the production of greater amounts of seed accounting for the significant difference displayed in this trait. To fully evaluate the comparative fecundity of populations controlled greenhouse experiments would need to be carried out to eliminate environmental effects.

In terms of fecundity and population survival, the most significant result is the linear relationship between population size and mean seed number per capsule / total seed number per plant. Despite the relatively small samples investigated, the significance level of this result means that it has far-reaching implications for the conservation of *Campanula trachelium* and for other out-breeding plant species existing in small populations. Seed number is an important component of fecundity and the occurrence of lower levels of seed production in smaller populations is significant, particularly as the absence of any other causal factor indicates that genetic effects may be causing the low fecundity. The same positive relationship between population size and seed number per

fruit has been found in many other species including *Lupinus sulphureus* ssp. *kincaidii* C.P.Smith (Fabaceae) (Severns, 2003), *Gentianella germanica* (Fischer & Matthies, 1998), *Primula veris* L. (Primulaceae) and *Gentiana lutea* L. (Gentianaceae) (Kéry *et al.*, 2000).

Decrease in pollinator activity is often responsible for reduced seed set (Menges, 1991a) but the pollen supplementation experiment indicated that this was not the case in either of populations 4 (Dunmore) or 5 (Archer's Grove). Dunmore was the smallest of the populations investigated for reproductive traits and the density of plants is also low at this site. The ground flora of the woodland in which *Campanula trachelium* occurs at this site is also sparse, with little vegetation to attract pollinators. It is therefore susceptible to the effects of pollen limitation and if lack of pollination were the causal factor in reduced seed set, the effect of supplemental pollination would be expected to be pronounced and highly significant. The non-significant result therefore suggests that pollen limitation is not occurring at Dunmore and other factors must therefore be responsible for the lower reproductive fitness.

In order to fully evaluate the effect of population size on reproductive fitness, post-zygotic fitness would also need to be considered. The germination success of seeds and subsequent survival and reproduction of the F1 generation would need to be assessed to evaluate whether small population size in the parent generation translates into reduced fitness of the population through reduced recruitment, reduced survival of seedlings or reduced fecundity in the next generation of adult plants. However, these effects are difficult to monitor, requiring detailed knowledge of the dormancy-breaking and germination requirements of the species and long-term study of seedling development. A previous study by Martin (1998) on germination success in a number of threatened Irish plant species found that populations of *Campanula trachelium* were highly variable with respect to final percentage germination and that dormancy in this species is complex. A chilling pre-treatment and the application of gibberellic acid did not remove dormancy in all populations. Storage at -18^{0}C removed dormancy and increased the rate of germination in all but one of the populations, but this population had not displayed dormancy in the initial test. Walsh *et al.* (2003) found that two of the four accessions re-tested for germination

success after two years storage at a level of 5% moisture and a temperature of -18°C, showed a significant increase in germination percentage. They concluded that this may be a result of the dormancy being broken by cold storage and this further illustrates the complex nature of germination conditions in this species. To compare the relative rates and final percentages of germination among the populations of *Campanula trachelium* included in this study, detailed prior knowledge of their dormancy would be needed and populations would have to be subjected to different treatments based on this knowledge. This was beyond the scope of this project and germination was therefore not investigated.

In conclusion, each new study species in which significant correlations are found between population size and reproductive fitness lends weight to the importance of genetic effects in the conservation of rare and threatened plant species and this has important implications for management. Small population size has long been recognised as impacting the survival probability of populations but due to other possible factors the role of the genetic structure of the population in reducing fitness was often overlooked. The cyclical nature of interacting factors is also often overlooked and while some studies may focus on the effects of one causal factor, there may be a system of factors, all producing the same outcome. For instance, decreased pollinator activity may cause reduced seed set but may also result in greater inbreeding and reproductive deterioration due to inbreeding depression if plants are forced to self (Ellstrand & Elam, 1993). The genetic causes and consequences of small population size are complex but the relationship between small population size and lowered reproductive fitness due to genetic effects, highlights the need to investigate reproductive and genetic components of population biology.

Plate 7.1. Comparison of the style and anthers of male-sterile (left) and hermaphrodite (right) flowers.
The anthers of the male sterile flower are disfunctional and do not produce any pollen whereas the anthers of the hermaphrodite flower have deposited pollen onto the stylar hairs prior to the opening of the flower.

CHAPTER 8

CONSERVATION GENETICS OF *CAMPANULA TRACHELIUM* IN IRELAND

8.1 INTRODUCTION

Two of the most significant causes of genetic deterioration and inbreeding depression in plant populations are the reduction in population sizes and increase in isolation caused by habitat destruction and fragmentation of populations. Habitat destruction has the effect of lowering population sizes and reducing the area of potential habitat available to a species. Where this destruction results in the fragmentation of habitat, populations become isolated from the beneficial effects of gene flow and are at the mercy of stochastic genetic processes. The acceleration of habitat loss in the last century has caused population decline in both rare and formerly widespread species and as conservation biologists struggle to understand the processes involved, populations are rapidly disappearing. The need for information is critical in threatened species but while action often has to be taken immediately for globally rare species, the study of less threatened species offers a chance to understand the processes involved in population decline, without the necessity of immediate action. Conservation biology is a crisis discipline (Soulé, 1985) but many of the theories that have given insight to the discipline were developed in isolation from this crisis through the study of common species. Plant species that are not critically endangered but that are experiencing declining distributions or decreasing population sizes are excellent subjects for research into the population-level effects associated with declining species, and the results can have implications for a broad spectrum of species.

Small population size has a number of consequences for the genetic structure of plant populations. The theoretical effect is a loss of genetic variation through genetic drift and inbreeding (Barrett & Kohn, 1991; Ellstrand & Elam, 1993), and this genetic erosion has been demonstrated in a translocated population of one of the world's rarest plants, *Grevillea scapigera* A.S.George (Proteaceae) (Krauss *et al.*, 2002). Genetic decline between the founder and F1 generations, measured with AFLP genetic markers, was significant, despite an increase in population size. The theoretical loss of genetic variation in small populations is also supported by indirect evidence through a large number of studies where significant correlations have been observed between population size and various estimates of genetic variability. Many of the studies are based on the

investigation of genetic variation in enzyme loci and significant positive correlations have been found between population size and the proportion of polymorphic loci, mean effective number of alleles per locus (Bi *at al.*, 2003; Tomamitsu & Ohara, 2003) and observed heterozygosity (Bi *at al.*, 2003). Allelic richness was shown to decrease with decreasing log population size in an endangered pea species, *Swainsona recta* A.Lee (Fabaceae) (Buza et al., 2000), and the percentage polymorphic loci, number of alleles per locus and number of alleles per polymorphic loci, were significantly correlated with population size in *Viola pubescens* Ait. (Violaceae) (Culley and Grubb, 2003). Populations of both these species have undergone fragmentation as a result of the destruction of their habitats. The same relationship is observed with the use of neutral DNA markers, and population size was significantly positively correlated with the percentage of polymorphic RAPD bands within populations in *Saxifraga paniculata* P. Mill. (Saxifragaceae) (Reisch *et al.*, 2003) and with Nei's expected heterozygosity and the Shannon Index in populations of *Eryngium alpinum* L. (Apiaceae) analysed with AFLPs (Gaudeul, *et al.*, 2000). In an AFLP study on three populations of *Banksia saxicola* A.J.George (Proteaceae), the smallest population had the lowest genetic diversity (Evans *et al.*, 2001).

Many of the species in which such a relationship is found do not exist naturally in small populations and have declined due to habitat destruction and fragmentation. The consequences of the loss of genetic diversity in such populations are complex and include the onset of inbreeding depression and the possible interruption of systems such as mating type incompatibility systems. Demauro (1993) found that the only remaining Illinois population of the lakeside daisy (*Hymenoxys acaulis* (Pursh) K.L.Parker *var. glabra* (A.Gray)K.L.Parker (Asteraceae)) contained plants that all belonged to the same mating type and were therefore unable to reproduce sexually. This situation brings the effective population size of a sexually reproducing plant species to a value of 1 and implies rapid extinction in the absence of pollen or seed dispersal from another population. The breeding system and degree of rarity of a species also has other implications for the response of individuals to loss of genetic variation. If a species has always occurred in populations of small number, it may be adapted to this condition (Milligan *et al.*, 1994). Species

that are self-compatible and highly inbreeding have often purged their genetic load and are therefore less susceptible to the increased inbreeding that can occur following a decrease in population size. Conversely, the mating systems of outbreeding plant species that have undergone population reduction and decline may not be adapted to deal with the genetic consequences of rarity (Barrett & Kohn, 1991; Schmidt & Jensen, 2000) and they may display inbreeding depression. This theory was supported by Husband and Schemske (1996), who compiled data for 35 angiosperm species and 9 gymnosperms and found a significant negative correlation between cumulative inbreeding depression (measured across three life stages) and population size.

The genetic basis for the observed loss in reproductive vigour observed in small isolated populations is supported by evidence from natural populations of *Lupinus sulphureus* ssp. *kincaidii* where hand outcrossed pollinations on bagged inflorescences significantly increased seed production and seed fitness compared with the open pollination treatment, and with artificial pollination between plants in the same colony (Severns, 2003). This confirms that the reduced fitness is directly related to genetic effects and the fragmentation of the habitat is thought to be responsible for this incidence of inbreeding depression. Experimental evidence also suggests that genetic effects are responsible for reduced seed mass and percentage germination in small populations of *Ipomopsis aggregata* as pollen introduced from distant populations increased the magnitude of these reproductive traits in small but not large populations (Heschel & Paige, 1995). A significant relationship is therefore expected to occur between estimates of genetic diversity and measures of reproductive fitness in populations exhibiting inbreeding depression. This was found to be the case in remnant populations of *Pedicularis palustris* L. (Scrophulariaceae) (Schmidt & Jensen, 2000), where molecular variance derived from AFLP data was correlated with the number of seeds per plant and number of seedlings per plant. However, genetic diversity was independent of population size and this study emphasises the influence that factors such as the genetic history of the population may have on the genetic outcome of small population size.

Not all study species follow the relationships described between population size, genetic variability and reproductive vigour, due to the many complex, interacting influences on all these

factors. However, there is enough support for the general trends described to make them important subjects for investigation with regard to rare and threatened species. A central question is therefore whether low genetic variability translates into significant impacts on plant populations. This question was addressed by Newman and Pilson (1997), who carried out an experimental manipulation of genetic effective population size (N_e) in *Clarkia pulchella* Pursh (Onagraceae), a self-compatible but predominantly outcrossing winter annual. Their objective was to test the hypothesis that the genetic variability of a population influences fitness, thereby affecting survival probability. The experiment involved the creation of populations with contrasting N_e and this was achieved by establishing 32 populations of 12 seedlings, from either 12 unrelated plants or from three groups of full-sibling families. This resulted in two treatments, high and low N_e, and two replicate populations of each were established in eight spatial blocks. To retain full knowledge of the genetic structure of the experimental populations, inbreeding from selfing was prevented by the emasculation of flowers assigned as 'females' and crosses were carried out by the experimenters in order to evaluate reproductive output in relation to the degree of inbreeding in the population. After three generations, germination and survival rates were significantly lower in the population with low genetic variability and estimated mean fitness was 21% of that in the more variable population. The significance of this is revealed by the survival of populations; decreased genetic effective population size dramatically increased the probability of population extinction within the first three years. At the end of the three years, only 31% of the low N_e populations had survived, compared to 75% of the high N_e treatments. This has important implications for conservation and suggests that low levels of genetic variation may potentially have a significant impact on the probability of population extinction in outcrossing species.

In view of the impact that the genetic characteristics of a population can have on reproduction and survival, the aims of this study were to assess the levels of genetic variation within and between populations of *Campanula trachelium* and to elucidate genetic relationships between populations. Through the analysis of genetic structure as revealed by AFLP fingerprints, specific objectives were to evaluate whether depleted genetic diversity and inbreeding depression occurs in small

populations, to examine correlations between population size, genetic diversity and reproductive vigour, to assess the degree of population differentiation and gene flow between populations as a possible indication of whether the species exhibits a metapopulation structure in the Nore Valley, to evaluate the native status of *Campanula trachelium* in Ireland through comparison with Welsh and French populations and samples from a garden cultivar, and to assess levels of genetic variation in the context of the evolutionary potential of the Irish populations.

8.2 METHODS

8.2.1 Sampling

To adequately answer the questions regarding the conservation genetic status of *Campanula trachelium* in Ireland, a large number of populations needed to be sampled. Of the known Irish populations, only three were not sampled due to unsuitability of the leaf material for DNA extraction and the re-appearance of plants at one site after sampling had taken place. The Irish populations sampled for genetic analysis are shown in Figure 8.1. This map shows the location of the sites, the county boundaries and the River Nore. Sites are numbered with the population number as specified in Chapter 6 and these numbers will be used throughout this chapter.

Populations were sampled during July 2000 with the exception of Mount Juliet (6), Henllan (21) and Strawberry Wood (22), which were sampled in 2001. In total, 19 Irish populations of *Campanula trachelium* were sampled for genetic analysis and in addition, one garden population in County Dublin was sampled. Leaf material was also taken from an isolated plant discovered in a hedgerow in Monmouthshire, Wales and a population at Strawberry Wood Nature Reserve in Monmouthshire was also sampled. Seed obtained from the Musée National d'Histoire Naturelle, Paris was germinated to obtain individuals from a French population. Where population size was smaller than 50 individuals five samples were collected and 12 samples per population were collected from larger populations. The sample number taken from all other population was restricted by the number of plants present or the unsuitable condition of the leaves. A herbarium

Figure 8.1 Location of Irish populations of *Campanula trachelium* sampled for genetic analysis
Sites are numbered with the population number as specified in chapter 6.
County boundaries are displayed and the River Nore is shown in blue.

voucher specimen from the Inistioge population has been lodged in the Trinity College Herbarium.

8.2.2 Genetic Analysis

DNA extraction and quantification was carried out on the samples using the protocols described in Chapter 2. Primer trials were then conducted using a sample of ten individuals from across the range of populations and 8 primer pairs were tested. Table 8.1 lists the three primers selected on the basis of the number and intensity of bands produced.

Table 8.1. The sequence of selective nucleotides selected for the analysis of *Campanula trachelium*

Eco RI primer (end sequence)	Mse I primer (end sequence)
ACA	CTT
AAG	CTC
ACC	CAC

Ten samples per population (or the maximum number of samples for smaller populations) were analysed using AFLPs, following the protocols specified in Chapter 2. If any reaction failures occurred in the larger populations, additional samples were analysed to bring the number of successful samples to 10. The numbers of amplifiable AFLP reactions used in the analysis of fingerprinting patterns are shown in Table 8.2.

Table 8.2 The number of samples used in the genetic analysis of *Campanula trachelium* populations.

Population no.	Population size	Population name	No. samples
1	600	Tulla	10
2	79	Eagle Hill	10
3	84	Knocknagroagh	10
4	29	Dunmore	5
5	347	Archer's Grove	10
6	53	Mount Juliet	10
7	60	Boherduff	10
8	69	Thomastown	10
9	175	Brownsbarn	10
10	412	Ballyduff	10
11	82	Ballygallon	10
12	1	Oldcourt Island	1
13	281	Woodstock	10
14	10	Clonamery	3

Population no.	Population size	Population name	No. samples
15	2	Murphy's of the river	1
16	3	Rathsnagadan	2
17	2	Pollmonty	1
18	1	Tintine	1
19	54	Tankardstown Cross	3
20	35	Clonlea	2
21	2	Henllan	1
22	20	Strawberry Wood	5
23	8	Fôret de Chatillon	9

All samples were run on an ABI Prism Genetic Analyser and the results were interpreted by Genescan® 3.1.2 software. The Genescan files were imported into Genotyper® 2.5.2 where the electropherograms were viewed and the presence and absence of bands were scored by eye. A macro for the automatic scoring of bands was available but was not used due to significant errors inherent within the band size classification method. A presence/absence matrix was obtained from the scoring of bands and this was analysed using a number of different software packages to allow comparison of the results derived from different methods of analysis. Details of the analyses performed are presented below.

Due to the significant error in the calculation of gene diversity statistics for small sample sizes, populations with less than 5 sampled individuals were excluded from the AFLPSURV and POPGENE analyses and as a result, bands that were unique to any of these populations were removed from the analyses.

PAUP v.4.0b10

Neighbour-joining analyses were performed on the presence-absence matrix using mean character difference and Nei-Li's restriction site difference (Nei and Li, 1979) for comparison. A genetic distance matrix computed between samples from 14 of the populations, using a Bayesian estimation of allele frequency, was also used as the basis for a neighbour-joining analysis. Bootstrap analyses were performed on all the datasets to obtain confidence estimates for the tree groupings. These were carried out using 10,000 replicates. Trees were drawn using midpoint rooting so as not to assume relationships between samples.

POPGENE version 1.32

POPGENE was used to calculate diversity estimates based on Nei's (1973) genetic diversity, the proportion of polymorphic loci and Shannon's Index (Shannon & Weaver, 1949). UPGMA dendrograms of populations, based on Nei's (1978) genetic distance estimates were plotted. The multi-population genic variation statistics were calculated as described in Nei (1987b) and total gene diversity and within population diversity were based on Nei's analysis of gene diversity in sub-divided populations (Nei, 1987a). The population statistics obtained are as described for the analysis of *Colchicum autumnale* in Chapter 5.

AFLP-SURV v.1.0

AFLP-SURV was used to calculate population statistics and to generate a genetic distance matrix derived from the estimation of allele frequencies using a Bayesian estimate with non-uniform prior distribution (Zhivotovsky, 1999). The distance matrix was used as the basis for a UPGMA dendrogram using PAUP. The gene diversity statistics obtained are Ht, the total genetic diversity, which is partitioned into Hw the within population diversity and Hb, the between-population diversity. An overall F-statistic for the differentiation of populations is provided by AFLP-SURV following the methodology of Lynch and Milligan (1994).

Arlequin Ver 2.000

Analysis of Molecular Variance (AMOVA) was carried out on the samples to obtain estimates of population structure. Three separate analyses were performed. The first included all populations, the second included all populations with a sub-structure of three groups; Irish populations, Welsh populations, and the French population. The third was performed on all Nore Valley populations. A Mantel test with 1000 permutations was carried out on all Irish populations to determine the correlation between genetic and geographic distance matrices.

Le Progiciel R v4·0d

Using Le Progiciel, Principal Coordinates Analysis was performed on the data to show spatial relationships between samples. The analysis was carried out on a distance matrix derived from

the original presence-absence matrix using the Dice similarity measure and subtracting all values in the matrix from 1.

8.3 RESULTS

The AFLP analysis of *Campanula trachelium* populations yielded 153 markers that could be scored unambiguously for all samples. Marker size ranged from 39 to 203 base pairs in length. Gene diversity calculations were based on 14 populations with more than 5 individuals and 139 AFLP loci.

8.3.1 Gene diversity

The three genetic diversity measures derived from POPGENE are plotted for all populations in Figure 8.2, with populations arranged in increasing order of population size. Population size is unknown for Fôret de Chatillon (population 23). Total gene diversity (Ht), mean gene diversity averaged over all loci (H), mean within population gene diversity (Hs) and the mean Shannon's Index (I) were calculated for three levels: all populations, all Irish populations and all Nore Valley populations. The diversity measures are summarised in Tables 8.3 and 8.4.

Table 8.3 Genetic diversity within 14 populations of *Campanula trachelium*

Population	Population size	No. samples	PPL	Shannon's Index	Gene diversity
1	600	10	0.4892	0.2608	0.1761
2	79	10	0.3525	0.1930	0.1311
3	84	10	0.2734	0.1443	0.0972
4	29	5	0.2878	0.1575	0.1063
5	347	10	0.4964	0.2716	0.1844
6	53	10	0.2806	0.1516	0.1025
7	60	10	0.3022	0.1684	0.1145
8	69	10	0.4604	0.2411	0.1643
9	175	10	0.5755	0.2960	0.1976
10	412	10	0.3022	0.1631	0.1095
11	82	10	0.3597	0.1978	0.1350
13	281	10	0.3453	0.1760	0.1173
22	20	5	0.2302	0.1166	0.0765
23	not known	10	0.2806	0.1538	0.1041

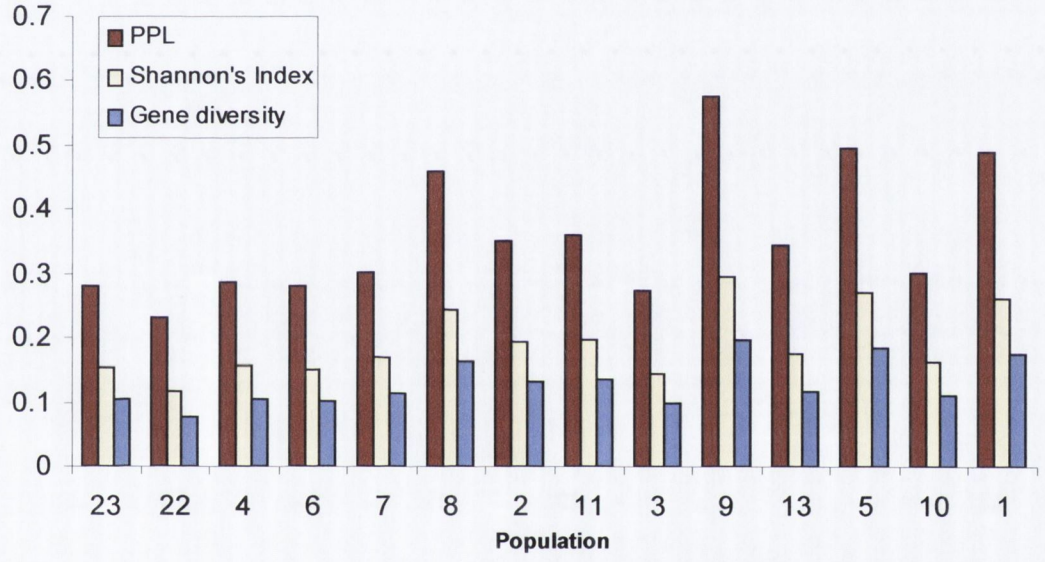
PPL = Proportion of polymorphic loci
Gene diversity (Nei, 1973)
Population size is the number of flowering stems estimated from the survey of populations described in Chapter 7.

Table 8.4 Summary of genetic diversity in populations of *Campanula trachelium*

Population level	No. samples	PPL	I	Ht	Hs
All populations	128	0.8921	0.3397	0.2191	0.1297
Irish populations	115	0.8129	0.3194	0.2042	0.1363
Non-Irish populations	14	0.5324	0.2758	0.1884	0.0903
Nore Valley populations	85	0.7626	0.3097	0.1991	0.1368

PPL = Proportion of polymorphic loci I = Shannon's Index Ht = Total gene diversity Hs = Within-population diversity

Figure 8.2 Measures of genetic diversity in 14 populations of *Campanula trachelium* arranged in increasing order of population size with the exception of population 23 for which population size is unknown.



PPL = Proportion of polymorphic loci

The three diversity measures give different estimates of the level of diversity within populations but the relative magnitudes between populations are consistent and the patterns of diversity are therefore similar for all three. The overall proportion of polymorphic loci is 89% (Table 8.4) and varies from 23% to 58% in individual populations (Table 8.3). 81% of loci present in Irish populations and 76% of those in Nore Valley populations are polymorphic, indicating high levels of polymorphism within these groups in comparison to the Welsh and French populations where only 53% of loci are variable (Table 8.4). The Shannon Information Index ranges from 0.1166 to 0.2960 (Table 8.3) and is 0.3397 over all populations (Table 8.4). Gene diversity (Nei, 1973)

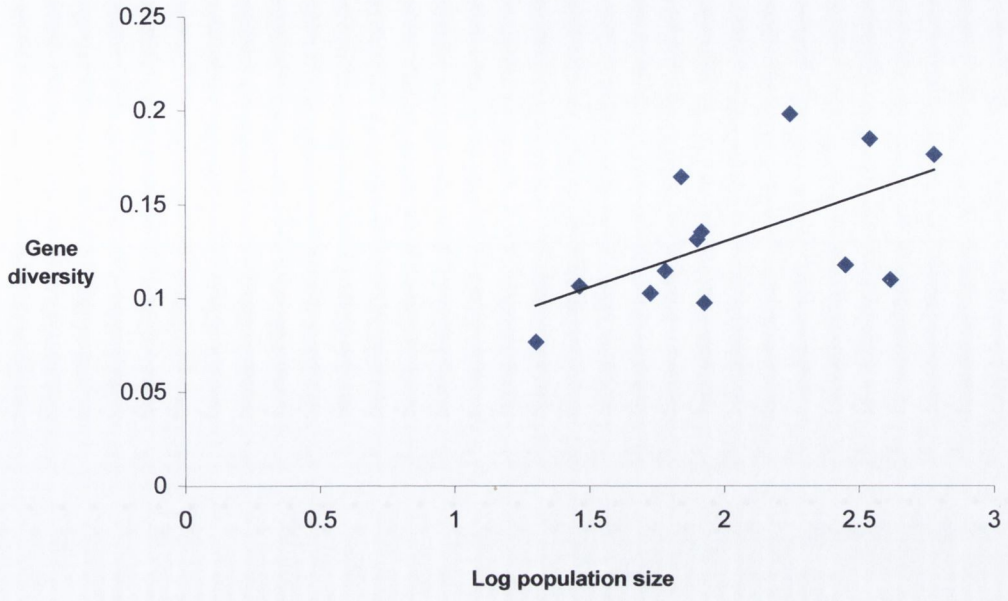
varies from 0.0765 to 0.1976 within populations (Table 8.3) and the total gene diversity is 0.2191 (Table 8.4). Irish populations contain a significant amount of the overall diversity for all the indices but the levels in individual populations vary greatly. Mean within population diversity is 0.1297 for all samples but the Nore Valley populations contain more of the diversity than other groups, with a mean population diversity of 0.1368 (Table 8.4). Within-population diversity in the two non-Irish populations is very low (Mean Hs=0.0903).

All of the genetic diversity measures were significantly correlated with population size and all showed a significant positive linear dependence on log population size as shown in Table 8.5 below. The relationship between log population size and gene diversity is shown in Figure 8.3. The smallest of the sampled populations (22 – Strawberry Wood), occurs in Wales and has the lowest genetic diversity. This is also observed for the proportion of polymorphic loci and the Shannon's Index. The three Irish populations that have least genetic diversity are populations Knocknagroagh (3), Dunmore (4) and Mount Juliet (6). Dunmore and Mount Juliet are the smallest Irish populations and the former was identified as potentially suffering from the effects of inbreeding depression on the basis of the study of reproductive characteristics detailed in Chapter 7. Knocknagroagh is an isolated population occurring on a tributary of the River Barrow.

Table 8.5 Results of correlation and regression analyses between log population size and three genetic diversity measures; the proportion of polymorphic loci, Shannon's Index and Nei's (1973) gene diversity. n = 13 for all correlations.

	Pearson Product Moment Correlation		Regression Analysis		
Diversity measure	**Correlation co-efficient**	**Significance**	**R-Squared (%)**	**F ratio** $_{df}$	**Significance**
Prop polymorphic loci	0.585	$p<0.05$	34.3	5.74 $_{1,11}$	$p = 0.0346$
Shannon's Index	0.588	$p<0.05$	34.5	5.80 $_{1,11}$	$p = 0.0347$
Gene diversity	0.588	$p<0.05$	34.6	5.81 $_{1,11}$	$p = 0.0355$

Figure 8.3 Relationship between log population size and gene diversity in 13 populations of *Campanula trachelium*.



Nei's (1973) genetic diversity was not significantly correlated with any of the reproductive variables obtained from the study of five *Campanula trachelium* populations described in Chapter 7 (Table 8.6) but this may be a result of the small number of data points. However, plots of gene diversity against all measured reproductive variables (Figure 8.4) show that the stem height, number of seeds per capsule and total seed output per plant do show increasing trends as gene diversity increases. In all three plots, the lowest reproductive fitness occurs in the population with the lowest gene diversity and for both stem height and the number of seeds per capsule the opposite is also true in that the population with the highest gene diversity also has the higher value for the reproductive fitness trait. In population terms, of the five Nore Valley populations that were included in the study of reproductive fitness, the smallest, (Dunmore), has the lowest genetic diversity and the lowest reproductive vigour. The largest population, (Archer's Grove) has the highest genetic diversity and was also found to have the greatest stem height and to yield the highest number of seeds per capsule and second highest total seed output. However, it also displayed the lowest mean number of stems, the mean lowest seed mass measured for capsules and the second lowest seed mass measured after dehiscence. It is possible that a re-allocation of

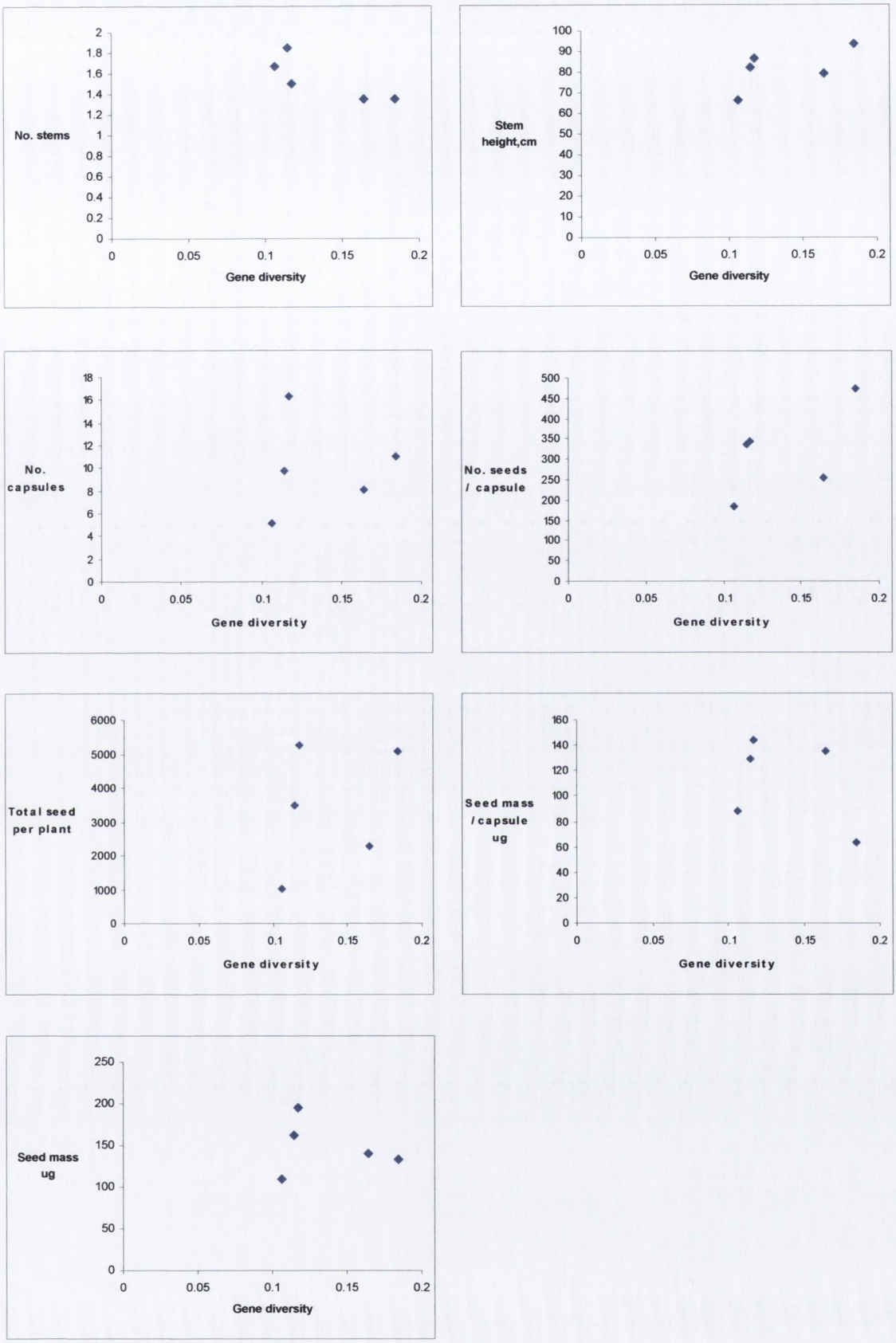


Figure 8.4 Relationship between genetic diversity and reproductive traits in five populations of *Campanula trachelium*.

resources has occurred, away from producing seeds of high mass and many stems towards increasing the quantity of seeds produced.

Table 8.6 Correlation between Nei's (1973) gene diversity and mean population values of the reproductive measures investigated in Chapter 7. A Spearman Rank correlation test was performed on the number of flowering stems due to non-conformity to the assumptions of a parametric test. Pearson Product Moment tests were carried out on all other variables. n=5 for all correlations.

Fecundity measure	Correlation Co-efficient	Significance
Number of flowering stems	-0.872	ns
Stem height	0.614	ns
Number of capsules	0.042	ns
Number of seeds per capsule	0.580	ns
Total number of seeds per plant	0.323	ns
Mean seed mass before dehiscence	-0.405	ns
Mean seed mass after dehiscence	-0.222	ns

ns = not significant ($p<0.05$)

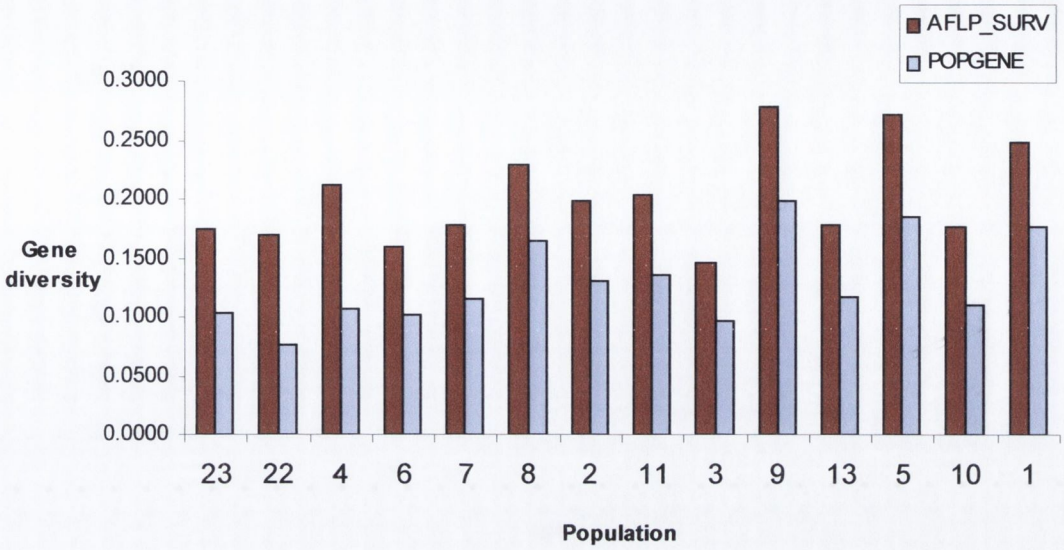
Bayesian estimation

Gene diversity statistics were also calculated using the AFLP-SURV analytical program, which employs a Bayesian estimate of allele frequency estimation. Estimates of Nei's genetic diversity were then calculated using the estimated gene frequencies and following the treatment of Lynch and Milligan (1994). The population values obtained for gene diversity are shown in Table 8.7 alongside the estimates of gene diversity obtained from POPGENE and are plotted in Figure 8.5.

Table 8.7 Comparison of diversity estimates obtained from POPGENE and AFLP-SURV programs

Pop no.	No. samples	Gene diversity (Nei 1973 - POPGENE)	Gene diversity (Lynch and Milligan (1994), with Bayesian estimate - AFLPSURV)
1	10	0.1761	0.2474
2	10	0.1311	0.1986
3	10	0.0972	0.1458
4	5	0.1063	0.2122
5	10	0.1844	0.2707
6	10	0.1025	0.1599
7	10	0.1145	0.1776
8	10	0.1643	0.2282
9	10	0.1976	0.1773
10	10	0.1095	0.1766
11	10	0.1350	0.2027
13	10	0.1173	0.1778
22	5	0.0765	0.1689
23	10	0.1041	0.1741

Figure 8.5 Comparison of genetic diversity estimates derived from square root (POPGENE) and Bayesian (AFLP-SURV) estimates of gene frequency



Gene diversity values derived from the Bayesian estimation of allele frequency are significantly higher than the previous values and range from 0.1458 to 0.2707 (Table 8.7) with a mean of 0.1941. The highest gene diversity is still found in the largest population, Archer's Grove (5) but the lowest gene diversity is found in Knocknagroagh (population 3). The greatest difference can be seen in the populations that previously had the lowest gene diversity, Strawberry Wood (22) and Dunmore (4), which have greatly elevated values using the Bayesian method. However, the error associated with this estimation procedure is known to be significant for small population size and as both these populations were represented by only five samples, this should be taken into consideration. Overall, there was a significant positive linear correlation between the two estimates of diversity (Pearson Product-Moment co-efficient = 0.686, $n=14$, $p<0.05$). Using the Bayesian estimation, total genetic diversity was 0.2651 (0.2191 with POPGENE) and mean within population diversity was 0.2013 (0.1297 with POPGENE). Gene diversity was not significantly correlated with population size or any of the measures of reproductive fitness (Table 8.8).

Table 8.8 Correlation between gene diversity (as calculated by the approach of Lynch and Milligan (1994) using a Bayesian estimate of allele frequency), and mean population values of the reproductive measures investigated in Chapter 7. A Spearman Rank correlation test was performed on the number of flowering stems due to non-conformity to the assumptions of a parametric test. Pearson Product Moment tests were carried out on all other variables. n=5 for all correlations.

Fecundity measure	Correlation Co-efficient	Significance
Number of flowering stems	-0.667	ns
Stem height	0.258	ns
Number of capsules	-0.349	ns
Number of seeds per capsule	0.349	ns
Total number of seeds per plant	-0.019	ns
Mean seed mass before dehiscence	-0.784	ns
Mean seed mass after dehiscence	-0.666	ns

ns = not significant ($p>0.05$)

8.3.2 Genetic distance

The presence-absence matrix of AFLP markers for the 14 populations used in the calculation of genetic diversity estimates was used to elucidate relationships between populations. A matrix of genetic distances between populations (Nei, 1978 – Table 8.9) was calculated using POPGENE 1.32 and used to construct a UPGMA of populations (Figure 8.6). The largest genetic distances occur between the Irish populations and the Welsh and French populations and this is reflected in a clear separation between these groups in the dendrogram. However, groupings within the Irish populations do not follow any clear geographic pattern and are not well-supported, and this structure may not be properly resolved.

As a further comparison, a genetic distance matrix (Table 8.10) was obtained from a Bayesian method of gene frequency estimation (Zhivotovsky, 1999) using the AFLP-SURV 1.0 analytical freeware (Vekemans, 2002) and this was also used to construct a UPGMA (Figure 8.7). The structure displayed in this tree is similar to the UPGMA dendrogram in Figure 8.6 in that the lowest level of structuring reveals similar population groupings. The overall structure is also similar in that the large genetic distances between the Irish and non-Irish populations (Table 8.10) are reflected in the placement of the Welsh and French populations outside the Irish grouo. Once again, the grouping of Irish populations does not appear to reflect their geographic proximity. The only structure of note is the presence of Eagle Hill and Knocknagroagh in the same major

	1	2	3	4	5	6	7	8	9	10	11	13	22	23
1	0													
2	0.0734	0												
3	0.0799	0.0613	0											
4	0.0833	0.0875	0.0597	0										
5	0.0565	0.0703	0.0983	0.0937	0									
6	0.1169	0.0961	0.1554	0.1645	0.0737	0								
7	0.0878	0.0810	0.1287	0.1245	0.0326	0.0398	0							
8	0.0369	0.0867	0.1017	0.0952	0.0611	0.1195	0.0968	0						
9	0.0774	0.1194	0.1253	0.1391	0.0451	0.0851	0.0720	0.0676	0					
10	0.0916	0.0733	0.1177	0.1347	0.0487	0.0333	0.0211	0.0986	0.0724	0				
11	0.0753	0.0811	0.0901	0.0854	0.0377	0.0766	0.0583	0.0663	0.0504	0.0538	0			
13	0.0573	0.0589	0.0660	0.0611	0.0605	0.0970	0.0715	0.0698	0.0902	0.0646	0.0503	0		
22	0.1391	0.1792	0.1886	0.1650	0.1470	0.2128	0.1850	0.1548	0.1776	0.1795	0.1575	0.1340	0	
23	0.1621	0.1882	0.2171	0.2199	0.1819	0.2045	0.2147	0.1645	0.1824	0.1804	0.1956	0.2046	0.2348	0

Table 8.9 Matrix of genetic distance (Nei, 1978) between 14 populations of *Campanula trachelium,* numbered as in figure 8.1.

	1	2	3	4	5	6	7	8	9	10	11	13	22	23
1	0													
2	0.0548	0												
3	0.0612	0.0449	0											
4	0.046	0.0586	0.0357	0										
5	0.0316	0.0473	0.0751	0.0569	0									
6	0.0932	0.0756	0.1313	0.1309	0.0501	0								
7	0.0662	0.0612	0.1061	0.0934	0.0158	0.0216	0							
8	0.0185	0.0681	0.0838	0.0601	0.0353	0.0947	0.0736	0						
9	0.048	0.0893	0.1007	0.0921	0.0212	0.0615	0.0488	0.0385	0					
10	0.0687	0.0561	0.0968	0.1	0.0277	0.0153	0.0058	0.0735	0.0474	0				
11	0.0521	0.0623	0.072	0.0534	0.0159	0.0559	0.0374	0.0442	0.0282	0.0336	0			
13	0.0382	0.0428	0.0493	0.0351	0.04	0.0781	0.0527	0.051	0.0663	0.048	0.0325	0		
22	0.1065	0.1492	0.1569	0.1239	0.1126	0.1789	0.1558	0.1227	0.1353	0.1483	0.1248	0.1087	0	
23	0.1344	0.1625	0.1936	0.1755	0.1451	0.1787	0.184	0.137	0.1469	0.1537	0.1657	0.1757	0.1944	0

Table 8.10 Matrix of genetic distance between populations 14 populations of *Campanula trachelium,* numbered as in figure 8.1. (Nei's genetic distance after Lynch and Milligan (1994) and based on Bayesian estimate of null allele frequency.)

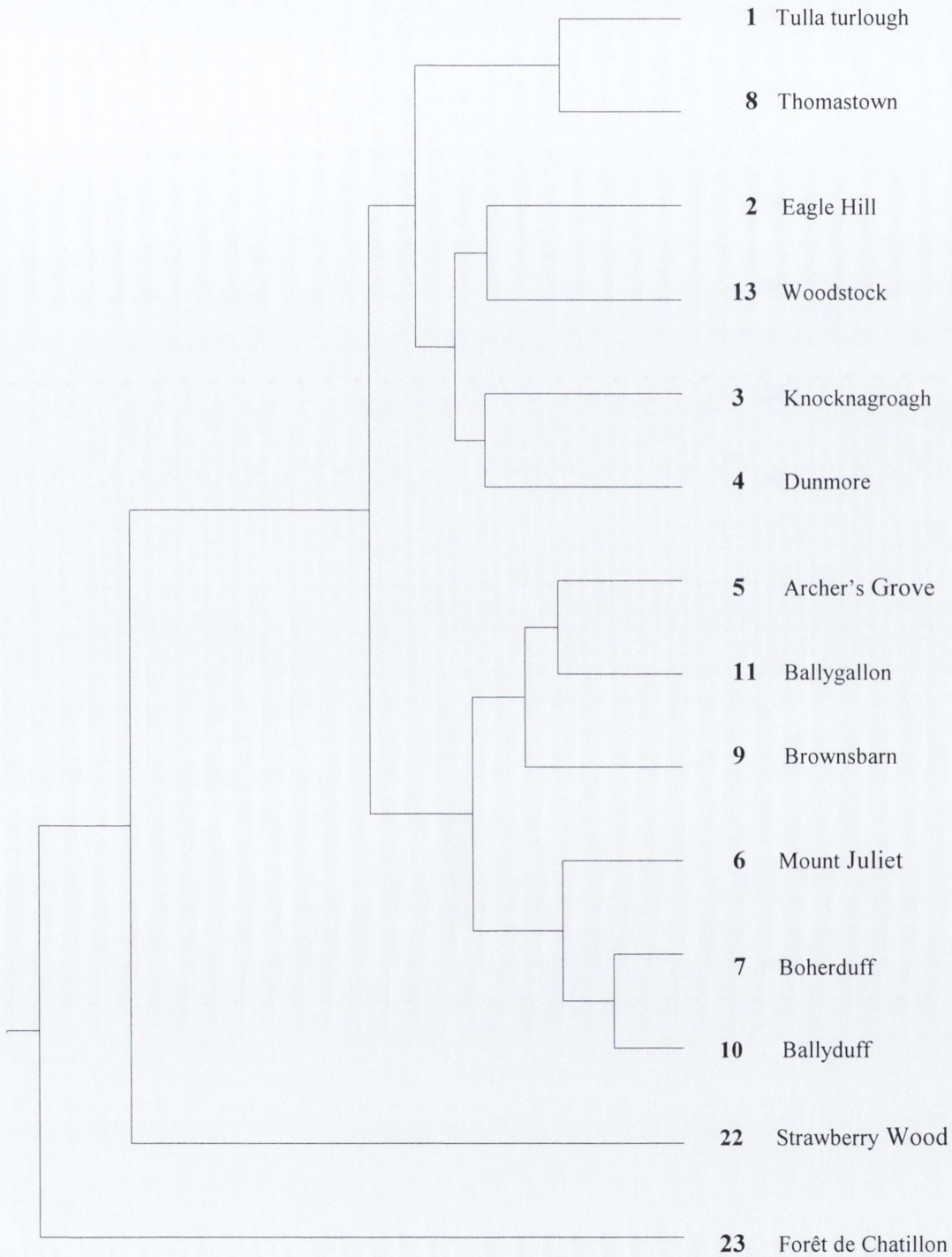


Figure 8.6 UPGMA dendrogram of 14 populations of *Campanula trachelium* using Nei's (1978) genetic distance

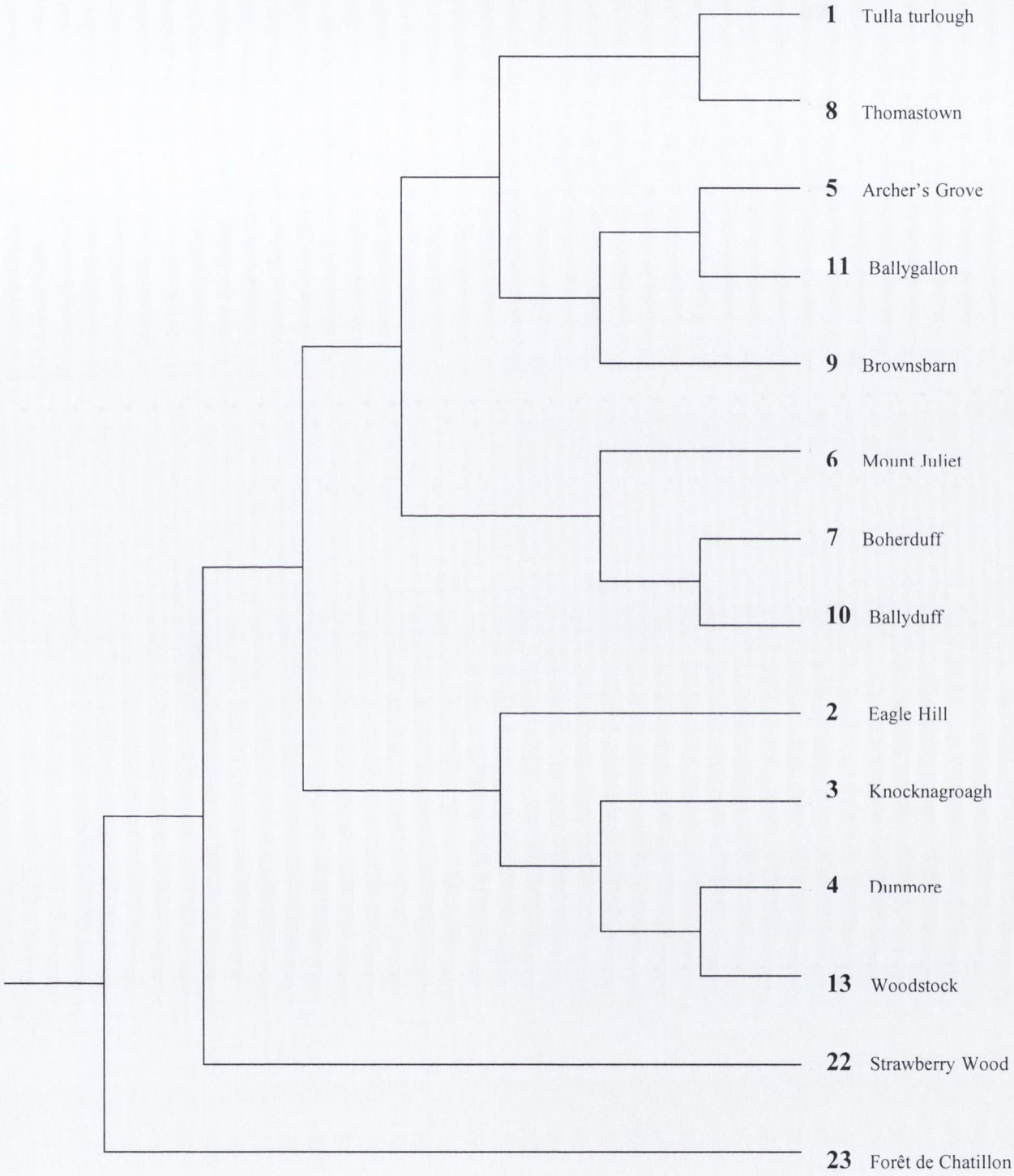


Figure 8.7 UPGMA dendrogram of 14 populations of *Campanula trachelium* using Nei's genetic distance after Lynch and Milligan (1994) based on a Bayesian estimate of allele frequency

group, as these are both populations occurring on esker ridges to the North of the River Nore. However, their geographic separation is significant and they occur on different river systems.

The relationships between samples from different populations were investigated more closely using neighbour-joining analyses performed in PAUP version 4.0b10. The original presence-absence matrix was used in the neighbour-joining analyses and individual samples were therefore grouped based on the similarity of the banding patterns. As this does not involve estimation of allele frequencies, all individuals were included in these analyses. Figure 8.8 shows a neighbour-joining phylogram of samples, based on mean character difference and plotted using mid-point rooting. The branch lengths of this phylogram correspond to genetic distance as determined by mean character difference and each character difference between samples corresponds to a distance of 0.006536. The scale of 0.05 corresponds to 7.65 character differences. The maximum character difference between two samples of *Campanula trachelium* was 82 differences and this occurred between a sample from the River Barrow Pollmonty population (17) and a sample from the River Nore Mount Juliet population (6). The minimum character difference between samples was 5 and this occurred between two pairs of samples from population 13 (Woodstock) and a pair of samples from population 3 (Knocknagroagh).

Figure 8.8 shows that the genetic structure of the individuals within populations is poorly supported. Bootstrap values, indicating the support for a particular group, are only greater than 80% for the grouping together of individuals from non-Irish populations, with the exception of the pairing of two samples from Brownsbarn (population 9). Individuals from populations 22, Strawberry Wood (97% bootstrap support) and 23, Forêt de Chatillon (95% bootstrap support) are grouped in population-specific sub-groups, indicating the high degree of genetic similarity within these populations. Individuals from populations 2 (Eagle Hill) and 3 (Knocknagroagh) also form population clusters but bootstrap support for these groups is not significant. The pairing of two sets of individuals within the cluster of Woodstock samples (population 13) is well supported but this population group is not monophyletic. In the overall structure there are a number of major outliers consisting of individuals from populations 1 (Tulla turlough), 8 (Thomastown), 17

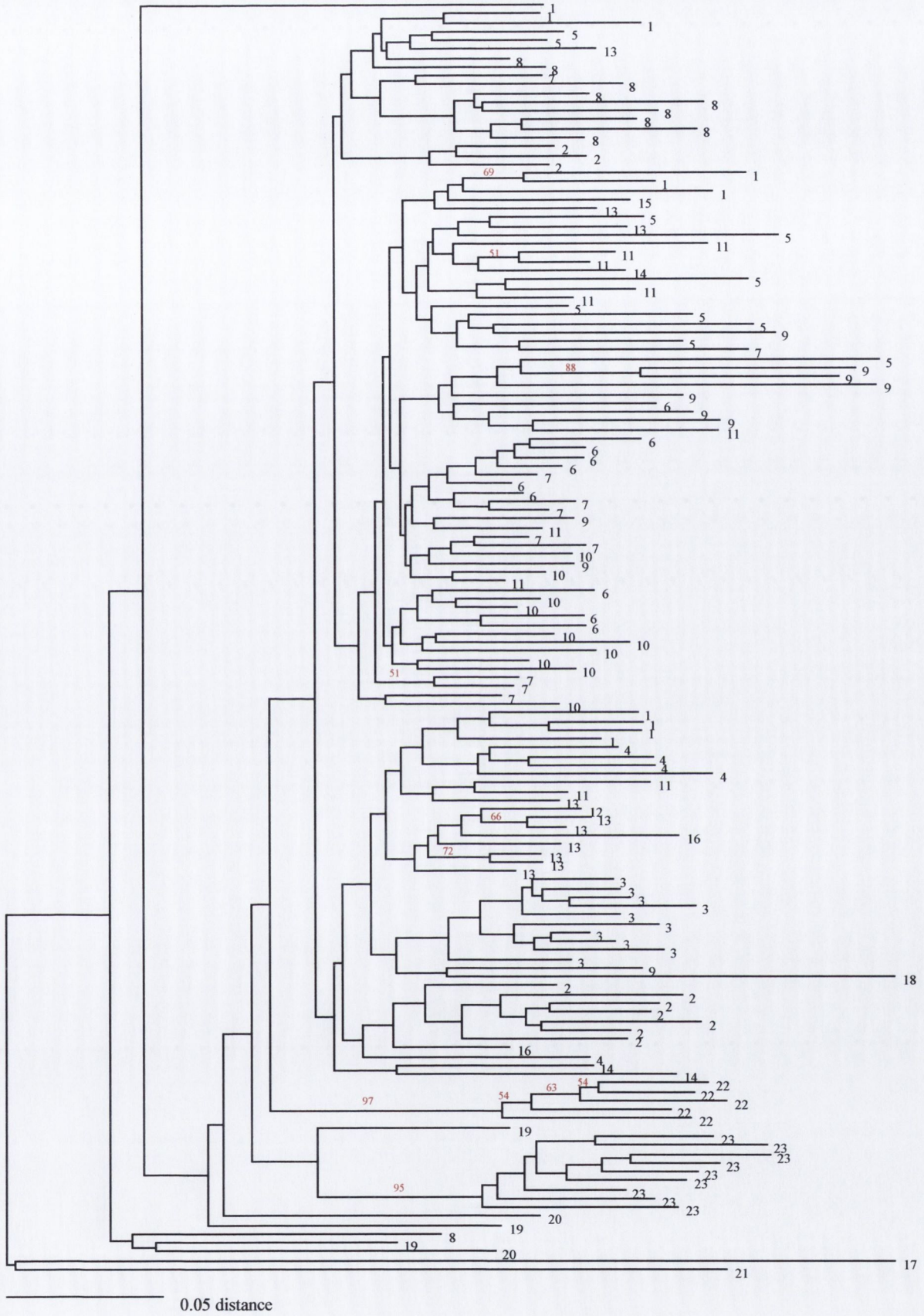


Figure 8.8 Neighbour-joining phylogram of individuals from 23 populations of *Campanula trachelium,* based on mean character difference. Bootstrap support values over 50%, from an analysis of 10,000 replicates, are shown in red on the relevant branches. Population numbers correspond to the populations as mapped in Figure 8.1.

(Pollmonty), 19 (Tankardstown Cross), 20 (Clonlea) and 21 (Henllan), and these are separated out from all other individuals.

The neighbour-joining analysis was repeated using the assumption that each difference in the amplification of an AFLP marker results from a restriction or amplification site difference in the DNA at that locus (Nei and Li, 1979). The neighbour-joining tree based on this calculation of distance using Nei-Li's restriction site distance (Figure 8.9) shows the same general pattern of relationships as the previous phylogram but with much smaller estimations of distance between the samples. The Welsh and French populations form exclusive population sub-groups once again but bootstrap support is not as high in this analysis (71%). Population 22, Strawberry Wood in Wales, separates out completely from all other samples, indicating that it is genetically distinct from all other populations. Although the French population forms a distinct group, it is placed in a sister group to the Irish populations and is more similar to these individuals than to the Welsh individuals, which appear to be significantly differentiated from all other populations. Once again, samples from Eagle Hill (2), Knocknagroagh (3) and Woodstock (13) show a tendency to group together with other samples from the same population only two of the Woodstock pairings have bootstrap support greater than 50% and the population groups are not well supported. Samples from Eagle Hill (2) and Knocknagroagh (3) share a common node and therefore show a high degree of similarity. There is only one major outlier in this analysis and this is an individual from Pollmonty (17).

The genetic relationships between populations were also subjected to a multivariate ordination analysis. Principal coordinates analysis was carried out using Le Progiciel. The first four axes accounted for only 15.92% of the variation in the dataset and plots of eigenvectors for axes one and two reveal very little structuring of populations other than the spatial separation of the Irish and non-Irish populations (Figure 8.10). The first axis accounted for 5.6% of the variance and the second axis accounted for 4.58% of the variance. The genetic structuring of the samples is poorly supported.

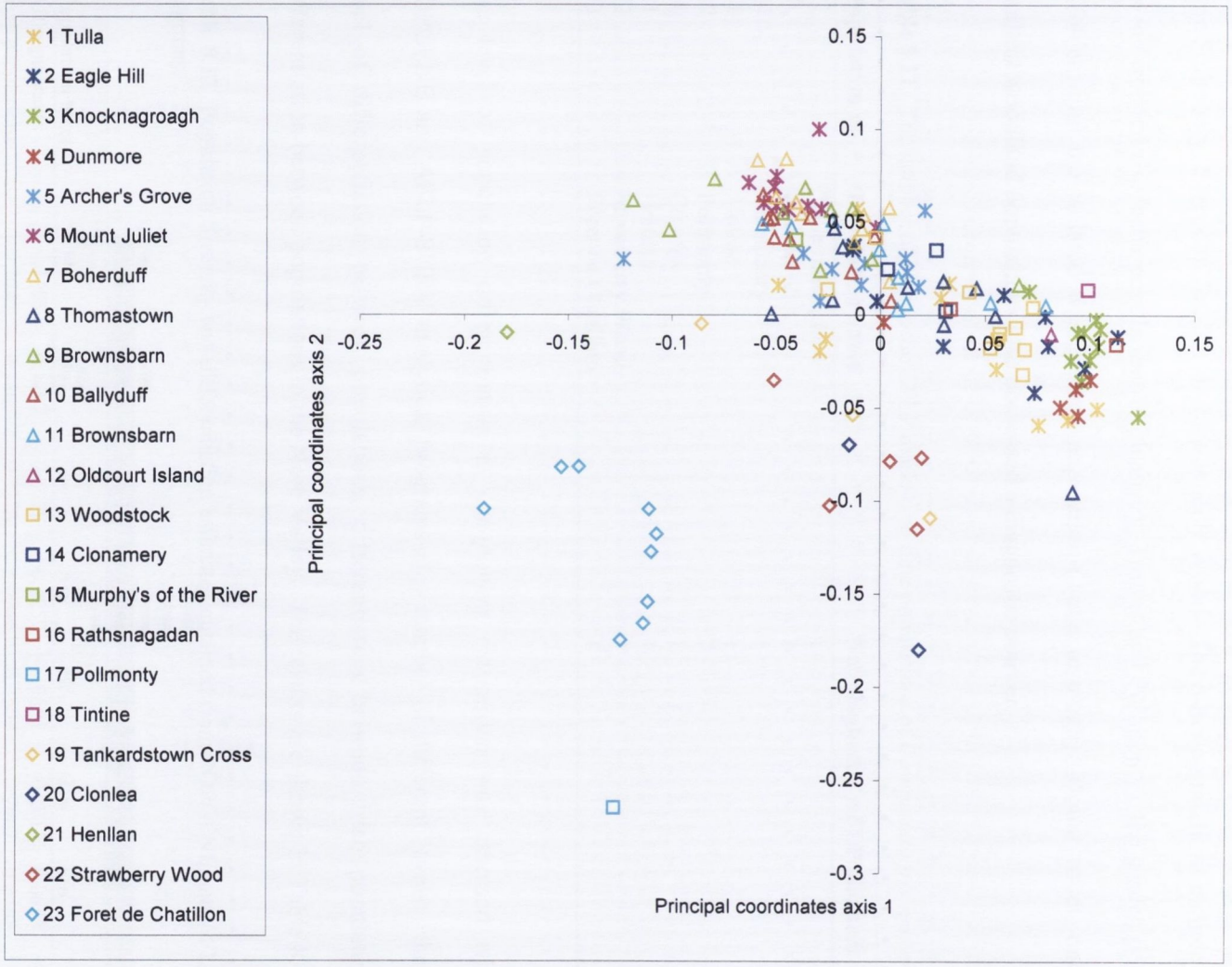


Figure 8.10 Plot of axis one and two from a Principal Coordinates Analysis based on a presence/absence matrix from the AFLP analysis of 23 populations of *Campanula trachelium*

8.3.3 Population differentiation

Significant genetic differentiation is often reflected in the number of unique alleles held by a population. Nine populations of *Campanula trachelium* possessed diagnostic AFLP markers and these are shown in Table 8.11. The three populations with the largest number of unique markers were Pollmonty, Fôret de Chatillon and Strawberry Wood. The other populations include three of the six isolated riverbank populations (Clonamery, Tintine and Pollmonty), the garden population (Clonlea) and the isolated west Ireland population (Tulla turlough). Mount Juliet, one of the smallest woodland populations also possessed a diagnostic marker.

Table 8.11 The number of diagnostic AFLP markers in nine populations of *Campanula trachelium*

Population no.	Population name	No. diagnostic AFLP markers
1	Tulla turlough	1
7	Mount Juliet	1
14	Clonamery	1
18	Tintine	1
17	Pollmonty	7
20	Clonlea	1
21	Henllan	1
22	Strawberry Wood	2
23	Fôret de Chatillon	4

The level of population differentiation in *Campanula trachelium* was assessed with both POPGENE and AFLP-SURV analytical programs at a number of different levels: the overall level of differentiation between populations, differentiation between Irish populations and differentiation between populations in the Nore Valley. The results are shown in Table 8.12.

Table 8.12 Population diversity and differentiation estimates obtained from POPGENE and AFLP-SURV programs.

	POPGENE analysis				AFLP-SURV analysis			
Population level	**Ht**	**Hs**	**G_{ST}**	**Nm**	**Ht**	**Hw**	**Hb**	**F_{ST}**
All populations	0.2191	0.1297	0.4079	0.7259	0.2651	0.2013	0.0638	0.2406
Irish populations	0.2042	0.1363	0.3323	1.0046	0.2509	0.2062	0.0447	0.1777
Nore Valley populations	0.1991	0.1368	0.3129	1.0982	0.2490	0.2092	0.0398	0.1597

Ht Total gene diversity
Hs / Hw Within-population diversity
Hb Between-population diversity
G_{ST} Proportion of between-population diversity
F_{ST} F-statistic for differentiation of populations (Lynch and Milligan, 1994)
Nm Gene flow

Estimates of total and within-population gene diversity are consistently higher in the AFLP-SURV analysis based on the estimation of allele frequencies using a Bayesian method with non-uniform prior distribution (Table 8.12). Conversely, as expected, estimates of genetic differentiation are higher in the POPGENE analysis than in the AFLP-SURV analysis. The G_{ST} statistic ranges from 0.3129 for Nore Valley populations to 0.4079 for all populations. The F_{ST} statistic ranges from 0.1597 for Nore Valley populations to 0.2046 for all populations. In both treatments, the level of differentiation decreases at each sub-population level but the G_{ST} and F_{ST} statistics indicate high population differentiation at all levels. A permutation test for the significance of population differentiation (10,000 permutations) was carried out in AFLPSURV and the F_{ST} values were found to be significant at all hierarchical levels ($p<0.05$). The estimates of gene flow provided by POPGENE are low (0.7259 – 1.0982, Table 8.12) and suggest that there is little exchange between populations, even within the Nore valley.

The partitioning of molecular variance was investigated using Arlequin ver. 2.000 analytical software and an analysis of molecular variance (AMOVA) was carried out at a number of different levels. The overall partitioning of variance was investigated hierarchically by analysing all populations, with a sub-structure of three groups based on the results of earlier neighbour-joining analyses; the Irish populations, the Welsh population and the French population were analysed as three separate sub-populations within the overall sample. Another two analyses were performed on the Irish populations, one with no structure imposed on the dataset and the other with a sub-structure based on regions or River Catchments; the Nore Valley populations, Tulla turlough population, River Shannon catchment population and River Barrow catchment population were specified as sub-populations of the Irish *Campanula trachelium* population. A further analysis was carried out solely on the Nore Valley populations to investigate the level of differentiation between these populations. The results are shown in Table 8.13.

Table 8.13 Partitioning of molecular variance within and between regions, river catchments and populations

Component of variation		df	SS	Variance component	% Variation	Fixation index ϕ	Significance
All populations	Among countries	2	231.201	6.2414	32.2	0.322	p=0.012
	Among pops within countries	11	489.187	3.65799	18.87	0.2784	p<0.00001
	Within populations	115	1090.433	9.48203	48.92	0.5108	p<0.00001
Irish populations	Among populations	11	489.187	3.63683	27.3	0.27301	p<0.00001
	Within populations	103	997.500	9.68447	72.7		
Irish populations	Among catchments	3	197.216	1.67292	11.78	0.1178	p<0.00001
	Among pops within catchments	8	291.971	2.84876	20.05	0.2273	p<0.00001
	Within populations	103	997.5	9.68447	68.17	0.3183	p<0.00001
Nore Valley pops.	Among populations	8	291.971	2.82348	22.15	0.2215	p<0.00001
	Within populations	76	754.1	9.92237	77.85		

df=degrees of freedom **SS**= sum of squares

Molecular variance components reveal that there is significant differentiation between populations at all hierarchical levels of population structure. Although most of the variation is found within populations, a large percentage of the variation is distributed among the country groups, among Irish populations, among groups of Irish populations based on River catchments and among populations in the Nore Valley. The fixation index among populations in the Nore Valley is 0.2215 indicating a high level of differentiation. This analysis supports the results obtained from the analyses carried out using POPGENE and AFLP-SURV.

The Arlequin population genetics freeware package was used to perform Mantel tests with 1000 permutations to investigation correlations between matrices of pairwise genetic distances (F_{ST} – Table 8.14) and geographic distances between populations (Table 8.15). Mantel tests were not significant for Irish populations (p=0.153) or for Nore Valley populations (p=0.145), suggesting a lack of genetic structuring based on geographical distance.

	1	2	3	4	5	6	7	8	9	10	11	13
1	0.00000											
2	0.28143	0.00000										
3	0.32040	0.31344	0.00000									
4	0.16858	0.31238	0.28821	0.00000								
5	0.17516	0.24658	0.37366	0.23605	0.00000							
6	0.35674	0.40903	0.55489	0.50458	0.21793	0.00000						
7	0.27730	0.36185	0.50004	0.40389	0.12106	0.11203	0.00000					
8	0.15090	0.34001	0.40463	0.26392	0.19459	0.35159	0.29024	0.00000				
9	0.20335	0.31225	0.38744	0.28164	0.09677	0.18586	0.14936	0.16630	0.00000			
10	0.27762	0.34347	0.47993	0.40449	0.13940	0.07849	0.06669	0.28618	0.12005	0.00000		
11	0.22719	0.35818	0.42808	0.25888	0.09205	0.29280	0.19540	0.22112	0.10885	0.18815	0.00000	
13	0.17800	0.27906	0.33520	0.17977	0.21389	0.41143	0.30435	0.24939	0.24843	0.31290	0.21186	0.00000

Table 8.14 Matrix of pairwise F_{ST} values for 12 Irish populations of *Campanula trachelium*. Population numbers are given and correspond to those displayed and listed in Figure 8.1.

	1	2	3	4	5	6	7	8	9	10	11	13
1	0.00											
2	74.15	0.00										
3	110.30	38.29	0.00									
4	120.00	50.76	43.85	0.00								
5	125.40	57.09	49.54	6.51	0.00							
6	132.00	66.83	61.60	18.16	12.07	0.00						
7	133.20	68.00	62.37	19.10	12.89	1.24	0.00					
8	135.30	69.87	63.46	20.56	14.19	3.28	2.04	0.00				
9	138.70	73.22	65.94	23.58	17.19	6.65	5.42	3.41	0.00			
10	139.70	74.22	66.67	24.49	18.01	7.70	6.46	4.45	1.04	0.00		
11	142.00	76.05	67.31	25.90	19.39	10.02	8.79	6.75	3.52	2.59	0.00	
13	143.80	78.42	70.16	28.53	22.02	11.86	10.62	8.64	5.23	4.21	2.85	0.00

Table 8.15 Matrix of pairwise geographic distance (km) for 12 Irish populations of *Campanula trachelium*. Population numbers are given and correspond to those displayed and listed in Figure 8.1.

8.4 DISCUSSION

8.4.1 Gene diversity

Populations of plant species exist in a continuous state of demographic and genetic flux and the factors affecting the pattern of this flux are complex. However, all populations have certain thresholds below which stochastic, non-adaptive forces have more influence than deterministic forces (Soulé, 1985) and their relative input has implications for population survival. When these thresholds are crossed, the effect on population genetic processes is to reduce the influence of adaptive selection on genetic change and increase the random influence of genetic drift. Whenever such random processes prevail over deterministic ones, there is a potential for detrimental effects to occur and for the extinction probabilities of populations to be increased. Due to this potential, which can be realised through inbreeding depression and manifested in a reduction in reproductive fitness (Husband and Schemske, 1996), the estimation of levels of genetic diversity in threatened plants is one of the primary goals of conservation genetics.

Gene diversity (Nei, 1973) within populations of *Campanula trachelium* was found to range from 0.0765 to 0.1976 and this represents a large span in comparison to other AFLP studies such as Rottenberg and Parker (2003) where the range of gene diversity was 0.16 to 0.18. Although their study was based on the only two surviving populations of *Rumex rothschildianus* Aarons. (Polygonaceae), the population sizes were highly contrasting, one consisting of 80 and the other of 500 individuals, and a larger difference might have been expected. In other studies of threatened plant species, the range of within-population gene diversity is closer to that observed in *Campanula trachelium* but is still noticeably less. Gene diversity ranged from 0.0373 to 0.1337 in the critically endangered *Astragalus cremnophylax* Barnaby (Fabaceae) (Travis *et al*, 1996), and from 0.083 to 0.160 in a wild palm, *Euterpe edulis* Mart. (Arecaceae) (Cardoso *et al.*, 2000). The large range of gene diversity in this study of *Campanula trachelium* reflects the large variation in population size, as shown by the significant linear dependence of gene diversity on the log of census population size. Although some populations exhibit very low levels of genetic diversity, the mean gene diversity is high (0.1297 in contrast to 0.0629 in *Astragalus*

cremnophylax and this is due to the fact that although rare, *Campanula trachelium* still exists in a number of large populations in the Nore valley and a relatively small number of populations contain less than 50 individuals. As a predominantly outcrossing species, *Campanula trachelium* is expected to maintain high levels of genetic diversity but as a consequence of this breeding system it is also likely to be less adapted to deal with the genetic consequences of population decline and isolation (Barrett & Kohn, 1991), and this may explain the low genetic diversity in small and isolated populations.

Gene diversity, as assessed using a Bayesian method of allele estimation with non-uniform prior distribution, showed a significant linear correlation with the square root estimation of gene diversity. The conclusions that can be drawn from both estimates are therefore similar and the only significant difference occurs in the magnitudes of the estimates. The Bayesian estimate consistently gives higher estimates of gene diversity than the square root estimation and although this is likely to be a more accurate estimate of true population diversity, I have discussed gene diversity with respect to the values as estimated by POPGENE as the square root method of allele frequency estimation has been most widely used in the past and comparisons between species are therefore more reliable for this estimate. However, more studies are beginning to use Bayesian methods and it is therefore advantageous to derive estimates using both procedures to allow for comparisons with a large number of studies. Using the Bayesian estimation, the range of gene diversity was 0.1458 to 0.2707 and mean within-population diversity was 0.2013. The major difference in the estimates obtained from this method is the greatly increased estimate of gene diversity in populations 4 and 22 but as these populations were represented by only five samples this may cause significant estimation errors (Vekemans, X., pers. comm.). Tero *et al* (2003) also used a Bayesian estimate in their study of genetic structuring in *Silene tatarica,* and also found that it produced elevated estimates of gene diversity. Mean within-population diversity was 0.127 using Nei's estimate of diversity and 0.165 with a Bayesian estimate. The two diversity estimates were also significantly correlated, in agreement with the correlation found in this study.

The other diversity measures also showed significant positive correlations with gene diversity and the patterns of diversity shown by the Shannon Index and proportion of polymorphic loci were therefore similar to that of gene diversity. The proportion of polymorphic loci was high overall with 89% of the loci showing variation among samples and 76% of the loci were also polymorphic in the Nore valley populations, reflecting the high proportion of diversity held in these populations. Despite this high overall diversity, polymorphism within populations is low (23.02 to 57.55%) showing a similar range to the levels of polymorphism found in the endangered *Silene tatarica*, a perennial plant showing 25.9 to 54.9 % polymorphism within populations (Tero *et al.*, 2003). The proportion of polymorphic loci is analogous to the allelic richness of a population and those populations that exhibit a high proportion of monomorphic loci have little variation at the restriction or amplification site. Due to the action of genetic drift in removing alleles from a population, the proportion of polymorphic loci is expected to decrease with population size, and the proportion of polymorphic loci showed a significant positive linear dependence on log population size in this study of *Campanula trachelium.* The log of population size was found to be significantly positively correlated with the proportion of polymorphic loci and also the mean number of alleles per loci in *Trillium camschatcense* Ker Gawl. (Liliaceae) (Tomimatsu & Ohara, 2003). It was also significantly correlated with allelic richness in *Swainsona recta.* (Buza et al., 2000) and with observed heterozygosity in *Succisa pratensis* Moench (Dipsacaceae) (Vergeer *et al.*, 2003a). The relationship between genetic measures of diversity and population size is not always linear due to the different demographic and genetic histories of the populations but even where there is no linear dependence of diversity on population size, the largest populations are often found to contain the most diversity and the smallest the least. This was the case with respect to the proportion of polymorphic loci, estimated heterozygosity and Shannon's Index in *Colubrina oppositifolia* Brongn.ex H.Mann (Rhamnaceae) and *Alphitonia ponderosa* Hillebr. (Rhamnaceae) (Kwon and Morden, 2002). Tomimatsu and Ohara (2003) similarly found that some small populations of *Trillium camschatcense* had relatively high inbreeding co-efficients. As previously mentioned, this relationship has been found with a variety of markers and population size was significantly positively correlated with

the percentage of polymorphic RAPD bands within populations in *Saxifraga paniculata* (Saxifragaceae) (Reisch *et al.*, 2003), with Nei's expected heterozygosity and the Shannon Index in populations of *Eryngium alpinum* analysed with AFLPs (Gaudeul, *et al.*, 2000), and with the proportion of polymorphic loci, expected heterozygosity and a dissimilarity index in reintroduced populations of *Abronia umbellata* subsp *breviflora* (Standl.)Munz (Nyctaginaceae) using ISSRs (McGlaughlin *et al.*, 2002).

The three Irish populations containing the lowest proportion of polymorphic loci, Shannon's Index and Nei's (1973) gene diversity were populations Knocknagroagh, Dunmore and Mount Juliet. Although Dunmore and Mount Juliet contain the lowest number of individuals, the census size of Knocknagroagh was 84 flowering individuals and yet this contained the lowest gene diversity of the Irish populations. It is possible that the isolation of this population, which occurs on a tributary of the upper reaches of the River Barrow, may be responsible for this reduction in diversity but other factors such as a past bottleneck followed by subsequent expansion may also be responsible. A survey of the population in 1991 recorded only six plants and although the survey was carried out in September when the species is easy to miss and only mentions one of the areas where the plant is found at this site, it is still likely that the population has expanded rapidly since then. Another factor that would affect the diversity is the separation of patches at this site. The population is highly fragmented and occurs in patches of hedgerow spanning a distance of 3 kilometres. There are two main sub-populations and if gene flow is sufficiently limited between them for them to be considered separate populations, this dramatically reduces the estimate of population size and could explain the low gene diversity despite the relatively large population size. Both neighbour-joining phylograms split this population into two groups with a shared node, also suggesting the divergence of two groups.

Although high levels of genetic diversity can persist in small populations of plant species eg *Leucadendron elimense* Phillips (Proteaceae) (Tansley & Brown, 2000) and *Microseris lanceolata* (Walp.)Sch.Bip (Asteraceae) (Prober *et al.*, 1998), this study has shown that *Campanula trachelium* is susceptible to the detrimental genetic effects of small population size and the

populations with small numbers of individuals therefore require careful monitoring, while care should also be taken to prevent the larger populations from declining. Large Nore Valley populations such as population 5 (Archer's Grove) contain significant levels of gene diversity and in the event of the extinction of the smaller populations they may act as an important seed source for re-colonisation of suitable habitats. Small populations also require careful monitoring due to the valuable contribution they make to the allelic diversity of the Irish populations. A high proportion of the small and/or isolated populations contained unique alleles and Pollmonty, an isolated population of only two individuals possessed seven unique alleles. This and other populations such as Tintine, Clonamery, Mount Juliet and Tulla should be monitored closely in an effort to preserve allelic diversity in the Irish populations.

As Irish populations of *Campanula trachelium* represent peripheral European populations, a comparison with other populations from throughout the range of the species should have provided an estimate of gene diversity against which Irish populations could be evaluated. However, the inclusion of populations from Wales and France did not provide a useful comparison due to the small size of the Welsh population and the fact that the French samples were obtained from seed with no information on the size of the population or the number of individuals sampled. This could therefore represent a very small and biased sample of the genetic diversity in the entire population and cannot be used to compare relative levels of gene diversity in different parts of the European range. The study would have benefited greatly from the inclusion of more non-Irish populations but despite this, the occurrence of the lowest gene diversity estimates in the Welsh population, which contained the smallest number of individuals, helped to confirm the relationship between population size and genetic diversity and this has important implications for conservation.

One of the most serious consequences of genetic deterioration is the occurrence of inbreeding depression, which has been shown to have a detrimental effect on reproductive fitness in a wide range of plant species (eg. Newman & Pilson, 1997; Culley *et al.*, 1999; Vogler *et al.*, 1999; Buza *et al.*, 2000; Severns, 2003). Population size was shown to be significantly correlated with a

number of estimates of reproductive fitness in the reproductive study of *Campanula trachelium* populations detailed in Chapter 7 but these same variables were not significantly correlated with the estimates of genetic diversity obtained in this study. In consideration of the low sample size (only five populations were part of the reproductive study), a small deviation from the expected effect could greatly influence the probability of detecting a relationship. Deviations from a linear relationship would be expected in natural populations of plant species as the manifestation of reproductive depression depends on the particular genetic constitution of the population and interactions with the environment (Hedrick & Kalinowski, 2000). The effect of inbreeding depression on reproductive fitness is known to vary in different environments and this is supported by the finding that inbreeding depression is greater when plants are under stress (Heschel and Paige, 1995) and greater when plants are in harsh environments (Roff, 1997). The impact of low genetic diversity on reproductive fitness will also depend on the breeding pattern within populations and a multitude of other factors. A population could have a moderate level of diversity but this diversity could represent several localised inbreeding lines. There may also be a time lag between the change in the level of diversity and its manifestation through inbreeding depression and this could also account for the lack of a significant relationship. However, the fact that the stem height, number of seeds per capsule and number of seeds per plant show an increasing trend with increasing gene diversity suggests that a relationship may occur between these variables and the addition of a larger number of populations to the analysis may increase the strength of the relationship. Another consideration when evaluating this relationship is that although a decrease in population size correlates with a decrease in genetic diversity, inbreeding depression only occurs beneath a certain threshold level of diversity and a linear relationship would not be expected in this case. The fact that the lowest reproductive fitness estimates occur in the smallest population lends weight to the conjecture that inbreeding depression is occurring in the small populations.

Whether or not the populations are suffering from inbreeding depression, the low genetic diversity in Knocknagroagh, Dunmore and Mount Juliet is a cause for concern due to the lack of

evolutionary potential inherent within them. This leaves them susceptible to future changes in the environment and to population reduction by pathogens (Schmid, 1994). The low within-population gene diversity also suggests that inbreeding depression could potentially be affecting more populations than those covered by the reproductive study and this could have implications for the demographic structure of populations through reduced seed input, and could affect the response of the populations to environmental stress (Kery et al 2000; Vergeer *et al.*, 2003).

8.4.2 Genetic distance and population differentiation

The analysis of genetic distance broadens the view given solely by the estimates of genetic diversity and allows an understanding of the genetic relationships between populations. In this case, the UPGMA dendrograms based on genetic distances between populations separated the Welsh and French populations from the Irish populations and showed that these have diverged significantly from the Irish populations and from each other. Within the Irish populations the two major groups were not characterised by the grouping of geographically close populations together and the lack of structuring suggests that the relationships between populations are not well resolved in the AFLP dataset. This is supported by the neighbour-joining phylograms of samples, which show little bootstrap support for groupings other than the major separation of the two non-Irish and Irish populations. In the rest of the phylogram, samples from Knocknagroagh (2), Eagle Hill (3) and Woodstock (13) are well defined into groups but the remainder of the samples are clustered into smaller groups spread across many branches. The only relationship that suggests any geographical structuring is the grouping of Knocknagroagh (2) and Eagle Hill (3) in the Nei-Li neighbour-joining phylogram and in the UPGMA dendrogram based on Nei's genetic diversity with Bayesian estimation of null allele frequency. These populations both occur on esker ridges to the North of the Nore Valley. However, these populations occur along different river systems.

The inclusion in the neighbour-joining analysis of data from the populations with small numbers of samples provided additional information on the relationships between populations. Some doubts have been voiced on the native status of *Campanula trachelium* in Ireland so the inclusion of the two samples from the garden population (population 20) gave an important insight into this

suggestion. In both the mean character difference and the Nei-Li phylograms the two garden samples separated from the main body of Irish populations and this suggests that they are genetically different from the rest of the Irish plants. In the mean character difference phylogram, they are placed outside the group containing samples from all other populations and this reinforces the genetic differences, which may have resulted from cultivation. However, in both phylograms, the garden samples form a group with two samples from population 19 and this suggests a possible genetic link, although the grouping does not have significant bootstrap support. Population 19 is an isolated hedgebank population occurring near a tributary of the Slaney River, outside the main range of the plant and may therefore have been introduced into this location. Other features of interest include the sample from population 17, which is a major outlier in both phylograms reflecting its unique pattern of marker presence/absence.

The distribution of diagnostic AFLP markers reflects the genetic structure shown in the neighbour-joining phylograms in that the majority of populations possessing unique markers formed distinct sub-groups, or displayed long branch lengths in the neighbour-joining phylograms. Isolation appears to be a common feature of a large proportion of these populations and the Welsh and French populations were expected to possess unique markers due to their large geographic separation from the majority of sampled populations. However, Pollmonty possessed an unusually high number of unique markers possibly reflecting the large influence of stochastic genetic processes in the history of the population. This population of only 2 plants is probably a relict of one of many populations previously occurring along the River Barrow that have since declined and disappeared, and its isolation and small size may have resulted in large genetic change. The large genetic difference between this population and all other populations is also reflected in the exclusion of the sample from all other groups in the neighbour-joining phylogram.

Although phylograms should be unrooted, so as not to infer derivative relationships between populations, with the inclusion of so many samples the relationship between samples would be difficult to elucidate in this format. The proximity of similar samples remains the same when plotted as an unrooted phylogram but due to the spreading branching pattern the trees can be

difficult to interpret when presented in this way. The conclusions that can be drawn from the examination of the trees are limited due to the lack of bootstrap support for the majority of branches and this indicates that the structure is unstable and could easily collapse on the addition of more samples or the inclusion of more marker data. However, the differences between Irish and French samples indicate that the Irish populations may be significantly differentiated from other European populations and may represent a unique component of the European diversity, an hypothesis that requires further testing.

This is also supported by the investigation of the partitioning of molecular variance among and within populations. The proportion of between-population diversity revealed by the calculated values of G_{ST} were high for all tested levels and this indicates that significant differentiation occurs between Nore Valley populations, between Irish populations and between all the sampled populations. This same conclusion can be drawn from the F_{ST} values derived from the Bayesian estimate of allele frequency, all of which were significant, and from the Analysis of Molecular Variance (AMOVA). Although AMOVA was not designed for use with dominant data it is a useful tool, providing an indication of comparative levels of variation at different hierarchical levels of population structure. It has been used successfully in many other dominant marker studies on rare plants (eg. Travis *et al.*, 1996; Fischer & Matthies, 1998; Schmidt & Jensen, 2000; Tansley & Brown, 2000). Over all sampled populations of *Campanula trachelium*, 32.2 % of the variation was found among groups ($p=0.012$), indicating that the differentiation between the Irish, Welsh and French groups was significant. Species at the periphery of their range can undergo significant differentiation, diverging from central populations (Lesica & Allendorf, 1995). 18.87% of the variation was partitioned among populations within groups, indicating significant differentiation between the Irish populations and 48.92% was found within populations.

Although the structuring of the analysis into regional groups was useful, the presence of only one population in each of the Welsh and French groups limits the conclusions below the regional level to Irish populations, so the analysis was repeated excluding all non-Irish populations. The differentiation of Irish populations was significant ($p<0.0001$), with 27.3% of the variation

occurring among populations and 72.7% within populations. Perennial outcrossing plant species are expected to maintain the larger proportion of variation within populations and the fixation index of 0.273 is typical of an outcrossing species with some degree of selfing (Frankham *et al.*, 2002) and is similar to the Bayesian estimate of 0.287 obtained by Tero *et al.* (2003) for *Silene tatarica*. Typically, selfing species are found to contain 50% of their variation among populations in comparison to 20% for outcrossing species (Hamrick & Godt, 1996; Bussell,1999).

The significant differentiation at all population levels indicates that a metapopulation system is unlikely to be operating in populations of *Campanula trachelium* and sporadic appearances in hedgerows may be the result of recruitment from a seed bank rather than from recent colonisation from a large source population. The small, isolated populations that occur may be remnants of more widespread populations that existed in the past that have since differentiated due to isolation, inbreeding and genetic drift. However, due to the significant differentiation between populations it is more likely that they have always been distinct. The estimates of gene flow obtained from the analysis of data using POPGENE suggest that gene flow between populations in all hierarchical levels is low. The genetic input from immigrants to a population has the potential to ameliorate the effects of genetic drift in small populations and to prevent the incidence of inbreeding depression. The high level of differentiation between populations in the Nore Valley, coupled with limited gene flow, therefore have important implications for the conservation of the smaller populations such as Dunmore (population 4). Without genetic input to populations, genetic drift and inbreeding depression may reduce the population size and genetic effective population size to levels below the threshold at which survival can be maintained. Although an input of even one migrant per generation can compensate for genetic drift (Menges, 1991), one of the main populations of concern (Dunmore) occurs at the northern extreme of the Nore Valley distribution and is therefore isolated, in addition to containing a small number of individuals and low genetic diversity.

However, these estimates of gene flow are indirect measures subject to error and further study therefore needs to be undertaken to confirm or invalidate these results. Estimates among loci can

vary by orders of magnitude so an estimate that is averaged over all loci may not be informative (Bossart & Prowell, 1998). They may also be flawed on account of the inherent assumptions that are made when a genetic model is used. Estimation of gene flow from molecular marker data assumes that genetic structure represents an equilibrium between gene flow and neutral genetic drift. Where historical events such as fragmentation and bottlenecks have taken place, the genetic similarities between populations will be a reflection of a common ancestry rather than present levels of gene flow between populations (Schaal *et al.*, 1998). If the assumption of equilibrium is not met, gene flow estimates will be biased but they can still serve as a starting point on which to base further investigation (Bohonak *et al*, 1998).

CHAPTER NINE

OVERALL DISCUSSION

9.1 An Evaluation of the Utility of AFLPs in Species Conservation

Genetic evidence has formed the basis of many of the conclusions drawn from this study but the use of molecular markers in the field of conservation biology has limitations that must be carefully considered. Estimations of genetic diversity statistics are based on a number of assumptions, imposing limitations on the certainty with which the results can be relied upon to make effective conservation decisions. In the case of dominant markers such as AFLPs and RAPDs the main source of error is introduced through the estimation of allele frequencies. The absence of a particular fragment gives the proportion of null homozygotes, but the presence of a fragment does not allow a distinction to be made between dominant homozygotes and heterozygotes, as amplification of the fragment occurs in both these genotypes. Some researchers have claimed that separation of these components is possible based on the assumption that the relative intensity of the band indicates whether the amplification products were derived from one or both chromosomes (Castiglioni *et al,* 1999). However, the reliability of this method has not been stringently tested and it is subject to significant error, as weakly-concentrated or impure DNA may also result in reduced intensities of bands in some samples. The estimation of allele frequencies therefore remains the only viable option and is used universally in plant population genetic studies.

The square root transformation method was used in this study primarily to allow comparisons with earlier studies. However, an alternative procedure was also used and it was shown that the Bayesian method of allele estimation with non-uniform prior distribution of allele frequencies produced consistently higher estimates of gene diversity than the square root transform method. Bayesian methods are becoming more commonly used in plant conservation biology (Marin *et al.*, 2003) and the application of Bayesian statistics to population genetics has been shown to greatly improve the accuracy of estimation of heterozygosity, genetic distances and F-statistics (Zhivotovsky, 1999). The Bayesian estimates for genetic diversity in *Colchicum autumnale* and *Campanula trachelium* are therefore likely to be closer to the true population parameters but the

conclusions based on comparative levels between populations are still valid due to the significant correlation between the estimates.

Apart from the biases that can arise from the estimation of population parameters such as gene frequencies, the use of the AFLP technique is based on a number of assumptions that may not reflect the true genetic structure of the populations under study. In particular, the assumption of Hardy-Weinberg equilibrium may not apply to all populations, and deviations from this may occur, particularly in the small populations of *Campanula trachelium* that may be suffering from inbreeding depression, and also in *Colchicum autumnale* where inbreeding may be high due to clonal spread. However, not all estimates of diversity rely on this assumption and the congruence of the Shannon Index and percentage of polymorphic loci with the gene diversity estimates indicates that any violations of the Hardy-Weinberg assumption have not significantly affected the conclusions drawn from the analysis of populations in the studies presented in this thesis. Shannon's Index does not require assumptions to be made regarding heterozygosity (Bussell, 1999) and was therefore a useful index to use alongside the other genetic diversity measures.

Another inherent assumption associated with dominant molecular markers is that there is only a single amplifiable gene per locus. However, it is possible that two different alleles at a locus may have the same primer sequences but differ in internal length. In this case they would both be amplified, but being of different molecular weight, would be treated as alleles from two different loci. In an inheritance study on soybean, Maughan and co-workers (1996) found an AFLP marker that was inherited in a co-dominant fashion indicating the possible presence of a microsatellite. AFLPs may therefore not be completely independent. Although this influences the accuracy of diversity estimates, the low frequency at which this would be expected to occur would not result in a large influence in the results of an analysis.

The theoretical limitations of dominant markers have led some researchers to conclude that they are not as efficient as co-dominant markers for population studies (Lewis and Snow, 1992; Lynch and Milligan, 1994). Co-dominant markers, such as microsatellites, yield genotypic data and

analysis of genetic structure does not therefore require the estimation of allele frequency. Lynch and Milligan estimated that two to ten times more individuals are required when dominant as opposed to co-dominant markers are used but Krauss and Peakall (1998) state that the large number of polymorphisms generated by the AFLP technique may overcome this disadvantage. Another consideration is that AFLPs are more beneficial to conservation studies because they sample the whole genome thereby including regions under selection, which may be of conservation interest. However, in a study by Ribeiro *et al.* (2002), on chloroplast microsatellite and AFLP diversity in maritime pine, a mantel test showed that the genetic distance matrices calculated from the two different sets of markers were significantly correlated. The two techniques may therefore be comparable.

Aside from the inaccuracies that arise from the inherent assumptions in the use of dominant markers for the estimation of population genetic parameters, there are a number of other sources of error. Krauss and Peakall (1998) encountered a 'disappearing fragment,' one that was present in the original analysis but not in any subsequent analyses despite repeated extraction and molecular analysis. However, in consideration of the large number of fragments produced by AFLP analysis, the presence of one erroneous fragment would have a minimal effect on the outcome of any statistical analyses and this is therefore not a significant limitation. Explanations for the disappearing fragment include the possibility that the genomic template DNA was not fully digested, that the fragment was not amplified properly during the PCR reactions or that the DNA was contaminated with DNA from another source. Lin and Kuo, (1995) suggested that partial digestion was the most common cause of 'artefactual polymorphism' and Dowling *et al.* (1997) suggested that it can also be caused by dirty or methylated DNA. In this study, every attempt was made to guard against contamination but there is always a small possibility that it may occur. Methylated DNA can result in errors where material is collected from different organs or at different times due to the occurrence of organ-specific methylated restriction sites during tissue ontogenesis (Donini *et al.*, 1997). However, all collections of leaf material in this study were made at the same time of year from the same part of each plant.

Other criticisms of the AFLP method relate to the methods of interpretation and analysis of the banding patterns. The selection of 'scorable' bands, based on the intensity of the fragment, and the condition for the scoring of the band to be unambiguous at all loci, means that the markers chosen for the fingerprint will vary depending on the individual conducting the study. Robinson and Harris (1999) have called for the parameters that determine the suitability of a band to be clearly defined and as the field of conservation genetics develops, this issue will have to be addressed. The scoring of bands by eye can also be viewed as a potentially subjective procedure but while computer detection of fragments is more efficient and accurate than scoring bands by eye from autoradiographs (Krauss and Peakall, 1998), the automated scoring procedure in Genotyper carries a substantially greater risk of error due to the rounding up and down of fragment lengths to whole numbers of base pairs. As discussed in the methods section, this results in the same fragment being scored as different-sized fragments between individuals. The data *must* therefore be scored by eye to ensure accuracy, while using a standard minimum intensity of the band to determine presence or absence.

The advantages of the AFLP technique far outweigh the shortcomings and one of the major benefits of their use in the study of threatened species is the small amount of DNA required to obtain results. Only 0.25µg DNA is needed from each individual plant and this enables widespread sampling of populations without the destruction of plants. The execution of the AFLP technique also requires no prior sequence knowledge and does not require a large input of time and money to develop primers. The first two stages of the technique need only be performed once on each sample and thereafter the pre-selective primer products can be used repeatedly to test the suitability of primers. AFLPs also have a high multiplex ratio and the whole genome is sampled (Robinson and Harris, 1999).

Many researchers regard AFLPs as reliable and informative markers (eg Winfield *et al.*, 1998). They provide high levels of resolution (Powell *et al.*, 1996) and are also highly reproducible (Jones *et al.*, 1997). They have been shown to exhibit close to 100% reproducibility (Winfield *et al.*, 1998; Bagley *et al.*, 2001), and are more efficient than RAPDS (Kjolner *et al.*, 2004). The

fingerprint obtained is largely independent of DNA concentration (Vos *et al.*, 1995 and pers. obs.), virtually free from artefacts (Jones et al 1997), and comigration of non-allelic fragments occurs at extremely low levels (Rouppe Van Der Voort *et al.*, 1997). A large number of markers are generated and the sampling errors associated with estimates of population genetic parameters from other techniques can be compensated for by the analysis of a large number of loci (Falk, 1991). The precision of genetic distance estimates increases with increasing numbers of markers and Keim *et al.* (1992) have suggested that if more than 90 markers are used, standard error is reduced to acceptable levels. The number of markers used in this study greatly exceeded this for both species and the error due to variation among loci should therefore be reduced.

The AFLP technique clearly provides a robust and reproducible analysis of genetic variation within a species in comparison to other fingerprinting techniques. However, one final criticism relates to the broader issue of the use of neutral genetic markers in evaluating the evolutionary potential of a species. Although the adaptability of a population is dependent on variation in traits that have the potential for selective advantage, many studies have found no correlation between variation in neutral molecular markers and variation in coding regions (eg. Steinger, 2002; Bekessy *et al.*, 2003). One reason for this may be that loss of neutral allelic diversity is a product of the same processes as the loss of gene diversity but occurs more rapidly (Land & Barrowclough, 1987). A loss in marker diversity may therefore be detectable before any significant change has occurred within coding regions. Another consideration is that most studies on the correlation between diversity detected by quantitative traits and molecular markers focus on only one or a small number of traits (eg. Bekessy *et al.*, 2003). These are unlikely to be representative of patterns of diversity across the range of adaptive traits and therefore cannot be used as evidence that neutral markers do not reflect variation of evolutionary importance. Focussing on a small number of adaptive traits also ignores the possibility that neutral characters may become selectively advantageous at some future point in the life span of a population.

Whether or not neutral markers effectively predict exact levels of diversity, they are a powerful comparative tool and are effective in the identification of conservation units (Heywood & Iriondo,

2003). They can reveal levels of differentiation between populations and are useful for inferring phylogeographic relationships (eg. Despres *et al*, 2002; Schönswetter *et al.*, 2002). With respect to the divergence of populations, neutral markers and adaptive traits often reveal the same relationships. Merila and Crnokrak (2001) reviewed data from eighteen independent studies of plants and animals and found that the degree of divergence in neutral marker loci between populations was strongly correlated with divergence in genes coding for quantitative traits. Although AFLPs may not give exact estimates of gene diversity and differentiation, through a comparative approach among populations the technique has made a valuable contribution to species conservation through the production of baseline data on which future conservation decisions can be founded. This has been achieved in the population genetic studies of *Colchicum autumnale* and *Campanula trachelium* and the relevance of genetic data to practical conservation will now be discussed.

9.2 Genetic Contributions to Conservation Management

Conservation management is ineffective without an understanding of the biodiversity it aims to protect (Soulé, 1991), and the genetic structure of populations is a fundamental component of this biodiversity. The result of a *lack* of genetic information in conservation management can be clearly seen in an example of plant conservation resource planning in the Canary Islands, Spain (Marrero-Gomez *et al.*, 2003). The recommendations repeatedly contain the phrase 'unless genetic studies prove otherwise' and this shows that without genetic information management recommendations make assumptions about genetic structure that may not be accurate. Genetic data on the endangered Californian plant *Cordylanthus palmatus* (Ferris) J.F. Macbr. (Scrophulariaceae) showed that contrary to many other similar studies, genetic diversity was not significantly correlated with population size (Fleishman *et al.*, 2001). The common assumption that larger populations are more diverse and contain greater evolutionary potential and conservation value than smaller populations would therefore have not been a correct one to make in this situation and, if taken, may have resulted in the loss of a large proportion of the genetic variability of the species.

Molecular methods allow the identification of populations at risk from genetic deterioration and this approach can lead to specific management recommendations that can guard against the detrimental effects of genetic deterioration and associated fitness losses. Godt *et al.* (1996) analysed genetic diversity in *Geum radiatum* Michx. (Rosaceae) and *Calamagrostis cainii* A.S.Hitchc. (Poaceae) and identified several genetically depauperate populations. It was concluded that these populations were unlikely to regain genetic variation without artificial supplementation of their gene pool through the addition of more individuals to the population. Reinforcement may also be undertaken for demographic rather than genetic reasons in small populations but in both cases a knowledge of the molecular structure of populations is beneficial to guard against the onset of outbreeding depression. This is a reduction in fitness that can result from the break-up of locally adapted gene complexes or from sub-optimal adaptation to the environment when individuals from two genetically differentiated populations are mixed (Templeton, 1986; Fenster & Dudash, 1994). Although gene flow between populations is encouraged and sometimes facilitated by conservation managers, outbreeding depression can have detrimental effects in plant populations (Barrett & Kohn, 1991; Fenster & Dudash, 1994) and if outbreeding depression is significant in small populations it can greatly increase the probability of population extinction (Templeton, 1986). To avoid this situation, where genetic information is not available, reinforcement can be carried out by introducing individuals grown from seed from the population itself, as this increases population size without introducing a risk of outbreeding depression. However, where data are available on the genetic relationships between populations, informed judgements can be made on the likelihood of detrimental effects resulting from the mixing of gene pools from different populations.

RAPD analysis of *Acacia raddiana* Savi (Fabaceae) in the Negev desert revealed that the Negev and Arava valley populations are significantly differentiated (Shrestha *et al.,* 2002). In addition to informing the investigators of the importance of conserving both populations to effectively conserve the diversity within the species, this information also led to the conclusion that the mixing of populations could lead to outbreeding depression. The management outcome of this

was the decision that the separate evolutionary histories of the populations should be maintained and therefore the most effective way of conserving the two populations and their inherent genetic diversity would be to treat the two populations as two separate management units (Shrestha *et al.*, 2002). The decision to preserve the evolutionary history and genetic integrity of differentiated populations is a common practice in conservation management and has been applied to many threatened plant species. The critically endangered plant *Rumex rothschildianus,* endemic to Israel, occurs in only two populations and AFLP analysis of these populations revealed a moderate level of differentiation between the two gene pools (Rottenberg & Parker, 2003). Once again, to avoid risks of outbreeding depression and to maintain genetic diversity in the remaining populations it was recommended that only intrapopulation reinforcement should be considered to preserve the separate gene pools.

In the case of *Rumex rothschildianus*, reintroduction into protected areas of suitable habitat was also suggested as an additional and complementary conservation strategy to the maintenance of diversity in the wild populations (Rottenberg & Parker, 2003). Reintroduction has been used with varying success in threatened plant species but it is clear from results such as the increased probability of extinction in experimental populations of *Clarkia pulchella* with low genetic effective population size, N_e (Newman & Pilson, 1997), that genetic factors can influence the success of reintroduction or translocation programmes. The monitoring of genetic variation in reintroduced populations should therefore form an integral part of any species recovery programme and if necessary, repeated introductions should be made until the population becomes genetically diverse and stable. Providing a genetic analysis of native populations has been undertaken, the genetic monitoring of reintroduced populations also allows an assessment to be made of the success of a recovery programme in re-creating populations with similar genetic characteristics to the native populations (McGlaughlin *et al.*, 2002). Based on an analysis of Inter Simple Sequence Repeats (ISSRs) in reintroduced populations of *Abronia umbellata* subsp. *breviflora*, McGlaughlin and co-workers were able to predict that 90% of the genetic variation of

the source population could be maintained if the reintroduced populations contained at least a thousand individuals.

In the majority of cases reintroductions rely on the availability of *ex situ* collections and on the quality of these collections. A genetically diverse population is required as a source of donor propagules (Fleishman *et al.*, 2001), as a large initial effective population size reduces the risk of genetic decline and its associated short and long-term fitness effects. The *ex situ* seed or plant source must therefore adequately represent the genetic diversity present within the population or, when multiple reintroductions are considered, within the species. It has been suggested that seed collection for *ex situ* storage must be based on the minimum viable population estimates based on genetic considerations (Menges 1991b). If the goal is to maintain evolutionary flexibility then it has been suggested that an MVP of 500 is required whereas this is reduced to 50 if the goal is simply to evade the effects of inbreeding depression (Franklin, 1980). However, these figures are purely a guideline and vary in their applicability to different species. Genetic studies provide far more accurate estimates of the number of samples required to adequately represent the genetic variability within the species.

To conclude, as a result of limited resources conservation management frequently involves the selective conservation of populations or the selective use of propagules to establish new populations and these decisions have inevitable genetic consequences (Neel & Cummings, 2003). Genetic data can remove the ambiguity from conservation decisions and can ensure that *ex situ* collections adequately represent the diversity present within a species so that these resources can be used to maximum effect if required in the recovery of a threatened species. Molecular methods therefore have a vital role to play in conservation and this role can be efficiently and effectively executed with the use of fingerprinting techniques such as AFLP.

9.3 POPULATION BIOLOGY OF *COLCHICUM AUTUMNALE* AND *CAMPANULA TRACHELIUM* IN IRELAND: IMPLICATIONS FOR CONSERVATION

This study of *Colchicum autumnale* and *Campanula trachelium* has reinforced the usefulness of the genetic component to conservation but has also shown the importance of an integrated approach, where information is gathered on many different aspects of population biology. The investigation of population processes in *Campanula trachelium* has also emphasised that conservation biology should not just proceed in a reactionary manner, investigating and conserving only those species that are under severe threat. Such an approach limits the capacity of the discipline to understand the processes that lead to the decline of populations and therefore prevents the identification of species and populations that are potentially at risk.

One of the major aims of the project was to assess evidence relating to the native status of the two species in Ireland, particularly in the case of *Colchicum autumnale*, where historical evidence provides a connection with the medicinal use of the plant. The genetic studies suggest that *Colchicum autumnale* is a native component of the Irish flora and provide limited evidence to suggest that *Campanula trachelium* may also be native, and this justifies the allocation of resources to their conservation. Although the hypothesis that *Colchicum autumnale* was introduced by monastic sources links it to the historical heritage of Ireland, the native Irish flora clearly has to take priority over introduced species and if this study had supported the hypothesis of introduction, the conservation status of *Colchicum autumnale* would require re-evaluation. However, the genetic evidence supports the native status of the species, or at least refutes a recent introduction of either on account of the moderate to high levels of diversity, the long branch lengths in neighbour-joining analyses and the nature of the genetic relationships with other non-Irish populations. The conservation of both species is therefore important to the protection of Irish biodiversity.

The evaluation of Irish populations of *Colchicum autumnale* in a European context was crucial to the assessment of the conservation status of this species. There are a number of reasons why the population genetics of threatened plant species should not be restricted to populations in one

province or country. Values for genetic diversity are meaningless unless placed in the context of population statistics from other parts of the species range, and in a biogeographical context, the relationships between populations can also be important to the evaluation of conservation status. This wider perspective ensures that species conservation is not restricted by the assumptions concerning 'normal' levels of variability that must be made if populations are viewed in the isolation of a within-country study. If Irish populations were examined in isolation and low levels of genetic variation were found, there would be no indication of whether this was a phenomenon of the species or just a characteristic of the Irish populations. The level of population differentiation should also always be assessed in this broader context, as if populations in one locality have differentiated significantly enough to be regarded as genetically distinct, they may be of high conservation priority in a European context. Genetic data for *Colchicum autumnale* showed a significant biogeographic split in the western European populations, suggesting lineages arising from separate glacial refugia, and thus identifying two units with regard to the conservation of genetic variability in the species.

In the case of *Campanula trachelium,* samples were collected from Wales but the French population was sampled entirely from a seed collection from an unknown number of individuals and no other populations were sampled. Although there were a larger number of Irish populations, providing a detailed analysis of population genetic structure and relationships among Irish populations, the lack of a broader European perspective placed limitations on the wider assessment of genetic variation in *Campanula trachelium.* It would have been preferable to have a wider spectrum of populations for a comparative analysis but the striking genetic differences between the Irish and non-Irish populations still provided valuable information.

The demographic and reproductive assessment of populations in both species greatly enhanced the interpretation of the genetic data. In particular, the estimates of population size enabled the interaction between genetic and demographic parameters to be evaluated and the assessment of reproductive output enabled possible fitness consequences of low population size and genetic

variability to be investigated. In addition to this, these data provided information that can be utilised as an aid to the conservation of the species in Ireland.

In *Colchicum autumnale* the detailed study of plants through the use of permanent quadrats yielded a large amount of data. Although this was not sufficient to perform a full PVA, it enabled general trends to be assessed. The largest populations showed an overall decrease in the number of genets over the study period and this may be an indication of a deterioration in habitat quality or may simply be part of a natural cycle in the population numbers. The difficulty with evaluating trends in demography in *Colchicum autumnale* is that the ramets and genets have a high longevity and also exhibit dormancy, as revealed by this study. This places limitations on the ability to draw conclusions about demographic trends in the Irish populations and emphasises the need for long-term monitoring of such species.

One of the significant results of the study of *Colchicum autumnale* was the discovery that population size was positively correlated with mean vegetation height. The management of the sites has been consistent over a long period of time and the vegetation height is therefore likely to have been an important factor in the development of the populations. The ratio of ramets to genets displayed a relationship with mean vegetation height larger clonal sizes were also observed in abandoned compared to managed grassland in populations of *Knautia arvensis* (Vange, 2002). At Jerpoint, the site with the highest mean vegetation height, the genets are composed of a large number of highly clumped ramets and much of the site is un-colonised by *Colchicum autumnale*. A possible cause of the lack of spread into potential habitat is the absence of grazing animals at the site. It is likely that the primary mode of dispersal of the gelatinous wet seeds may be transportation in the hooves of grazing animals and without these agents, seeds at Jerpoint are unable to spread. Alternatively, the seeds may simply be less successful at Jerpoint due to environmental and biotic conditions and not through restricted dispersal. It is clear however, that the grazed habitats of Thomastown and Inistioge are more conducive to the successful spread of the populations and this has implications for future conservation management of the site, and of

the site at Inistioge Lower where the field is only occasionally grazed by a single horse and the vegetation is also high.

The investigation of reproductive biology also pointed to the unsuitability of the current management regime at Jerpoint as the population has failed to flower for three consecutive years due to the dense mat of *Filipendula ulmaria, Pteridium aquilinum* and *Urtica dioica* at the site. In contrast, the Thomastown, Fourpenny Rock and Inistioge populations were reproductively successful and large numbers of fruits and seeds were produced. Plants at Inistioge Lower produced fruit but the number of fruits per ramet and the number of seeds per ramet were significantly lower than at Thomastown and Inistioge. This may also have been a result of the taller vegetation, which may obstruct pollinators or may have been a result of the smaller above-ground mass of plants at Inistioge Lower, as indicated by leaf width which was found to significantly affect fecundity. One final implication of the study of the reproductive biology of *Colchicum autumnale* is that the difficulties encountered in attempts to germinate seed mean that the *ex situ* cultivation of plants is hindered and the possible re-introduction of populations into suitable areas in the future may be restricted to the spreading of seed into potential habitat.

The study of reproductive and population biology also had important conservation implications for *Campanula trachelium* and greatly enhanced the interpretation of genetic variation within the species. The significant linear dependence of gene diversity on log population size and the significant differences in fecundity between large and small populations point to the possible expression of inbreeding depression in the smaller populations, particularly Dunmore. The genetic and reproductive deficiency in this population, along with the small population size, suggest that it may have a higher risk of extinction than other populations and may therefore be in need of reinforcement. It is widely accepted that large, continuous populations with high levels of gene diversity, large numbers of alleles, high levels of differentiation and low levels of inbreeding have greater conservation value than smaller less diverse populations (Fleishman *et al.*, 2001) and are therefore conservation priorities (Neel & Ellstrand, 2001). However, in a situation where the larger populations are not at threat and where selective conservation of some populations over

others is not necessary, conservation resources can be directed towards the smaller populations that have higher extinction risks. Small, isolated populations often contain unique alleles (as was the case in Pollmonty) and therefore their conservation ensures the maintenance of genetic diversity within the species.

Based on population, reproductive and genetic data, Dunmore is the population that is most at risk from demographic and genetic stochasticity and it is likely that it is already suffering from inbreeding depression. In Ireland as a whole, the population that is likely to be most vulnerable is Knocknagroagh as it contained the lowest genetic diversity of all the Irish populations, is fragmented and consists of a small number of individuals. The remedy for inbreeding depression is to outcross the inbred population to another unrelated population (Falconer and Mackay, 1996) but the introduction of new genetic material to a population presents a risk of outbreeding depression (Templeton, 1986; Barrett & Kohn, 1991). The breakdown of local adaptation, through break-up of gene complexes or introduction of new, less adaptive genes may result in reduction in population size if this is not carefully considered. However, the weak bootstrap support for the groupings within the neighbour-joining phylograms of samples suggests that although significant, population differentiation is not sufficiently high to resolve population groups, other than for Eagle Hill, and the risk of outbreeding depression is therefore low if this population is avoided as a seed or pollen donor.

The integrated approach allowed the conservation status of both species to be viewed in a broader context and also highlighted the differences between the species. Although small population size was of concern in both species, the relationship between log population size and genetic diversity in *Campanula trachelium,* in addition to the low reproductive output in small populations, suggested that genetic stochasticity was the major concern for some of these populations. While small population size and low reproductive output were also of concern in small populations of *Colchicum autumnale*, the major problems appear to be more related to habitat quality resulting from the different management regimes than from the risk of inbreeding depression, although more detailed study on reproductive output would need to be carried out to verify this. Another

difference between the two species was that *Colchicum autumnale* populations contained no population-specific AFLP markers in contrast to *Campanula trachelium* where five Irish populations contained unique components of genetic diversity.

The results of the analysis of population biology in *Colchicum autumnale* and *Campanula trachelium* clearly point to the development of conservation strategies for these species in Ireland and these will be specified in the following section.

9.4 Management Recommendations and Suggestions for Further Study

9.4.1 Colchicum autumnale

One of the most effective methods of population recovery is to instigate changes in management based on conjectures about the causes of population decline or reproductive failure and to monitor the population's response to these changes (Heywood & Iriondo, 2003). At Jerpoint there is a clear need to reduce the dominance of *Pteridium aquilinum, Urtica dioica* and *Filipendula ulmaria* and to open up the vegetation. The spread of genets is likely to be hindered by the dense vegetation and in addition to this the large aggregation of ramets may result in a large degree of geitonogamy between flowers on different ramets of the same individual. Due to the large number of ramets per genet in abandoned grassland in *Knautia arvensis* (L.) Coult. (Dipsacaceae), it was suggested that geitonogamy between ramets of the same clone may be greater than in managed grassland and may result in inbreeding depression (Vange, 2002). The wide separation of genets in the Jerpoint population and the clustering of ramets within genets would also result in a high degree of inbreeding and management should therefore focus on encouraging the spread of genets.

The failure of the population to flower during three years of the study period is a more serious issue but this is also likely to be a direct result of the tall, dense vegetation that was allowed to develop at the site during these years. However, as the population flowered successfully during the first two years of study, a return to the original rolling regime should be instigated, but the possibility of introducing a grazing regime should also be considered. This would reduce the

density of the vegetation and encourage sexual spread within the populations through increased pollination and increased dispersal of seed by grazing animals. This strategy has been previously suggested by staff from the National Parks and Wildlife Service but was met with opposition from Teagasc, the Irish Agriculture and Food Development Authority on the basis of regulations that prevent farmers under Rural Environment Protection Schemes (REPS) from keeping livestock where they have access to a river. The River Nore flows adjacent to the *Colchicum autumnale* site and as this is a highly sensitive river of international importance for salmonid populations, objections were made to the introduction of livestock to the field. Further discussion is required to resolve this conflict of interests but the reversion to the late rolling regime can be immediately applied and will have a positive effect on flowering. Ideally, continued research would investigate a variety of management techniques with close monitoring of the demography and reproductive biology of the population in subsequent years to evaluate their effects.

At Thomastown and at Inistioge the management regime is successful and the cycle of grazing and hay/silage cutting should be maintained. However, the overall decline in the number of genets at these populations over the study period is of slight concern and although at this stage it is difficult to tell whether this is a natural fluctuation in numbers caused by an increase in dormancy or by internal demographic stochasticity or whether there is an external deterministic factor, the populations should be monitored over a longer time period to ensure that the decline does not continue. These two sites have been fertilised and re-seeded with *Lolium perenne* in the past and while *Colchicum autumnale* appears to have adapted to this environment, the cessation of fertiliser application may eventually encourage the development of a species-rich grassland, thereby diversifying the habitat, adding to the ecological value of the sites and also attracting a greater concentration of pollinators. This should improve outcrossing in the populations and will also enhance the ecological value of the habitat.

At Inistioge Lower, the habitat is occasionally grazed by a single horse and occasionally by cattle but there is no set management regime and the grazing is sporadic. To ensure the successful reproduction and spread of *Colchicum autumnale* at this site, a management regime of grazing or

cutting should be put in place that will result in a shorter vegetation height during late spring and early summer when the leaves and fruits are present, and during the flowering period in late August and early September. The vegetation requires at least two cuts, rolls, or grazing periods: one at the end of January before the emergence of leaves and one at the end of July before the emergence of the flowers.

It has been suggested that where reproduction is predominantly clonal, management should focus on the protection of mature plants (Burne *et al.* 2003). However, despite the ability of *Colchicum autumnale* to spread clonally, this study has found that sexual recruitment was at a similar level to vegetative recruitment in Inistioge and Thomastown. The protection of seedlings is therefore an important part of the conservation of Irish populations of *Colchicum autumnale.* Although grazing may result in the accidental herbivory of seedlings (sheep generally avoid consuming the leaves due to their poisonous nature), it can increase seedling emergence due to the removal of competition from dense vegetation. Intensive winter grazing increased seedling emergence in *Cirsium vulgare* (Bullock *et al,* 1994) and at Inistioge Lower and Jerpoint the instigation of a management regime designed to reduce the height of the vegetation at critical times of the year may encourage the spread of the populations. At Fourpenny Rock the population occurs under light shading from mature trees within a private garden that extends to the riverbank. This site is therefore not suitable for grazing and a cutting regime should therefore be adopted based on strimming the grass-dominated bank after the dehiscence of fruits in late June and again at the beginning of August. No cutting of the vegetation should take place during vegetative growth (from February to June) or during the flowering period.

The degree of genetic variation detected within Irish populations of *Colchicum autumnale* suggests that the evolutionary potential of these populations is not threatened and can be protected by maintaining population sizes at their current level. The lowest levels of genetic diversity were found at Fourpenny Rock and Inistioge. At Fourpenny Rock, the small number of individuals in addition to the low level of genetic variation means that the population is at risk from both demographic and genetic stochasticity. If further decline is observed, augmentation of the

effective population size could be attempted by introducing more individuals through translocation or seed supplementation. Fourpenny Rock was found to be closely related to Inistioge and this would therefore be a suitable seed or ramet source. However, genetic variation was low in Inistioge and although the population dendrogram grouped the two populations, the neighbour-joining tree showed that samples from Fourpenny Rock also showed affinities with the other Irish populations and the resolution was poor. Outbreeding depression is highly improbable from the mixing of gene pools from any combination of the Irish populations.

At some point in the future management of *Colchicum autumnale* in Ireland it may become ethically acceptable to introduce or reintroduce the species to potential habitat within its past range. Reintroductions are generally reserved for situations where the species is likely to disappear from a region and a future decline in the number of populations of *Colchicum autumnale* in the Nore Valley or in the size of these populations may result in a need for such action to be taken. In such an event, the most suitable areas for the establishment of populations would be the riverside meadow habitats along the Nore Valley between Inistioge and Kilkenny. Sites could also be considered along the King's River on account of the past distribution of the species.

Thomastown was found to be the most diverse Irish population and as this is the largest population it would be the most suitable source for translocation material. However, reintroduction of a species is usually only considered when all other options have been exhausted and it usually therefore relies on the utilisation of *ex situ* resources. As a result of the Irish Threatened Plant Genebank Project, two accessions of *Colchicum autumnale* seed are held at Trinity College Botanic Garden (Waldren *et al.*, 1998) and one of these consists of over 1000 seeds. In terms of introducing Irish populations into new habitats, the most genetically diverse populations would be most suitable as donor populations due to the wide adaptability of the gene pool. Therefore, Thomastown would be the most suitable seed source to utilise due to the high levels of genetic diversity and high levels of polymorphic loci. However, as genetic

differentiation of Irish populations was found to be relatively low, the risk of outbreeding depression is low and multiple seed sources could be used to ensure a diverse starting population.

As germination under artificial conditions is time and energy inefficient and not guaranteed success, the most suitable strategy would be to introduce seed directly to the population rather than attempting to develop *ex situ* collections of seedlings, juveniles or mature plants for introduction. Because of the low genetic difference between Irish populations, the mixing of individuals from different populations would not be likely to result in outbreeding depression. This was also found to be the case in *Grevillea scapigera* (Rossetto *et al.*, 1995) where RAPD analysis enabled the selection of a small number of plants with a high proportion of the overall genetic diversity to be used in the recovery program for the species. In the event of significant population decline in Ireland, translocation sourced from large British populations such as Asham Wood could be considered, as the differentiation of Irish and British populations appears to be a recent event and the samples were not fully resolved into population or even country-specific groups. Alternatively, small numbers of ramets could be collected from any remaining populations and propagated at Trinity College Botanic Garden, or elsewhere, to produce an adequate population size for introduction to suitable habitat. However, the removal of plants from threatened populations should be a secondary strategy and should only be considered upon failure of the introduction of seed to the habitat. Seed sources are more diverse than propagated ramets and such reintroductions are also more efficient in terms of resources. Seed should be introduced to areas of suitable habitat during the month of June, which is the time of capsule dehiscence in existing Irish populations.

The establishment of e*x situ* conservation resources such as the Irish Threatened Plant Genebank Project is clearly an important preparatory tool for future conservation work but although there are a large number of *Colchicum autumnale* seeds held in the genebank, these only represent two of the Irish populations and also the viability of these seeds over time cannot be assessed due to problems involved with germination. It is therefore important to renew this resource through

further collection of seeds from all the Irish populations of *Colchicum autumnale* at regular intervals.

External threats to the populations

Deterministic threats to the populations of *Colchicum autumnale* include the possible draining, ploughing or fertilisation of the meadows, the cessation of grazing at Thomastown or Inistioge and the failure of farmers to follow recommended management regimes. However, the legal protection afforded to the species and its habitat by the Flora Protection Order and by the designation of the Nore and Barrow Special Area of Conservation under the EU Habitats Directive means that the landowners have a legal obligation to protect the populations and to follow prescribed management plans set out by the National Parks and Wildlife Service. The difficulty with legally-enforced conservation measures is that they are often met with opposition from landowners due to conflicts of agricultural and conservation interests and due to the lack of consultation with landowners on the management of the sites. Threats to populations of rare species from non-compliance with legal requirements can therefore be reduced by educating landowners on the value of biodiversity and of the importance of the species occurring on their land, by extensive consultation with landowners during the development of management plans and by increasing the compensatory payments to farmers that have their land designated under the Habitats Directive.

Further study

Priorities for further study include a population survey and genetic evaluation of the Tintern population in County Wexford. This will allow it to be placed in context with the other Irish populations and will also add to the European dimension. The European evaluation of genetic variation within the species could also be extended to include populations from the central and eastern European parts of its range. This would allow a full assessment to be made of the conservation genetics of *Colchicum autumnale* in Europe.

In terms of the Irish populations, this study detected a decline in the number of genets in the larger populations over the study period. The permanent quadrats at these populations should therefore be monitored over a longer time period to detect whether the decline is sustained over the next five years and evaluate the need for action. In addition to this, Fourpenny Rock, the smallest Irish population with the least genetic variation, should be closely monitored and its conservation status should be regularly re-assessed. The management recommendations for the Jerpoint and Inistioge Lower populations should be instigated and monitoring of their effects on demography and reproductive biology should be carried out.

A study could also be carried out to determine whether the species forms a seed bank or whether the seeds are lost through predation or decomposition. However, predation is unlikely in view of the toxicity of the seeds due to the presence of colchicine and other alkaloids.

Finally, further detailed work on germination requirements of the species should be undertaken to ensure the viability and utility of the Irish Threatened Genebank resource and to ensure the success of future *ex situ* conservation activities.

9.4.2 *Campanula trachelium*

Based on the combined review of demographic, reproductive and genetic data in *Campanula trachelium*, inbreeding depression is a potential threat to small populations and this must be carefully considered in the conservation evaluation of populations. The evidence supports the conclusion that low reproductive output at Dunmore is result of genetic effects rather than pollen limitation and it is likely that this population is suffering from inbreeding depression. Dunmore should therefore be monitored closely and in the event of a decline in numbers, supplementation of the population with seed or plants from a closely related population should be considered. The Woodstock population showed the greatest genetic identity with Dunmore and this should therefore be considered as the donor population. However, the resolution of population groupings was poorly supported by bootstrap analyses, and different groupings were suggested by each different analysis indicating weak differentiation. As outbreeding depression is therefore unlikely

to occur, populations from throughout the Nore Valley could be used as source populations for translocations. Mount Juliet was also identified as containing low genetic diversity and supplementation of the gene pool could also be considered at this site, although it is possible that the species may have been introduced into the estate.

An important consideration is how to conserve the small isolated clumps of *Campanula trachelium* that occur along the River Barrow and the lower reaches of the Nore. Due to the isolation of the River Barrow populations at Tintine and Pollmonty, they have become significantly differentiated from other individuals and the Pollmonty samples contain many unique markers that indicate the presence of unique alleles in these individuals. Enhancement of these 'populations' with individuals from other populations is not a viable option due to genetic differences between populations and the high risk of outbreeding depression. However, the collection of seed from Pollmonty and the *ex situ* propagation of individuals at Trinity College Botanic Garden could provide genetically variable seedlings or mature rosettes that could be planted at the site to guard against demographic, environmental and genetic stochasticity. The disturbance of the vegetation in a small part of the site may also result in recruitment from the seed bank. Many of the isolated clumps of *Campanula trachelium* discovered during this study contained a high proportion of female individuals and some were entirely female. The introduction of hermaphrodites at these locations would enhance the probability of outbreeding and may result in the successful establishment of populations at these locations.

With respect to demographic and environmental stochasticity one of the most threatened Irish populations of *Campanula trachelium* occurs adjacent to the River Nore at Inistioge. The population size was only 18 flowering individuals in 2003 (reduced from 35 in 1999) and from personal observations this was largely due to the erosion of the riverbank at this site by anglers. Supplementation of the population at this site may be necessary if the population size drops further.

In the event of a future decline in the distribution of the species in Ireland, establishment or re-introduction of populations could be considered as part of a species recovery plan. *Ex situ* sources may be useful in this instance as in the establishment of *Gymnocarpium robertianum* in the Burren from propagated cuttings (Waldren *et al.*, 2002) and from plants raised from spores (Waldren *et al.*, unpublished data). In contrast to the situation in *Colchicum autumnale*, seed from multiple populations of *Campanula trachelium* has been successfully germinated *ex situ* and plants can therefore be propagated at Trinity College Botanic Garden and translocated into suitable woodland or hedgerow habitats within the known past range of the species. This is a more efficient use of the resource than simply spreading seed in potential sites as germination can be carried out in carefully controlled environment, yielding a higher number of seedlings than would be recruited *in situ*.

Information on the genetic variability of the source populations is a vital part of any reintroduction programme due to the risks of inbreeding and outbreeding depression developing in the established populations. This study suggests that although the Nore Valley populations were significantly differentiated, the genetic distances between populations were not high and outbreeding depression is unlikely to occur through the mixing of gene pools. However, as the level of differentiation above which outbreeding depression may occur has not been experimentally evaluated, this is purely conjecture and as a precaution, the genetic distance between populations should be used as a guide to the compatibility of their gene pools. Alternatively, single diverse populations could be used as donors. The populations with the largest degree of genetic diversity were Tulla turlough, Archer's Grove, Thomastown and Brownsbarn and these are therefore suitable source populations.

An additional consideration in reintroducing populations of *Campanula trachelium* is the ratio of female to hermaphrodite plants in the source population. In the establishment of a new population, both females and hermaphrodites should be present and they should be introduced in proportions that fall within the range detected in the existing Nore Valley populations. Any reintroductions must be carefully monitored but this is particularly important in *Campanula*

trachelium due to the possible susceptibility to inbreeding depression as suggested by the results presented in this study.

Ex situ resources are clearly important in future management considerations for *Campanula trachelium* and the enhancement of existing *ex situ* seed collections is required. There are currently four accessions of *Campanula trachelium* held at Trinity College Botanic Garden as part of the Irish Threatened Genebank Project and all comprise more than 1000 seeds. Although this is a valuable resource, a wider collection is needed with representation from all Irish populations. This will ensure that all available genetic diversity has been sampled. The discovery of gynodioecy in the populations also has implications for seed collection and where possible the proportion of female plants sampled for seed collection should reflect the frequency of female plants in the population.

Threats to the populations

A large proportion of Irish populations of *Campanula trachelium* are protected as part of the Nore and Barrow Special Area of Conservation. However, although the species is listed in the Red Data Book of Vascular Plants (Curtis & McGough, 1988) and was protected under the 1987 Flora Protection Order, its conservation status was revised and it was not included in the revision of this order in 1999. It therefore has no legal protection in the sites that occur outside designated areas and conservation of the species therefore relies on educating landowners and the general public with regard to its conservation value.

Even populations within the candidate Special Area of Conservation are susceptible to deterioration, as at Inistioge where the riverbank has eroded due to heavy use by anglers. This population is under severe threat due to the low number of individuals and the environmental stochasticity associated with the riverbank habitat and efforts should therefore be made to reduce erosion on all angler paths along the Nore Valley as they run adjacent to many of the populations.

Other threats are related to trampling by livestock, which was observed at the Tulla turlough site and resulted in the destruction of the flowering stems before seed-set, and recreational picking of

the flowers. The livestock issue is another issue that requires discussion between the National Parks and Wildlife Service and the landowner, but this may not be seen as a priority due to the lack of legal protection surrounding the plant. However, the picking of flowers can be prevented by public education on the benefits of leaving wild flowers *in situ* and where the plants are contained within designated areas, information boards on the rare species found at the site would be a valuable contribution to public awareness and conservation in the area.

Further study

Priorities for future study of *Campanula trachelium* populations include monitoring of all populations containing less than one hundred individuals at regular yearly or two-yearly intervals and monitoring of Archer's Grove and Woodstock due to their declining population sizes over the study period. The Dunmore population should be monitored more closely and detailed surveys of the number of seedlings, non-mature rosettes and flowering rosettes should be carried out yearly. The study of fecundity could also be repeated for other small populations such as Mount Juliet in order to determine which populations are suffering from inbreeding depression.

The evaluation of genetic diversity in Irish populations and assessment of native status would be greatly enhanced by more extensive sampling of European populations for genetic analysis. Leaf material could also be collected from the Abbeyleix population and these samples could be subjected to AFLP fingerprinting and included in the genetic analysis of Irish populations. This population had disappeared from the site for many years (or was reduced to a small number of undetected individuals) and it is therefore likely that it was re-established from the seed bank. Seed bank influences can have significant effects on the genetic diversity of populations, acting to remedy the genetic problems of small population size. In a study of the seed bank of *Clarkia springvillensis*, total genetic diversity was significantly higher in the seed bank than in the adult population and the seedbank populations exhibited significantly less differentiation (McCue & Holtsford 1998). An interesting line of study would therefore be to determine whether the species forms a natural seedbank, to assess genetic variability in the seed bank of a selection of Irish

populations and evaluate the future contribution that this could make to genetic diversity in the species.

Further work could also be carried out in determining the specific dormancy mechanism within the species. In a previous study by Martin (1998) a large degree of variability was detected among populations in the response of seeds to different germination-inducing techniques. The genetic variability within the seed bank of the Irish Threatened Genebank Project and the viability of the seed over time should also be assessed to safeguard future conservation efforts. Walsh *et al.* (2003) have assessed seed viability since the establishment of the seed bank and this should be repeated at regular intervals, with more seed collections made where possible.

9.5 Concluding Remarks

Where conservation resources are limited and the number of critically endangered species is high, the only option is to react to this and in this sense conservation biology is indeed a 'crisis discipline'. However, where the study of less threatened species can be incorporated into the research of high priority species, important information can be gained with a minimal increase in resource-use. This study has shown that a way of achieving this is to study species that have similar geographic distributions so that maximum use can be made of field visits. For example, the collection of leaf material for genetic analysis does not require a large time input and so for the same travel and accommodation cost, leaf material can be collected from a number of populations from a number of species rather than just from one highly threatened species. The concurrent study of *Colchicum autumnale* and *Campanula trachelium* has contributed greatly to the conservation of Irish threatened plant species and has therefore made efficient and effective use of conservation resources.

Despite this, the translation of genetic information into practical conservation management can often be difficult. The policy makers and personnel involved in practical conservation are inclined to underestimate the importance of genetic factors in conservation due to the fact that molecular markers are an abstract concept in terms of conservation management (Oostermeijer et

al, 2003). However, molecular data are more informative when used in conjunction with other types of data (Haig, 1998) and if genetic variation is related to real population fitness parameters such as reproductive output, the information becomes much more relevant to conservation planners and genetic concerns become 'real' threats to population persistence. This study of *Campanula trachelium* has shown that genetic variation is reduced in the smallest populations and also that reproductive otuput is reduced, and these findings help to reinforce the importance of genetic studies in species conservation.

This study is the first to successfully integrate demographic, reproductive and genetic data in the assessment of the conservation status of Irish threatened plant species. The concurrent study of *Colchicum autumnale* and *Campanula trachelium* has proved to be both efficient and productive and has led to informed recommendations for the present and future conservation of these valuable constituents of the Irish flora.

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APPENDIX I. AFLP ADAPTOR AND PRIMER SEQUENCES

Adpators

EcoRI adaptor sequence 5' -CTCGTAGACTGCGTACC
CATCTGACGCATGGTTAA- 5'

MseI adaptor sequence 5' -GACGATGAGTCCTGAG
TACTCAGGACTCAT -5'

Pre-selective primer sequences

EcoRI primer sequence 5' -GACTGCGTACCAATTCA- 3'
MseI primer sequence 5' -GATGAGTCCTGAGTAAC- 3'

Selective primer sequences

EcoRI primer sequence 5' -GACTGCGTACCAATTCANN- 3'
MseI primer sequence 5' -GATGAGTCCTGAGTAACNN- 3'

Letters in red are the selective nucleotides. The chosen end sequences for *Colchicum autumnale* are given in Chapter 5 and for *Campanula trachelium* are given in Chapter 8.