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The Biogeography and Conservation Biology of *Spiranthes romanzoffiana* Chamisso

Darach Lupton

January 2008

Thesis submitted in fulfilment for the degree of PhD

to

University of Dublin, Trinity College

Declaration

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Darach Lupton

Acknowledgements

Throughout this thesis I have had the honour of meeting many wonderfully kind, enthusiastic and helpful people. I owe the following individuals my sincerest gratitude to Mr. Gerry Sharkey, Miss Clare Herdman and the other NPWS field staff who helped me to locate populations in the first year of my research. I owe a very special thanks to Mr. John Mathews of the NPWS. John was always helpful above and beyond the call of duty and has remained a vital source of information on *Spiranthes romanzoffiana* in Roscommon and Leitrim, without his help many of the plants in these areas would not have been recorded. To Dr. Naomi Kingston and Dr. Mike Wyse Jackson thank you for helping me to access the records of *S. romanzoffiana* in Ireland and for assisting me with the procurement of a licence to collect specimens in the wild. I would also like to thank you for allowing me to input into the development and publication of Ireland's first vascular plant Species Action Plan.

I extend a huge debt of gratitude to Dr. Richard Gulliver for his tireless enthusiasm and generosity with his data and his endless ideas on the species ecology and conservation management. To the *S. romanzoffiana* UK and Ireland steering group committee and in particular Dr. Deborah Long, thank you so much for giving me the opportunity to share my work with you and for giving me the chance to put my results to some practical use. To Dr Chuck Sheviak of the New York State Museum and Dr Mike Fay of the Royal Botanic gardens Kew, thank you both for your assistance with my samples and for sharing your thoughts with me.

After a worrying start my field trip to eastern Canada in 2005 was eventually successful, I owe much of this to the wonderfully eccentric and highly talented Mr. Roger Latour. His boundless energy and positivity ensured that I kept going on the long road hunting for *S. romanzoffiana*. I am also indebted to Roger for his fondness for cool French Canadian beer and fine Portuguese wine. Without his help things would have been very, very different, so merci beaucoup Roger. I would also like to thank Sean Blaney in New Brunswick for his botanical assistance and his fine hospitality.

To my good friends in room 9 in the Botany dept at Trinity College Dublin, all I can say is that I have loved every minute of it and that I will miss you all very much. To my friends and colleagues at TCD, particularly Miss. (soon to be Dr.) Noeleen Smyth thanks for the companionship and moral support during the more diffucult times. To Dr. Rhian Smith where do I begin, thank you so much for holding my hand in the lab and for guiding me deftly through the quagmire. To Dr. Trevor Hodkinson thanks for answering all of my many inane questions as I peered around your office door. Thanks also all my good friends in the lab and herbarium at TCD, you have all been wonderful. My supervisor Dr. Steve Waldren, cheers Steve for all of your guidance and help. I really enjoyed working with you, I like our rapport and the fact that we get there in the end even if it is at the very last minute. I did not realise it at the time but I have learned a great deal over the last few years, so thanks again, I promise I will put it to good use.

To my, father cheers for putting up with me. I am really glad that you can share in my achievement; it's a long way from Portmarnock Community School eh? To my darling mother I hope me reaching this high point in my life can go some way in helping you through this very difficult time. I know you will pull through so hang on in there, there is still plenty to see and do. To my new family the Kellys, thank you all for your warmth, hospitality and generosity and thanks so much for the use of the room upstairs, it was great sanctuary during my thesis write up. I'm sure you are glad I have this thesis behind me.

To my darling wife Ashling, words fail me when I attempt to describe how I much I love and appreciate all that you have done for me over the last few years. You have displayed incredible patience with me and my *Spiranthes* obsession and have been continually supportive and understanding right the way through. Without question I could not have finished this endeavour without you, so thank you from the bottom of my heart.

Finally to my beautiful new baby boy Ruben, you have no idea how much you mean to me. Apart from anything else your entrance in to this world put some well-needed perspective on this work and really helped me to see the big picture. I hope some day you will read this work and smile at your old father as I bore you with stories of how difficult the whole thing was.

If I have forgotten anybody I apologise sincerely, I promise I will thank you in the future.

List of Abbreviations

AFLP	- Amplified Fragment Length Polymorphism
AMOVA	- Analysis of Molecular Variance
BSBI	- Botanical Society of the British Isles
CITES	-Convention on International Trade in Endangered Species
cpDNA	- Chloroplast DNA
DNA	- Deoxyribonucleic Acid
GPPC	-Global Partnership for Plant Conservation
GSPC	-Global Strategy for Plant Conservation
INSPC	-Ireland's National Strategy for Plant Conservation (www.botanicgardens.ie/gspc/inspc.htm)
IUCN	-World Conservation Union (International Union for the Conservation of Nature and Natural resources)
NPWS	- National Parks and wildlife Service
NMS	-Non-metric Multidimensional Scaling
NVC	-National Vegetation Classification
PAUP	-Phylogenetic Analysis Using Parsimony
PCO	- Principal Co-ordinate analysis
PCR	- Polymerase Chain Reaction
pers. comm.	- personal communication
SNH	-Scottish Natural Heritage
SSR	- Simple Sequence Repeat
UPGMA	- Unweighted Pair Group method with Arithmetic Mean

The Biogeography and Conservation Biology of *Spiranthes romanzoffiana* Chamisso

Abstract

The aim of this research was to gather baseline data on Irish populations of *Spiranthes romanzoffiana,* a species with an uneven amphi-Atlantic distribution. The species is widespread in North America. In Europe, populations are confined to the west and north east of Ireland and to the Hebridean islands in western Scotland. *S. romanzoffiana* is protected in the Republic of Ireland by the Wildlife Act (1967), under the Flora Protection Order [SI 94 of 1999] and is an Irish Red List species. Four sample populations in the Republic of Ireland were studied to determine the ecology, population, reproductive and pollination biology of *S. romanzoffiana*. Threats to the species' persistence were assessed at each site. Amplified Fragment Length Polymorphism (AFLP) and chloroplast microsatellite markers were used to determine the level of genetic diversity and differentiation within and between Irish, Scottish and North American samples.

Results suggest that *Spiranthes romanzoffiana* is confined to lakeshore habitats in the Republic of Ireland. The species appears to tolerate a range of soil pH levels from mildly acidic to mildly alkaline, with a very high mineral content. The vegetation communities are all within the range of lake-shore, mire and rush to wet grassland. Four new populations of *S. romanzoffiana* were recorded during this research. The population demographic data imply that current population census methods, which to date have relied solely on the presence of flowering plants, are under-recording the true extent of populations. The proportion of vegetative to flowering plants was 0.37, suggesting that populations may be larger than previous data indicate. The census data also suggests that Irish populations of *S. romanzoffiana* may be under recorded at present. The fluctuation in annual flower spike production detected in this research suggests that population monitoring should be conducted over a minimum of five consecutive years. Irish populations of *S. romanzoffiana* appear to reproduce vegetatively by the production of twin lateral buds during the summer months. However, the discovery of seed, natural pollinia removal and the detection of potential pollinator species suggest that Irish populations are not totally reliant on vegetative spread as a mode of reproduction. The small quantities of viable seed found in the capsules suggest that recruitment by seed is low in Irish populations. Possible threats to Irish populations include grazing by cattle and sheep during the flowering season and habitat disturbance by vehicles and pedestrians.

The total number of unambiguous markers yielded from the combination of the two AFLP primer pairs was 191. DNA was extracted from 121 individual plants from a total of 16 populations: six North American populations, nine Irish and a single Scottish population. No private alleles diagnostic of any individual population were detected with the AFLP markers. Total gene diversity (Ht) in Irish samples was 0.26, in North American samples it was 0.22. Within population diversity (Hs) was 0.23 and 0.16 in Irish and North American samples respectively. Between population diversity (Gst) for all populations was 0.22. North American Gst was 0.25 the Gst for Irish samples was 0.11. Partitioning of molecular variance (AMOVA) among and within groups in North America and Ireland showed that 15.99% of the variation was distributed among all the groups and 72.3% within. Variation among the Irish populations was 14.43% within variation was 85.57%. Variation among the North American populations was 22.5%, variation within was 77.5%. Ordination of the samples using PCO (Principal Coordinate Analysis) and distance measures using neighbour joining and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) revealed a genetic geographic split between European and North American samples and populations. (The pattern of genetic diversity, the discovery of seed and the recording of potential pollinator species suggest that Irish populations are sexually out-crossing and are not genetically depauperate.

Chloroplast microsatellite analyses revealed one unique haplotype in the Irish samples. Scotland and North America share a single haplotype, which was not detected in the Irish samples. All of the four haplotypes recorded in the North American samples are found in the Irish samples, though one of these was absent from the Scottish samples. The highest allelic diversity for the European samples was found in the Lough Mask population. Based on the levels of genetic divergence between the North American and Irish samples and low levels of population differentiation, this research suggests two possible hypotheses to explain the occurrence of *S. romanzoffiana* in Ireland. Irish populations may be of recent geological origin and may have originated from populations in North Eastern Canada, possibly Newfoundland or Irish populations represent a remnant of a formerly more widespread pre-glacial, European distribution. This research concludes that *S. romanzoffiana* is most likely native to Ireland.

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Chapter 1.

1.1 Brief Introduction to Conservation biology

The conservation of global biodiversity is reliant upon the conservation of individual species and their inherent genetic variation. It is therefore necessary for conservation strategies to be based upon fundamental scientific knowledge of the species they aim to conserve (Falk and Holsinger, 1991). It is the goal of conservation biology to provide principals and methods for preserving biological diversity.

Conservation biology began to emerge in the mid-twentieth century when a growing social and political interest in environmental issues directed scientists towards the problems associated with the survival of threatened species. The discipline has grown at a rapid pace since then. Its most prominent journal, Conservation Biology was created in only 1987 and the Society for Conservation Biology (created by Michael Soulé, Paul Ehrlich and Jared Diamond) was founded in 1985.

Conservation biology differs from most other biological sciences in one important way, it is often a crisis discipline (Soulé, 1985). A conservation biologist may have to make decisions or recommendations about design and management before he or she is completely comfortable with the theoretical or empirical basis of the analysis. Tolerating uncertainly is often necessary (May, 1984). As a discipline, conservation biology is a relatively new addition to the biological sciences, however the science in itself borrows from a number of biological and social disciplines. Conservation biology is a synthetic science, built from ecology, population biology, population genetics, biogeography, economics, anthropology, philosophy, and probably other disciplines, with the intention of developing principles and strategies to preserve diversity (Diamond, 1975; Simberloff and Abele, 1976). Different approaches attempt to maintain the diversity of species directly, or through maintenance of a diversity of habitats. It must maintain a balance between the potential desire to preserve everything in a pristine natural state, and the political desire to permit intensive development (Meffe and Carroll, 1997).

Perhaps one of the most important contributions to modern conservation biology is 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 emphasises another dimension to the preservation of biodiversity: the preservation of genetic diversity inherent within populations and species. The documentation of inbreeding depression (the lowered fitness of inbred compared to outbred individuals) in small populations was one of the first discoveries to hammer home the importance of understanding the genetic diversity contained within and among 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 20th century (Moore, 1962). Subsequent years saw the development 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. Karp *et al.*, 1998; Frankham *et al.*, 2002).

With the advancements in genetics came an understanding of the long-term consequences of the loss of genetic diversity of species. 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 a defining feature of conservation biology, which essentially aims to conserve species whilst preserving the raw material for their continued evolution.

1.2 Fundamental Concepts of Conservation Biology

1.2.1 Biodiversity

As a shorthand description of this great variety of life, the term "biodiversity" is a contraction of "biological diversity", and was first coined by Walter Rosen for the 1986

National Forum on BioDiversity. However, biodiversity refers to more than just an accumulation of species. Instead, biodiversity also refers to organisms' existence in situ, and incorporates the ecological and evolutionary interactions among them. For example, the UN Convention on Biological Diversity defines biodiversity as "the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems" (United Nations Environment Programme, 1992). Another more concise definition describes biodiversity as 'the structural and functional variety of life-forms at genetic, population, community and ecosystem level' (Sandlund *et al.*, 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 species as a whole. The all-encompassing concept of the conservation of biodiversity is a fundamental concept at the core of modern conservation biology. Biodiversity has unified the different fragments of biology under a common objective and has provided a conceptual focus for the science of conservation biology and its practice (Jeffries, 1997).

1.2.2 Population paradigms

Caughley (1994) in his review of the direction of modern conservation biology suggests that the discipline is advancing in a somewhat dichotomous manner with two distinct approaches or paradigms emerging. The first of these ideas is referred to as the 'small population paradigm' and deals with the risk of extinction in small populations and aims to manage threats to the persistence of small populations (Van Dyke, 2003). The second idea is termed the 'declining population paradigm' is concerned with the processes by which populations are driven to extinction and aims to reverse the process through the identification of causal factors (Caughley, 1994; Caughley and Gunn, 1996). Although Caughley supports an integration of the two paradigms he has been criticised for constructing a false dichotomy and 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.

Another important paradigm, which is highly relevant to conservation biology, is that of metapopulation theory. A metapopulation consists of a group of spatially separated populations of the same species which interact at some level. Levins was the first 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). A metapopulation is generally considered to consist of several distinct populations together with areas of suitable habitat, which are currently unoccupied. Each population cycles in relative independence of the other populations and eventually goes extinct as a consequence of demographic stochasticity 'fluctuations in population size due to random demographic events'; the smaller the population, the more prone it is to extinction (Hanski, 1999).

Although individual populations have finite life spans, the population as a whole is often stable because immigrants from one population (which may, for example, be experiencing a population boom) are likely to re-colonize habitat that has been left open by the extinction of another population. They may also immigrate into another small population and so rescue it from extinction. The development of metapopulation theory emphasized the importance of connectivity between seemingly isolated populations. Although no single population may be able to guarantee the long-term survival of a given species, the combined effect of many populations may be able to do this. Metapopulation structure has important implications for conservation biology. It forms the conceptual framework for designing a reserve system and managing populations whose habitat is fragmented. Metapopulations also potentially have the ability to rescue declining local populations by dispersal from larger local populations.

A standard definition of a population is 'a group of individuals of the same species occupying a defined area at the same time' (Hunter, 1996). Two procedures are commonly used for evaluating the viability of a population, or the probability that the population will survive for some specified time. Population viability analysis (PVA) is the methodology of estimating the probability that a population of a specified size will persist for a specified length of time. The minimum viable population (MVP) is the smallest population size that will persist for some specified length of time with a

specified probability. In the first case, the probability of extinction is estimated, whereas in the second, the number of individuals is estimated that is needed in the population to meet a specified probability of persistence. For a population that is expected to go extinct, the time to extinction is the expected time the population will persist. Both PVA and MVP require a time horizon, i.e., a specified, but arbitrary, time to which the probability of extinction pertains (Morris and Doak, 2002). The value of a PVA is restricted by the quantity and quality of data available and the accuracy of the models that are used; as result it is at best an approximate science and at worst it is a hugely inaccurate estimator of extinction probability. Although the final outcome of the process may be questionable, the process itself provides useful information that can be used to make informed conservation decisions. A full PVA was not carried out during this research but the concept of population viability has directed the method of the study.

1.3 A brief introduction to the conservation biology of orchids

There is extensive variation in the ability of individual species and groups of species to deal with potential threats to their survival. Relative to other plant families orchids for example are subject to high levels of threat, through both anthropogenic and natural causes (Hutchings, 1989). Orchids are a species rich family (ca 25,000 species), with representatives capable of occupying almost every conceivable ecological situation. However orchids also feature prominently in many red data book lists and the abundance of many orchid species is believed to have fallen to critical levels in recent years (Kull, 2006). The complex life history of orchids require long term studies to fully understand the processes taking place, because of this conservation initiatives are often difficult to implement.

There is a dichotomy associated with orchid conservation, they have had a long history of attraction for amateur and professional breeders, artists, photographers and this attraction has in turn lead to over enthusiastic collectors removing large quantities of species form the wild. The attraction is undoubtedly attributed to the beauty inherent in many of the species. The problem is that much of the data available regarding orchids are often anecdotal and contrasting. Relatively speaking there are only a small number of truly detailed studies on the population ecology and conservation biology of orchid

species e.g. Wells (1981), Wells and Cox (1991), Hutchings (1987), Hutchings *et al.*, (1998).

In 1990 a series of workshops were initiated to synthesize scientific information on aspects of orchid biology and conservation and to discuss future research, management and conservation of orchids. The first Orchid Conservation Congress was held in Perth, Australia in 2001 and a volume of papers was written on a wide range of topics related to issues facing orchid conservation. The most recent Orchid Conservation Congress was held in Haapsalu, Estonia in 2005, and again a large volume of papers were written on topics such as orchid biodiversity (Pillon *et al.* 2006), hybridization (Cozzolino *et al.* 2006), and orchid soil seed banks (Whigham *et al.* 2006). The management of orchid populations is a topic that stimulates much debate within the conservation community. Research into the management of mowing regimes on populations of *Dactylorhiza majalis* concluded that annual mowing after fruit set produces the greatest benefits for individual plants and populations (Janeckova *et al.* 2006). Examination of the relationships between survival, plant size, life stages and dormancy in populations has shown some interesting results. Using capture-recapture models, Greg and Kery (2006) reported that models based on the classification of orchids using both size and life stages produce the most accurate information for evaluating different management regimes for conservation. Many notable papers on a wide range of orchid related topics from population dynamics (e.g. Tremblay *et al.*, 2006) to fire management of orchid populations (e.g. Coates *et al.*, 2006) have been recently published. These highlight the increasing sophistication in research techniques and the growing interest in the subject of orchid conservation. This thesis utilizes some of these techniques and theories and in doing so aims to better understand the conservation requirements of *Spiranthes romanzoffiana* in Ireland.

1.4 Plant Conservation in Ireland

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. 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 0f 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 angiosperms (Webb *et al.,* 1985) and of these, 68 are currently protected under this legislation. Species are included on the order if they have a restricted distribution in the Republic of Ireland or if, whether common or not in the Republic, they are rare in a European context.

1.4.1 Plant Conservation Programmes

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 Strategy for Plant Conservation (GSPC). The Global Partnership for Plant Conservation (GPPC) was established to promote and support the implementation of the Global Strategy for Plant Conservation. The primary aim of the GSPC is to halt the decline in the world's plant diversity and it was adopted unanimously by 187 governments present at the 6th Conference of the Parties to the Convention on Biological Diversity in the Hague, Netherlands in April 2002. It aims to achieve its ultimate objective to the implementation of 16 targets to be met by 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 these.

In response to the GSPC a stakeholder meeting was held at the National Botanic Gardens, Glasnevin, Dublin in September 2005 to discuss the establishment and development of a National Strategy for Plant Conservation in Ireland. The 16 targets that had been developed from the GSPC were adopted following brainstorming sessions on plant records, important areas of plant diversity, invasive alien species, and public awareness. Ireland's National Strategy for Plant Conservation comprises a set of 16 targets modelled on the GSPC. These targets include; a preliminary assessment of the conservation status of all known plant species in Ireland completed and made widely

available, at least 10 per cent of each of Ireland's plant habitats should be effectively conserved and at least 30 per cent of production lands in Ireland managed consistent with the conservation of plant diversity, The conservation of at least 60 per cent of Ireland's threatened plant species assured *in-situ* and ensure that plant conservation and biodiversity issues are incorporated into the formal educational curricula at all levels in Ireland, and in informal education and national public awareness programmes.

In 2002 Ireland's National Biodiversity Plan was published, this comprises a set of 91 actions to halt the current and continuing loss of plant species, as well as the vegetation and habitats they compose by the year 2010. Following on from this, the National Platform for Biodiversity Research was set up under the auspices of National Parks and Wildlife Service and the Environmental Protection Agency. The Platform aims to facilitate biodiversity research in Ireland, taking into account the needs of the research community, stakeholders, policy makers and the public.

There are a number of practical plant conservation strategies currently being managed in the Republic of Ireland. A gene and seed bank is housed at Trinity College Botanic Gardens, Dublin, which was set up as a co-operative venture between National Parks and Wildlife, Trinity College Botanic Gardens and Genetic Heritage Ireland, with funding from the Heritage Council. A number of recovery plans have been implemented in recent years, involving the reintroduction of selected threatened plant species back in to the wild, e.g. *Inula salicina*, *Asparagus officinalis* ssp. *prostratus, Crambe maritima*, *Gymnocarpium robertianum and Otanthus maritimus.* Projects on the conservation of land-races of crops are being carried out by Genetic Heritage Ireland, the Department of Agriculture, Trinity College Dublin, and Irish Seed Savers.

Plant conservation in Ireland is currently undergoing a positive shift forward. For many years the problems of plant conservation suffered through a lack of funding and sluggish governmental commitment. Through pressures from the EU and Ireland's recent financial prosperity this situation appears to be changing for the better. It is hoped that this impetus continues long in to the future and that research projects such as this one continue to receive financial backing so that the issues facing Irish plant conservation can be managed in a coherent and meaningful manner.

1.5 Introduction to Orchidaceae, Spiranthes and the species Spiranthes romanzoffiana

Orchidaceae are among the most diverse of the flowering plant families, with over a 1000 described genera and 25,000 species, and perhaps another 60,000 hybrids and varieties (Heywood, 1985). The Kew checklist gives about 24,000 accepted names. Many new species are added to the list each year. Whilst the majority of these are found in the tropics, many growing as epiphytes on trees, approximately 300- all of them terrestrial- occur in Europe. Fifty-four species are found in the British Isles (Foley and Clarke, 2005).

1.5.1 Characteristics of the orchid family

Orchids share many features with related groups of monocots: scattered vascular bundles, parallel leaf venation, flower parts in threes and inferior ovary. However there are a number of features that distinguish orchids from other vascular plants (Dressler, 1981) as listed below.

Distinguishing features of orchids:

(1) The **Column**, the fusion of male and female organs within a single structure located at the centre of the flower.

(2) **Pollinia**, tightly packed masses of pollen found in most orchids, transported as a unit by pollinators (Freudenstein and Rasmussen, 1997; Pacini and Hesse, 2002); a single pollinator visit is potentially sufficient to produce a full seed complement (Proctor and Harder, 1994; Nazarov and Gerlach, 1997).

(3) **Zygomorphy**, whereby the **labellum** (modified petal) is often highly adapted to serve different functions (Van der Pijl and Dodson, 1966).

(4) **Protocorms** A protocorm is the structure formed after the germination of the seed and before the development of the seedling plant. The protocorm has no radicle and instead has mycotrophic tissue (and hence differs from other flowering plant seedlings).

The diversity of floral shapes and functional modifications found across the family is largely a result of variation in these four features (Tremblay *et al.*, 2005). Like other higher plants, orchids begin life as a seed, but unlike most other higher plants they require the presence of a fungus, usually a *Rhizoctonia,* before the seed will germinate. Symbiotic germination can be achieved in a laboratory with many species of terrestrial orchid (Stoutamire, 1974). The smallest orchid is thought to be *Bulbophyllum minutissimum,* at 3-4 mm tall, but many other orchids approach it in size. Vanillas, which are lianas that reach into the crowns of rain-forest trees, are deemed to be the largest orchids and grow to over 20 m in length. Some tropical orchids also form very large clumps on rocks or in trees. *Grammatophyllum speciosum* plants of several hundred kilograms have been reported from Southeast Asia. Some orchids live on the ground while others grow perched on trees as epiphytes or on rocks as lithophytes and in some instances orchids spend their entire life below ground e.g. *Rhizanthella gardneri* of Australia (George, 1981)

Phytogeography of Orchids

Orchids are a cosmopolitan family ranging from Northern Sweden and Alaska to Tierra del Fuego. Orchids can therefore be found very near the limits of vegetation development. The epiphytes among them are however limited to the tropical and subtropical environments. Orchids are lacking, also, in the most extreme desert environments, though they may be found in oases, in sheltered desert canyons and in cactus scrub vegetation (Dressler, 1981).

The origin and affinities of orchids

The orchid family (Orchidaceae) demonstrates one of the most specialized lines of flowering plant evolution. It is likely that all orchids derived from *Hypoxis*-like ancestors had six tepals (three tepals of an outer whorl called sepals and three tepals of an inner whorl called petals) and six stamens (again three in an outer whorl and three in an inner one). Several trends of morphological specialization can be observed in the evolution and formation of different orchid groups. However, a reduction in the number of stamens and fusion of the remaining fertile stamen(s) with the pistil is the main general floral

transformation that led to the evolution of the family. Successive reduction in the number of stamens has led to formation of groups of orchids with three, two and one stamen remaining in the flower (Heywood, 1993). More than 99 % of all orchid species have only a single stamen in the flower and this is one of the main features of the orchid family. In the most recent taxonomic treatments, orchids with only one stamen are separated into three subfamilies: Vanilloideae, Orchidoideae and Epidendroideae (Pridgeon *et al.*, 1998). The median petal (lip/labellum) in these orchid subfamilies usually plays a role as a landing platform for pollinators and can also provide attraction for pollinators, such as nectar, and a convenient path to the anther.

Currently *five* subfamilies of orchids are recognized:

Apostasioideae

The apostasioid orchids, placed into subfamily Apostasioideae, are considered to be the most primitive group of orchids. They have two or three stamens in their flowers. They comprise two genera, *Apostasia* and *Neuwiedia*, with approximately 16 species. All are terrestrial orchids confined to tropical Asia, the adjacent archipelagos and northern Australia. Their flowers are almost regular and somewhat resembles those of *Hypoxis* itself (Pridgeon *et al.*, 1998).

Cypripedioideae

The second group, subfamily Cypripedioideae, also retains two stamens in its flower. They are popularly called slipper orchids and represent a distinct lineage. This subfamily is widely distributed in Eurasia and throughout the Americas. It includes five genera; *Cypripedium*, *Mexipedium*, *Paphiopedilum*, *Phragmipedium* and *Selenipedium* totaling about 150 species. In the slipper orchids (*Paphiopedilum*), two fertile anthers are placed on either side of the column. The central stamen is sterile and is borne at the apex of the column. It is curiously modified into a large shield-like organ, the staminode that

prevents direct access by the pollinator from the front to the centre of flower. The two other stamens are hidden behind the staminode, one on each side of the column. The saccate lip of slipper orchids has evolved as a trap for pollinators. The inside walls of the lip are very slippery but a ladder of hairs lies on the interior dorsal wall. This leads under the stalked ventral stigma to one of two exits at the base of the lip on either side of the column (Cox, *et al.*, 1997)

Vanilloideae

The vanilloids are a small group that includes *Vanilla*, a genus of about 70 species of lianas. The subfamily comprises approximately 16 genera, and about 200 species. Most Vanilloideae species are tropical in their distribution (Pridgeon *et al.*, 2003)

Orchidoideae

The orchidoids are mostly terrestrials with tubers or fleshy rhizomes and include the majority of European, Mediterranean, North American, terrestrial African and temperate Australian orchids. The type genus *Orchis* and the bee orchids (*Ophrys*) belong here as do the terrestrial Australian, temperate North and South American and many African and Madagascan ground orchids, including *Spiranthes* (Pridgeon *et al.*, 2003).

Epidendroideae

The epidendroid orchids, the largest group, are predominantly epiphytes or lithophytes and include all the showy tropical genera, such as *Cattleya*, *Oncidium*, *Phalaenopsis* and *Vanda*. Two growth habits are found in this subfamily. Some produce each new growth from the base of the old growth (sympodial growth habit). Others continue to grow each year from the same point (monopodial growth habit) (Pridgeon *et al.*, 2003).

The Orchid Flower

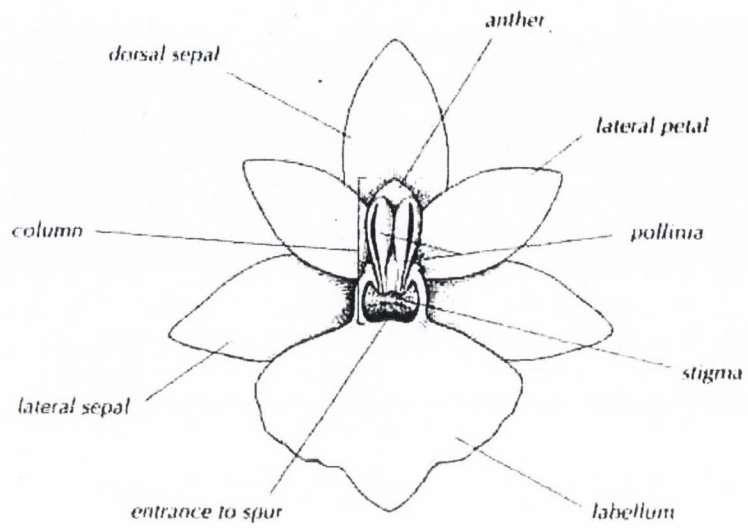

Figure 1.1 Basic segments of a typical orchid flower. Taken from Foley and Clarke (2005).

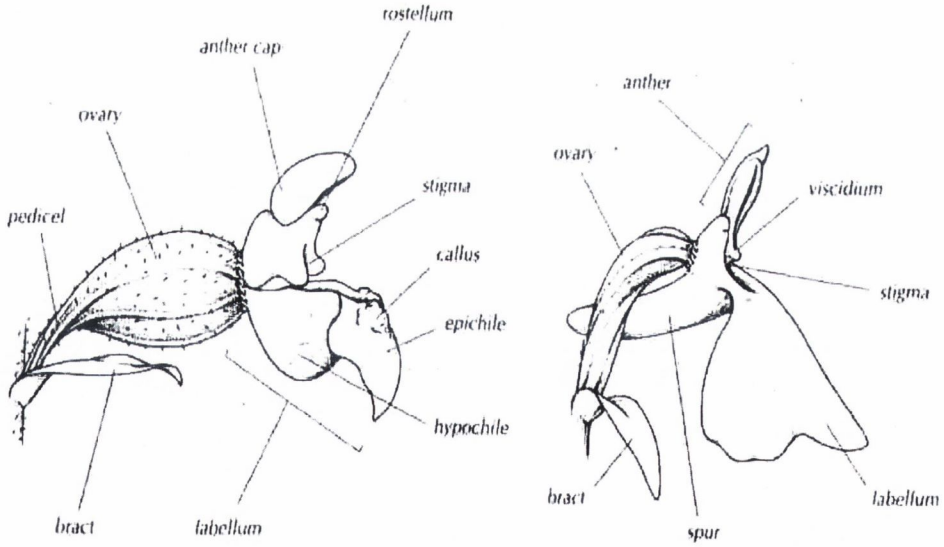

Figure 1.2 A typical orchid flower with sepals and petals (except the labellum) removed. The male and female reproductive organs fuse together to form the column. Taken from Foley and Clarke (2005).

Orchid flowers are simple in structure, yet highly modified from the more typical monocotyledon flower as exemplified by a *Trillium* or *Lilium*, to which orchids are very distantly allied. These characteristically have their floral parts arranged in threes or multiples of three. Orchids are no exception. This can most easily be seen in the two outer whorls of the flower. The central part of the orchid flower shows the greatest modifications to the basic monocotyledon pattern (Luer, 1975).

The major evolutionary forces at work in orchids have been reduction in the number of floral parts and fusion of the male and female organs into a single structure (Dressler, 1981). The fused organ in the centre of an orchid flower is called the column. The pollen in the anther is not powdery as in most plants, but is borne in discrete masses, called pollinia (single pollinium). The pollinia are attached to a sticky mass called a viscidium. The number of pollinia per flower may be two, four or six and these are attached to the viscidium either directly or by a stalk called a stipe in most epiphytic orchids, and by a caudicle in most terrestrial species. The stigma, the receptive surface on which pollen alights and germinates, is also positioned on the column in the centre of the orchid flower, on its ventral surface. The stigma is a sticky lobed depression situated below and behind the anther in most orchids. However in some terrestrial genera such as *Habenaria* and *Peristylus* the stigma is bilobed with the receptive surfaces at the apex of each lobe. In many species the pollen masses are transferred to the stigmatic surface by a modified lobe of the stigma called the rostellum (Delforge, 1994).

An interesting feature in the development of most orchid flowers is the phenomenon of resupination. In bud, the lip lies uppermost in the flower while the column lies lowermost. In species with a pendent inflorescence the lip will, therefore, naturally lie lowermost in the flower when it opens. In most species the lip is lowermost in the flower. This position is achieved by means of a twisting of the flower stalk or ovary through 180 degrees as the bud develops. This twisting is termed resupination (Nyman *et al.*, 1987).

1.5.2 The Orchid Inflorescence

The orchid inflorescence is normally racemose, with the flowers axillary on the rachis and usually flowering from the base upwards. There are a few orchids, such as *Orchis*

simia, that flower from the top down but the inflorescence is still a raceme. Inflorescences may be branched (paniculate), in which case the ultimate branches are racemose. There are also many orchids that bear one-flowered inflorescences, such as *Lycaste* and *Maxillaria* (Heywood, 1993). In all cases the flower is subtended by a bract. The bract is usually inconspicuous but may be large or coloured as in *Lockhartia.* The flowers are often spirally arranged on the rachis even when the leaves are distichous, but the bracts and flowers are distichous in a number of groups and the flowers are whorled in a few cases. The inflorescence may arise from any part of the stem.

In the primitive condition the inflorescence is terminal and is simply a continuation of the shoot axis. In other cases the inflorescence is lateral from the side or base of the shoot or from the rhizome. In the monopodial growth habit, it is always lateral (Dressler, 1981). While the normal inflorescence is produced from the axil of a leaf or bract, there are some notable exceptions.

1.5.3 Vegetative characteristics of Orchidaceae

The vegetative features of orchids are, if anything, more variable than their floral ones. This is scarcely surprising when the variety of habitats in which orchids are found is considered. Orchids grow in almost every situation: on the permanently moist floor of the lowland tropical rain forest; in the uppermost branches of tall forest trees where heavy rainfall is followed by scorching sun for hours on end; on rocks; and in the grassy areas found on landslips and roadsides. The major adaptations seen in orchid vegetative morphology allow them to withstand adverse environmental conditions, in particular, the problems of water conservation on a daily and seasonal basis (Heywood, 1985).

Many orchids have marked adaptations of one or more organs, which allow them to survive these periodic droughts. Cactaceae display quite dramatic adaptations, whereby the stem develops into a water-storage organ. This is so common in tropical orchids that the resulting structure has been given a technical name, a pseudobulb (Arditti, 1992) In *Dendrobium* the pseudobulbs comprise several internodes, while in *Bulbophyllum* they are of one internode only. Pseudobulbs are also found in many terrestrial orchids and can

grow either above the ground as in *Hammarbya paludosa* or underground as in *Geodorum*. Many terrestrial orchids, such as *Orchis*, *Ophrys* and *Disa*, lack pseudobulbs but instead have underground tubers in order to survive drought (Johnson, *et al.*, 1998). The new growth extends from one end of the tuber in suitable conditions. In others, such as jewel orchids and the creeping lady's tresses, *Zeuxine* and *Goodyera,* the stems are succulent but not swollen. The horizontal stem or rhizome creeps along the ground in the leaf litter, and erect shoots bearing the leaves are sent up periodically.

The leaf is another organ that has undergone dramatic modification in the orchids. Fleshy or leathery leaves with restricted stomata, such as those of *Dendrobium* and *Bulbophyllum* species are common. A number of orchids have no green leaves. In some epiphytic orchids, such as *Chiloschista* and *Dendrophylax*, the leaves have been reduced to scales and photosynthesis takes place in the flattened green roots (Arditti, 1992). Some terrestrial orchids, such as the ghost orchid *Epipogium aphyllum*, are leafless and lack chlorophyll altogether. These are termed saprophytes or mycotrophs. Lacking chlorophyll, they cannot photosynthesize and must obtain all of their nutrition from the mycorrhizal fungus with which they are associated. Terrestrial species usually have leaves of a much thinner texture than their epiphytic cousins (Arditti, 1992).

Orchid roots are highly modified in most epiphytic orchids. They provide both attachment to the substrate and also uptake of water and nutrients in a periodically dry environment. The roots have an actively growing tip; an envelope of dead empty cells, called a velamen, covers the older parts. The velamen protects the inner conductive tissue of the roots and may also aid the uptake of moisture from the atmosphere, acting almost as blotting paper for the orchid (Dycus and Knudson, 1957).

Orchids have mycorrhizal associations with soil fungi believed to be essential for seed germination and to assist growth in adult plants (Rasmussen, 1995; Currah *et al.*, 1997). Most orchids have specific fungal associates that vary between host species and habitat (Brundrett, 2002). The majority of these fungi are assigned to the anamorphic form genus *Rhizoctonia*. It is not clear whether orchid fungi from different regions are more closely related to each other or to saprophytic or parasitic groups of *Rhizoctonia* species (Currah *et al.*, 1997). However, it seems likely that orchid fungi are a disparate group with many separate origins and the recruitment of new fungal lineages by orchids continues today (Brundrett, 2002).

The benefits provided by orchids to their mycorrhizal fungi, if any, are not clear, as these fungi seem to grow equally well with or without their orchid hosts (Brundrett, 2000). The minute seeds of orchids are considered to be fully dependent on mycorrhizal fungi for germination. However the adult plants of some species at thought to be fully autotrophic (Hadley 1982; Rasmussen, 1995). Evidence that mycorrhizas of green orchids are partially exploitative is provided by ^{14}C transfer experiments, the survival of achlorophyllous mutants of some orchid species and the apparent below ground persistence of other orchids for years (Alexander and Hadley, 1985). Saprophytic (myco-heterotrophic) orchids without chlorophyll have fully exploitative mycorrhizal associations that supply both the energy and nutrient requirements of the host (Leake, 1994). Many of these plants associate with fungi that are not related to the mycorrhizal fungi of green orchids. These associations have a high degree of specificity and the species of *Corallorhiza* and *Galeola* may only associate with a single fungal genus (Brundrett, 2002).

1.6 Overview of the genus Spiranthes.

Etymology

From the Greek speira, coil and anthos, flower, in reference to the spiral arrangement of the flowers in the raceme (Salazar *et al.*, 2003).

Description

Spiranthes species are terrestrial, acualescent, sometimes stoloniferous herbs. The roots are typically several, relatively slender to thick, fleshy, fusiform, cylindrical – fusiform or long cylindrical glabrous. The leaves form a basal rosette and are sometimes found on the lower half of the inflorescence, often absent at anthesis; when present it is usually on the same shoot as the inflorescence, but sometimes the leaves of the flowering shoot are absent and the ones present are those of the new shoot (e.g. *Spiranthes spiralis*). Leaves are sessile to more or less distinctly petiolate; blade elliptic, ovate, linear, linear-oblanceolate or linear elliptic, acute to acuminate, often channelled in various shades of green, dull to glossy (Delforge, 1994).

Inflorescence

The inflorescence of *Spiranthes* is glabrous below, variously pubescent above and is typically partially concealed by herbaceous tubular, acuminate bracts. The flowers are many, single to several ranked, spirally arranged (e.g. *Spiranthes lacera*). The floral bracts are herbaceous, ovate to lanceolate, acute to acuminate, glabrous to pubescent (Heywood, 1985)

Flowers

Spiranthes flowers are held horizontally to nodding. They are tubular, often with the apices of the floral parts flared, recurved or spreading to various degrees. Typically fragrant, the flower colour varies from white to pale yellow or pale green, rarely pink above the middle (e.g. *S. sinensis*). The labellum often has green marks or veins, the throat coloured yellow to green, rarely completely pink. The sepals are glabrous to sparsely pubescent outside. The dorsal sepal is erect, recurved near the apex and is usually lanceolate. Lateral sepals are free, spreading, obliquely lanceolate acute. The petals are erect, recurved near the apex, obliquely oblanceaolate, obtuse to acute. The labellum is typically clawed, arcuate, sometimes a short blade provided with a conical, fleshy, pubescent nectar gland at each side of the base. This is adherent to the sides of the column, forming a tunnel like access to the nectary (Arditti, 1992).

The column is claviform, straight, ventrally shortly pubescent, with prominent, irregular membranceous margins (Delforge, 1994). The anther is ovate, obtuse to acute; pollinarium ovate to lanceolate in outline, formed by deeply cleft yellow pollinia with or without distinct stalks. Pollinarium may be joined to a ligulate to linear ventral viscidium that leaves a V-shaped notch in the rostellum on removal. The rostellum is narrowly triangular; the stigma is elliptic or transversely elliptic. The ovary is pubescent, erect and obliquely fusiform. The capsule is typically ellipsoid. Seeds are shortly fusiform, curved or sigmoid, with transversely oblong ridged testa cells; the median ones more elongate than the polar cells (Salazar *et al.*, 2003).

Distribution

Spiranthes is virtually a cosmopolitan genus of about 40 species, attaining its maximum diversity in North America with fewer species in Mexico, Central America, Europe, temperate and tropical Asia, Australia, New Zealand and the southwest Pacific islands (Salazar *et al.*, 2003).

Palynology

The pollinia of some species have more or less differentiated stalks, but in others these are absent or indistinct (Balogh, 1982). Where a distinct stalk is present, such as in *Spiranthes cernua*, the tetrads of the stalk portion are imbricating, linear to ligulate, with the pollen grains arranged linearly. The fertile tetrads are orbicular, oval or somewhat tetragonal and compressed. In some species there is little or no differentiation into pollinium stalks, such as *Spirathes sinensis,* and all tetrads are more or less like the tetrads of the stalked species. According to Balogh (1982), there are species-specific differences in the pollen of *Spiranthes*, although the general validity of this assertion has not been demonstrated (Salazar *et al.*, 2003).

Cytogenetics

The genus includes two cytological groups of species; a large number with basic chromosome number $x = 15$ and a smaller group where $x = 22$. Within the former most are diploid, but *Spiranthes cernua* encompasses a tetraploid complex in which some populations undergo regular or irregular chromosome pairing during meiosis (Sheviak, 1982). Martinez (1985) reported $2n = 30$ for a plant of *S. cernua* from Rio de Janeiro, Brazil (probably cultivated), but this number is discordant with those provided in the comprehensive study of Sheviak (1984) and may actually represent *S. odorata* (Salazar *et al.*, 2003). Sheviak (1984) postulated a possible allotetraploid origin for *S. diluvialis* through a hybridisation between two diploid species, *S. magnicamporum* ($2n = 30$) and *S. romanzoffiana* ($2n = 44$), on the basis of morphological and cytological evidence for the origin of the autogamous *S. hongkongensis* from *S. sinesis* and *S. spiralis*. His report of triploid ($3n = 45$) morphologically and phenologically intermediate natural hybrids

between the self pollinating, tetraploid *S. hongkonensis* (4n = 60) and the outcrossing diploid *S. sinensis* (2n = 30) may be seen as an indication of reproductive isolation in *S. hongkongensis* and its recognition as a species in its own right (Salazar *et al.*, 2003). However, autogamous forms of *S. sinensis* appear to have arisen independently in distant locations throughout its wide geographical range e.g. *Spiranthes novae-zelandiae* (Garay, 1982; Catling, 1990). Clarification of the taxonomic and evolutionary meaning of these variants will require cytogenetic studies on a much broader scale (Salazar *et al.*, 2003).

Phylogenetics

A phylogenetic analysis of plastid and nuclear DNA sequence data has confirmed that *Spiranthes* is sister to a mostly Mexican and Mesoamerican clade including the genera *Schiedeella, Mesadenus* and *Dichromanthus* (Salazar *et al.*, 2003).

General ecology

The species of *Spiranthes* are exclusively terrestrial, living in a variety of soil types in open areas under natural or disturbed conditions (Sheviak, 1982). Most species grow in mesic or xeric ground in grassland, tall grass prairies, meadows, grassy and rocky slopes, open or scrubby pine and oak forests, abandoned fields and roadsides. However some show a preference for continuously or intermittently inundated terrain, such as marsh or stream borders, bogs and fens. The species are found from sea level to about 3600m. Flowering occurs throughout the year. Yet each species has a restricted blooming season, with a generally recognised blooming peak during the spring and summer months (in the northern hemisphere, mostly April to September) (Sheviak, 1982).

Uses

A number of *Spiranthes* species have been used for centuries in traditional medicine, as love charms, aphrodisiacs, food and food flavouring, although probably to a limited extent (Arditti, 1992). A few easily grown species, such as *S. cernua* and *S. sinensis*, are relatively common in cultivation (Salazar *et al.*, 2003).

1.7 General introduction to Spiranthes romanzoffiana Chamisso.

Etymology

Named after Romanzoff, a Russian Minister (1754-1826). The type specimen was collected in Alaska, which at that time belonged to Russia (Delforge, 1994)

Synonyms

Neottia gemmipara Smith., *Gyrostachys stricta* Rydberg.

Gaelic translation of *Spiranthes romanzoffiana*: Cuilin gealach

Morphological description

The plant is typically 15-25cm in height, the stem being puberulent above. Basal leaves, numbering 3-6, are linear-lanceolate, pointed and erect. The leaves vary from 7-12cm in length and 0.5-1.3cm in width, often reaching to the base of the inflorescence. The cauline leaves vary from 1-3 per plant. The bracts are puberulent, 10-17mm long, oval and acuminate with sheathing exceeding the ovary. The inflorescence is compact, 3-8cm tall, with flowers arranged into 3 vertical rows in a tight spiral (Plate 1.3). Each inflorescence of *S. romanzoffiana* displays between 12-35 small, white or yellowish flowers. The perianth is typically 8-12mm with the lobes converging into a tube for half of its length. The sepals and petals are elongate. The labellum is oblong, concave, grooved and pinched in the middle, striped with green. Its tip is widened and bent backwards with wavy margins. There are two visible nectaries at the base, the ovary is erect, sessile and typically 8mm long (Delforge, 1994).

Plate 1.3 Three ranked inflorescence of *Spiranthes romanzoffiana*. Photograph taken on the shores of Lough Conn in county Mayo in the west of Ireland in early August 2005.

Root structure

All orchids have a rhizome in the strictest botanical meaning of the word. In *Spiranthes romanzoffiana* it is vertically orientated and can range from several millimetres to several centimetres in length. Work carried out by Gulliver *et al.* (2000) showed that all the underground structures of any size within the species are in fact roots. *Spiranthes romanzoffiana* does not have horizontal rhizomes like those present in its close relative *Goodyera repens*. The genus *Spiranthes* is unique among the British and Irish members of the tribe Cranichideae in only having roots and not a horizontally spreading rhizome. The number of roots on an individual plant varies from between one to five per plant (Sheviak, 1982).

Plate 1.4 Root structure of a single specimen of *S. romanzoffiana*. The plate shows two swollen roots approximately 5cm long. Results from this research show that the number of these swollen roots can vary from 1- 4 and the size ranges from 1cm to 6cm in length. The plant in the image was removed from the shores of Lough Cuilin in county Mayo and translocated to the Irish National Botanic Gardens Glasnevin, Dublin.

Life cycle of Spiranthes romanzoffiana

The green to yellowish-green leaves of *Spiranthes romanzoffiana* expand in the spring, having been originally produced the previous year. They are long and narrow with a hooded tip and have a characteristically rubbery feel (R. Gulliver, 2003, *pers. comm.*). The flower stem emerges from the centre of the rosette in late June, initially protected by a large bract. The inflorescences are normally visible by mid July and are usually fully developed by late July, though the development of new emerging inflorescences can continue into mid August. In September the flowering stems wither and die. In the absence of fertilization the seed capsules become brittle. In some cases the capsule splits, revealing an empty ovary with numerous withered ovules. In North America there have been numerous observations of capsule formation and seed set. The capsule is light brown, ellipsoid, typically 0.8x0.4cm, ascending. The seeds are light brown and are released by mid to late September (Reddoch and Reddoch, 1997).

Many plants produce a lateral bud underground during the months of July and August. This bud emerges close to the stem of the mature plant and is visible by late August. The leaves of the bud expand slowly throughout the autumn, winter and early spring, developing more rapidly in late spring (Gulliver, 2002). As the lateral bud is produced in July and August, there is an overlap in growing years i.e. above ground tissue from a mature plant occurs alongside a shoot, which will reach maturity in the following year. Occasionally two lateral buds are produced. The survival of both of these buds results in the development of twin plants the following year. In a number of cases, plants with three or four buds have been recorded (Gulliver, 2002).

Plate 1.5 A specimen of *S. romanzoffiana* with bud emerging from the base of the plant. This image was taken at Lough Cuilin, Co. Mayo in late July 2004. Observations suggest that this emerging bud will remain as seen above until approximately May / June of the following year, at which point it will grow to form the flowering stem.

Geographic distribution of Spiranthes romanzoffiana.

Irish ladies-tresses orchid is one of the few British and Irish natives with an amphi-atlantic distribution, being found widespread in North America and limited to the western fringes of Europe (Preston and Hill, 1997). Indeed within Europe it is almost entirely confined to the west of Scotland and south western, western and Northern Ireland.

Although not discovered until 1810 in Ireland (Hackney, 1992), it is widely accepted that *S. romanzoffiana* is native, though deliberate or accidental introduction has also been suggested (T. Curtis 2002 *pers. comm.*) Theories to explain the amphi-atlantic distribution of *S. romanzoffiana* include migration across a former land connection between Europe and North America (Dahl, 1963; Hulten, 1953) followed by a contraction in the European distribution during glaciation, with survival in or near the present localities. Another common theory suggests that *S. romanzoffiana* arrived in Europe via long distance dispersal from North America e.g. migratory birds. It is unlikely that humans are responsible for propagule transportation, given the species' remote location (Webb, 1985). The above hypotheses are examined in detail in Chapter 4.

The main populations of *S. romanzoffiana* occur around the Galway-Mayo lakes and Lough Neagh in Ireland, and on Colonsay, Coll and the Outer Hebrides of Scotland. *S. romanzoffiana* has also been noted in one outlying post in Devon, discovered in 1957, though this population has not been recorded since 1987 (Gulliver, 2003 *Pers.comm*) Stewart and Excoffier (1996) categorise the species as scarce, found only in eighteen 10-km squares in Britain. As the only orchid species in Britain widely recognised as absent elsewhere in Europe and with a stronghold in Scotland, Scottish Natural Heritage has designated *S. romanzoffiana* a priority species for conservation (Gulliver, 2002). None of the Scottish localities recorded before 1981 now show the orchid, although new sites have since been discovered. While little is known of the natural fluctuations of the populations, these results have increased concern over the orchid's status in the British Isles.

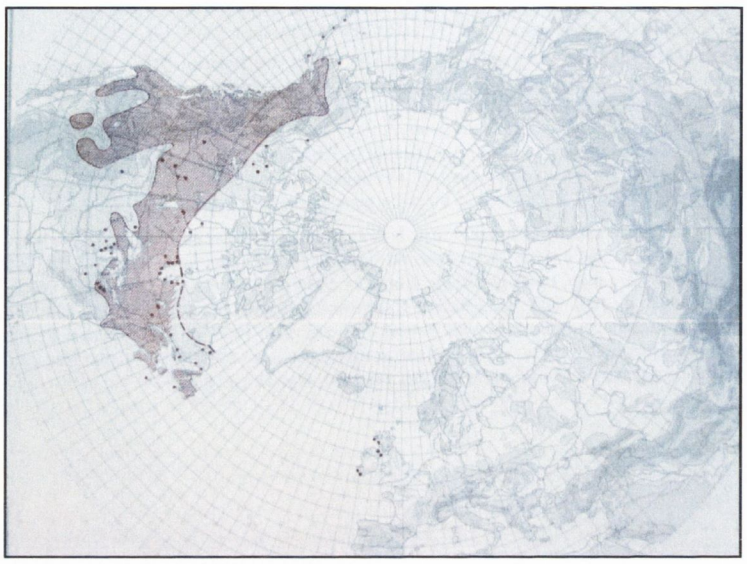

Plate 1.6 The global distribution of *S. romanzoffiana*. The distribution map clearly shows the uneven amphi-Atlantic distribution of the species. Taken from (Hulten, 1953).

Plate 1.7 The European distribution of *S. romanzoffiana*. Taken from (Preston and Hill, 1997). Some uncertainty exists regarding the existence of the population in Devon. New populations discovered during the current research (not included on the map above) increase the range and number of populations in Ireland.

1.7.1 Conservation status in Ireland

Spiranthes romanzoffiana is protected in the Republic of Ireland by the Wildlife Act (1967), under the Flora Protection Order [SI 94 of 1999] and is an Irish Red List species (Curtis and McGough, 1988). In Northern Ireland the species is protected by Schedule 8 of the Wildlife (NI) Order. Despite being classified as 'near threatened', *S. romanzoffiana* is not afforded special protection in Great Britain. It was once included in the British Red Data Book, but was removed on discovery of additional sites in the 1980's. However the species undoubtedly remains a priority for conservation action due to the European importance of its UK populations. In Europe the species is considered a Medium Risk with critically low populations (IUCN/Orchid specialist group). As with all orchids *Spiranthes romanzoffiana* is afforded general protection under CITES.

1.7.2 Current Irish status

Given the restricted distribution of *Spiranthes romanzoffiana* in Europe, Ireland has an international obligation to protect the species. Unfortunately much needed conservation information about this species is lacking, as the biological and genetic characteristics have not been examined in Ireland to date. A notable feature of Irish and Scottish populations is an apparent fluctuation in population size and location. Evidence suggests that population turnover is high, as the species is now absent from several previously known sites, while simultaneously recorded in a number of new sites (Gulliver *et al.*, 2000). Unfortunately due to the lack of detailed mapping of the species in Ireland, variation in recording cannot be ruled out as a possible reason for this apparent high population turnover. A further problem with the Irish data is the lack of any reliable census of populations, including demographic analysis of class sizes.

To date, no information exists in relation to seed set or reproductive biology of Irish populations, however seed set in Irish and British populations has been perceived to be low or non-existent. Seed set has been documented in North American populations (Catling 1982, Larson and Larson, 1997). Limited studies suggest that there is some morphological variation between northern and southern populations in Ireland (Summerhayes, 1968) but this requires further confirmation. Whether this perceived morphological difference has any reproductive or genetic basis is not known. It is clear

that data on habitat specificity, population differentiation, and the perceived difficulties of successful reproduction are urgently needed if *Spiranthes romanzoffiana* is to be successfully conserved in Ireland.

1.8 Aims of this work

This research project aims to gather baseline data on Irish populations of *S. romanzoffiana*. A number of key questions are tackled throughout this study. What habitat or range of habitats do Irish populations of *S. romanzoffiana* occupy? Can they be classified using habitat descriptions available in the literature? What is the current distribution of *S. romanzoffiana* in Ireland? How many populations exist and how has this changed since the species was first recorded in 1810?

Using two types of molecular markers (AFLP and a chloroplast microsatellite) attempts were made to determine the levels of genetic diversity and population differentiation within and between Irish populations of *S. romanzoffiana*. Populations of *S. romanzoffiana* in Scotland and North America were also examined using the same techniques, for comparative purposes. Results of molecular analyses within the study were applied to the varying hypotheses regarding the uneven amphi-atlantic distribution of the species in an attempt to elucidate the most likely explanations for this anomalous distribution. The research further aims to determine the dominant mode of reproduction in Irish populations and ascertain whether there is any evidence of seed production in *S. romanzoffiana*.

The study also aims to clarify the major threats to the future of *S. romanzoffiana* in Ireland. Inferences gleaned from results will input to recommendations for the conservation of *S. romanzoffiana* in Ireland and will be used to update the all-Ireland Species Action Plan (National Parks and Wildlife Service, 2005). The thesis consists of six chapters. Chapter two looks at the ecological characteristics and vegetation association of *S. romanzoffiana* in Ireland. Chapters three and four assess the genetic diversity within and among Irish and North American individuals and use the results to postulate biogeographical relationships between the two continents. Chapter five focuses on the population and reproductive biology, focusing particularly on the possible modes of sexual reproduction and the production of seed in Irish populations.

Chapter 2

2.1 Ecology and Habitats of Spiranthes romanzoffiana in Britain and North America

In Britain *Spiranthes romanzoffiana* is described as occurring on low-lying sites that are at least periodically irrigated and sometimes inundated (Scottish Natural Heritage, 1995). Habitats of *S. romanzoffiana* were surveyed on the Hebridian islands of Coll and Colonsay (Scottish Natural Heritage 1995; Gulliver 1996) using the National Vegetation Classification (NVC) system (Rodwell, 1992). All sites surveyed fall into the categories for mire, wet heath and rush pasture. Many studies in the U.K on the habitat of *S. romanzoffiana* have relied on the Rodwell NVC system. The problem, however, is that much of this work has been conducted on a micro-scale. The NVC system requires larger sample sizes (>2m x 2m) to gain an accurate description on the scale and pattern within the vegetation. To mitigate against potential inaccuracies in describing the habitat and vegetation type based entirely on Rodwell (1992), further vegetation descriptions were employed in this thesis using White and Doyle (1982).

In Ireland, *Spiranthes romanzoffiana* appears to be restricted to low lying damp meadows (<20m in altitude), lakeshores, in seasonally flooded pastures and valley bogs (Curtis and McGough, 1988). In the north of Ireland the distribution is similar, with the plant preferring low lying, damp, seasonally flushed grasslands and lakeshores. One known exception occurs at Gortnagory, County Antrim. This is an area of upland grassland and flush and is the highest known site for *Spiranthes romanzoffiana* in Ireland, at between 225 and 260m ASL.

Horsman (1994) described *S. romanzoffiana* in Scotland as having a distinct habitat of *Molinia caerulea* carpet on old lazy beds grazed by cattle. *M. caerulea* is also listed as the closest associate in 17 Scottish sites surveyed in 1995 (Scottish Natural Heritage 1995). However a study on Colonsay found only one site on a lazy bed (Gulliver 1996). Furthermore, work by Henderson (2001) found that despite being abundant and the second most frequently associated species with *S. romanzoffiana* at the micro-scale,

many sites contained no *M. caerulea* at all. Henderson (2001) found that all site types have an abundance and constancy of the generally low growing *Carex panicea* and an abundance of *Hydrocotyle vulgaris, Ranunculus flammula*, and or *Anagallis tenella.* These species are characteristic of unshaded, soligenous mire on peaty, mildly acidic soils where growth of potential dominants is suppressed by low fertility and grazing pressure (Grime *et al.*, 1990).

In habitats where *Spiranthes romanzoffiana* was most frequent, Henderson (2001) also found a species component tolerant of a degree of waterlogging and typical of neutral/ mildly acidic conditions (Grime *et al.*, 1990). *Caltha palustris*, *Potentilla palustris* and *Equisetum fluviatile* define this habitat and are largely restricted to it. Other wetland species present in many of the Henderson (2001) sites include *Mentha aquatica, Myosotis laxa, Filipendula ulmaria, Senecio aquaticus* and *Iris pseudacorus. Hydrocotyle vulgaris* is constant and is a wetland species suggested to have an ability to exploit sites which are waterlogged all year (Grime *et al.*, 1990), while *Ranunculus flammula,* also constant, is adapted to both submergence and desiccation (Henderson, 2001). The evidence suggests that this habitat type is periodically flooded. Indeed many of the samples used to derive these results were found around a periodically flooded pasture and flat, poorly drained lakeshores (Henderson, 2001).

Henderson (2001) considered *Carex panicea* to be the most widely associated species with *S. romanzoffiana,* at both the large and small scale. *Carex panicea* is characteristically a wetland species, relatively low growing, with a pH range from 4-7.5 (Grime *et al.*, 1990). The low association of *Juncus articulatus* with *S. romanzoffiana* at the micro-scale, despite the presence of the rush in 76% of the samples at the meso-scale, may reflect an ecological preference of the orchid (Henderson, 2001). *J. articulatus* is morphologically variable (ranging in height from 20mm to 600mm), often depending on the level of grazing (Grime *et al.*, 1990). As alluded too earlier it is possible that *S. romanzoffiana* cannot grow in the shade of the tall rushes, but can survive when *J. articulatus* is relatively short. Alternatively, the tufted nature of the rush may make survival and growth of the orchid amongst its stems difficult.

Henderson's (2001) results provide a more accurate depiction of the immediate habitat of *S. romanzoffiana* in Scotland than the NVC descriptions. The study suggests that *S. romanzoffiana* has a preference for seasonally flooded habitats, most notably, inundated lakeshores. The data also demonstrates the species ecological affinity for *Carex panicea* and avoidance of tussock forming species at the micro-scale. It further suggests that the species does not have an affinity to *Molinia caerulea*. However, Henderson (2001) does not account for possible environmental limitations and colonisation abilities of *Spiranthes romanzoffiana*. Some understanding of the habitat preferences of this species can be elucidated by combining the classification techniques described above, though the descriptions fall short of definitively describing the detailed habitat requirements for the species.

Typical habitats of *Spiranthes romanzoffiana* in North America are moist bogs, marshes, meadows, salt flats, thickets, on sandy gravely beaches, but also occasionally in dry woods and dry open hill sides (Luer 1975; Correll 1978; Case 1987; Homoya 1993). Typically a northern species, in the more southern parts of its range in America it is a mountain plant where it occurs up to 3000m and flowers until October (Correll, 1978).

Currently no reliable quantitative information exists on the ecology of *Spiranthes romanzoffiana* in the Republic of Ireland. The paucity of data relating to the species is a concerning issue and represents a serious impediment to its future conservation. Lack of resources and poor knowledge of the species' habitat has most likely been contributory factors here. However the nature of the plant itself must also be considered. It is notoriously difficult to find, particularly without a flower spike. The leaf blades are grass like and blend in well with the surrounding vegetation, thus requiring a very keen eye. Even in full bloom the plant can be difficult to spot. Nevertheless it is important that an effort is made to remedy the current lack of data on the species. It is critical that thorough and precise information is gathered to provide a base for future conservation.

This section of the study focuses on the ecology of *Spiranthes romanzoffiana* in Ireland. Data in this chapter were collected during two field seasons, from 2003 to 2004. During the first field season of 2003, a considerable portion of the time was spent searching for historical and existing populations and surveying individual populations. Due to the

remote location of *S. romanzoffiana* populations and the scarce data available on the species' distribution, this resulted in many hours of frustrating and at times unsuccessful endeavours to locate current populations of *Spiranthes romanzoffiana* in Ireland. It is the aim of this chapter to begin the collection and analysis of data relating to the ecology and vegetation associated with *Spiranthes romanzoffiana* in Ireland.

2.2 Aims

The main aims of this chapter were largely to provide descriptive data, focusing in on the following areas:

- Gather baseline data on the habitat type occupied by *S. romanzoffiana* in Ireland.
- Describe the fundamental soil characteristics of the sites surveyed.
- Assess and describe the vegetation associated with *S. romanzoffiana* in Ireland.
- Determine the land use at the four survey sites and assess whether current management regimes favour the persistence of the species.
- To use the sample sites to help elucidate the most pernicious threats to *S. romanzoffiana* in Ireland.

2.3 Materials and Methods

2.3.1 Survey Area

The initial objective was to gather all available data on the recording and distribution of *Spiranthes romanzoffiana* in the republic of Ireland. Historical data were gathered from the herbaria at Trinity College and the National Botanic Gardens, Glasnevin. Distribution maps and contemporary data were supplied by the National Parks and Wildlife Service (NPWS).

By cross-referencing published information on the species with historical maps it was possible to produce a database and a map of distribution, dating back to the first recording in Co. Cork in 1810. Four historic sites (> 20 years since records were made) were visited. These sites were selected based on reliable data and anecdotal evidence from individuals interested in the status of the species in Ireland. Four new, potentially suitable sites were surveyed for the presence of *S. romanzoffiana*. Visits were also made to sites where *S. romanzoffiana* had been recorded in the last ten years (Table 2.3.1). Contact was initiated with NPWS rangers in these areas, who proved to be an invaluable source of information. By contacting members of the B.S.B.I. (Botanical Survey of the British Isles) it was possible to acquire the most up to date information on population locations and sizes.

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Table 2.3.1 Sites visited between 2003 - 2005. Sites in bold were selected for detailed analysis

County	Location	Grid ref
Known sites		
Mayo	**Knockmore** (Lough Conn)	G2284 / 0815
Mayo	**Corysola bridge** (Lough Conn)	G1980 / 0453
Mayo	**Carraig-a-Moiltin** (Lough Conn)	G1764 / 0511
Mayo	**Drummin wood** (Lough Cuilin)	G2321 / 0481
Historic sites		
Cork	Gouganbarra	W080 / 660
Cork	Lough Glenbeg	V747 / 545
Kerry	Lough Carragh	V7274 / 9297
Kerry	Lough Glanmore	V7720 / 5543
Potential sites		
Mayo	Lough Levally	I5026 / 04357
Galway	Lough Corrib	M4880 / 0885
Mayo	Lough Levalliree	M2065 / 9751
Mayo	Lough Beltra	G0745 / 97541

Criteria for site selection:

1. Reliable information on exact locations.
2. Time since the population was last observed (using NPWS records).
3. Accessibility to site.
4. Potential of site to represent putative habitat for the species in Ireland.

Plate 2.3.1 Map of the four sample populations. The four sites are located in Co. Mayo in the west of Ireland. (Pink:Carraig-a-Moiltin, Green:Corrysola bridge, Yellow:Drummin wood, Red:Knockmore).

2.3.2 Habitat description

The habitat was classified using Rodwell (1991) British Plant Communities Vol. 2: Mires and Heaths, Vol. 3 (1992): Grassland and Montane Communities, Vol. 4: Aquatic Communities, Swamps, and Tall-Herb Fens and White and Doyle (1982): The vegetation of Ireland.

2.3.3 Field sampling method

The number of quadrats sampled at each of the four study sites (Plate 2.3.1) varied according to the number of flowering spikes of *Spiranthes romanzoffiana* observed and

the area of the site being studied. A $1m^2$ quadrat was placed around spikes of *S. romanzoffiana*. Where possible the quadrat was positioned so the flowering stem was in the centre. Due to the low number of flowering spikes available, a series of extra quadrats were randomly placed around the study sites. Positioning of the quadrats was determined using a random walking procedure (Kent and Coker 1992). In this instance a sample point is located taking a random number between 0^{o} and 360^{o} to give a compass bearing, followed by another random number between 1 and 30, to give the number of paces. The point reached by the compass bearing and the number of paces becomes the centre point of the next quadrat to be placed. The procedure is repeated until the required number of random quadrats has been recorded. As the location of each next point is still to some extent dependent on the previous one, this technique is not strictly random. However it was deemed sufficiently unbiased for the purposes of the study

2.3.4 Soil sampling and analysis

Soil samples were collected from the four study sites (Plate 2.3.1) using a steel cylindrical corer, 3cm in diameter and 10cm deep. Where necessary the above ground vegetation was removed prior to insertion of the corer. Three separate soil samples were taken per $1m^2$ quadrat (Table 2.3.2). To ensure consistency, the same procedure was carried out with each soil sample. The three soil samples from each quadrat were then mixed together in a plastic bag to form a composite of the soil sample unit. Compositing sample units into a single sample for analysis is an effective method for obtaining an accurate estimate of the mean value for the population, while reducing cost and analytical time (Hodgson, 1976). The samples were transferred to the laboratory in plastic bags, where 10g of fresh soil was removed for pH measurement. Large roots and vegetation were removed from the sample and the remainder was air-dried overnight at room temperature on tinfoil trays, then 2mm sieved and stored in sealed plastic containers in a dark cupboard at laboratory temperature. The soil was later removed from storage and the samples were analysed for pH and soil organic matter content (loss on ignition).

Table 2.3.2 Number of soil samples collected per site.

Site	No. of quadrats	Total soil samples	Total composites
Knockmore	20	60	20
Corrysola bridge	10	30	10
Carraig-a- Moiltin	20	60	20
Drummin Wood	10	40	10

2.3.5 Soil water status

The soil water status was assessed in the field and was used to describe the moisture condition of the soil profile around the root zone. The wetness of the soil was determined by inspecting randomly sampled cores from within the 1m x 1m quadrats. Excavation below a wet horizon causes water to flow down the exposed face. Flow in some instances was extremely slow and confined to major pores and gaps between large aggregate gravel (>6mm < 4cm). The rate of flow was assessed according to the volume of water lost from a core over a period of five minutes, e.g. flow rate = 100ml / 5 minutes. In some cases a known volume of water was added to a core and the subsequent outflow was then determined using the method above.

2.3.6 Loss on ignition (organic matter content) and pH analysis techniques.

- pH

pH was read using an ion sensitive electrode on three replicates from each soil sample. The pH meter was calibrated daily before use. An automatic temperature probe connected to the pH meter was used to account for variation in temperature. 5g of soil was weighed out, large lumps were removed and 10ml of distilled water were added. The mixture was stirred well in order to form a slurry and left to settle for 15 minutes before reading the pH. As pH is measured on a logarithmic scale, in order to calculate the mean pH for each sample it was first necessary to determine the anti-log (to the

base 10) for each value, calculate the mean of these anti-logged values, and then determine the log (to the base of 10) of the resulting mean value.

- *Loss on ignition*

Loss on ignition gives an estimate of organic matter in the soil by raising the temperature of the soil gradually and causing the ashing of organic matter. The air-dried soil samples were oven dried to a constant weight at 80 ^{0}C. Crucibles that had been stored in a desiccator were weighed and labelled with a pencil. Crucibles were half filled with oven-dry soil and re-weighed, and three replicates were prepared for each soil sample. The crucibles were then placed in a Thermolyne 6000 furnace and heated [1 hour @ 150^{o} C, 30mins @ 180^{o} C, 30mins @ 200^{o}C, 30mins @ 240^{o}C, 1 hour @ 300^{o}C, 5 hours @ 550^{o}C]. The crucibles were then left in the furnace over night to cool. The cool crucibles were then reweighed. The percentage loss on ignition was calculated using the weights of the soil sample before and after being heated in the furnace.

2.3.7 Vegetation description

- A 1m^{2} quadrat was placed around every individual *Spiranthes romanzoffiana* recorded at each of the four study sites (Plate2.3.1).
- All vascular plants within the quadrat were identified, either in the field or in the Herbarium at Trinity College. Species present in the quadrats were identified and their associated abundance values were recorded using the Domin scale, table 2.3.3.
- Height of tallest plant within the quadrat was recorded.

Table 2.3.3 Domin scale (Kent and Coker, 1992)

Value	Domin Scale
+	A single individual, no measurable cover
1	1-2 individuals, no measurable cover
2	Several individuals but less than 1% cover
3	1 - 4% cover
4	4 - 10% cover
5	11 – 25% cover
6	26 – 33% cover
7	34 – 50% cover
8	51 – 75% cover
9	76 – 90% cover
10	91 – 100% cover

2.3.8 Vegetation association analysis

Software employed: PC-ORD version 5 (statistical software compatible with Windows that allows the researcher to perform multivariate analysis of data).

Non-metric Multidimensional Scaling (NMS)

This multivariate analysis measures the association (or similarity) between quadrats and species, and allows study of spatial patterns in vegetation. Ordination places samples in relationship to each other such that samples with similar subjects occupy similar ordination space. The assumption in ordination of vegetation samples is that environmental variables determine the variation among samples. Ordination provides views into high-dimensional space by seeking and displaying the strongest structure (McCune and Grace, 2002). Advantages of NMS are that, being based on ranked distances, it tends to linearise the relation between environmental distance and species distance, relieving the "zero-truncation problem", a problem, which plagues all ordinations of heterogeneous data sets structure. NMS can use a variety of distance measures as input, an appropriate distance measure can therefore be selected for the particular data being analysed. NMS also performs more effectively, with less distortion of model data than for example PCA (Principal Component Analysis), DCA (Detrended Correspondence Analysis). Methods such as PCA and DCA see only a portion of the configuration that fits a limited perspective, as specified by the particular underlying

model. NMS on the other hand can "see" a much wider range of structures (McCune and Grace, 2002).

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

Graphical output is provided from two sources: graphs generated as NMS is running and graphs generated by Graph in the PC-ORD menu. The graph is viewed as a two or multiple dimensional image. The order of the dimensions in NMS is arbitrary, they are not extracted in order of the amount of variance explained by the eigenvalues as in for example, Principal Coordinates Analysis (PCA). Each NMS output is a unique solution.

Parameters selected for NMS analysis:

- Distance measure – Bray-Curtis
- Number of axes (max. = 6)
- 400 - Maximum number of iterations
- Starting coordinates - RANDOM
- 1 - Reduction in dimensionality at each cycle
- 0.20 - Step length (rate of movement toward minimum stress)
- Random number seeds – Used current time
- Number of runs with real data - 40
- Number of runs with randomized data - 50
- Auto-pilot - Slow and thorough
- Stability criterion, standard deviations in stress over last 15 iterations - 0.000010

2.3.9 Other environmental variables measured

Altitude

The altitude at each site was measured using a hand held Garmin GPS 12 XL unit. Accuracy of the readings were verified using Ordinance Survey maps (1:50,000).

Extent and duration of site inundation.

Position of the population in relation to the low and high water mark was assessed at the survey sites. These data were gathered to help determine the extent and duration of inundation of the populations surveyed. The high water mark was determined by observing the limit of silt deposition from the lake water. These data were corroborated by information from local landowners and repeat visits to the sites throughout the winter months. Photographic evidence of high and low lake water levels were gathered throughout the winter and summer months. These were used to verify the extent of lakeshore flooding and the duration of inundation experienced by *S. romanzoffiana* at the study sites.

Land use

Land use was determined by consulting local landowners and county council records. From a conservation point of view this raised some interesting questions. On a number of occasions there appeared to be conflicting information regarding the ownership and use of the land. Observations of land use were made throughout each year from 2002-2005. Ease of accessibility to the general public, the presence of vehicular traffic and recreational usage (e.g. angling) were all noted at each of the four study sites. Data from these observations although somewhat anecdotal are useful in the assessment and implementation of habitat management plans.

Grazing

The level of grazing was measured on a subjective scale. Observations made at each site and information acquired from the local farmers and landowners provided adequate data

on the extent and timing of grazing at all of the surveyed sites. Grazing by invertebrates was determined by close observations on randomly selected plants at each of the four study sites. 20% of all flowering plants were inspected for invertebrate grazing. Damage by invertebrates, where it occurred was distinctive and easily detected with the naked eye.

Grazing scale from 1-4

1. Lightly grazed by invertebrates.
2. Heavily grazed by invertebrates.
3. Lightly grazed by livestock (Cattle, sheep and Goats).
4. Heavily grazed by livestock.

Table 2.3.4 Summary of variables measured

Variable	Methods	Data type	Category scale
Vegetation description	Observed in field and lab	Categorical	Height (cm) of associated species.
Soil pH	Ion electrode in lab	Quantitative	Proportion of hydrogen ions
Soil loss on ignition	Furnace in lab	Quantitative	% organic matter content
Soil particle characteristics	In the field	Categorical	Small (<2mm) - large (>6mm), Rounded to angular.
Extent of grazing	Observed in field	Categorical	Scale from 1 –4 / Site.
Possible threats	Observed in field	Anecdotal	Anecdotal
Water status	Observed in field	Quantitative	Water loss (ml) / 5mins /39.25cm3)
Altitude	Observed in field	Quantitative	Metres above sea level
Inundation	Observed in field	Quantitative	Duration of flooding in months.

2.4.1 Results

The four sites surveyed were in sheltered bays, situated on low-lying, (<20m ASL) lakeshores. The plants were found growing in a narrow strip of open habitat (<10m wide), between the lakeshore line and the adjacent Birch, Alder and Willow stands. The

soil at the four sites was moist to wet throughout the survey period. In August 2003, after a period of 10 days without rain the soil still remained moist. The substrate was extremely coarse and had a high mineral content (Table 2.4.1). The soil at Corrysola bridge was almost exclusively lake sediment with no trace of clay particles. The median soil pH values for the four sites ranged from 5.8 – 6.8 and the organic matter values ranged from 0.36% – 1.9% (Table 2.4.1). This suggests that the species favours mineral rich soils, with a neutral to high pH value.

2.4.1 Summary of site descriptions

Knockmore, Lough Conn

Habitat description

This site is located on a sheltered bay on the north Eastern Shore of Lough Conn, it is periodically flooded during the winter and flooding can persist for up to four – five months. *Spiranthes romanzoffiana* is found in an open area between the goal posts at the end of a disused soccer pitch (Plate 2.7a) where there appears to be little competition from the surrounding Alder (*Alnus glutinosa*) stand. The median soil pH value for Knockmore was 6.8. The median organic matter content was 0.75%. The soil particles are gravely, rounded to sub-rounded and vary in size from very small stones (2-6mm) to medium stones (Table 2.4.1).

Using Rodwell (1992) this habitat can be loosely described as fluctuating between M 22 *Juncus subnodulosus- Cirsium palustre* fen-meadow and an M29 *Hypericum elodes – Potamogeton polygonifolius* soakway. On a more micro scale, that is the $1m^2$ quadrat scale, White and Doyle's (1982) work on the "Vegetation of Ireland" provides a useful vegetation classification type. They describe *Hydrocotyle – Baldellion* as a vegetation type of mesotrophic and oligotrophic habitats with periodic alternation between wet and dry phases. The strong fluctuating water table is very distinctive in their description. *Baldellia ranunculoides, Hypericum eloides, Hydrocotyle vulgaris, Eleocharis multicaulis,* and *Anagalus tenella* are also used by White and Doyle (1982) in the classification of this type of habitat. Rodwell (1992) describes the *Hydrocotyle –*

Baldellion (M30) habitat type as being floristically similar to the M29 habitat described above. The *Hydrocotyle – Baldellion* description is the most appropriate description of the habitat occupied by *Spiranthes romanzoffiana* at Knockmore.

Current Site Use and Management

The area is predominantly used for recreation, including walking, boating, and fishing. It is an open and unprotected amenity area and therefore the species is vulnerable to potential damage - intentional or accidental. The area in front of the goalposts (formerly a *Spiranthes romanzoffiana* site) was mown by the county council in late August, thus any potential reproduction by seed was eliminated. Although the species is protected in Ireland there appears to currently be little regard for the species at this site.

Potential threats

As an amenity area, there is a definite threat of physical damage from the pressure of visitors. Evidence of vehicular damage was noted at the site, where large track marks from heavy diggers were observed in late August 2003. This physical damage appeared to contribute significantly to the destruction of *Spiranthes romanzoffiana* plants, before seed capsules had formed. The presence of vehicles on the site may also cause damage to any lateral buds forming below the ground. Mowing of the grass on the football pitch at inappropriate times of the year may also contribute to the decline of the species at this site. Grazing on this site appears to be restricted to invertebrates and therefore is considered to be a low threat.

Corysola bridge (Sandybay)

Habitat description

This site is located on a sheltered bay on the southern shores of Lough Conn, 200m west of Corrysola Bridge. The site is intersected by a river from the Attiapleton lough as it flows into Lough Conn at this point. It is periodically flooded during the winter and flooding can persist for up to four to five consecutive months, typically between

December and April. The median soil pH value was 6.4. The median value for soil organic matter content was 0.36%. The soil particles were gritty, rounded to sub-rounded to sub angular. The size range from very small stones (2-6mm) to small stones (6mm-2cm) (Table 2.4.1). Using Rodwell (1992) the descriptions most appropriate range from an M 25 *Molinia caerulea-Potentilla erecta, Angelica sylvestris* sub-community to an M 26 *Molinia caerulea-Crepis paludosa* mire *Sanguisorba officianalis* sub-community.

Current Site Use and Management

As this is a sandy cove and seen as an amenity area, there is some recreational use. There is currently no specific management visible, apart from the grazing action of transient goat populations. Observations suggest that grazing by goats is frequent and may be detrimental to the population.

Potential threats

Potential threats to this population include unmanaged grazing by goats, and disturbance by tourists.

Carraig-a-Moiltin

Habitat description

This site is located on a sheltered bay on the eastern-shore of Lough Conn. It is periodically flooded during the winter for a period of four – five months. *Spiranthes romanzoffiana* was recorded from the lakeshore all the way up to the Birch (*Betula pubescens*) and Alder (*Alnus glutinosa*) stand, 60m inland from the Lough. The median soil pH for the site was 5.8. The median organic matter content was 1.5%. Soil particles were gravely, rounded to sub-rounded. The particles range in size from very small (2-6mm) to medium stones (2-6cm) (Table 2.4.1).

Using the Rodwell (1992) and White and Doyle (1982) classification systems the Carraig-a-Moiltin site can be described as similar in its composition to the Knockmore site.

Current Site Use and Management

The habitat at this site is heavily grazed by cattle and is used to moor small boats for anglers. Management appears to be minimal and there has been some discussion locally regarding potential development at the site. The possible construction of a lakeshore marina (local farmer, *pers comm.*) would in all likelihood destroy this *S. romanzoffiana* habitat. Intermittent cattle grazing during the flowering season appears to be problematic for *S. romanzoffiana* at this site as the cattle completely remove the flowering spike thus eliminating the opportunity for sexual reproduction.

Threats

During several visits to the site in August and September 2003 and 2004, it was obvious that vehicles had crushed several of the flowering spikes. Cattle grazing on the land in late summer 2003 also appear to have had a serious detrimental affect on the *Spiranthes romanzoffiana* population. Of the 13 plants recorded in July and August 2003, just two remained in September 2003. Cowpats were abundant throughout the site. It seems likely that the flowering spikes had been grazed or trampled, rendering seed production impossible.

Drummin Wood, Lough Cuilin.

Habitat description

The site is located on the Northwestern shores of Lough Cuilin, Co Mayo and is inundated throughout the winter for up to four - five months. The site is surrounded by a stand of *Betula pubescens* and *Alnus glutinosa*. The alder stand appears to be encroaching on the open space between the low and high water mark. Observations made in 2004 and 2005 suggest that the alder Carr was becoming the dominant vegetation type

on the site. The median soil pH value was 6.2. The median soil organic matter content was 1.9%. The soil particles are gravely, rounded to sub-rounded and vary in size from very small stones (2-6mm) to medium stones (2-6cm) (Table 2.4.1). The phytosociological assemblage of the Drummin wood site was difficult to classify. The habitat is somewhat linear along the lakeshore and is interspersed with pockets of grassland and rush pasture. Using Rodwell (1992) and White and Doyle (1982) the habitat ranges from an MG 9 *Holcus lanatus-Deschampsia cespitosa* grassland to MG 10 *Holcus lanatus-Juncus effusus* rush pasture to *Hydrocotylo – Baldellion* seasonally inundated habitat.

Current Site Use and Management

The site is not easily accessible and therefore does not appear to suffer from trampling by anglers or walkers. No grazing by livestock was noted at the site, grazing scale was 1. Thus the Drummin wood site appears to be the least disturbed of all sites surveyed and therefore represents the most intact and stable population of *S. romanzoffiana*. This is reflected in the relatively large number of plants present at this site. There does not appear to be any existing management regime in place at the site.

Threats

Competition and potential shading caused by the encroachment of *Alnus glutinosa* and *Betula pubescens* onto the site may reduce the population size of *S. romanzoffiana* at this site.

Table 2.4.1 Environmental variables recorded at the four study sites. n = the number of replicate quadrats per site. The ranges for each site are included in brackets.

Location	Knockmore	Carraig-a-Moiltin	Corysola bridge	Drummin wood
Median associated veg height (cm)	**18** (n=5, 15 -22)	**8** (n=6, 2-13)	**6** (n=5, 2-10)	**20** (n=5, 10 – 30)
Median soil pH	**6.8** (n=20, 5.3-7.8)	**6.4** (n=20, 5.8 – 6.9)	**5.8** (n=10. 5.4- 6.2)	**6.2** (n=10 5.8-6.8)
Median soil loss on ignition (O.M.C. %)	**0.75** (n=20, 0.27-1.17	**1.5** (n=20, 1.2-2.0)	**0.36** (n=10, 0.3-0.5)	**2** (n=10, 1.6 –2.3)
Soil particle characteristics	2mm-6cm-gravel-Rounded	2mm-6cm-gravel-rounded	2-6mm-grit-rounded to angular	2mm-6cm - gravel-rounded to angular
Extent of grazing (scale 1-4) / site	1	3	3	1
Water status	Moist to wet	Moist to wet	Moist to wet	Wet
Altitude	<20m	<20m	<20m	<20m
Inundation	4-5 months/year	4-5 months/year	4-5 months/year	4-5 months/year

2.4.1 Ordination

An NMS ordination using a 2 un-rooted axes gave the lowest stress values (table 2.4.2) for the data set of the four studied populations. The data set consisted of the species recorded within $1m^2$ quadrats at each of the four sites (Plate 2.3.1). The final stress for a 2-dimensional solution = 16.5 (table 2.4.2). The two axes are displayed in figures (2.4.1 and 2.4.2). The species and site data are presented in two separate figures to aid clarity. Figure 2.4.1 shows the sites separated into three clear groups. Knockmore and Carraig-a-Moiltin are spread across axis 1 but show only moderate separation on axis 2. The Drummin wood population is clustered together on both axes 1 and 2. The Corysola bridge population also cluster together. Corysola bridge and Drummin wood are not widely separated on axis 1 though they separated on axis 2.

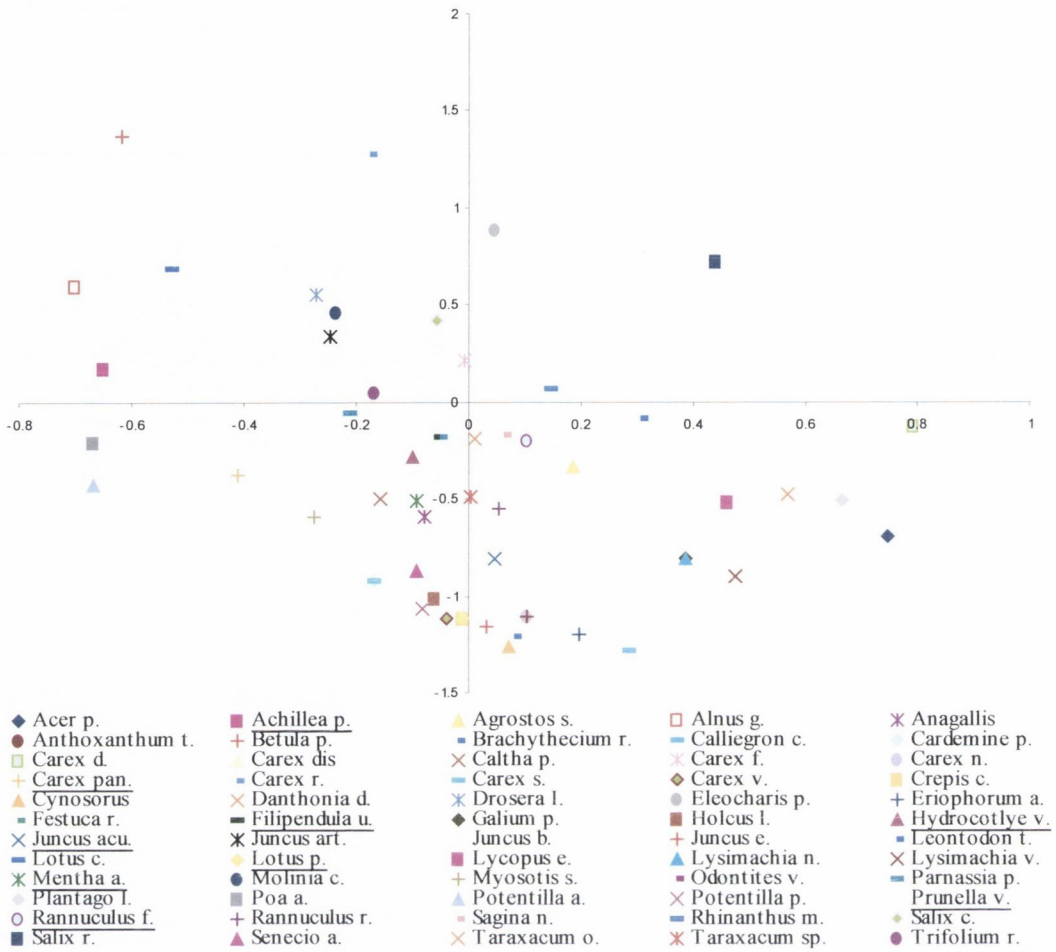


Figure 2.4.1 NMS graph of the associated species distribution at the four study sites. The underlined species were consistently associated with *S. romanzoffiana* at all four sites. See appendix 1 for list of complete species names.

Table 2.4.2 Results for the NMS ordination.

	Stress in real data 40 runs			Stress in randomised data Monte Carlo test, 50 runs			
	Minimum	Mean	Maximum	Minimum	Mean	Maximum	p value
Axes							
1	32.044	40.019	54.994	36.848	47.179	55.766	0.0196
2	16.476	18.395	22.477	21.845	28.097	34.625	0.0196
3	11.995	13.134	14.608	16.47	21.8	31.511	0.0196
4	9.772	10.718	14.065	11.532	19.674	53.615	0.0196
5	7.492	9.927	15.983	8.883	16.807	45.028	0.0196
6	6.194	10.477	13.651	7.769	16.109	33.728	0.0196

Final stress for 2-dimensional solution = 16.47568

Final instability = 0.00033

Number of iterations = 400

p = proportion of randomized runs with stress < or = observed stress

i.e., p = (1 + no. permutations <= observed)/(1 + no. permutations)

2.5 Discussion

All sites visited experience inundation during the winter for a period of four to five months (plates 2.7 a, b and c). Winter flooding on the sites may be an important factor in maintaining populations of *Spiranthes romanzoffiana*. Work by Henderson (2001) suggests that *S. romanzoffiana* is unable to tolerate the shade of tall growing rushes, e.g. *Juncus articulatus*. Henderson (2001) goes on to postulate that *S. romanzoffiana* may be able to grow alongside tall rush species, if the rushes are kept short by grazing. It is possible that the seasonal inundation acts as a thinning mechanism for more invasive and dominant species, thus, in effect allowing *S. romanzoffiana* to grow uninhibited by surrounding vegetation. However results from this study suggest that *S. romanzoffiana* may not be affected by surrounding taller vegetation. The median height of the associated vegetation at the Knockmore and Drummin wood sites was 18 cm and 20 cm respectively (Table 2.4.1). Further study is needed to assess the species' ability to cope with more vigorous surrounding species and to determine whether seasonal inundation is an integral part of the species ecological requirements. In turn more data on community structure, vegetation association and the ability of *S. romanzoffiana* to use gaps or microsites for colonisation between taller vegetation is required.

Results from this research suggest that *S. romanzoffiana* in Ireland occupies a number of marginally variable habitat types associated with flooded lakeshore habitats. The Corysola bridge site is an open site dominated by *Calluna vulgaris*, *Drosera intermedia* and intermittent clumps of *Sanguisorba officinalis*. The soil pH at this site was 5.8 (Table 2.4.1) almost an order of magnitude lower than recorded from the other three sites. *In vitro* germination experiments by Arditti (1992) suggest that North American specimens of *S. romanzoffiana* prefer a soil pH from 4.7-5. At the other end of the scale the mean pH value at Knockmore was 6.8 (Table 2.4.1), a value more typical of base-rich soils. These data suggest that *S. romanzoffiana* is tolerant of a large range of soil pH levels. These baseline results suggest that soil pH may not be a limiting factor in the ecological requirements of *S. romanzoffiana.*

Corysola Bridge had the lowest percentage organic matter content (0.36%). The other three sites ranged in value from 0.75% to 1.9% (Table 2.4.1). The overall assumption from the data suggests that Irish populations of *S. romanzoffiana* may have a preference for soils very low in organic matter content, though this is far from conclusive at present. Further analysis of a broader range of samples from across the species Irish distribution would help to clarify this issue.

The effect of grazing on the populations studied was carried out in a somewhat subjective manner. The occurrence and extent of grazing on each site was determined by field observations and through information obtained from local farmers and landowners. Carraig-a-Moiltin and Corysola bridge both experience relatively high levels of grazing by sheep and cattle during the summer months. Whether this is impacting on the species persistence in Irish populations is not fully understood at present. Gulliver (2003) in his work on the relationship of *Spiranthes romanzoffiana* to grazing in the West of Scotland suggests that the species is tolerant of a wide range of grazing regimes. Gulliver (2003) demonstrated an increase in population size on two heavily grazed sites between 1999 and 2001. These results however need to be viewed with some caution, as plants in 1999 may have been dormant and belowground or may not have been in flower and were therefore overlooked. The current data for the Irish populations is not extensive enough to determine the negative or positive effects of grazing. It must however be noted that the populations at Knockmore and Drummin wood in terms of flowering plant numbers were

consistently higher than the two other sites surveyed (2.3.1). This may be associated with very low levels of grazing at Knockmore and Drummin wood.

The ordination of the vegetation data (Figure 2.4.1 and 2.4.2) suggests that the four sites form three floristic groups. This is consistent with the site description summaries in section in (section 2.4.1). Knockmore and Carriag-a-Moiltin form a floristic group with very strong association on Axis 2 the two sites show a wide spread along Axis 1. Drummin wood and Corysola bridge form two distinct groups along Axis 1 and Axis 2, though the separation is minimal along Axis 1.

2.6 Conclusion

The vegetation and ecological survey of the four sample populations of *Spiranthes romanzoffiana* in the Republic of Ireland suggest that the species is confined to lakeshore habitats with varying plant community groups (section 2.4.1). The species appears to tolerate a range of soil pH levels from mildly acidic to mildly alkaline, with a very high mineral content (table 2.4.1). The vegetation associated with populations of *S. romanzoffiana* in the Republic of Ireland fits into a number of NVC communities (Rodwell, 1992) and White and Doyle (1982) 'Vegetation of Ireland' communities. However these vegetation communities are all within the range of lakeshore, mire and rush to wet grassland types. There are a number of key associated species found consistently with *S. romanzoffiana* in the Republic of Ireland (figure 2.4.2). These key species are similar to those described by Henderson (2001) and suggest some ecological similarity between southern Irish and Scottish habitats.

Survey work carried out in Northern Ireland in 2001 by the Environmental Heritage Service (EHS) at a number of key *S. romanzoffiana* sites (Gortnagory, Corraslough Point, Brookend and Lough Beg). The EHS work revealed vegetation types more akin to the Scottish communities described by Henderson (2001) and Horsman (2002) then to the communities described for the Republic of Ireland. The Gortnagory site for example is the highest known site for the species in Ireland (225-260m ASL). There is no lakeshore inundation at this site, however most of the plants observed at this site are found growing in flushes or in marshland. Using the Rodwell (1992) the EHS site survey report suggests this community to be broadly equivalent to NVC type M10a, *Carex*

dioica-Pinguicula vulgaris Carex demissa- Juncus bulbosus sub-community. This is described as a distinctively, calcicolous flush vegetation in which small sedges and dicotyledons predominate (Rodwell 1992). To date *S. romanzoffiana* has not been recorded in this vegetation type in the Republic of Ireland. A more extensive all Ireland and UK vegetation survey may reveal a northerly shift in the species distribution. Data from Gulliver (2003) and Scottish Natural Heritage (1995) indicate that Scottish populations of *S. romanzoffiana* can tolerate a wider range of ecological niches (e.g. dune slacks and old disused lazy beds) then those associated with the species in the Republic of Ireland. It may be that there are number of ecotypes found across the species European range. The perceived narrow ecological range occupied by *S. romanzoffiana* in the Republic of Ireland and its conservation implications are discussed in chapter 6.

A.

B.

C.

Plate 2.7 (A,B,C). Three pictures taken at the Knockmore field site: (A) August 2004- Low water, (B) November 2004 –lake water level rising, (C) February 2005- site is completely flooded, all plants are submerged under water.

Chapter 3

Assessment of genetic diversity in Irish and North American populations of *Spiranthes romanzoffiana*.

3.1 Introduction

Very little is known about the patterns of genetic variation in species of Orchidaceae (Wong and Sun, 1999). This lack of data may be associated with the many difficulties in locating and studying orchid populations. Many orchids defoliate and remain dormant after a flowering season such that only an inconspicuous tuber below ground or partly above ground can be found. Their sporadic occurrence makes it especially difficult to locate them because individuals are usually found in small colonies or scattered singularly over wide areas (Rogaly, 1975). The sporadic occurrence of orchids may well be a product of their obligate mycorrhizal association, whereby the underground parts of the plant assimilate nutrients and carbon from the fungal host. Consequently, the aerial photosynthetic parts of the plant may not be present every season. Indeed some orchids are wholly saprophytic, hence the sporadic appearance of for example, *Epigonium aphyllum* in the U.K. (Delforge, 1994).

In general, successful management and preservation of rare, threatened or endangered species depends on the complete understanding of the species, including levels of genetic structure and variation (Wallace, 2002). Knowledge of population genetics provides an historical perspective of evolutionary changes that characterises a species and allows us to predict how populations will respond to future events of natural or artificial origin (Berry 1971; Vrijenhoek 1987; Huenneke, 1991). When used in conjunction with other information about a species' ecological requirements, studies of population genetic structure provide effective foci for conservation and management by defining evolutionary significant units (Ryder, 1986). This is often imperative, particularly as there are rarely sufficient resources to protect all the genetic diversity in a species, making the choice of what species are conserved very important.

Molecular markers are now used regularly to provide genetic information for use in programmes concerned with the conservation of rare or endangered species. Conservation genetic research has been used to elucidate phylogeographical relationships among populations (e.g. Evans *et al.*, 2001; Loeffler and Morden, 2003), assess the extent and consequences of genetic erosion, describe genetic structure and differentiation of populations of threatened plant species (e.g. Ayres *et al.*, 1997; Cardoso *et al.*, 1998; Archibald *et al.*, 2001; Bonnin *et al.*, 2002; England *et al.*, 2002), clarify taxonomy to identify which lineages to conserve (e.g. Soltis and Gitzendanner 1998; Forrest *et al.*, 2004), identify clonal diversity in rare plant species (Bushakra, 1999; Brzosko, 2002), determine the cause and effects of low genetic variation in many threatened plant species (e.g. Lammi *et al.*, 1999; Buza *et al.*, 2000; Lutz *et al.*, 2000), and determine the origins and or population genetic structure of some species, including *Spiranthes* (e.g. Arft and Ranker 1998; Szalanski *et al.*, 2001).

Many rare and endangered plants possess reduced genetic variability compared with widespread taxa and are differentiated into genetically unique populations adapted to local conditions for survival and growth (Ellstrand and Elam, 1993; Krauss, 2000). Other species have been shown to display high levels of genetic diversity with high or low population differentiation despite small numbers (Rosetto *et al.*, 1995; Maguire and Sedgley, 1997). Out-crossing species commonly have higher levels of genetic diversity and lower levels of differentiation between populations than selfing or clonal plants (Rosetto *et al.*, 1995; Hamrick and Godt 1996; Palacios *et al.*, 1999).

Work by Forrest *et al.* (2004) on the population genetic structure of *Spiranthes romanzoffiana* revealed a genetic-geographic split in the species' European range. Using cpDNA and AFLP markers they concluded that the Northern populations (Scotland, including Coll, Vatersay and Barra) were fixed for one chloroplast haplotype but showed high levels of genotypic diversity consistent with sexual reproduction (proportion of distinguishable genotypes, P_D = 0.98). Their more southerly populations (Scotland including Colonsay; Ireland, including Antrim, Fermanagh, Derry, Tyrone, Galway and Mayo) showed fixed differences from their northerly populations in their chloroplast haplotype and for 10 AFLP markers. They suggest that the genetic uniformity detected in their southern group was consistent with vegetative, agamospermous or autogamous

reproduction, and/or extreme population bottlenecks, but were unable to distinguish between the relevant possible explanations.

Spiranthes romanzoffiana in North America has been described as a protandrous, out-crossing species, reliant on long-tongued bumblebees for successful cross-pollination. Observed levels of autogamy in the species in its North American range are extremely low (Catling, 1982). Thus the occurrence of purely vegetative, agamospermous or autogamous plants in all the Irish populations seems unlikely. To examine this conclusion, leaf tissue samples were collected from a broad Irish geographical range. Using AFLP markers the genetic structuring of a larger geographic range of Irish populations then studied by Forrest *et al.* (2004) was examined. The data obtained from the genetic fingerprinting allows a number of complementary analyses to be performed. Genetic distance measures, gene diversity and population differentiation estimates were used to describe the genetic variation within and among Irish populations of *Spiranthes romanzoffiana*.

3.1.1 Amplified Fragment Length Polymorphism (AFLP) Marker Analysis

AFLP is a DNA fingerprinting technique that uses a combination of fragmentation of genomic DNA through the action of restriction enzymes, and the Polymerase chain reaction (PCR) to amplify the fragments. AFLPs are dominant markers. The technique, first described by Vos *et al.* (1995), exploits the advantages of technical simplicity and generation of large numbers of markers spanning the whole genome without any prior knowledge about it. Thus AFLP allows the relatively quick development of markers, which is often important in conservation and endangered species management (Gaudel *et al.*, 2000).

AFLP analysis requires only a small amount of DNA (typically 250ng). Thus it is a useful technique in the study of rare plant species, where the availability of plant material may be limited (Qamaraz-Zaman *et al.*, 1998). The AFLP band patterns, or fingerprints, can be used for many purposes, such as monitoring the identity of an isolate or the degree of similarity among isolates. Polymorphisms in band patterns map to specific loci, allowing the individuals to be genotyped (AFLP ^{TM} Plant Mapping Protocol). The

process uses restriction enzyme digests, ligation of adaptors to fragments to modify genomic DNA and amplification using PCR (Ridout and Donni, 1999). Visualisation and analysis of the resulting polymorphic fingerprints is possible using radioactive labelling, silver staining or fluorescent labelling (Karp *et al.*, 1996; Mueller and Wolfenbarger, 1999).

Issues with the use of AFLP markers.

A major assumption with dominant marker analysis and many population genetic statistics in general, is that genotypic frequencies are in Hardy-Weinberg equilibrium at each locus. 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). Small, inbreeding populations or populations with a high degree of immigration, may exhibit a departure from the Hardy-Weinberg equilibrium, though there is no way of evaluating this in the AFLP analysis of a single generation (Lynch and Milligan, 1994). The assumption that Hardy-Weinberg equilibrium exists is therefore a limitation to the use of dominant markers to describe population genetic structure. There are a number of additional problematic assumptions that exist with the use of dominant markers. 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 the locus is amplifiable so each band represents a different loci.
- there is no co-migration of fragments from different loci.
- loci are independent

Applications of AFLP.

AFLP was originally developed as a method of genomic mapping to "bridge the gap between genetic and physical mapping" (Vos *et al.*, 1995). The technique has since proven to have a wide range of applications, ranging from assessment of the degree of

relatedness or variability among cultivars (AFLP ™ Plant Mapping Protocol) to diversity studies and phylogenetic reconstruction (Hodkinson *et al.*, 2000; Hodkinson *et al.*, 2002). AFLP techniques have been used to construct genetic maps of a wide range of crop species (Becker *et al.*, 1995; Wang *et al.*, 1994; Levi and Rowland, 1997; Costa *et al.*, 2000; Arcade *et al.*, 2000; Cato *et al.*, 1999; Remmington and O' Malley, 2000). Identification of hybrids and the extent of hybridisation have also been detected (Hodkinson *et al.*, 2002).

AFLP has proved very useful in distinguishing closely related taxa, in particular those found to have few distinguishing morphological traits or insufficient variation for sequencing analysis (De Reik *et al.*, 2001; Hodkinson *et al.*, 2002). AFLPs provide a powerful means of fingerprinting individual clones as well as establishing genetic distances and relationships among clones based on genetic similarities (Hills *et al*, 1996). AFLP techniques have allowed the phylogenetic reconstruction of relationships within closely related species (Mueller and Wolfenbarger, 1999) and have helped contribute to diversity analysis for breeding programmes and conservation strategies (Webber *et al.*, 1999; De Reik *et al.*, 2000).

Advantages of AFLP

- The technique requires small quantities of DNA to produce high resolution, reproducible data with low error levels (Vos *et al.*, 1995; Muller and Wolfenbarger, 1999; AFLP ™ Plant Mapping Protocol).
- Unlike randomly amplified polymorphic DNAs (RAPDs) which use multiple, arbitrary primers and may lead to unreliable results, the AFLP technique uses two primers and gives reproducible results.
- The multiple loci can be screened at random along the genome (Vos *et al.*, 1995)
- Many restriction fragment subsets can be amplified by changing the nucleotide extensions on the adaptor sequences. Hundreds of markers can be generated reliably.
- High resolution can be obtained because of the stringent PCR conditions.
- The AFLP technique works on a variety of genomic DNA samples.
- No prior knowledge of the genomic sequence is required.

Potential problems associated with AFLP

- Practical problems

- The relatively high cost of reactions
- Fragment scoring in fingerprint is subject to "interpretation" which can lead to errors in the analysis (Travis *et al.*, 1996).
- Different primer combinations produce fingerprints of varying information quality.
- Scoring of fragments from the fingerprint data is often time-consuming.

Data output problems

The majority of AFLP markers are dominant. A dominant marker is one, which is detected as either present or absent (the AFLP investigator produces data in the form of a presence/absence matrix). This limits the usefulness of the data, as the ability to distinguish between homozygotes and heterozygotes is lost (Queller *et al.*, 1993, Mueller and Wolfebarger, 1999). A small proportion of AFLP markers (4-15%) have however been found to be co-dominant (Mueller and Wolfebarger, 1999). A technique has been developed to utilise co-dominant markers and is known as "microsatellite AFLP" or Selective Amplification of Microsatellite Polymorphic DNA (SAMPL); Mueller and Wolfebarger, 1999; Singh *et al.*, 2002). Homology can be a problem with AFLP data, as co-migrating bands are assumed to be homologous, though this may not always be the case. AFLPs may not be appropriate for analysis of unrelated or distantly related organisms due to high levels of polymorphism that is routinely detected, since this can lead to errors in data interpretation (Hodkinson *et al.*, 2000).

3.2 Aims

This section of the research aims to compare levels of genetic variability using AFLP markers within and between a number of sample populations from Ireland, Scotland and North America in an attempt to determine if the level of diversity in Irish populations is equivalent to diversity levels in the species putative natural range of North America. Data from this study inputs to a broader understanding of the population and reproductive

biology of the Irish plants chapter 5. These data will contribute to positive conservation initiatives for the species and the species habitats on the island of Ireland and will also be used to provide information relevant to the updating of an all-Ireland species action plan for *Spiranthes romanzoffiana*.

3.3 Methods

3.3.1 Sampling

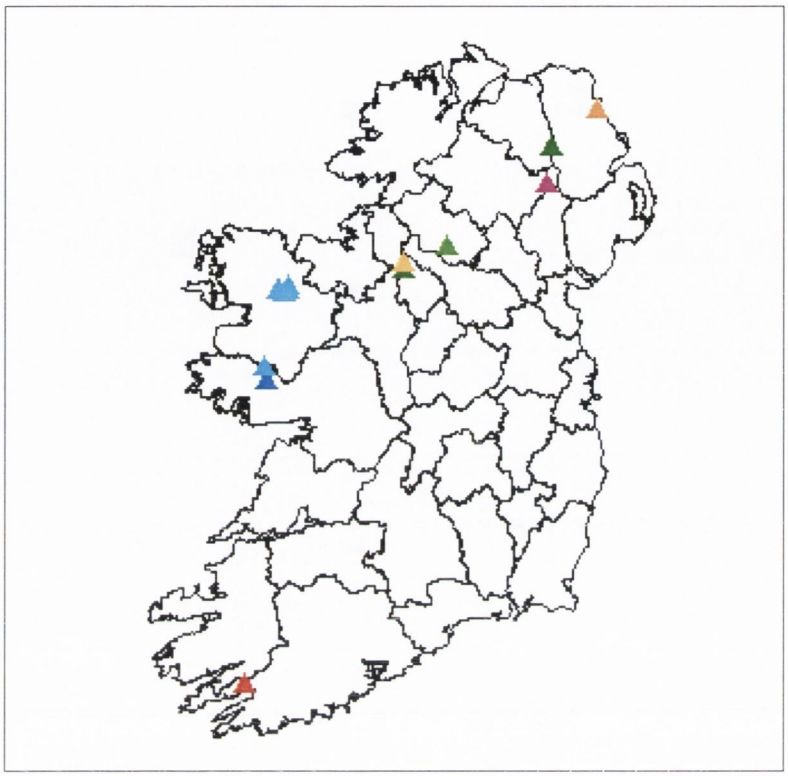
To investigate the comparative levels of genetic diversity between populations in a taxon, a large number of populations must be sampled (Kay and John, 1997). Populations across the range of the species should be incorporated into the analysis; this will allow the results to be placed in the context of the species as a whole. Samples were therefore collected from as many Irish, Scottish and North American populations as possible. Where possible, i.e. in populations where there were more than five plants, sampling was carried out randomly. Without the random sampling strategy, samples would contravene the assumptions of the population genetic analysis. In populations with less then five plants, the appropriate leaf material was removed from all the individuals. Only intact, undamaged and disease free leaves were collected. Leaf samples were harvested from the newest growth on each individual. Approximately 3–4cm^2 of intact leaf was carefully removed and placed into clear, sealable plastic sample bags, containing Type III indicating silica gel for rapid desiccation of leaf material. This method allowed a period of time between leaf collection and DNA extraction. In most cases DNA was extracted within 12 months of collection.

Samples from the Republic of Ireland were collected during August 2003 and August 2004. Leaf samples from the North of Ireland and Scotland were donated to this project in 2003 by Dr. Pete Hollingsworth. These samples were collected in 2001 as a part of an M.Sc research project, and had been stored at the Royal Botanic Gardens, Edinburgh. DNA was extracted from these samples in late 2003. At the time of extraction the samples had been in silica gel for almost two years. Unfortunately the resulting DNA was of poor quality and could not be used in the analysis of genetic variation in Irish populations.

Samples from Canada were collected during a two-week period from late July to early August 2005. In planning the Canadian fieldtrip and choosing sample sites, contact was made with a number of reliable local Canadian botanists. A Canadian field guide (Newcomb, 1977) and plant records from the herbarium at the Montreal Botanical Gardens were also consulted to gather information on the location of populations throughout the maritime states of North Eastern Canada. This region of North America was selected for DNA sampling, as it is the closest to Europe and is a potential source of bird or wind transported seed. Using the maps and prior information, a 4000km round trip was mapped out along the Trans-Canadian Highway between Montreal, Quebec and Halifax, Nova Scotia. Potential sites were visited along the way. The collecting and sampling protocol followed the same criteria as described above for the Irish samples. To ensure correct plant identification, a voucher specimen was collected at each population. These specimens were pressed, labeled and were delivered to Dr. Stewart Hay at the herbarium at Montreal Botanic Gardens, where they were verified as *S. romanzoffiana*. The leaf specimens were stored in silica gel and remained in Montreal until the relevant (CITES) certificates had been organised. The samples eventually arrived in Dublin in December 2005. Total genomic DNA was immediately extracted and the samples were stored at -20^{0} C.

Table 3.3.1 List of sites sampled for genetic analysis of Spiranthes romanzoffiana. Number of samples indicates the actual number samples in which DNA was successful y extracted

Country	Region	Location	No. of samples	Habitat	Collector
Ireland	Mayo	Lough Conn (Cuingbeg)	5	Lakeshore/grassland	D. Lupton
Ireland	Mayo	Lough Conn (Knockmore)	10	Lakeshore/grassland	D. Lupton
Ireland	Mayo	Lough Conn (Carraig a Moiltin)	5	Lakeshore/grassland	D. Lupton
Ireland	Mayo	Lough Conn (Corysola bridge)	5	Lakeshore/grassland	D. Lupton
Ireland	Mayo	Lough Cuilin	10	Lakeshore/grassland	D. Lupton
Ireland	Mayo	Lough Mask	10	Lakeshore/grassland	D. Lupton
Ireland	Mayo	Lough Levally	5	Lakeshore/grassland	D. Lupton
Ireland	Galway	Lough Corrib	10	Lakeshore/grassland	D. Lupton
Ireland	Leitrim	Lough Allen	10	Lakeshore/grassland	D. Lupton
Ireland	Roscommon	Lough Allen	8	Lakeshore/grassland	D. Lupton
Ireland	Cork	Glengarriff	1	Lakeshore/grassland	D. Lupton
Northern Ireland	Antrim	Gortnagory	4	Upland pasture/flush	D. Lupton
Northern Ireland	Derry	Long Point, Lough Beg	4	Lakeshore/grassland	D. Lupton
Northern Ireland	Fermanagh	Upper Lough Erne	2	Lakeshore/grassland	D. Lupton
Northern Ireland	Tyrone	Lough Beg	3	Lakeshore/grassland	D. Lupton
Scotland	Tiree	?	2	Grassland	J. Bowler
Scotland	Colonsay	Kiloran dunes	5	Dune slack	A. Forrest
Scotland	Coll	Arileod field	5	Lakeshore/grassland	A. Forrest
Scotland	Vatersay	Causeway	5	Dune slack	A. Forrest
Scotland	Barra	Bruernish	4	Lakeshore/grassland	A. Forrest
Canada	Quebec	Laurentian Mtns	10	Birch wood	R. Latour / D. Lupton
Canada	New Brunswick	Grand Falls	10	Pine forest	D. Lupton
Canada	New Brunswick	Lepreau	7	Gravel pits	D. Lupton
Canada	Newfoundland	Iles St Pierre et Miquelon	7	Lakeshore/grassland	R. Etchberry
Canada	Nova Scotia	Brier Island	8	Dune slack	D. Lupton
U.S.A	Alaska	?	6	?	RBG Kew



County	Location
Antrim	Gortnagory
Cork	Glengarriff
Fermanagh	Upper Lough Erne
Galway	Lough Corrib
Leitrim	Lough Allen
Derry	Long Point, Lough beg
Mayo	Lough, Conn, L. Levally, L. Cuilin and L. Mask
Roscommon	Lough Allen
Tyrone	Lough Beg

Figure 3.3.1 Location of *Spiranthes romanzoffiana* sites sampled for genetic analysis on the island of Ireland.

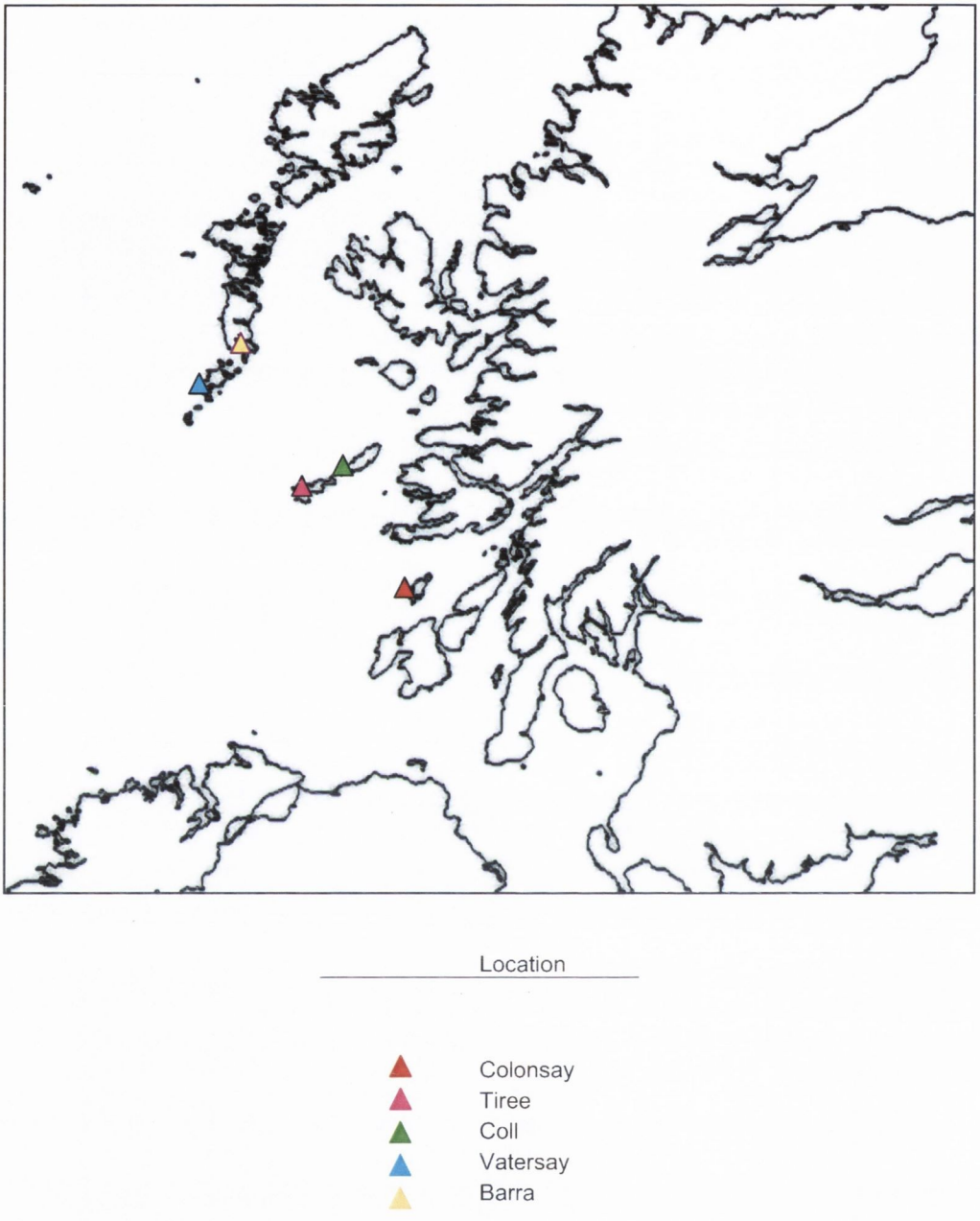


Figure 3.3.2 Location of *Spiranthes romanzoffiana* sites sampled for genetic analysis on the Hebridean islands in Western Scotland.

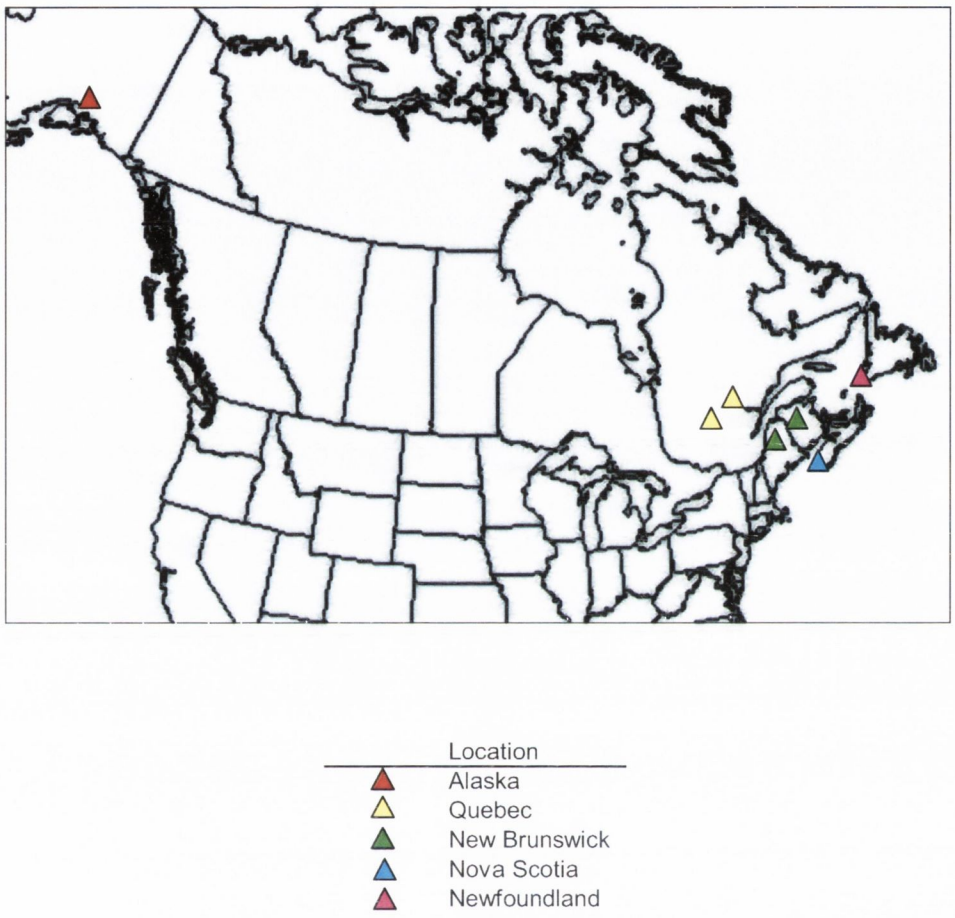


Figure 3.3.3 Location of sites sampled for genetic analysis of *Spiranthes romanzoffiana* in the North America.

3.3.2 DNA Extraction

DNA was extracted from the leaf material following two different extraction methods; **1)** a modified CTAB (hexadecyltrimethylammonium bromide) extraction method (Doyle and Doyle, 1988) and **2)** DNeasy^{R} Plant Mini Kits (Qiagen LTD UK). Both extraction methods were tested for efficiency, reliability and quality of total genomic DNA in the end product (section 3.3.3). Testing was carried out using gel electrophoresis, as described in section 3.3.3. A random sample of total DNA from each extraction

technique was run out on a gel. The quantity of DNA was measured in relation to the distance each sample migrated along the gel, i.e. the shorter the distance travelled the greater the amount of total DNA within the sample.

CTAB

The CTAB method involved adding 10ml of 2X CTAB[1] extraction buffer to re-usable 30ml centrifuge tubes, which were then preheated to 65° C in a water bath. Mercaptoethanol (40µl) was added to each tube prior to use. Between 0.09g and 0.15g of dried leaf material was added to a heated pestle and mortar with a small amount of the 2XCTAB buffer. The leaf fragments were ground to a slurry until no visible leaf fragments remained. The slurry was then poured back into the centrifuge tube and placed in the water bath at 65° C for 10 minutes.

Following this 10ml of CI (24:1 chloroform:isoamyl alcohol) was added to each tube. The tubes were then inverted a number of times, the lids subsequently loosened to release any built up gas. The tubes were placed on a shaker to gently mix the contents and then centrifuged at 4000g rotating centrifugal force (rcf) on a 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 samples were then placed in a freezer for at least 24 hours to allow the DNA to precipitate.

Following this the samples were centrifuged at 2000g (rcf) for 10 minutes in a MSE Harrier 15/80 centrifuge to pellet the DNA. The supernantant was drained off and 3ml of wash buffer (70% ethanol) was added to the tubes and gently mixed. Samples were centrifuged once more for five minutes at 2000g (rcf) to re-pellet the DNA. The tubes were placed upside down on paper towels for 20 minutes to drain excess ethanol and placed in a fume cupboard for 30 minutes until all the ethanol was evaporated. The DNA pellets were then suspended in 0.5ml of TE buffer, transferred into labelled 1.5ml Eppendorf tubes and stored at –20° C.

2X CTAB extraction buffer

The buffer has a pH of approximately 8 and was preheated to 65 ^{0}C before use so that the optimum conditions for DNases 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-dependant enzymes and mercaptoethanol limits the effects of polyphenols, quinines and tannins.

QIAGEN DNeasy Plant Mini kit

A 1.5ml Eppendorf tube was filled with 50µl of elution buffer (Buffer AE) and placed on a heating block at 65 ^{0}C. Another clean, labeled 1.5ml Eppendorf tube was filled with 1.5mg of dried leaf material and a metal ball was placed in the tube with the sample and 400µl of lysis buffer (Buffer AP1) and 4µl of RNAase stock solution were added.

The labelled tubes were then placed on a mixer mill rack for 1.5 minutes at 30 H_Z. The racks were turned and milling repeated twice in order to sufficiently break the leaf material down into a fine slurry. The labelled samples were then placed in a micro-centrifuge and pulsed to 3000rpm, which spins down froth formed in mixer mill from the lids of the tubes. The tubes were tapped and mixed gently and incubated for 10 minutes on a heating block at 65 ^{0}C

Precipitation buffer (Buffer AP2) was added to each labelled tube and the samples were placed on ice for 10 minutes (original protocol suggests 5 minutes, the additional time allows for the formation of more precipitates with difficult material). The samples were then centrifuged for 5 minutes at 13,000 rpm to spin down precipitates. The supernatant from the labelled tubes was then placed in labeled QIAGEN shredder spin columns and centrifuged at 13,000 rpm for 2 minutes.

The lysate, which was in the bottom of the tube, was transferred to a clean 1.5ml tube and the volume was recorded. Binding buffer (Buffer AP3/E) was added at 1.5 times the recorded volume of lysate for each sample and the buffer was mixed with the sample by drawing in and out with a pipette three times. DNeasy spin columns with collection tubes

were labeled and 650µl of the lysate and buffer mix was added to the spin columns and centrifuged at 9,000 rpm for 1 minute. The flow through was discarded and any extra remaining sample and buffer mix was added and centrifuged at 8,000rpm for 1 minute. The remaining flow through was discarded and the 50µl of total genomic DNA remaining in the bottom of the tube was stored at -20^{0}C until further use.

Further purification of total genomic DNA

Further purification of the DNA was carried out in order to ensure successful amplification of the samples using PCR (Polymerase Chain Reaction). This was done according to the protocol from Conert^{TM} Nucleic Acid Purification System (Life Technologies-Gibco-BRL^{R}). The manufacturer provided the following information regarding the constituents of the kit: Binding solution (H1) contained concentrated guanidine hydrochloride, EDTA and Tris-HCL. Wash buffer (H2) contained NaCl, EDTA and Tris-HCL and TE buffer contained 10mM Tris-HCL (pH 8) and 0.1mM EDTA.

Protocol for the further purification of total genomic DNA

A mixture of 400µl binding solution and 100µl sample were added to the centre of labelled spin cartridges (in 2ml wash tubes). The tubes were then centrifuged at 12,000g for 1 minute. The flow through was discarded and 700ml of wash buffer (containing ethanol) was added to the centre of the spin cartridges. The samples were centrifuged once more at 12,000g for 1 minute and the flow through was once again discarded. The samples were centrifuged again to remove the remaining wash buffer from the tubes and the spin cartridges were placed into labelled recovery tubes. 50µl of warm TE (68^{o} C) was added to each to dissolve the purified DNA. The tubes were allowed to incubate at room temperature for 1 minute and centrifuged again at 12,000 or 2 minutes. The spin cartridges were then discarded and the samples were labelled and stored in a freezer until required.

3.3.3 DNA quantification

Two different quantification methods were used to determine the quality and quantity of total genomic DNA. 1) Gel electrophoresis, 2) Colorimetry.

Gel electrophoresis

A crude assessment of the quality and quantity of each total DNA sample was determined using electrophoresis on an agarose gel containing ethidium bromide stain (stained DNA fluoresces in the presence of UV light). This was done under the assumption that there is a direct correlation between the amount of DNA present and the degree of fluorescence. 5µl of each total genomic DNA sample was mixed with 1µl of loading dye [40% sucrose, 0.25% bromophenol blue] and run out on a 1.5% agarose gel (7 x 14cm) stained with ethidium bromide in 1xTBE [45mM tris-HCL, 44mM boric acid, 1mM EDTA], at 110 volts for 30 minutes in a Horizon^{R} 11.14 gel rig. The DNA samples were run against a standard of known molecular weight (Gibco BRL 1kb ladder). The gels were illuminated under UV light and the concentration of the resulting DNA bands was estimated by comparing with a ladder of known concentration. A digital photograph was taken of each run and stored for later use.

Colorimetric determination

A spectrophotometer (Eppendorf BioPhotometer) was used for a direct measurement of nucleic acids at 260nm, where one absorbance value is equal to 50µg/ml DNA. A blank consisting of 5µl TE and 95µl of distilled water was placed in an Eppendorf disposable single-sealed 50-2000 µl cuvette, which was then placed in the spectrophotometer. 5µl of each DNA sample and 95µl of distilled water was placed in a clean cuvette, the solution was mixed thoroughly to ensure accurate readings. The correct dilution rate was entered, allowing the spectrophotometer to automatically calculate the concentration of DNA, based on the absorbance of light at 260nm. DNA concentrations in ng/ul were recorded for each sample. Purity (DNA versus RNA) of each sample was assessed by the ratio of absorbance at 260nm and 280nm.

3.3.4 AFLP reaction sequences

Restriction-Ligation Reaction

The first stage of the process involved 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 was carried out in the same reaction and they provide a recognition sequence for the primers in the subsequent selective PCRs. The ligation step did not recreate the restriction enzyme recognition site and therefore did not affect the restriction reaction. It was also advantageous to combine the two steps in the same reaction, as addition of the adaptor pairs to the cut ends ensures that artefacts were not produced by the linkage of two cut fragments (Vos, *et al.*, 1995).

Restriction-Ligation steps carried out in this research.

0.25µg of total DNA was added to labelled 0.2ml PCR tubes and the volume was made up to 2.75µl with ultra-pure water. 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 to 95 ^{0}C for five minutes and allowed to cool over 10 minutes. The next stage involved making up a master mix for all of the samples. Two extra samples (2.75µl each) were factored into the master mix volumes, to allow for any error. Master mix 1 was prepared by adding the components per reaction to a 0.2ml tube.

Table 3.3.3 Components of Master Mix 1 for the restriction-ligation step of the AFLP analysis

Reagent	Volume per sample
T4 DNA Ligase	0.05 µl
10X T4 DNA ligase buffer with ATP	0.05µl
0.5M NaCl	0.05µl
1mg/ml BSA	0.025µl
Mse1(10U/µl)	0.5µl
EcoR1(100/µl)	0.025µl
Ultra-pure water H_2O	0.295µl

Table 3.3.4 Components of Master Mix 2 for the restriction-ligation step of the AFLP analysis.

Reagent	Volume per sample
10X T4 DNA ligase buffer with ATP	0.5µl
0.5M NaCl	0.5µl
1mg/ml BSA	0.25µl
Mse1 Adaptor	0.5µl
EcoR1 Adaptor	0.5µl
Master mix 1	0.5µl
Total volume	2.75µl

The total volume of the Master Mix was then made up to 0.5µl with ultra-pure water. The mixture was mixed thoroughly and spun down by pulsing to approximately 5000g in a MSE MicroCentaur microcentrifuge and stored on ice. All samples were kept on ice throughout all stages to prevent degradation of the DNA. Master mix 2 was prepared from the components described in Table 3.3.4. The mixture was thoroughly mixed after the addition of the EcoR1 and Mse1 adaptor pairs. Master mix 1 (vol. 2.75µl) was then added, the total volume in the 0.5ml tube was spun down again. 2.75µl of this total combined mix was then added to each 2.75µl of DNA. The contents of the tubes were mixed and spun down as before. The tubes and contents were then incubated at 37 ^{0}C for two hours. Following this the products were diluted by adding 94.5µl $TE_{0.1}$ buffer to each

reaction, all tubes were thoroughly mixed. The restriction products were stored on ice until needed.

Pre-selective Amplification

The second stage of the process was a pre-selective PCR designed to reduce the number of fragments for selective amplification. The EcoRI primer recognises fragment ends with the EcoR1 sequence and adjacent guanine and thymine bases, and the Mse1 primer recognises fragment ends with the complimentary Mse1 adaptor sequence and adjacent thymine then guanine bases. Fragments with sequences matching that of the pre-selective primers at each end are amplified.

Pre-selective amplification steps carried out in this research

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 and spun down in a microcentrifuge. 4 µl of the master mix was added to each of the restriction products, making a total reaction volume of 5. µl. The tubes were finally placed in a MJ Research PTC200 Peltier Thermo cycler and run through the PCR sequence described in Table 3.3.5. Verification of amplification was carried out using agarose gel electrophoresis as described in section 3.3.3.

Table 3.3.5 Conditions of the pre-selective amplification of restricted and ligated DNA fragments

Process	Temperature ^{0}C	Time	Number of Cycles
Extension	72	2 minutes	1
Denaturing	94	20 seconds	
Annealing	56	30 seconds	20
Extension	72	2 minutes	
Final extension	60	30 seconds	1
Soak	4	∞	

Selective Amplification

Selective amplification involves the use of primers with an additional sequence of two or more bases for further reduction of fragments. The fragments used in this study contain two additional selective base pairs. Primer pair combinations were chosen from eight available EcoR1 and eight Mse1 primers in the Applied Biosystems AFLP I primer kit. There were therefore 64 possible combinations to choose from. The available Mse1 and Eco1 selective sequences are listed below:

Mse1 primer (end sequence) CAA, CAC, CAG, CAT, CTA, CTC, CTG, CTT
Eco1 primer (end sequence) AAC, AAG, ACA, ACC, ACG, ACT, AGC, AGG

Selective amplification primers consist of a core sequence, an enzyme-specific sequence and a selective extension sequence. EcoR1 primers also include a fluorescent label, which is used in the detection process. Only the fragments ending in EcoR1-Mse1 are detected during this step as Mse1-Mse1 ended fragments do not have any fluorescent labelling and EcoR1-EcoR1 ended fragments tend to not amplify as the distance between the rare cutter sites is too great.

Selective amplification steps carried out in this research

Three new sets of PCR tubes were labelled per sample to enable the reactions to be carried out with three different fluorescently labelled dye primers. In each pair of primers, those that are complementary to the ecoR1 sequence were labelled at the 5' end with blue (FAM-labelled), green (JOE-labelled) or yellow (NED-labelled) fluorescent dyes. This fluorescent labelling allows the fragments to be detected by laser in an automated sequencer. This allows fragments from the primer pair combinations to be determined simultaneously, therefore saving a considerable amout of time. 1.5µl of diluted pre-selective products were added to each set of tubes, these tubes were then placed on ice. The master mix described in Table 3.3.3 was prepared for each primer pair.

Table 3.3.6 Components and volumes of reagents added to each of the primer pair combinations.

Component		Volume per sample
MseI	primer at 5μM	0.5μl
EcoR1	primer at 1μM	0.5μl
AFLP	core mix	7.5μl

The three master mixes were mixed and spun down on a microcentrifuge. 8.5μl of each master mix was added to each of the 2μl diluted pre-selective products, these were further mixed and spun down in a microcentrifuge. The samples were then placed in an MJ Research PTC200 Peltier Thermo Cycler and run through the sequence described in table 3.3.7.

Table 3.3.7 Conditions of selective amplification (annealing temperature becomes progressively less stringent).

Denaturing		Annealing		Extension		Number of Cycles
Temp	*Time*	*Temp*	*Time*	*Temp*	*Time*	
94°C	2min	65°C	30 sec	72°C	2 mins	1
94°C	1sec	64°C	30 sec	72°C	2 mins	1
94°C	1sec	63°C	30 sec	72°C	2 mins	1
94°C	1sec	62°C	30 sec	72°C	2 mins	1
94°C	1sec	61°C	30 sec	72°C	2 mins	1
94°C	1sec	60°C	30 sec	72°C	2 mins	1
94°C	1sec	59°C	30 sec	72°C	2 mins	1
94°C	1sec	58°C	30 sec	72°C	2 mins	1
94°C	1sec	57°C	30 sec	72°C	2 mins	1
94°C	1sec	56°C	30 sec	72°C	2 mins	23
-	-	-	-	60°C	30 mins	1
			Soak 4°C			

3.3.5 Preparation of samples for Automated genotyping

The selective amplification products from the three 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

24μl of formamide (CH_3NO – Applied Biosystems, used to denature the samples) and 0.20μl of ROX (Applied Biosystems – a fluorescently-labelled 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.

3.3.6 Primer Selection

The number and quality of bands produced by AFLP analysis varied depending on the primer combination used. Preliminary tests were carried out to select three primer pairs from the 64 possible primer pair combinations. Three primer pairs makes for efficient genotyping using an automated sequencer, hence efforts were made to select a suitable number of variable bands from three primer pairs. A sample of ten individuals from a selection of populations covering a wide geographic distribution were used to test 12 primer pairs (Table 3.3.8). Primers were chosen based on the quantity and quality of the bands produced. The optimum primers yielded a large number (>60) of obviously differentiated bands with a high intensity. The primer combinations and the samples used in the trials were labelled separated and analysed randomly to avoid any bias that may arise due to preconceived ideas of population structure.

Table 3.3.8 Primer combinations tested prior to analysis. Primer combinations in bold were selected based on their quality and quantity of bands.

Combination	EcoRI + Fluorescent dye	MseI
1	-ACG + JOE	-CAG
2	**-ACT +FAM**	**-CTA**
3	-ACT + FAM	-CAA
4	**-ACA + FAM**	**-CAG**
5	-AAC + NED	-CTG
6	-AGC + NED	-CTC
7	-ACC +NED	-CTA
8	-AGG + JOE	-CTC
9	-AAG + JOE	-CTT
10	–ACG +JOE	-CTG
11	–ACC + NED	-CAC
12	-ACA +FAM	-CAC

3.3.7 Band scoring

Sizing of fragments was performed by Applied Biosystems Genescan® software version 3.1. Only fragments between 40 and 250bp (base pairs) were scored, bands outside this range being considered unreliable. The scoring of bands was then checked manually on a electropherogram using Genotyper™ (figure 3.3.4) due to inconsistencies reported in the literature regarding the accuracy of automated techniques. Smith (2005) reported that inaccuracies resulted from the incorrect classification of fragments falling close to the demarcation limits of the size classes: for example, a fragment with a size of 12.49bp was classified as being different from a marker with a size of 12.51bp. The Genescan™ sample files were imported into Genotyper™. Size standards (ROX) were checked for consistency. Incorrectly aligned size standards were manually re-aligned and the Genescan™ sample file was re-analysed. Once the size standard was accurately aligned the peaks were labelled with the corresponding fragment size to assist scoring. Fragment sizes were rounded up or down to the nearest whole number of base pairs. The method was consistent throughout the procedure. Results were converted to a zero/one matrix based on the presence or absence of markers in the samples. The default Genotyper™

setting does not label peaks below a fluorescence intensity threshold of 50. This threshold was adhered to throughout the analysis. Positive (*Miscanthus sinensis*) and negative (purified water) controls were used in each set of reactions. Any samples where the negative controls contained bands were discarded and the samples were re-run.

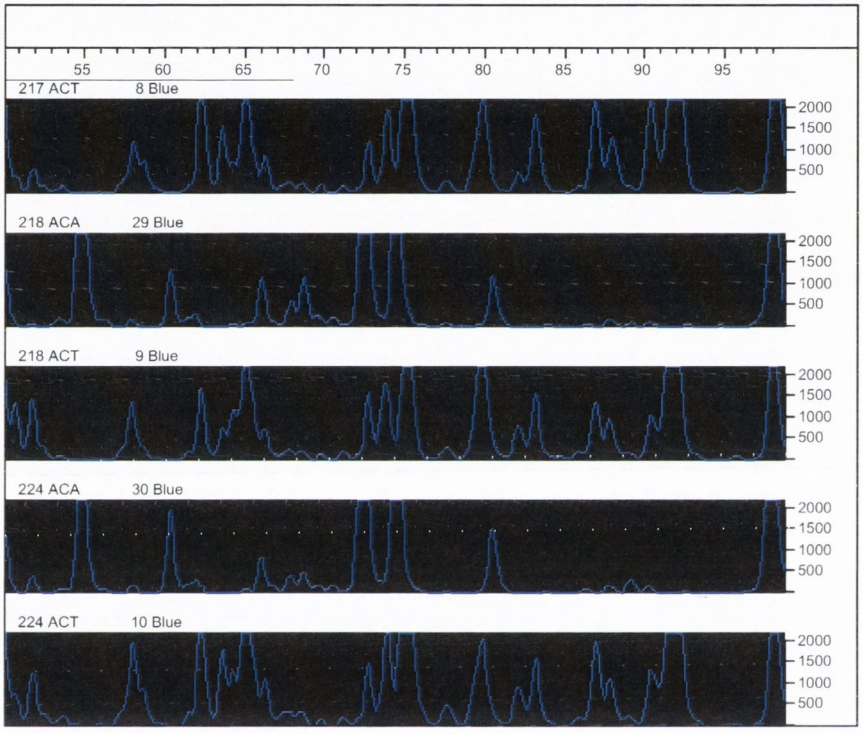


Figure 3.3.4 Example of AFLP banding patterns. Image copied from Genotyper™. Numbers of the left side of the image are the sample numbers. ACT and ACA represent the two primers used in the analysis. The numbers on the right side of the image determine the strength of each peak. Peaks below 50 were not included in the analysis, this scale can be manually adjusted in Genotyper. The numbers along the top of the image correspond to the base pair length of each fragment.

3.3.8 Data Analysis

A number of different methods of analysis were employed to process the data from the zero/one matrix.

Diversity Analysis

Population genetic structure can only be quantified exactly by the analysis of all structural loci present within all individuals (Nei, 1987). Populations can potentially contain many individuals with tens of thousands of loci per individual. In order to estimate the genetic variability of a population, sampling must therefore be performed on a subset of individuals and a subset of loci. There are a number of techniques widely used in population genetics to display data attained from such sample analyses. The methods described below have been used to analyse the genetic data set from this research:

% Polymorphic loci

This is a simple measure of the percentage of polymorphic loci in a population. It is a useful and popular statistic, though some caution is required as the technique is highly susceptible to sampling error with respect to the number of individuals and the number of loci sampled. It is not considered 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. Due to these potential sources of error, percentage polymorphic loci statistics should be used in conjunction with other population genetic statistics. Percentage polymorphism statistics were carried using Popgene version 1.32 (see section 3.3.12)

Shannon's Index $\mathbf{H = - \sum p_i \ln p_i}$

Equation 3.3.1 Shannon's Index, where p_i is the frequency of the ith polymorphic marker.

Nei's Gene diversity

This is a widely used technique to estimate gene diversity amongst and between populations. Gene diversity is also a measure of heterozygosity but is derived from the probability that two randomly chosen alleles from a population are different (Nei, 1973) and therefore can be applied to haploid, diploid or polyploid species.

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

where allele frequency is estimated by: $x_i = X_{ii} + \sum X_{ij}/2$

Equation 3.3.2 Nei's (1978) Gene diversity

Total gene diversity can be divided 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}) and can be calculated as follows:

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

Equation 3.3.3 Total Gene diversity

Gene diversity between populations can then be expressed as the proportion of the total diversity to give G_{ST}. This is a measure of population differentiation and can be calculated as follows:

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

Equation 3.3.4 Gene Diversity between populations

Finally, the proportion of gene diversity within populations can be calculated as follows:

$$1 - G_{ST}.$$

Equation 3.3.5 Gene Diversity within populations

3.3.9 Tree construction

Trees are mathematical structures used to infer and display evolutionary relationships between taxa and can be constructed using many different methods (Page and Holmes, 1998). Distance matrix methods are often used in tree construction and are widely used in population genetics. Commonly used trees include un-weighted pair-group method with arithmetic mean (UPGMA) and neighbour-joining. Both of these methods are widely available in the analytical software. There are a number of other techniques available, e.g. minimum evolution method. However, this method and others like it are computationally demanding and therefore impractical in many situations. Neighbour-joining provides a fast approximation of the minimum evolutionary tree and is the preferred method (Wen Hsuing, 1997). For comparative reasons both UPGMA and neighbour-joining methods were used in this research. For both analyses a distance matrix was calculated from the original zero/one matrix using Nei-Li genetic distance measure (Nei 1987). Nei-Li distance measure, which is similar to Sorensen-Dice similarity measure was chosen for its ability to deal with the dominant nature of the data set. UPGMA and neighbour joining analysis were performed on the data using PAUP *4.0b10* (see section 3.3.12).

UPGMA

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 is the same length (Swofford *et al.*, 1996). This is a simple method of tree construction and generates branches in a stepwise manner. The most similar units are combined first, so a tree is constructed in order of decreasing similarity. The analysis assumes that the individuals in question have a similar rate of evolution; if this hypothesis is not met errors can occur.

Neighbour-joining

Neighbour-joining is based on finding two samples (neighbours) that are most similar (i.e. that minimise the total length of the phylogenetic tree) and clustering them together. This is a progressive process in which the next pair of neighbours with the smallest

branch length are then clustered, and then continues additively until N-3 interior branches are defined (Li, 1997). The resulting tree therefore illustrates the arrangement of the samples so that the most similar are clustered together under the principal of minimum evolution (Saitou and Nei, 1987).

Confidence levels of the branching patterns and length were statistically tested using Bootstrap values. This works by re-sampling the character matrix a thousand times at random and creating new matrices of the same size that are treated as replicate data-sets. 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.

3.3.10 Ordination methods

Ordination techniques are used to simplify a complex multivariate data set into a smaller number of dimensions that explain most of the variation. In short, ordination is the ordering of objects along axes according to their similarities. The main purpose is to reduce the data set, by expressing many dimensional relationships in a small number of dimensions. Objects that come out close together on a given axis are generally more closely associated with one another. The inverse is generally true for objects furthest away from each other.

Principal Co-ordinate analysis (PCO)

PCO (Gower, 1966) works on distance or similarity matrices of all the samples and combines these values to give a representation of the data in an n dimensional space (where n = number of objects). Thus the dimensions are not reduced but are condensed, because generally the first few dimensions account for a greater proportion of the overall variation and dominant patterns in the data are reflected in these first few dimensions (Digby and Kempton, 1987). A major advantage of PCO over some ordination methods is that a distance or similarity measure appropriate to the data recorded can be utilised.

For a zero/one data of the sort generated by AFLP, Sorensen's similarity or Nei-Li genetic distance are appropriate measures.

The first step in PCO analysis is the construction of a similarity matrix of zero/one data. To account for the dominant nature of AFLP data the similarity coefficient had to be carefully chosen. This is because the dominant data, the heterozygous and dominant homozygous states, are indistinguishable leading to problems in the accurate estimation of similarity of individuals to each other (or the distances from each other) (Harbourne, 2005). PCO was carried out using the R package (Le Progeciel) (PCO-ORD) version 4.0d (Casgrain, 1999). The eigenvectors of the first two axes were copied into and graphed in Microsoft Excel.

3.3.11 Similarity Coefficients

A number of similarity coefficients appear throughout the relevant literature, though two in particular are used frequently; Jaccard's Similarity Coefficient (1908) and Sorensen-Dice Similarity Coefficient (Dice, 1945). The fundamental difference between the two methods is how they deal with zeros. Jaccard's Similarity does not account for negative occurrences in the data. In general the Jaccard's distance has not performed as well as the Sorensen distance (Beals, 1984).

$$\text{Jaccard's similarity} = \frac{a}{a + b + c}$$

Equation 3.3.6 Jaccard's Similarity Coefficient where 'a' is the sum of shared fragments and 'b' and 'c' are the number of fragments, which have scored differently in individuals.

The second, Sorenson-Dice Similarity Coefficient assigns more weight to shared presence and not shared absences in the data. Sorenson-Dice Similarity Coefficient is the most appropriate similarity coefficient for use with dominant data (Duarte *et al*, 1999). For this research similarity was calculated using Sorenson-Dice Similarity Coefficient.

$$\text{Sorenson-Dice similarity} = \frac{2w}{2a + b + w}$$

*Equation 3.3.7 Sorenson-Dice Similarity Coefficient, where **W** is the sum of shared abundances and **A** and **B** are the sums of abundances in individual sample units.*

3.3.12 Statistical software used in the analyses

There are a wide variety of software packages available to carry out statistical analyses in population genetics, many of which are freely available on-line. The main differentiation between packages is their approach to statistical calculations. The results output format also varies greatly between the various packages. Repetition of the analysis using a number of different statistical approaches is advisable, as this can mitigate against limitations inherent in some statistical software packages and allow greater confidence to be placed in the conclusions drawn from the data. The software packages described below were used in the completion of this thesis.

POPGENE Version 1.32

This is Windows-based software for the analysis of population genetic data (Yeh *et al*, 1999). The software can be used to analyse diploid and haploid individuals and for dominant or co-dominant markers. It is capable of carrying out a number of tests, including gene diversity statistics (Nei, 1973), genetic distance (Nei, 1972; 1978) and F-statistics (G_{ST} – Nei, 1973). It can perform these calculations on populations, sub-populations or groups of populations. Dendrograms can be produced based on UPGMA analysis (Sokal and Michner, 1958) of Nei's distance matrices.

PAUP Version 4.0b10

PAUP (Phylogenetic Analysis Using Parsimony) (Swofford *et al.*, 1996) is software that allows the construction and examination of phylogenetic trees. A number of tree building methods are available including UPGMA and Neighbour Joining, and tree support values can be provided by bootstrap analysis. Trees can be constructed directly

from a presence/absence matrix, using mean or total character difference or Nei-Li's restriction site difference (Nei-Lei, 1979).

Arlequin Version 2.000

Arlequin is a free downloadable software package for population genetic data analysis (Sneider *et al*, 2000). Analyses are divided into four catagories: Diversity indices, Disequilibrium Tests, Neutrality Tests and Population structure. Population statistics by AMOVA (Analysis of Molecular Variance) is based on the analysis of variance of gene frequencies, taking into account the number of mutational differences between co-dominant molecular data such as microsatellites and allozymes (Michalakis and Excoffier, 1996). AMOVA partitions the total variance of the sample into its components, e.g. within individuals, among populations and within groups. Population pairwise measurements (F_{st}) can be computed and are outputted in matrix format. The exact test of population differentiation can be performed by testing the null hypothesis of the random distribution of haplotypes or genotypes among populations (Raymond and Rouset 1995b in Labate, 2000).

R- Package

The R Package is a software program that offers wide variety of tools for the exploration and analysis of multivariate and spatial data, including calculations of a wide variety of similarity and distance measures, selected ordination and other statistical methods (Casgrain and Legendre, 2001).

PC-ORD

PC-ORD performs multivariate analysis of ecological data. PC-ORD offers many ordination and classification techniques not available in major statistical packages. Virtually any multivariate data set consisting of a set of entities, each with a number of measured attributes is adaptable to PC-ORD. Very large data sets can be analyzed. Most operations accept a matrix up to 32,000 rows or 32,000 columns and up to 536,848,900 matrix elements (McCune and Mefford, 1997).

3.4 Results

Two primer pairs (Table 3.3.8) were successfully analysed during this research. The total number of unambiguous markers yielded from the combination of the two primer pairs was 191. The size range of the markers was between 35 and 300 base pairs in length. DNA was extracted from 125 individual samples from a total of 16 populations: six North American populations, nine Irish and a single Scottish population, (consisting of only two plants). No private alleles diagnostic of any individual population were detected with the AFLP markers. The level of population differentiation in all the sampled populations in North America and Ireland was assessed using POPGENE. The overall level of differentiation between and within all populations, the level of differentiation between and among North American populations and the level of differentiation between and among Irish were examined.

3.4.1 Gene diversity

The software package POPGENE was used to derive three gene diversity measures (results of proportion of polymorphic loci, Shannon's index and gene diversity are plotted in figures and in tabular format in table). Total gene diversity (Ht), mean gene diversity average over all loci (H), mean within population gene diversity (Hs) and the mean Shannon's index (I) were calculated on two levels: all populations and populations within geographic regions.

The total proportion of polymorphic loci (PPL) (table 3.4.1) was 1.00 across all samples from both Ireland and North America. The total PPL for Ireland was 0.97; this was lower in North American populations at 0.96. PPL values in Irish populations ranged from 0.24 (Antrim) to 0.8 (Lough Cuilin, Co Mayo). The range of PPL values from the North American populations varied from 0.52 (Alaska) to 0.69 (New Brunswick)

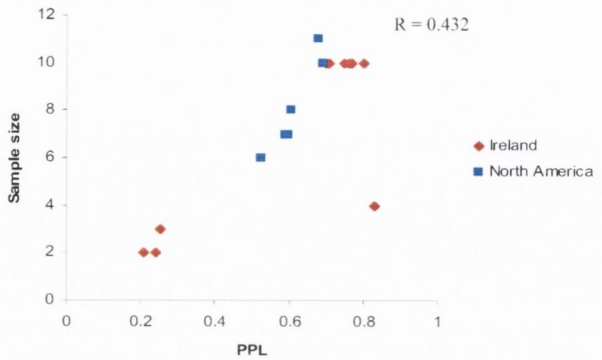
The total value for Shannon's index (table 3.4.1) from all samples was 0.41. Again the Irish populations showed a higher total value of 0.40 compared to the North American total population value of 0.37. Nei's gene diversity estimates ranged from 0.2587 for all populations of to 0.24 for North American populations to 0.26 for all Irish populations. In North America the lowest levels of gene diversity occurred in Newfoundland

populations 0.15, the highest levels were recorded in Quebec, 0.21. In Ireland the lowest gene diversity levels were recorded in Fermanagh, 0.035, though this is based on the analysis of only two samples, the highest levels were found in Lough Cuilin, Co Mayo, 0.25.

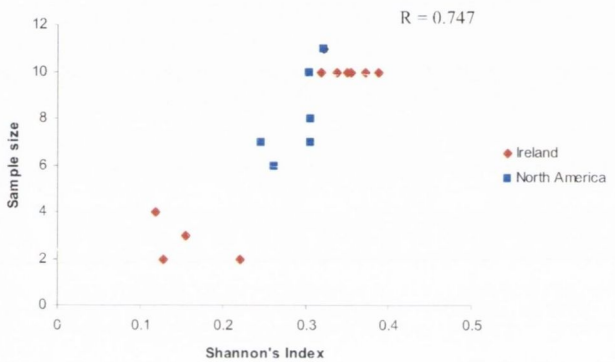
Total gene diversity (Ht) in Irish populations, 0.26, in North American populations the value was 0.22. Within population diversity (Hs) was again lower in North American populations, 0.17. In Ireland within gene diversity measure was 0.23.

Table 3.4.1 Genetic diversity estimates of 16 populations of *Spiranthes romanzoffiana*. The samples in bold font had < 5 individuals the diversity estimates for these individuals is questionable.

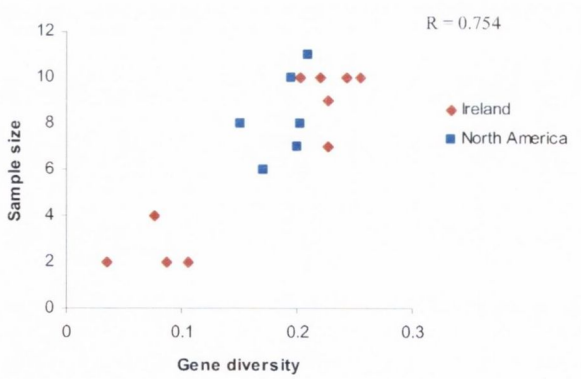
Geographic region	Location	No. of samples	PPL	Shannon's Index	Nei's Gene diversity
Ireland	Antrim	4	0.24	0.22	0.076
	Fermanagh	2	0.33	0.1183	0.0347
	Knockmore	10	0.701	0.3357	0.2196
	Drummin wood	10	0.8	0.3867	0.2546
	Allen	10	0.706	0.317	0.2031
	Car.-a-Moiltin	5	0.75	0.377	0.268
	Corysola bridge	5	0.759	0.3703	0.2428
	Corrib	10	0.748	0.3491	0.2273
	Levally	5	0.253	0.1535	0.1051
	Mask	10	0.769	0.3539	0.2267
Scotland	Tiree	2	0.209	0.22	0.0867
N. America					
	Quebec	12	0.68	0.32	0.209
	New Brunswick	10	0.69	0.3042	0.1944
	Alaska	6	0.523	0.2605	0.171
	Lepreau	10	0.607	0.3053	0.2004
	Newfoundland	10	0.596	0.2452	0.1512
	Nova Scotia	10	0.591	0.3058	0.2027
all Ireland		71	0.97	0.4	0.2624
all N. America		50	0.96	0.37	0.2388



(A)



(B)



(C)

Figure 3.4.1. Relationship between sample size and percentage polymorphism (A), Shannon's index (B) and gene diversity (C). (10-Irish and 6-North American populations)

3.4.2 Genetic distance

The presence/absence matrix of AFLP markers was used to determine relationships between populations. Analysis was carried out on the AFLP matrix data. The Irish samples (Figure 3.4.2) in the analyses are predominantly confined to one side of the X or Y axes, however the samples are more scattered and thus may be considered less similar to each other when compared to the North American samples. The analysis show the North American samples as a tight group with very little separation among samples (Figure 3.4.3).

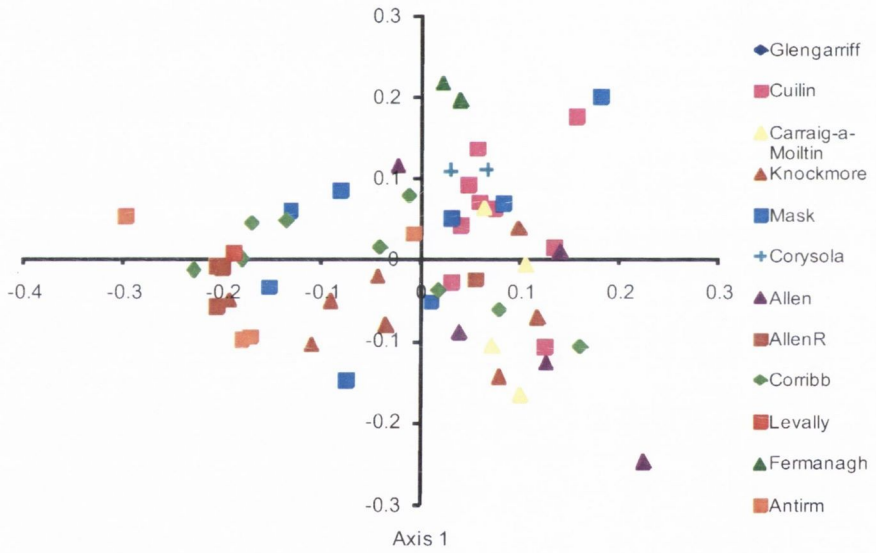


Figure 3.4.2 PCO graph of AFLP data from Irish samples Axis 1 shows (53%) of the variation, Axis 2 (36%) of variation. Axis 3 is not shown as it contains a very small percentage of the variation.

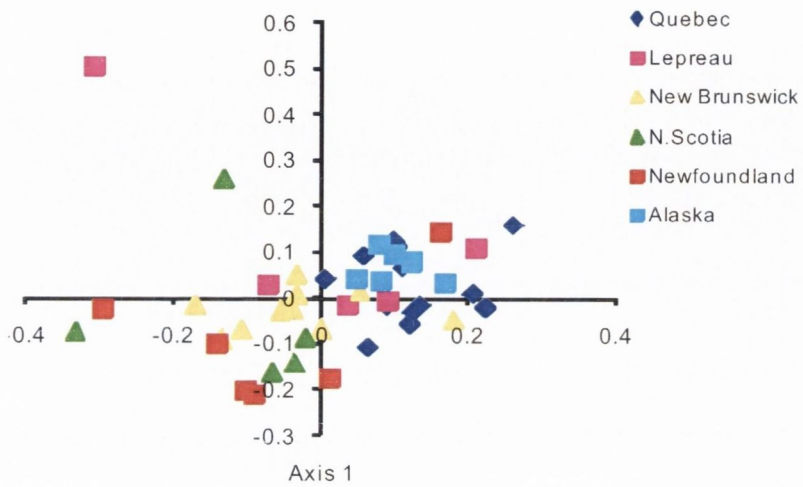


Figure 3.4.3 PCO graph of AFLP data from North American samples. Axis 1 shows (48 %) of the variation. Axis 3 is not displayed as it do not significantly alter the distribution of the samples.

This pattern was further investigated using POPGENE. Gene diversity estimates for the 13 sample populations were used to construct a genetic distance matrix between populations (Nei, 1978) and a UPGMA dendrogram of populations was produced. UPGMA analysis the North American samples (Figure 3.4.4) groups the Newfoundland and Lepreau together. Quebec, New Brunswick and Nova Scotia come out as broader members of this grouping. The Alaskan population is placed in a separate group, indicating these samples are somewhat genetically isolated from the other North American samples.

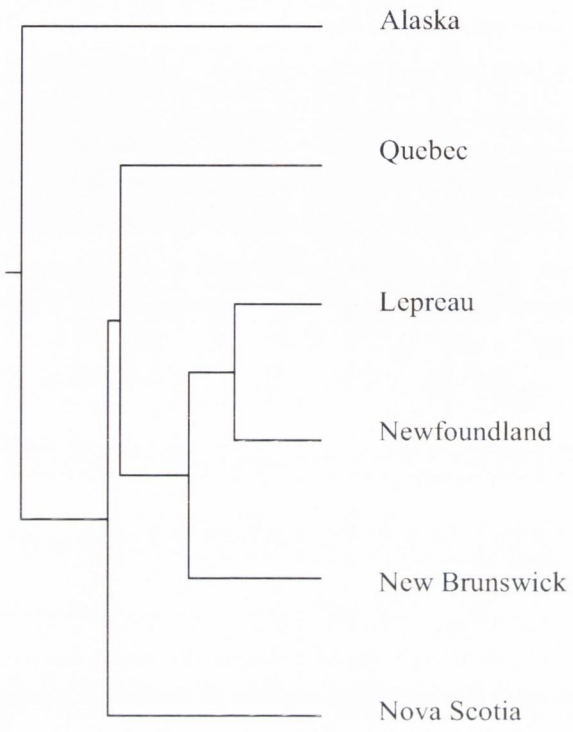


Figure 3.4.4 UPGMA tree derived from the AFLP data from the North American populations.

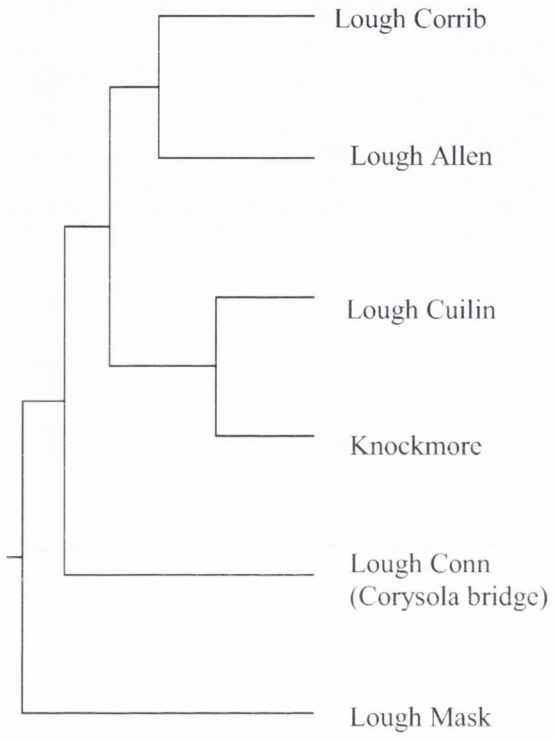


Figure 3.4.5 UPGMA tree derived from the AFLP data from the Irish populations.

UPGMA analysis of the Irish populations (Figure 3.4.5) reveals two groups, 1) the Lough Corrib and Lough Allen populations, and 2) the Lough Cuilin and Knockmore populations. The Lough Conn population is showing some association with the first two groups. Lough Mask however appears to be coming out as a potentially the most genetically distinct of the populations sampled.

The relationship between the individual samples from the 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, individuals were then grouped based on their individual banding patterns. As this does not involve estimation of allele frequencies, all individuals were included in this analysis. Figure 3.4.8 shows the neighbour-joining dendrogram of samples. Neighbour-joining analysis was carried out under the assumption that each difference in the amplification of an AFLP marker results from the restriction or amplification site difference in the DNA at the locus (Nei and Li, 1979). This neighbour-joining analysis is based on the calculation of Nei and Li (1979) and plotted using mid-point rooting

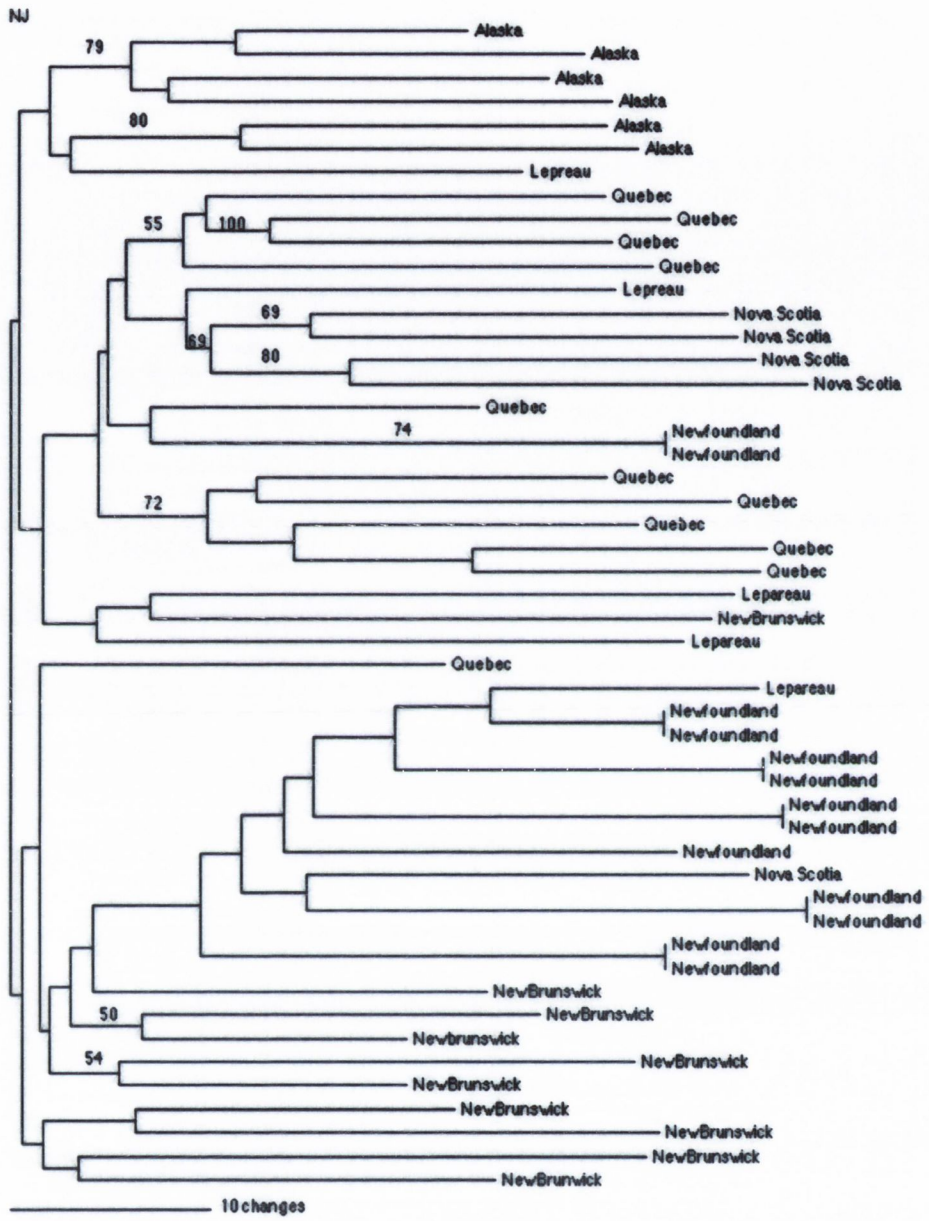


Figure 3.4.6 Neighbour-joining phylogram of North American samples of *Spiranthes romanzoffiana*. Phylogram construction was based on mid point rooting using data from the AFLP 0,1 matrix. The data from each of the two sets of primer pairs were interleaved to form a single large 0,1 matrix. Analysis was performed in PAUP version 4. Where they occur, the numbers above the lines represent the bootstrap value at that branching point.

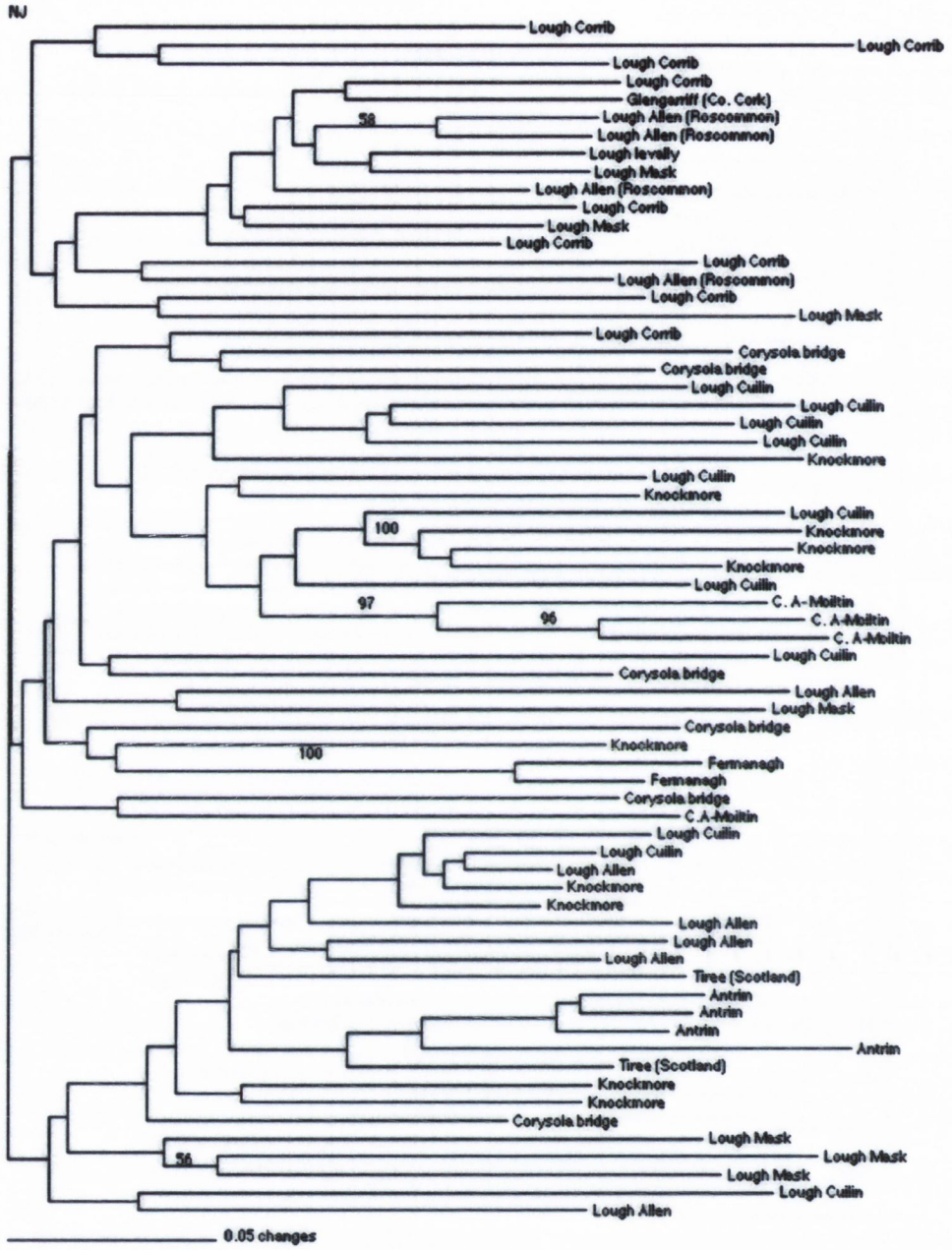


Figure 3.4.7 Neighbour-joining phylogram of Irish samples of *Spiranthes romanzoffiana*. Phylogram construction was based on mid point rooting using data from the AFLP 0,1 matrix. The data from each of the two sets of primer pairs were interleaved to form a single large 0,1 matrix. Analysis was performed in PAUP version 4 . Where they occur, the numbers above the lines represent the bootstrap value at that branching point.

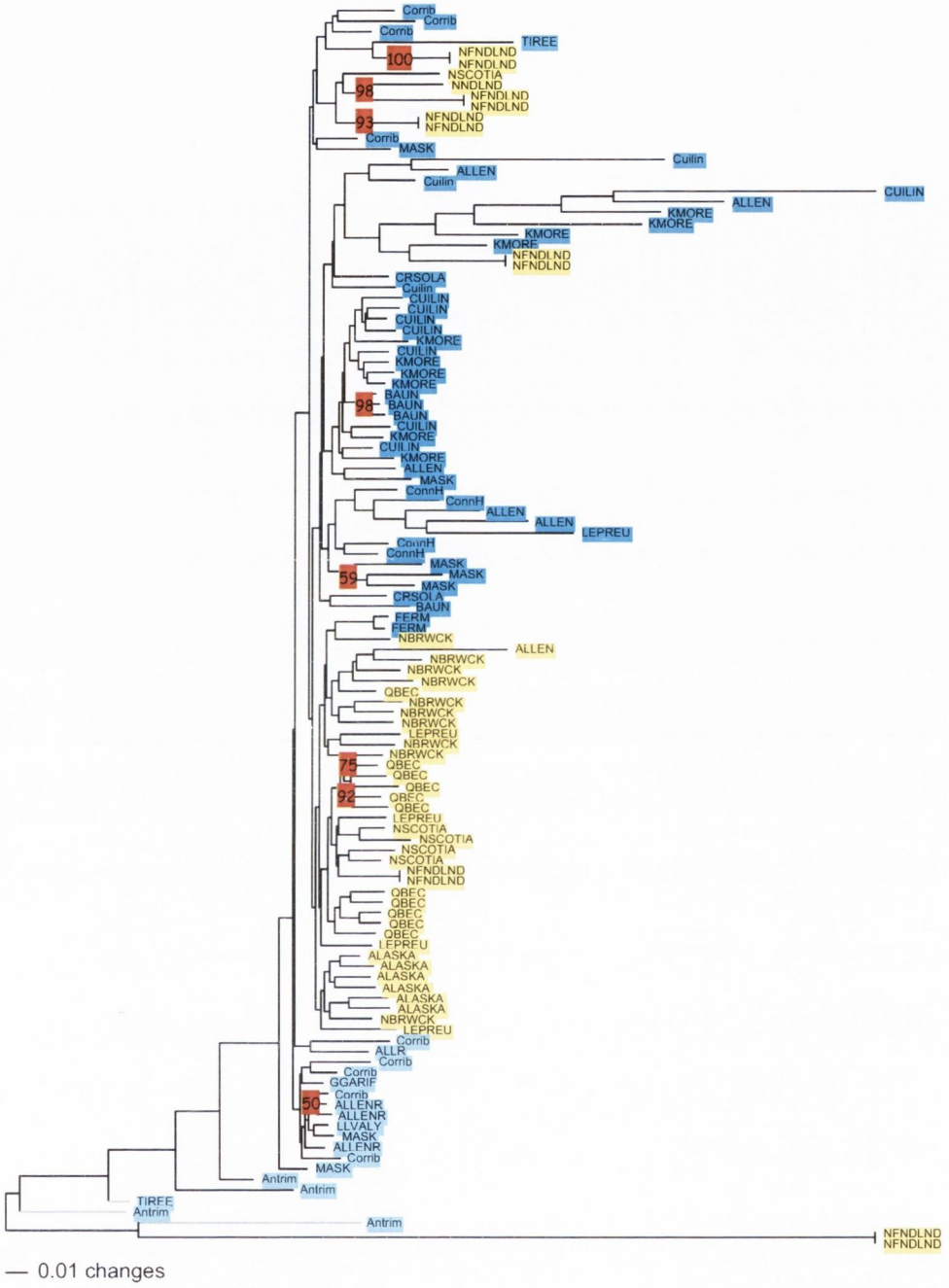


Figure 3.4.8 Neighbour-joining phylogram (PAUP) of all Irish, North American and a single Scottish population of *Spiranthes romanzoffiana*. Phylogram construction was mid point rooted, based on data from the AFLP 0,1 matrix. The data from each of the two sets of primer pairs were interleaved to form a single large 0,1 matirx. Analysis was performed in PAUP version 4. Samples in blue are Irish and Scottish. Samples in yellow are North American. Bootstrap values are in red.

The Neighbour-joining dendrogram of individual North American samples (Figure 3.4.6) shows a moderate level of population structuring. There are however three groups showing some moderate clustering. The Alaskan samples do form a group with high bootstrap support for the grouping. This grouping does however contain a sample from the Lepreau population. New Brunswick and Newfoundland both form distinct groups, though a number of samples from these populations occur within a number of different groups. The Quebec and Lepreau samples are interspersed among the other populations.

The Neighbour-joining dendrogram of individual Irish samples (Figure 3.4.7) shows very little supported structure. There is some bootstrap support for a number of population-specific groups. The grouping of three samples within the Knockmore population is supported by 100% of the bootstrap replications. The two samples from Fermanagh in the Northern Ireland are also supported by 100% bootstrap replications. The Antrim population, also in the North of Ireland, appears as a single group, though it does contain one of the samples from the only Scottish population (Tiree) in this analysis. The remaining Tiree sample comes out as a basal branch in a group containing samples from Lough Allen, Lough Cuilin and Knockmore. It is interesting that one of the Scottish samples is placed with a population from the North East of Ireland. This suggests that gene flow is occurring or has occurred between Irish and Scottish populations.

3.4.3 *Population differentiation*

Table 3.4.2 Summary of gene diversity estimates

Population level	Sample size	PPL	Shannon's index	Ht	Hs	Gst	Nm
All populations	125	1	0.413	0.2571	0.1984	0.2281	0.6883
Irish populations	68	0.9734	0.4029	0.2579	0.2526	0.1121	3.9584
North American populations	57	0.9634	0.377	0.1679	0.229	0.2526	1.7921

PPL = Proportion of polymorphic loci
Ht = Total gene diversity
Hs = Within population diversity
Gst = Proportion of between population diversity
Nm = Gene flow
S. index= Shannon's index

Estimates of total gene diversity (Ht) between all populations was (Ht = 0.26) (Table 3.4.2). North American populations showed a lower overall gene diversity (Ht = 0.17) compared with the value for Irish populations (Ht = 0.26). The overall within population diversity (Hs) for all populations was (Hs = 0.19). The within population gene diversity was again lower in the North American samples (Hs = 0.23) compared to the value for the Irish samples (Hs = 0.25). The proportion of between population diversity (Gst) for all populations is (Gst = 0.23). North American populations (Gst = 0.25) have a higher value higher than the Irish populations (Gst = 0.11). The high within population diversity (Hs) in the Irish populations and the low proportion of between population diversity (Gst) indicates that Irish populations are not differentiated as much as North American populations. The gene flow estimates (Nm) are considerably higher for Irish populations (Nm = 3.9) compared to the values for North American populations (Nm = 1.8).

The partitioning of genetic variation was further investigated using Analysis of Molecular Variation (AMOVA) at two different levels (table 3.4.3, 3.4.4 and 3.4.5). The overall partitioning of molecular variance was calculated among and within groups in North America and Ireland. 15.99% ($p < 0.0001$) of the variation was distributed among all the groups and 72.3% ($p<0.0001$) within. Variation among the North American samples was 22.5% ($p<0.0000$), variation within the samples was 77.5% ($p<0.0001$). Variation among the Irish samples was 14.43% ($p<0.0001$), within variation was 85.57% ($p<0.0001$

Table 3.4.3 Partitioning of variation among and within regions (North America and Ireland) using AMOVA.

Source of Variation	df	SS	Variance component	% Variation	Significance
Among groups (North America and Ireland)	1	274.06	4.1443	**11.72**	p< 0.0001
Among populations within groups	11	740.14	5.6539	**15.99**	p< 0.0001
Within populations	85	2173.33	25.568	**72.3**	p< 0.0001

df = degrees of freedom
SS = sum of squares

Table 3.4.4 Partitioning of molecular variation among and within North American populations of *Spiranthes romanzoffiana* using AMOVA.

North American populations

Source of variation	df	ss	Variance components	% of variation	Significance
Among populations	5	385.152	7.28012 Va	**22.5**	p<0.00001
Within populations	38	952.917	25.07675 Vb	**77.5**	p<0.00001

df = degrees of freedom
ss = sum of squares

Table 3.4.5 Partitioning of molecular variation among and within Irish populations of *Spiranthes romanzoffiana* using AMOVA.

Irish populations

Source of variation	df	ss	Variance components	% of variation	Significance
Among populations	6	354.995	4.37974 Va	**14.43**	p<0.00001
Within populations	47	1220.413	25.96623 Vb	**85.57**	p<0.00001

df = degrees of freedom
ss = sum of squares

3.5 Discussion

3.5.1 Genetic diversity and differentiation within and among Irish populations of Spiranthes romanzoffiana.

A number of key factors influence the distribution of genetic variation within and between populations, the breeding system and population size for example often affect the amount of within population variation and population differentiation (Sun, 1996; 1997). The preservation of genetic diversity both within and among natural populations is a fundamental goal of conservation biology (Hamrick *et al.*, 1991). Several aspects of conservation biology, such as determining the extent of genetic diversity, can only be addressed by detailed population genetic studies (Hamrick and Godt, 1995). The genetic diversity of a population is fundamental to the evolutionary potential and ultimate survival. In order to assess the diversity and evaluate the viability, information is required on the level of genetic diversity that is expected to occur within populations.

The gene diversity estimates (Proportion of polymorphic loci, Shannon's index and Nei's gene diversity) for the Irish populations of *S. romanzoffiana* were consistently higher than corresponding measures from the North American populations. Populations in Antrim (n = 4) and Fermanagh (n =2), displayed the lowest values for all three gene diversity measures. However, caution is required when interpreting these results as Antrim and Fermanagh had the smallest samples size in the analysis. There was a strong positive correlation between increasing log sample size and increasing diversity estimate values (Figure 3.4.1 a, b and c). However, Forrest *et al.* (2002) carried out AFLP work on a number of Irish populations, including the Antrim population analysed in this research. They found a low proportion of polymorphic loci value (0.03) in this population and had a sample size of n = 25. In there case a small sample size was not a contributing factor the low gene diversity value. There may be some justification for considering these populations as genetically depauperate relative to the other Irish populations sampled.

In contrast to this, the highest gene diversity values across the range of estimates were found in the Lough Cuilin (n = 10) population in Co. Mayo. The increase in diversity values is again strongly correlated with increasing sample size. The pattern and high proportion of gene diversity in the sampled populations may indicate that the southern Irish populations are not suffering from a genetic paucity. A larger sample size and further genetic analysis would help to elucidate this whether this statement is true or not. These data also suggest that the *Spiranthes romanzoffiana* in Ireland is certainly not exclusively clonal as has been suggested in the literature (Forrest, *et al.*, 2002), as considerable and significant variation exists within and among populations.

The UPGMA analysis of the Irish samples (Figure 3.4.5) indicate the possible presence of two moderately distinct groups, group 1; populations from Lough Corrib in Co. Galway and Lough Allen in Co. Leitrim. Group 2; Lough Cuilin and Knockmore, both in Co. Mayo. The remaining two populations, Knockmore on Lough Conn in Co. Mayo and Lough Mask in Co. Galway appear in the analysis as individual populations. Lough Mask is the outlying group and is possibly genetically distinct from the other Irish populations. The Lough Cuilin and Lough Conn populations are separated by < 5 km. The populations at Lough Corrib and Lough Allen are separated by >80 km. The analysis however, indicates that the populations within these groups are genetically similar. It is possible that the small dust-like seeds of *Spiranthes romanzoffiana* are dispersed between these populations. Pollen dispersal, though possible, would depend on relatively long distance flights by bumble bees (*Bombus* sp). Long distance pollinator flights could result in gene flow, but the extent to which this happens relative to seed flow is unknown (Squirrell *et al.*, 2001). Work by Neilson and Siegmund (1999) and Chung and Chung (2000) suggest that the small, dust like and potentially highly dispersible seeds, rather than pollen are the primary agent of the inter-population communication of genetic material in orchids.

Gene flow among orchid populations appears to be variable (Tremblay *et al.* 2005). The neighbour-joining dendrogram of the Irish samples (Figure 3.4.7) is less supported than the North American dendrogram (Figure 3.4.6). There are two small, but distinct groups shown on the tree. The two samples from Fermanagh are grouped together (100% bootstrap

support). The four samples from Antrim are grouped together, though this group has no bootstrap support and also contains a single sample from the Scottish population on Tiree. These data suggest that the Antrim population has or is sharing genetic material with this southern Scottish population. The remaining Irish samples are interspersed together, with no discernible pattern and only moderate levels of population differentiation. These data are consistent with the Amova results (Table 3.4.4) which indicate that the Irish populations are less differentiated than the North American populations analysed in this study.

3.5.2 Comparison of genetic diversity and differentiation within and among Irish and North American populations of Spiranthes romanzoffiana.

Comparative population studies using molecular genetic methods are needed to obtain information on the levels and patterns of genetic diversity in wild orchids (Wong and Sun, 1999). With this in mind the research expanded its genetic assessment to include a number of North American populations of *S. romanzoffiana.* The inclusion of North American populations also allowed the genetic diversity of the Irish populations to be viewed in the context of the species North American range. Genetic distance relationships were looked at on the individual sample level, using cluster analysis and neighbour-joining trees (Figures, 3.4.6, 3.4.7 and 3.4.8) based on Nei-Li 1979, restriction site distance measure. The results for the North American samples to some extent support the findings from the UPGMA (Figure 3.4.4) and PCO analyses (Figure 3.4.3). However the Lepreau and Newfoundland populations in this case are not grouped together. The overall shape and branch length of the neighbour-joining tree does suggest differentiation of populations, however the data do not support a complete divergence of populations. A complete divergence the populations may be hampered by, for example, infrequent gene flow. The gene flow estimates for the North American samples (Nm = 1.8) suggest that some level of gene flow is occurring or has occurred in the recent past. Long distance seed dispersal is possible though it would be difficult to determine the mode of dispersal involved. It may be the case that these populations once belonged to a single refuge population or a refuge meta-population during the last glaciation and have subsequently diverged. The current genetic structure may also

be a result of recent fragmentation (human disturbance) of a once continuous genetic system. Determining the answers to these questions, though extremely interesting, are beyond the scope of this research.

The North American population gene flow estimate is lower (Nm = 1.8) and suggests limited genetic communication between the North American populations sampled. This is further corroborated by the high proportion of between population diversity for the North American populations (Gst = 0.26) compared to that of the Irish populations (Gst = 0.11). These data are consistent with the dendrogram structure described in the neighbour-joining analysis. This genetic pattern is expected from animal-pollinated out-crossing orchids with wind dispersed seeds such as *Orchis papilionacea* (Arduino *et al.*, 1995), *Epipactis helleborine* (Hollingsworth and Dickson, 1997), *Spiranthes sinensis* (Sun, 1996). A previous study by Arft and Ranker (1998) revealed high levels of genetic variation within but low levels of genetic differentiation among 12 populations of *Spiranthes diluvialis* from Utah and Colorado. In contrast to this, inbreeding orchids, such as *Cephalanthera rubra* (Scacchi *et al.*, 1991) and *Spiranthes hongkongensis* (Sun, 1997) often lack variation both within and among populations.

The partitioning of genetic variation among and within Irish and North American populations was examined using Analysis of Molecular Variation (AMOVA). The majority of genetic variation in Irish populations of *Spiranthes romanzoffiana* is held within rather than among populations (85.57 %, $p<0.0000$). This was in contrast to the lower levels of within population variation detected in the North American samples (77.5%, $p<0.0000$). The among population value for Ireland was significant (14.43%, $p<0.0000$) but was lower than that recorded in the North American samples (22.5%, $p<0.0000$). The lower levels of population differentiation among Irish populations may suggest a relatively high level of gene flow (Nm = 3.9584), though is not necessarily the case. Caution is required when interpreting the Nm estimate of gene flow this is an indirect and assumed level of gene flow therefore it is not completely reliable. The results for the Irish populations suggest a lower degree of population differentiation compared North American populations. These trends however must be viewed with caution, as this may be a result of uneven sampling between

the two geographic regions. Figure 3.4.7 A,B and C suggests that there is a strong correlation between sample size and diversity estimates. The trend implies that with a higher rate of sampling the rate of genetic diversity would increase. This is particularly true with the North American samples as the trend is increasing; where as the Irish samples appear to plateau.

Hamrick and Godt (1996) summarised allozyme data from 16 orchid populations and obtained a mean Gst for orchids of (0.087) (*S. romanzoffiana* Irish mean Gst = 0.11; North American mean Gst = 0.26). They noted that orchids had an exceptionally low mean Gst and attributed this to species-specific pollinator characteristics of orchids and their tiny wind borne seeds. Forrest *et al* (2004) in their work on the genetic structuring of Scottish and Irish populations of *Spiranthes romanzoffiana* suggests that their Northern populations show a moderate degree of differentiation (Gst = 0.19) but high levels of within population diversity. They attribute this genotypic diversity to historic or contemporary occurrences of sexual reproduction and seed set in these populations. These data are consistent with the results from this research. In addition, Forrest *et al.*, (2004) found that levels of population differentiation (pairwise differences = 0.22) between two populations (separated by only 0.5km) on the island of Barra were in the same order of magnitude as the estimate for differentiation among Northern populations as a whole (involving inter-island distances of 70km between Coll and Barra). They suggest that this significant level of differentiation, evident in some cases over short distances, may be related to infrequent production of seeds, which will ultimately reduce opportunities for inter-population gene flow (Forrest *et al.*, 2004). Their results further suggest that their southern group, including Ireland, has very low levels of genetic variability and are consistent with agamospermous or autogamous species. This hypothesis conflicts with the conclusions drawn from this research which suggests that there are low levels of population differentiation and high levels of gene flow within and between Irish populations, consistent with an out-crossing species. Some caution is warranted with this conclusion as the gene flow estimate from the gene diversity statistics is based on the assumption that if populations are not differentiated, gene flow will be occurring. Of course this may be the case, further fine scale genetic analysis and reproductive investigations are required in order to elucidate this hypothesis.

It is clear from the literature that some caution is warranted when interpreting the patterns in genetic structure in orchids. Some orchids are in accord with theoretical expectations, i.e. high within population diversity and low between population diversity is typical of out-crossing species. Yet this is apparently not the case in all orchid species (Sun and Wong, 2001). Work by Squirrell *et al.* (2001) looked at the genetic variation in the North American introduced orchid, *Epipactis helleborine* and compared these to the genetic variation in the species' native range across Europe. Using cpDNA and RFLP's they showed equivalent or higher levels of within population genetic diversity and lower levels of among population diversity in introduced populations relative to native populations. Their research suggests that the high levels of observed heterozgosity in the introduced North American populations could be attributed to the high levels observed in the native populations. In other words, such variability could be captured within a small number of plants (Squirrell *et al*, 2001). The maintenance of this high level of within population diversity they suggest would rely on the rapid population expansion after the species introduction, which would mitigate against loss of allelic diversity by genetic drift.

This however does not appear to be the case with *Spiranthes romanzoffiana* in Ireland, as there does not appear to have been any rapid proliferation of the Irish populations. Though this statement may be erroneous as our knowledge of the species' arrival and subsequent dispersal and expansion is still unknown, it could be the case that *S. romanzoffiana* is a very recent introduction to Ireland and is in fact expanding its geographical range at present. It is clear that more research is required into the demographics and life history stages of the species is required if we are to coherently debate this hypothesis.

A more rigorous study into the levels of fine scale genetic structure within the Irish populations may allow us to better understand the levels of gene movement within these populations. To date very few fine-scale genetic studies have been conducted within terrestrial orchid populations. Two of the prominent studies in this area both found significant genetic clustering on a scale of less than 10m within 20 x 40m plots (*Caladenia tentaculata,* Peakall and Beattie, 1996; *Cymbidium goeringii*, Chung *et al.*, 1998). These studies suggest that gene flow either via seed or pollen is relatively restricted. These data

conflict somewhat with the finding of the current study which suggests that gene flow between populations with distances of over 50 km is occurring or has occurred at some time in the past.

Genetic structure in natural populations can often be a result of the extent of pollen and seed dispersal. There are, however a number of other possible influences that can shape the structure within and among populations. High within population diversity and low population differentiation has been attributed to a number of factors including, insufficient length of time for genetic diversity to be reduced or evolved following a natural reduction in population size and isolation, particularly without knowledge of the species longevity (Coates, 1988), adaptation of the species genetic system to small population conditions (Coates, 1988; Rosetto *et al.*, 1995; James, 2000), and extensive gene flow due to a combination of animal pollination and high out-crossing rates (Maguire and Sedgley, 1997).

If we consider the first point, that low population differentiation can be attributed to an insufficient length of time for genetic diversity to be reduced, then it maybe possible to elucidate an explanation for the current genetic structure in Irish populations. From the data we have at present it is impossible to date the arrival of the Irish populations. The genetic data derived from this research may suggest that the Irish populations are relatively recent colonisers. The low population differentiation may well be explained by the recent arrival and insufficient time for the differentiation of the species into genetically distinct populations. In addition to this hypothesis, it is known that *Spiranthes romanzoffiana* is a bee pollinated, out-crossing species (Catling, 1982). Levels of seed set are relatively high in Canadian populations.

Until the last couple of years it was generally accepted that *S. romanzoffiana* in Europe was a predominantly clonal species, reproducing annually by vegetative means. This based on a lack of evidence of seed rather than any tested hypothesis. The recent discovery of seed in both Irish and Scottish plants has changed this perception. This new evidence suggests the species may be successfully out-crossing within and between Irish populations. The low levels of seed detected however suggest that recruitment levels are low. It is possible that

seed set and subsequent dispersal occurred during historic times, resulting in the population distribution and genetic structure that exists in populations today. A complete cessation of out-crossing, seed production and dispersal seems unlikely. Instead the high levels of diversity suggest that the process is continual and is prevalent in contemporary populations. Further detailed analysis of mating patterns in Irish populations of *S. romanzoffiana*, such as the assignment of paternity to individual plants should be commenced to determine out-crossing and possible out-breeding depression and the importance of the genetic system's adaptation in resisting the loss of genetic resources due to small population size.

Chapter 4

Biogeographical relationships European and North American populations of *Spiranthes romanzoffiana*.

4.1. The study of Biogeography

Biogeography, as the term indicates, is both a biological and geographical science. Its field of study covers the multitudinous forms of plant and animal life which inhabit the biosphere, as well as the complex processes which control their activities. The approach and aim of the subject is geographical in so far as it is concerned with the distribution of organisms and biological processes (Tivy, 1979). Although this field of study is common to both biology and geography it is not the exclusive preserve of either of the sciences. By its very character, biogeography is situated at, and overlaps the boundaries of, a great number of disciplines, including climatology, evolutionary biology and systematics.

Spiranthes romanzoffiana is one of the few Irish 'natives' with uneven amphi-atlantic distribution, found widespread in North America but limited to the western fringes of Europe (Preston and Hill, 1997). Controversy persists over the explanation for the disjunct distribution of *S. romanzoffiana*. There are currently three contrasting hypotheses; 1) Populations of *Spiranthes romanzoffiana* in Europe are relictual populations from a more widespread periglacial distribution, 2) *Spiranthes romanzoffiana* established in Europe as a result of long distance transport of seed across the Atlantic, either through wind currents or as passengers on migratory bird species and 3) European populations of *Spiranthes romanzoffiana* are of recent origin and were introduced by humans during historic times (T. Curtis, 2002, *pers. comm*).

The first hypothesis, suggesting that *Spiranthes romanzoffiana* had a more widespread periglacial distribution than is evident in today's distribution, may well be possible.

However, given the available data it is very difficult to determine. The study of historical biogeography is fraught with difficulties. A major problem lies in floristic records, which are often incomplete. For example, the lack of continuous sedimentary records in Ireland and a deficiency in comprehensive biostratigraphical organic sequences has meant that the clarification of an agreed Pleistocene succession has not been possible (Coxon and Waldren, 1995). The absence of a particular species from fossil record does not necessarily mean that the species did not occur. It is possible that suitable sediment was not sampled or that the particular species does not preserve well.

Migratory birds and /or wind currents have been cited as possible vectors for the long-distance dispersal of propagules (Horsman, 2005). The alternative suggestion is that of an anthropogenic introduction (T. Curtis, *pers comm.*, 2002). Proving or disproving any of the three main hypotheses is extremely difficult. This section of the research attempts to tease out the hypotheses in an effort to build up arguments for and against the opposing theories on the disjunct distribution of *Spiranthes romanzoffiana*. The pattern and distribution of genetic diversity between North American and Irish individuals will help to ascertain whether the Irish individuals are a result of recent or historical (>1,000 years bp)dispersal event or (events).

4.1.2 Disjunct distributions

The interpretation of disjunct distributions has traditionally been centred on two hypotheses. First, the existence of closely related taxa or populations of the same species may result from the development of a barrier arising from a previously more widespread distribution of a single taxon (McGlone, 2001). Alternatively, disjunct distributions may result from the dispersal of organisms across pre-existing physical and /or ecological barriers from a central zone of origin (Thompson, 1999).

It is occasionally difficult to obtain accurate estimates of dispersal patterns using direct observations, as long-distance dispersal events are often missed (Ouberg *et al.*, 1999). The stochasticity and rarity of successful colonisations, displaced from the geographic range of a

species, makes them fundamentally difficult to trace back to their origin (McBreen and Cruzan, 2004). To add to these difficulties, disjunct populations which might be interpreted as having originated via long-distance dispersal may have a longer history of separation and an independent origin relative to populations in the primary range of the species (Cruzan and Templeton, 2000).

In addition to the difficult interpretation of long distance dispersal and colonisation events there are also problems in determining the effect of introduction or colonisation on the species itself. Dispersal events and introductions from one location to another will in all likelihood involve a population bottleneck, particularly if the source is from a single population. Theory predicts that introduced species will show lower levels of intra-population diversity and higher levels of population differentiation than their natural counterparts (Brown and Marshall, 1981). However on a case-by-case basis, empirical data show the magnitude of these changes varies greatly (Squirrell *et al.*, 2001).

Work by Neuffer and Hurka (1999) shows comparative genetic depauperacy in *Capsella bursa-pastoris* (Brassicaceae), between North American introduced plants and its native European populations. In contrast however, there was no evidence of a genetic bottleneck with the introduction of *Apera spica-venti* (Poaceae) to Canada from Europe (Warwick *et al.*, 1987). In *Bromus tectorum* (Poaceae), there were fewer alleles per locus in the introduced American range compared with the native Eurasian range and within individual introduced populations levels of allelic diversity and polymorphic loci were higher in the American range (Novak and Mack, 1993 in Squirrell *et al*, 2001). Caution is clearly needed before assumptions are made on the response of individual species to dispersal and colonisation events.

If the founder population contains only a few individuals, is genetically depauperate and remains small, the effects of loss of diversity will be pronounced due to potential random genetic drift or inbreeding depression for example. On the other hand if there has been multiple introduction events followed by a rapid expansion these effects maybe minimal (McBreen and Cruzan, 2004). Given sufficiently variable founders, higher intra-population

diversity and lower inter-population differentiation can occur in introduced relative to native ranges. The development of molecular markers has provided the study of biogeography, dispersal and colonisation studies with new powerful tools (Ouberg *et al.*, 1999). The current study employs two types of molecular markers (organelle cpDNA and nuclear AFLPs) in an effort to determine the levels of geographic-genetic divergence between North American, Scottish and Irish populations of *Spiranthes romanzoffiana.*

4.1.3 Molecular techniques and analyses

Plastid DNA as a molecular marker.

The current investigation into the biogeography of *Spiranthes romanzoffiana* used both plastid DNA and AFLP marker techniques to elucidate potential biogeographic relationships between European and North American populations.

DNA found within the organelles of plants is known as organellar DNA. Animal cells have only one type of organellar DNA (in the mitochondria), while plants cells have two types: mitochondrial DNA and plastid DNA. In addition to organellar DNA, all cells contain nuclear DNA. Therefore plants have three types of genome within their cells. A number of key differences exist between these genome types and should be noted before undertaking a molecular study. Nuclear genomes contain linear chromosomes, which tend to be bi-parentally inherited with the ability to assort freely (Watson and Murphy, 1993). The genomes of organelles are significantly smaller than nuclear genomes and are generally uni-parentally inherited and therefore effectively haploid (Ennos *et al.*, 1999). Genomes also differ in their rates of mutation and substitution. Substitution rates in plastid DNA have been inferred to be 3-5 times higher than plant mitochondrial DNA, while mutation rates of plastid DNA is thought to be only half that of nuclear DNA (Wolfe *et al.*, 1987). Organellar DNA is therefore not as variable as nuclear DNA (Ennos *et al.,* 1999). However it is this low rate of evolution and uni-parental inheritance that makes organeller DNA useful for detecting changes within and among species over long periods.

The plastid genome is a single, circular genome found in the chloroplast. The chloroplast region of several species has been totally sequenced. A gene map of rice (*Oryza sativa*) chloroplast genome is shown in plate 4.1 (Tsutsumi *et al.*, 1992). The circular structure is composed of two inverted repeats, one region of short single copy repeats and one region of large single copy repeats (Jonson and Soltis, 1995). They are uni-parentally inherited from the female in angiosperms (Corriveau and Coleman, 1988) and show relatively low levels of size variation. The gene sequence is well characterised and in many cases these genes contain introns and are separated by inter-genic spacers, both composed of non-coding regions of DNA (Fay and Cowan, 2001). Some of these regions can contain higher numbers of repeating units (generally 10 or more for single nucleotide repeats), referred to as plastid microsatellites or Simple Sequence Repeats (SSRs). Plastid microsatellites can demonstrate high levels of variability and have been shown to vary greatly both between and within populations (Fay and Cowan, 2001).

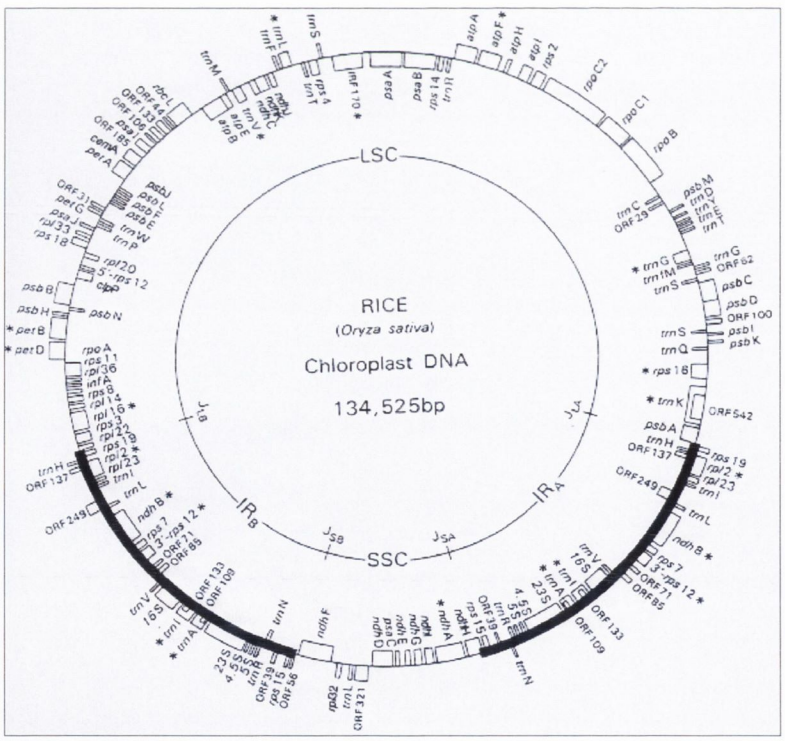


Plate 4.1 Genetic map of the entire rice chloroplast genome taken from N. Tsutsumi *et al.*, (1992)

The occurrence and position of microsatellites on the plastid genome varies from species to species. It is therefore necessary to survey the plastid genome to locate regions containing microsatellites. Once these regions have been identified it is possible to design primers to amplify the microsatellite. The amplification products can be assessed for all of the individuals being studied and any variation in base pairs can be determined.

This method has a general advantage over multi-locus genetic finger printing methods, such as AFLPs, in that only one short, well characterised region is amplified from each individual, which makes the technique less sensitive to DNA quality and quantity (Fay and Cowan, 2001). This is a useful characteristic as it helps to maximise the DNA quality from poor or degraded leaf material. This is often a problem with genetic studies where immediate extraction of the total DNA is not always possible. Storage of leaf samples for long periods in a desiccation media can lead to leaf degradation and poor DNA quality and quantity.

Chloroplast microsatellites have a number of other important applications. Chloroplast DNA can be used to assess the extent of sub-division within populations, for example the Norway spruce. This technique has been particularly useful in the study of biogeography. In Europe, chloroplast microsatellites have been used in the study of biogeographical patterns in a range of taxa, including species of *Orchis* (L). and *Liparis loeselli* (L). Rich. (Qamaruz-Zaman, 2000; in Fay and Cowan, 2001). They are also used for parentage analysis due to their polymorphic nature and uni-parental inheritance (Gillet, 1999). Variations in chloroplast DNA can be further applied in investigating the divergence of species over time from a common ancestor (Chang and Schaal, 2000). They are also employed in comparison of gene flow estimates between nuclear and plastid DNA to estimate the relative contributions of pollen and seed to the gene flow of a species (Ennos *et al.*, 1999).

Problems associated with chloroplast microsatellite primers

A major drawback associated with using chloroplast microsatellites for population studies is that, since the chloroplast genome is highly conserved, the levels of variation detected may not be sufficient to address the question studied. However with the detection of length

variable microsatellites in the chloroplast genome this is less of an issue. Plastid microsatellites are often sufficiently variable for population variability studies (Palme and Vendramin, 2002; Palme, 2003).

In addition chloroplast microsatellite primers developed for one species can rarely be used beyond the very closest relatives. Thus microsatellite primers need to be developed *de novo* for each new species (Mueller and Wolfenbarger, 1999). The development of microsatellite primers requires considerable molecular skills (i.e. cloning and sequencing) and a great deal of time.

4.2 Aims

Using a combination of AFLP and chloroplast microsatellite markers, the study aims to determine the scale of genetic divergence between European and North American populations of *Spiranthes romanzoffiana*. The resulting data will be used to investigate the current hypotheses concerning the present amphi-Atlantic distribution of the species.

4.3 Methods

4.3.1 Site selection and sampling

The samples used in this study were collected from the same locations as the samples used in Chapter 3 (see table 3.3.1).

4.3.2 Extraction and quantification of total DNA

Techniques used in this section of the research followed the same protocols as explained in sections 3.3.2 and 3.3.3. A larger number of individual samples were used in the microsatellite analysis. This was partly due to the poor quality of DNA from a number of the Scottish samples donated to this project by Forrest *et al.* (2004). Their samples were

collected in 2001/2 and were stored in silica gel to prevent desiccation. At the time of extraction these samples were three years old and the resulting DNA was of poor quality. During the research it was noted that this poorer quality DNA was unsuitable for AFLP analysis but was perfectly adequate for the chloroplast microsatellite work.

Target DNA regions and Primer trials

The sequencing of chloroplast DNA regions was carried out in RBG Edinburgh during a research project into the population genetic structure of European populations of *Spiranthes romanzoffiana* (Forrest, *et al.*, 2004). The following regions were sequenced for microsatellite loci by Forrest *et al.*(2004).

- *atpB-rbcL*
- *trnL*-intron-*trnL-trnF IGS*
- *trn-S-trnfM*
- *trnH-trnK*

Initial screening by Forrest *et al.* (2004) revealed 15 mononucleotide repeat loci >8bp in length, located on 4 of the 5-choroplast regions amplified. A polymorphic-A repeat was located on the *trnL*-intron. This was the only region to show any polymorphism in the 11 accessions studied (Forrest *et al.*, 2004). As these regions had already been screened it was decided that a number of other potential chloroplast regions should be sequenced in an effort to detect the presence of microsatellite markers. Table 4.3.1 shows the three gene regions screened during this research.

Table 4.3.1 Chloroplast gene regions and universal primer combinations used for amplification of *Spiranthes romanzoffiana* DNA.

Genes	Gene product	Gene region	Forward primer	Reverse primer	Reference
rpl 16	30S Ribosomal Protein CS16	Intron	1R	2R	Jordan *et al.*,1996
rps 16	50S Ribosomal Protein CL16	Intron	16F	2R	Oxelman *et al.*, 1997
trnT-trnL	30S Ribosomal Protein CL16	Intron	8F	2R	Taberlet *et al.*, 1991

The three cpDNA gene regions in table 4.3.1 were selected for analysis based on previously developed universal primers that are known to be reliable for amplification over a wide range of species. The amplification of each region was carried out using a range of annealing temperatures and cycle numbers as described in table 4.3.2.

Table 4.3.2 Steps in amplification of chloroplast regions.

Process	**Temperature (^{0}C)**	**Time**	**Cycles**
Premelt	95	1 minute	
Denaturation	95	45 seconds	
Annealing	50	45 seconds	30
Extension	72	3 minutes	
Final extension	72	27 minutes	
Soak	4	∞	

DNA quantification

Quantification was carried out as outlined in section 3.3.3.

4.3.3 PCR amplification of genes for sequencing and cycle sequencing

The amplified products were purified using the same column cleaning technique used in section 3.3.4. The cleaned samples were prepared for sequencing by aliquotting 3µl of the products into labelled flat-topped tubes. A master mix containing Applied Biosystems *Taq* DYE-Deoxy / Terminator cycle sequencing mix V.1.1 (Pink mix) and sequencing buffer along with sterile water was prepared following the steps in table 4.3.3. An aliquot of the master mix was added to each amplification product to make a total volume in each tube of 10µl. The samples were then mixed and placed on a MJ Research PTC200 Peltier Thermocycler. The temperature and time were set according to table 4.3.4.

Table 4.3.3 Components, volumes and concentrations per sample for amplification of target DNA regions prior to sequencing.

Component	Volume
Pink Mix	1ul
Sterile ultra-pure H_2O	1.8µl
Sequencing buffer	3.5µl
Primer (Forward/Reverse; 5ng/ml)	0.7µl
Total	7µl

Table 4.3.4 Temperature and time settings used throughout the thermocycling process.

Temperature (^{0}C)	Time	Cycles
96	10 seconds	1
50	5 seconds	25 cycles
61	4 minutes	1

Purification of samples prior to sequencing.

Each amplified sample was purified by mixing 50µl of ethanol (EtOH; 100%) with 2µl of sodium acetate (NaOAc; 3M). 52µl of the mixture was added to each amplified sample and incubated at room temperature for 5 minutes. The samples were then placed on ice and incubated for a further 10 minutes, after which the contents were mixed and centrifuged for 25-30 minutes at 12,000g.

Preparation for sequencing

The purified samples were prepared for sequencing by adding 25µl of a sample preparation agent called Template Suppression Reagent (TSR) into each tube. The content of the tubes were mixed on a vortex and incubated at 95^{0}C for 4 minutes. The samples were cooled on ice and centrifuged to ensure the contents were at the bottom of the tubes. The lids of the sample tubes were cut with a scissors and a rubber septa was placed into each one for use on the ABI prism 310 Genetic Analyser. The samples were then loaded onto a 310 Genetic Analyser. The analyser was set to the following specifications; Big Dye™ Terminator long-read, Run Module, Seq.Pop6 (1.0ml) using Pop 6 polymer for 130 minute per sample. The raw sequence data was automatically saved and compiled using Sequence Analysis version 3.4.1 (Applied Biosystems).

4.3.4 Sequencing of the chloroplast gene regions

The amplified regions of cpDNA were sequenced using *Taq* Dye-Deoxy terminator cycle sequencing kits (V.1.1; Applied Biosystems). There are a number of other methods commonly used, including Maxam-Gilbert sequencing (Maxam-Gilbert, 1977; Old and Primrose, 1994) and Sanger sequencing (Old and Primrose, 1994; Hillis *et al*, 1996). The Maxam-Gilbert method differs from Sanger sequencing as it uses chemicals to specifically cleave DNA instead of using enzymes to build DNA strands and terminators to stop synthesis (Maxam-Gilbert, 1977). The Applied Biosystem's cycle sequencing reaction utilises dideoynucleotide chain termination in a similar way to Sanger sequencing. However the Sanger sequencing PCR is used is used to amplify labelled strands of DNA that are complementary to labelled strands (Hillis *et al*, 1996).

The sequencing process was automated using an ABA Prism 310 Genetic Analyser (Applied Biosystems) where the fragments were automatically detected during electrophorsis. The successfully sequenced DNA samples were edited and assembled using the SEQUENCHER software package © (Gene codes corporation) version 3.1. SEQUENCHER allows assemblage of the forward and reverse primers and formation of a chromatograph of the sequenced DNA. The sequences were then imported into PAUP version 4.0b (Swofford, 1996) and aligned by eye. The resulting matrix was assessed by eye to look for regions that showed variation in terms of base composition.

4.3.5 Aligning the DNA sequences

The aim of aligning the sequences was to assemble the forward and reverse sequences for each sample together so that errors in the sequence could be rectified. The initial and final few bases (10-20b.p.) were deleted, as these base pairs are unreliable. Sequences were produced by combining sequences from both directions and checking ambiguities against each other. The complete sequence for each sample at each of the gene regions was then aligned with other samples from the same gene region to form a matrix. Samples were aligned using a combination of visual techniques using PAUP 4 (Swofford, 1996), and

automatic alignment software including: Seq-Al Version 2.0al, which was used to convert the sequences in the Nexus format. Mac Clade 4.0 (Maddison and Maddison, 2000) was used to convert the Nexus format into NBRF format and Clustal X 1.8 (Thompson, *et al.*, 1997) was then used to compile the sequences and align them to each other automatically. The aligned sequences were then imported into PAUP 4 (Swofford, 1999), checked by eye and alterations were made where required.

4.3.6 Identification of chloroplast microsatellite regions

Possible polymorphic regions were sought. None were found of sufficient length or polymorphism to be developed further into markers.

4.3.7 Microsatellite amplification

Sequencing and subsequent analysis of the three chloroplast regions from samples of *Spiranthes romanzoffiana* from Irish, Scottish and North American populations detected no repeat bases suitable for primer development. The forward and reverse primers designed to amplify the *trnL-F* microsatellite region by Forrest *et al.* (2004) were purchased from Primer3[1].

Forward primer: 5'- GGTAACTTCCAAATTCAGA – 3'.
Reverse primer: 5' – ACAGCTTCCGTTGAGTCTC – 3'.

A number of trials were carried out on the primer combination by varying the annealing temperatures and the number of cycles in order to achieve the optimum conditions for the amplification of the microsatellites. An amplification master mix was made up for the trnL-F microsatellite and aliquotted into 0.5µl total DNA. In order to assess the optimum concentration of total genomic DNA required for successful amplification a series of trials

were conducted on 20 samples; 10 Irish, 5 North American and 5 Scottish. Five different dilution rates were measured as shown in table 4.3.6.

Table 4.3.5 Components of the trnL-F microsatellite master mix.

Components	**Volume per sample**	**Concentration**
Sterile ultra-pure H_20	11.375µl	-
x10 Buffer (Promega)	5µl	x1
Forward primer	2.5µl	0.4µM
Reverse primer	2.5µl	0.4µM
dNTP's	0.5µl	0.2mM
$MgCl_2$	2.5µl	2.5mM
Taq (Promega)	0.125µl	1.5 units

[1](www.basic.northwestern.edu/biotools/Primer3.html)

Table 4.3.6 Total genomic DNA dilution trials (the dilution rate in bold print was determined as the optimum dilution rate).

Total genomic DNA	**Ultra-pure H_20**
1µl	20µl
1µl	60µl
1µl	100µl
1µl	150µl
1µl	**200**µl

Contents of the tubes were mixed and spun down and subsequently loaded onto an MJ Research PTC200 Peltier Thermocycler. The products were then checked for successful amplification using gel electrophoresis. Amplification products were mixed with 0.25µl Applied Biosystems ROX 500s size standard and 24µl of Formamide (CH_3NO), which is used to denature the DNA samples (Applied Biosystems).

The samples were denatured on a heating block for 10 minutes at 95^0C, transferred to an ice tray for 2 minutes and centrifuged. Analysis was performed on the samples using an ABI PRISM 310 Genetic Analyzer. Applied Biosystems Genescan analysis software version 3.1 was used to read and size each sample using comparisons with the ROX size standard and they were further assessed and then converted into tables using Genotyper Software version 3.7 (Applied Biosystems). Haplotype sizes (in base pairs) were recorded and proportions of haplotypes per geographic location were illustrated using pie charts. The overall haplotype frequency was assessed between Irish, Scottish and North American populations. For ease of interpretation pie charts were displayed on maps according to their location.

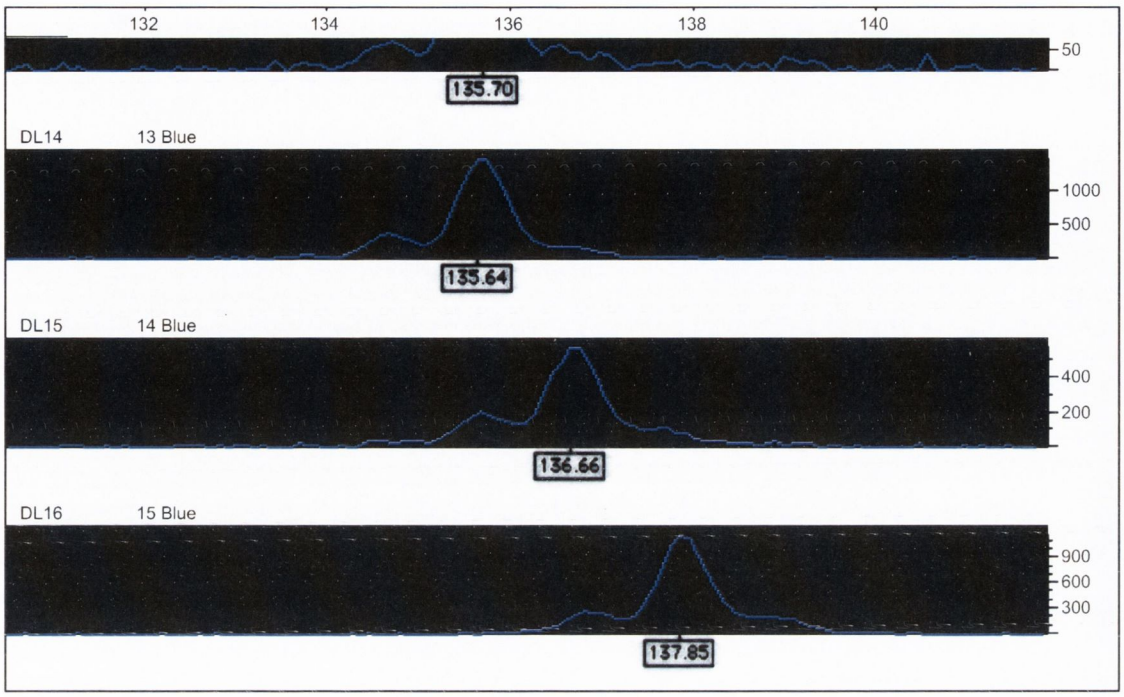


Figure 4.3.1 Results from a sample of the data obtained from the cp microsatellite analysis. Each of the three bands represent a potentially variable repeat differing by, in each case, a single base pair. Bands were scored based on their size in basepairs and closeness to the nearest whole number. Scoring was rigorous and was repeated twice to minimise error. Peaks <50 (see scale on the right of the graph) were disregarded Band DL 14 = Lough Mask, band DL 15 = Lough Mask and band DL 16 = Lough Corrib.

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4.3.8 Amplified Fragment Length Polymorhism (AFLP) molecular markers used in the assessment of the biogeogrpahical status of Spiranthes romanzoffiana.

See section 3.3 for complete account of the techniques used to collect leaf material, extract, quantify and analyse DNA and interpret the AFLP data.

4.4 Results

Three geographic regions were targeted in the sampling of populations of *Spiranthes romanzoffiana*. In total 23 populations were sampled; 6 from North America, 5 from

Scotland and 12 from Ireland. 145 individual samples were collected and successfully analysed (Table 4.4.1).

Table 4.4.1 Sites sampled for genetic / biogeographical study of *Spiranthes romanzoffiana*. Number of samples indicates the actual number of samples from which DNA was successfully extracted.

Country	Region	Location	No. of samples
Ireland	Mayo	Lough Conn	10
Ireland	Mayo	Lough Cuilin	10
Ireland	Mayo	Lough Mask	10
Ireland	Mayo	Lough Levally	5
Ireland	Galway	Lough Corrib	10
Ireland	Leitrim	Lough Allen	10
Ireland	Roscommon	Lough Allen	8
Ireland	Cork	Glengarriff	1
Northern Ireland	Antrim	Gortnagory	4
Northern Ireland	Derry	Long Point, Lough Beg	4
Northern Ireland	Fermanagh	Upper Lough Erne	2
Northern Ireland	Tyrone	Lough Beg	3
Scotland	Tiree	Unknown	2
Scotland	Colonsay	Kiloran dunes	5
Scotland	Coll	Arilcod field	5
Scotland	Vatersay	Causeway	5
Scotland	Barra	Bruernish	4
Canada	Quebec	Laurentian Mtns	10
Canada	New Brunswick	Grand Falls	10
Canada	New Brunswick	Lepreau	7
Canada	Newfoundland	Iles St Pierre et Miquelon	7
Canada	Nova Scotia	Brier Island	8
U.S.A	Alaska	Unknown	6

4.4.1 Chloroplast microsatellite analyses

Three regions of the chloroplast genome (*rps*16, *rpl*16 and *trn*t-*trn*L) were examined for the presence of variable microsatellites. No microsatellite or minisatellite repeats were detected

in these chloroplast regions. The time and resources required to further pursue this aim was beyond the scope of this project. The mononucleotide, polymorphic-A repeat on the *trn*L-intron developed by Forrest *et al.* (2004) was used to help elucidate the biogeographical relationships between Irish, Scottish and North American populations of *Spiranthes romanzoffiana* sampled. Data derived from AFLP analyses were used to expand and substantiate the results of microsatellite analysis. Analysis of the samples from the three geographic regions using the single locus marker (*trn*L-intron) revealed the occurrence of 6 distinct alleles/ haplotypes. The size range of the alleles was 134 – 139 base pairs. Five of the haplotypes occur in the Irish populations, four in the Scottish and four in the Canadian populations.

Table 4.4.2 Size and distribution of the haplotypes of the *trn*L-intron poly-A repeat. The size in base pairs of each allele corresponds to the numbered colums. The occurrence of an allele at a particular site is denoted by a coloured cell. The proportion of each allele is given within the appropriate cells. See maps 3.3.1, 3.3.2, 3.3.3 for details on the geographic location of the individual populations. The presence or absence of each haplotype in each geographic location is stated at the bottom of the table.

	Haplotype	1	2	3	4	5	6
	Size (bp)	134	135	136	137	138	139
Location	Population						
Ireland	G.garriff					1	
	L. Corrib					1	
	L. Allen		0.19			0.81	
	L. Mask	0.07	0.19			0.53	0.13
	L.Levally			0.2		0.8	
	L. Conn					1	
	L. Cuilin					1	
	Gortnagory					1	
	L. Beg					1	
	L. Erne					1	
	L.Beg					1	
Scotland	Colonsay					1	
	Coll			0.9	0.91		
	Tiree				0.67		0.33
	Vatersay				1		
	Barra			0.4	0.6		
N. America	Alaska		0.2	0.8			
	Quebec		0.2	0.07	0.4	0.33	
	New Brunswick		0.18	0.06	0.29	0.47	
	N. Scotia		0.57			0.43	
	Nfoundland				0.6	0.4	
Total	Ireland	1	2	1	0	11	1
	Scotland	0	0	2	4	1	1
	N. America	0	4	3	3	4	0
Haplotype present	Ireland	Yes	Yes	Yes	No	Yes	Yes
	Scotland	No	No	Yes	Yes	Yes	Yes
	N. America	No	Yes	Yes	Yes	Yes	No

4.4.2 Distribution of haplotypes.

A number of shared haplotypes were identified as occurring throughout the *Spiranthes romanzoffiana* range analysed. Haplotypes 1 (134 BP) and 6 (139 BP) were unique to the European populations. These haplotypes occurred in Lough Mask, county Mayo and on the Island of Tiree in the Inner Hebrides on the west-coast of Scotland. No unique haplotypes

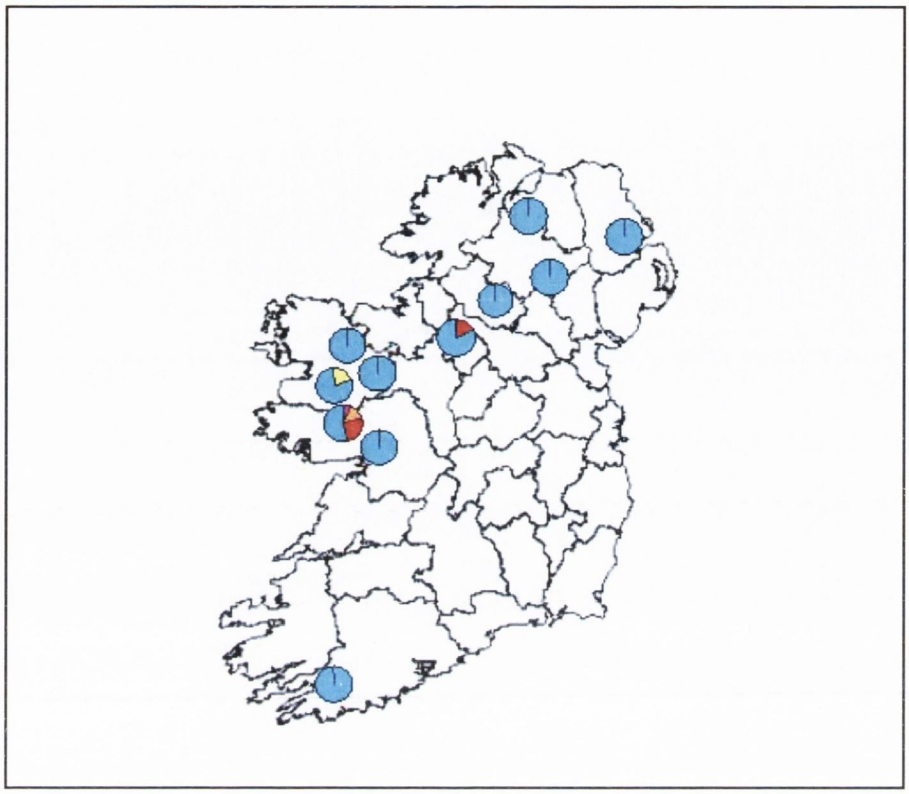
were recorded in the North American populations. However haplotype 2 (135 BP) was found in Ireland and North America but was absent in the Scotland. Haplotype 2 was found at low proportions in all North American populations except Newfoundland where it was absent. In Ireland this haplotype was found in Lough Allen and Lough Mask, where it again occurred in relatively low proportions (Figure 4.4.1). Haplotype 4 (137 BP) was found in all the populations in North America except Nova Scotia and Alaska. Haplotype 3 (136BP) is widespread and was found in all three geographic regions including the geographically isolated population in Alaska, however the proportions in all populations except Alaska were low. Haplotype 4 is found in all Scottish populations, except the most southerly of the sampled populations on Colonsay. Haplotype 4 is absent in the Irish populations. Haplotype 5 (138BP) is the most geographically widespread haplotype. It occurs in all North American populations except Alaska, it was present in all Irish populations and was present in the Colonsay population. The occurrence of haplotype 5 in the most southern Scottish populations shows some affinity with the high proportion of haplotype 5 in Northern Irish populations.

The geographic patterns of haplotype proportions and distributions are represented as pie charts and are placed on maps at their approximate locations. Figure 4.4.1 illustrates the pattern of distribution and proportion of haplotypes in Irish populations of *Spiranthes romanzoffiana.* Eight of the twelve populations examined are monomorphic for haplotype 5 (138 BP). Lough Allen, in county Leitrim in the north-west of the country contains two haplotypes (haplotypes 2 and 5). Lough Levally in County Mayo also contains two haplotypes (haplotypes 3 and 5). Lough Mask in County Mayo contains four haplotypes (Haplotypes 1, 2, 5 and 6), representing most of the allelic diversity recorded in the Irish samples. Lough Mask also contained haplotype 1, a unique allele not detected in any of the other Irish, Scottish or North American populations examined. The majority of allelic diversity in Irish populations detected in this research was found in the west of the country and appears to be concentrated in county Mayo.

Figure 4.4.2 shows the pattern of distribution and proportion of haplotypes in Scottish populations of *Spiranthes romanzoffiana.* Barra, the most northerly positioned population

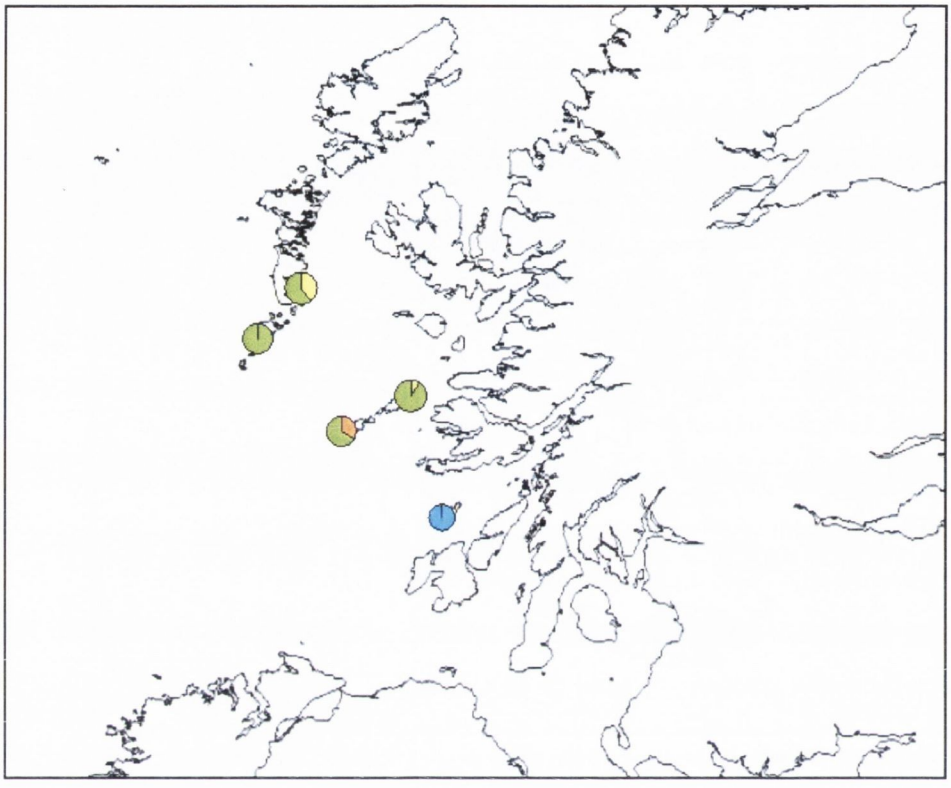
has two haplotypes (haplotypes 3 and 4). Just south of Barra lies the Island of Vatersay, where only one haplotype (haplotype 4) was recorded. The population on Coll contains two haplotypes (haplotypes 3 and 4). The population on Tiree also contains two haplotypes (haplotypes 4 and 6). The most southern of the Scottish populations contains one haplotype (halotype 5). Based on the single locus used in this research Colonsay appears to be genetically distinct from the other Scottish populations studied and more closely related to Irish plants.

Figure 4.4.3 shows the pattern of distribution and proportion of haplotypes in the North American populations of *Spiranthes romanzoffiana*. The Alaskan samples, geographically isolated from the remaining North American samples by >3000km, contain two haplotypes (haplotypes 2 and 3). Newfoundland has three haplotypes within the populations sampled (haplotypes 4 and 5). Nova Scotia also contains two haplotypes (haplotypes 2 and 5). Quebec and New Brunswick both contain all four haplotypes recorded in the North American samples and are therefore considered to have the highest allelic diversity within the North American populations sampled.



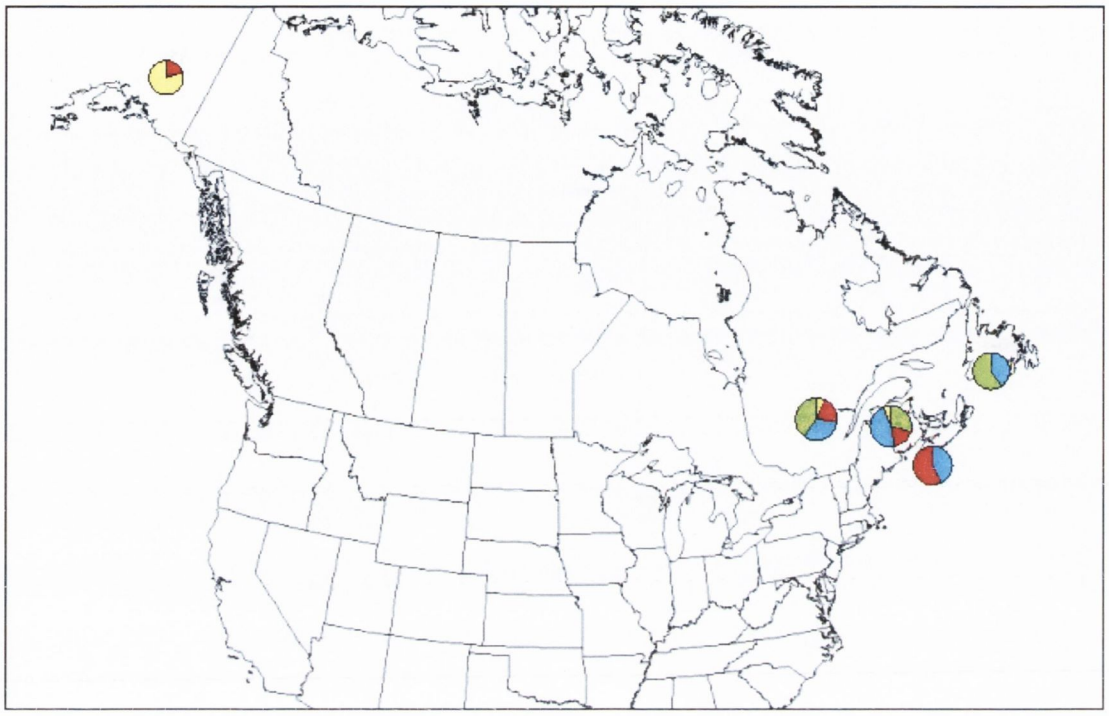
Haplotype	Allele size (bp)	
1	134	
2	135	
3	136	
4	137	
5	138	
6	139	

Figure 4.4.1 Proportion of haplotypes detected in Irish populations of *Spiranthes romanzoffiana*. Five haplotypes were detected, haplotype 4 was not detected in Irish samples (see key to symbols for haplotype number, size in base pairs and colour code. Lough Mask appears to contain most of the allelic diversity. The remaining Irish populations are dominated by haplotype 5 (138bp). Lough Conn contained haplotype 3 and 5 (136bp and 138bp), Lough Allen contained haplotype 2 and 5 (135bp and 138bp).



Haplotype	Allele size (bp)	
1	134	
2	135	
3	136	
4	137	
5	138	
6	139	

Figure 4.4.2 Proportion of haplotypes detected in Scottish populations of *Spiranthes romanzoffiana*. Four haplotypes were detected, haplotypes 1 and 2 were not detected in the Scottish samples (see key to symbols for haplotype number, size in base pairs and colour code. Haplotype 5 (138bp) is the only haplotype shared with the Irish samples.



Haplotype	Allele size (bp)	
1	134	
2	135	
3	136	
4	137	
5	138	
6	139	

Figure 4.4.3 Proportion of haplotypes detected in North American populations of *Spiranthes romanzoffiana*. Four haplotypes were detected, haplotypes 1 and 6 were not detected in the North American samples (see key to symbols for haplotype number, size in base pairs and colour code.

Summary of haplotype data

Haplotype 1 (**134**) was unique to Ireland, haplotype 2 (**135**) was in Ireland and North America, haplotype 3 (**136**) was in Ireland (Lough Mask) North America and Scotland, haplotype **137** was in Scotland and North America, haplotype 5 (**138**) was in Ireland, North America and Scotland (Colonsay) and haplotype 6 (**139**) was in Ireland and Scotland.

In summary, Ireland contains one unique haplotype (haplotype 1 (134 BP)). Scotland and Ireland share a single haplotype (haplotype 6 (139BP)), which was absent from the North American samples. Scotland and North America share a single haplotype (haplotype 4 (137BP)), which was not detected in the Irish samples. All of the four haplotypes recorded in the North American samples (haplotypes 2,3,4 and 5) are found in the Irish populations, haplotype 2 being absent from the Scottish populations. The highest allelic diversity for the European samples is found in the Lough Mask population. Haplotype 5 (138BP) is the most widespread haplotype, occurring in high proportions in all Irish populations, though in Scotland it was found only on Colonsay and was absent in the Alaskan population. Within population allelic diversity appears to be highest in the North American populations.

There appears to be a genetic-geographic split between the Irish and Colonsay populations and the Northern Scottish population on Tiree, Coll, Vatersay and Barra. The most diverse North American populations are Quebec and New Brunswick (haplotypes 2, 3, 4 and 5). Alaska has two haplotypes (haplotype 2 and 3) and is somewhat genetically distinct as the proportion of haplotype 3 is much higher than any of the other samples examined. Newfoundland has only two haplotypes (haplotypes 4 and 5). It shares haplotype 4 with Scotland but not with Ireland. Nova Scotia contains two haplotypes (haplotypes 2 and 5). It shares haplotype 2 with Ireland but not with Scotland.

4.4.3 Assessment of the biogeographical relationship between Irish, Scottish and North American populations of Spiranthes romanzoffiana using Amplified Fragment Length Polymorphism (AFLP) markers.

The biogeographical relationships between the three different regions studied were further examined using the data obtained from the AFLP analysis described in section 3.3. The Scottish samples, except the Tiree samples, and a number of the Irish samples were removed from the analyses. Samples were not included because they failed to produce DNA suitable for AFLP analyses. This was thought to be due the age of the samples involved, i.e. the DNA had degraded due the longevity of storage (>3 years). A number of techniques, including ordination, genetic distance measures and cluster analyses were employed to examine the relationships between the three geographic regions. Results obtained from the gene diversity, genetic distance measures and population differentiation estimates of Irish, Scottish and North American populations in section 3.4 were utilised in this section of the research. Where appropriate these measures were used to clarify and endorse any conclusions or theories discussed throughout this chapter.

Relationships between the populations of *Spiranthes romanzoffiana* in North America and Ireland were examined using Principal Co-ordinate analysis (PCO). The data on the PCO graph (figure 4.4.4) are somewhat congruent with the data depicted on the UPGMA dendrogram. There are two clear groups clustered on either side of axis 1 (38% variation). The Irish samples are positioned on the right side of axis 1 and show a high degree of scatter. The North American samples are confined to the left side of axis 1 and are less scattered than the Irish samples. There is some overlap of samples on axis 1. Two samples from Lough Mask in County Mayo and a single sample from Lough Corrib in county Galway show a strong association with two samples from the Newfoundland population.

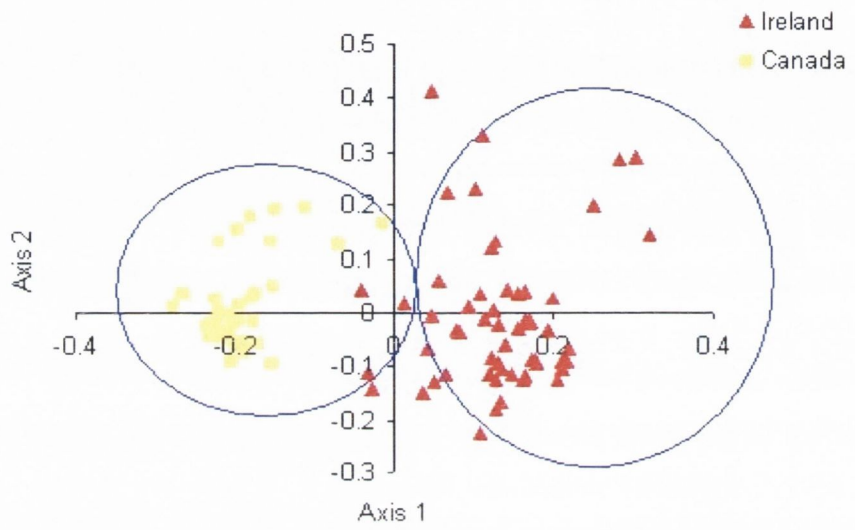


Fig 4.4.4 PCO graph of AFLP data matrix. Axis 1 shows 38 % of the variation. The areas within the circular outlines show the separation of the Irish and North American samples on axis 2.

UPGMA analysis was used to further examine the relationship between 13 of the Irish and North American populations of *Spiranthes romanzoffiana*. (Figure 4.4.4). The analysis is based on Nei's (1978) genetic distance measure. Thus a tree is constructed in order of decreasing similarity. The analysis produced a tree with two distinct groups. The first group (group 1) depicted in red (Figure 4.4.5) groups 6 of the 7 Irish populations together and includes the Newfoundland population. The Newfoundland and the Lough Allen populations are grouped together. This may indicate a level of genetic similarity between these two disjunct populations. Group 2, depicted in yellow consists of Quebec, Lepraeu and New Brunswick, this group shows some similarity to group 1. Nova Scotia, Alaska and Antrim are separated from the two main groups.

Group 2, depicted in yellow on the dendrogram (figure 4.4.5) consists of 5 of 6 North American populations sampled. The Antrim population is included in this group. The Antrim population contains haplotype 5, which is present in all the North American populations except Alaska.

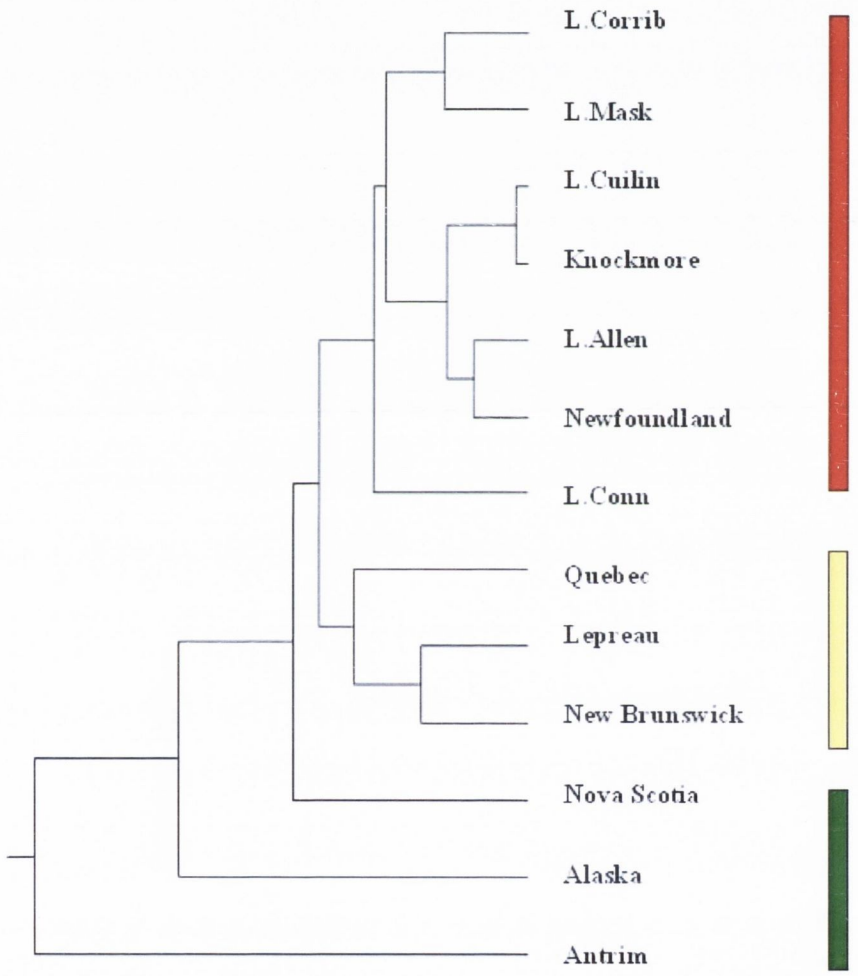


Figure 4.4.5 UPGMA dendrogram (POPGENE) of 13 populations of *Spiranthes romanzoffiana*. Analysis is based on Nei's (1978) genetic distance. Three broad groups are depicted on the dendrogram.. The red group consists of six of the seven Irish populations; this group also includes the Newfoundland population. Quebec, Lepreau and New Brunswick form a group (yellow). Nova Scotia, Alaska and Antrim all come out as separate branches, with Antrim forming the basal branch.

The final analysis of the AFLP data was performed in chapter 3 using a cluster analysis method called Neighbour-joining. Neighbour-joining is based on finding two samples (neighbours) that are most similar (i.e. that minimise the total length of the phylogenetic tree) and clustering them together based on mean character difference. The resulting tree illustrates the arrangement of the samples so that the most similar are clustered together under the principal of minimum evolution. The neighbour-joining tree structure (Figure 3.4.8) shows a similar structure to the UPGMA dendrogram (Figure 4.4.5) analysis of populations. The PCO scatterplot (figure 4.4.4) suggests a similar pattern of distribution.

The neighbour-joining tree splits the samples into four broad groups (Figure3.4.8) The Irish samples in blue, is made up of Irish individuals and the two individuals from the only Scottish sample (Tiree) included in this analysis. Group 2 consists of all of the North American samples (yellow). The division of these two groups is evident on the tree. However there is no bootstrap support for this apparent split. As with the previous sets of analyses there is an element of overlap between the two geographic-genetic groups. The Irish group contains 9 of the 12 Newfoundland samples. There is strong bootstrap support (>90%) within three of the Newfoundland branches. There is a single sample from the Nova Scotia population positioned within this group, though was no bootstrap for this split. The Newfoundland samples and the Nova Scotia sample are positioned between samples from Lough Corrib and Lough Mask and also with a single sample from Tiree in Scotland. The remaining two Newfoundland samples are positioned at the base of the tree and are grouped the four samples from Antrim and the other sample from Tiree. The remaining samples make up an almost exclusively North American group. However this group is interspersed by a single sample from the Lough Allen population. The Lough Allen sample is clustered with a number of samples from New Brunswick. This grouping is reflected in the chloroplast microsatellite analysis results, which reveal that Lough Allen and New Brunswick populations share haplotype 2 (135 BP). Haplotype 2 is also found in the Lough Mask population and is absent from the Scottish populations. There is very little bootstrap support for the tree structure. These issues may be related to statistical or sampling error.

Summary of AFLP results

The results of AFLP analysis highlight a number of trends between North American and Irish populations of *Spiranthes romanzoffiana*. The analyses used in the research show some split the Irish and North American populations into two broad geographic-genetic groups. In all cases, a number of populations consistently overlap these two putative groups. Furthermore, in all the analyses the Newfoundland population shares some similarity with the Irish populations and results of neighbour-joining analyses suggest that Newfoundland also shares some genetic similarity with the Scottish population on Tiree. These data indicate that the Irish populations with the most similarity to the North American populations are located in Lough Mask, Lough Allen and Lough Corrib. Lough Mask and to some extent Lough Allen show the highest degree of allelic diversity in all the Irish populations sampled.

4.5 Discussion

This chapter is essentially concerned with two issues; 1) Is there a genetic-geographic split between European and North American populations? 2) How did the current amphi-Atlantic distribution of *Spiranthes romanzoffiana* come about? These questions were tackled using a number of investigative approaches. Results from two molecular analyses techniques coupled with literature reviews and the consultation of historic plant records were examined and interpreted using contemporary statistical techniques. In the hope of maintaining clarity, the following section of this chapter deals with these questions separately.

Using a single chloroplast microsatellite locus and two AFLP (Amplified Fragment Length Polymorhism) markers, a total of 145 individual samples from 23 populations from three geographically distinct locations were successfully analysed. Results suggest that there is some genetic-geographic split between the North American and European populations. The data also indicate the occurrence of a possible genetic-geographic split between the Irish and Scottish populations. The chloroplast microsatellite data revealed the occurrence of six alleles or haplotypes throughout the sample range. A single, novel allele, Haplotype 1 was

detected in one population at Lough Mask in the west of Ireland. The occurrence of this novel allele in an Irish population has a number of possible explanations. Firstly the allele may be present in other European or North American populations but was undetected due the small sample size from each of the respective geographic locations. On the other hand haplotype 1 may have arisen separately during, for example, the Pleistocene or subsequently. Alternatively it could have arisen by a deletion of a base at a locus, this could have happened at anytime. It is possible that haplotype 1 is of a more recent origin. Given the restricted distribution of haplotype 1 in the Irish populations, it may well be that narrow range of this haplotype is due to a reduced amount of time for dispersal compared to older haplotypes. Haplotype 1 may have at one stage been more widespread in the Irish populations and has subsequently declined due to the deleterious effects of genetic drift, inbreeding or out-breeding depression.

4.5.1 Genetic-geographic split between Irish and Scottish samples.

As mentioned in the previous paragraph, results from this research indicate that haplotype 1 was unique to the Irish samples analysed. In addition to this, haplotype 6 (139 BP) was unique to the European samples. This haplotype occurred in Lough Mask, County Mayo and on the Island of Tiree in the Inner Hebrides on the West Coast of Scotland. As with haplotype 1, the origin of haplotype 6 and its possible uniqueness to European populations is open to scrutiny. Nevertheless the distribution of this allele suggests that the some of the Tiree and Lough Mask samples have a common ancestor, experienced some level of gene flow in the past or that the allele has arisen more than once in the species history. It is possible that haplotype 6 occurs throughout the species' Irish and Scottish and range but was undetected in this research due possibly to PCR error. A more thorough sampling regime would help to reduce the likelihood of missing alleles present in the species European range.

Forrest *et al* (2004) in their work on the on *Spiranthes romanzoffiana* detected two haplotypes (139 BP and 140 BP). Interestingly haplotype 140BP was not detected in the current research. Based on their results, Forrest *et al.* (2004) divided sample populations into two groups; one, a northern Scottish group and a southern group, including Colonsay and

Ireland. They suggested a lack of contemporary gene flow exists between Irish and Scottish populations. The results from the current study are somewhat congruent with the Forrest *et al.* (2004). For example, haplotype 4 (137 base pairs) was found in all Scottish populations, except the most southerly of the sampled populations on Colonsay. Haplotype 4 was not detected in any of the Irish populations surveyed. Thus it appears to be restricted to the Forrest *et al* (2004) "Northern group". Adding to this putative genetic pattern, haplotype 5 (138 base pairs) was widespread in Ireland (fixed for 8 of the 12 populations surveyed) though it was absent from all Scottish populations surveyed, except Colonsay, where it was fixed. This pattern fits with the Forrest *et al.* (2004) "Southern group" It is evident from the results obtained by Forrest *et al.* (2004) and the results from the current research that there is at least a modest genetic-geographic split between Irish and Scottish populations of *Spiranthes romanzoffiana.*

4.5.2 Genetic-geographic split between European and North American samples.

The microsatellite marker revealed no unique haplotypes in the North American populations. Haplotype 2 (135 BP) was found in Ireland and North America but was absent from Scotland. Haplotype 2 was found at low frequencies in all North American populations except Newfoundland, where it was not detected. In Ireland this haplotype was found in Lough Allen and Lough Mask, where it occurred again in relatively low proportions. There are a number of possible inferences that can be drawn from these results. It may be that the populations in the west of Ireland either shared a common ancestor with the North American populations or that the allele has arisen more than once during the species evolution. It may also be inferred that Irish individuals arrived at this location via long distance dispersal from one or several *Spiranthes romanzoffiana* populations in North Eastern America. It should further be noted that the populations at Lough Allen and particularly Lough Mask contain the highest allelic diversity in the European populations surveyed. This could be interpreted as being consistent with a colonization event by more than one propagule by sexual reproduction in a sexually out-crossing species (McBreen and Cruzan, 2004).

The occurrence of haplotype 4 in all the North American and Scottish populations (except Colonsay) and its absence from the Irish populations may be indicative of separate colonisation events during earlier times. It may also go some way to explaining the putative genetic-geographic split between Northern Scottish and Irish populations of *Spiranthes romanzoffiana*. Haplotype 3 (136 base pairs) is the only allele detected in Ireland, Scotland and North America. This haplotype is extremely widespread, as is evident by its presence at the extreme ranges of the species distribution, i.e. Alaska and Scotland. In Ireland, however this allele was only detected in the Lough Levally population, in Co Leitrim. This allele may of course have been undetected in the sampling. Based on these points it would seem possible that this site may have been the location of an introduction event from either Scotland or North America. As mentioned earlier with haplotype 1, the limited distribution of haplotype 3 in the Irish samples surveyed may be indicative of a recent origin, i.e. the allele has not had sufficient time to expand its range in Ireland. As with all of the inferences reached in this section of the research caution must be maintained, as sampling size may have an important influence on these findings.

The UPGMA (figure 4.4.5) analysis divided the populations into two broad groups. The Irish populations and the Newfoundland population make up a group on the dendrogram. The North American populations of Quebec, Lepraeu and New Brunswick make up the second group. Nova Scotia, Alaska and Antrim do not form good groups. From the data it appears that Antrim is sister to all the other populations. However there were only four samples analysed from Antrim so this inference has to be viewed with caution. There was no bootstrap support for the splitting up of the two groups on the neighbour-joining tree. Therefore, although there is evidence of a genetic divergence between the two continents, it appears that this divergence is weak. The AMOVA (table 3.4.3) analyses carried out on the AFLP data found that 72% of the variation was accounted for within the populations and only 11.72% of the variation was accounted for between the North American and Irish populations. These data suggest that the Irish and North American populations are poorly differentiated.

There are a number of possible explanations here. The supposed split maybe of recent origin and therefore the populations have not had sufficient time to completely diverge. Alternatively, there is a very low occurrence of seed set and recruitment in the Irish populations and thus very little opportunity for the evolution of divergent or unique genotypes. Infrequent seed set and recruitment would mean that some genetic divergence from the North American populations might occur but that the rate would be extremely slow. The recent discovery of seed in both the Irish and Scottish populations (A. Scobie and C. Wilcock, *pers comm.* 2006) suggests that this may not be the case. However although seed has been detected it is clear that the quantity is very low when compared to other orchid species. This will have an effect of reducing recruitment and also dispersal away from populations. Hence generation time is likely to be long and the rate of divergence very slow. The overall indication from the chloroplast microsatellite and AFLP data suggests that there is a genetic-geographic split between the European and North American populations of *Spiranthes romanzoffiana*, however the extent of this genetic differentiation is low. Also the microsatellite evidence (table 4.4.2 and figure 4.4.1, 4.4.3) suggests that genetic variation in the European populations is less than in the North American populations which suggests colonisation from North America. In addition to this it appears that allele frequencies are more even in North America. In Ireland three quarters of all populations are fixed for one allele, this suggests a founder effect and possibly different founder events in Ireland and Northern Scotland.

4.5.3 How did the current amphi-Atlantic distribution of Spiranthes romanzoffiana arise?

The tentative conclusions in relation to a genetic-geographic split between European and North American populations of *Spiranthes romanzoffiana* drawn thus far suggests a genetic split between the two disjunct populations, though with limited divergence. In addition to this pattern, molecular evidence analysed and discussed in chapter 3 suggests that the levels of genetic variation in the Irish samples is greater than that found in the North American samples examined. So how did this anomalous distribution arise? How did *Spiranthes*

romanzoffiana, a widespread, native North American orchid species come to exist in small, localised but genetically variable populations on the Atlantic coast of Ireland and Scotland?

Spiranthes romanzoffiana in Ireland – A periglacial relict species?

Past climatic and geomorphological events have shaped population genetic structure through their effects on species distributions and levels of gene flow among populations (Dodd *et al.*, 2002). In the terrestrial environment, migrations during the glacial events of the Pleistocene have had a profound effect on population genetic structure as a result of restrictions to refugial sites and subsequent migration by seed dispersal during interglacials (Hewitt, 2000). The first hypothesis suggests that the current distribution and genetic pattern of *Spiranthes romanzoffiana* in Europe has been shaped by the survival of refugial populations of *S. romanzoffiana* during the Pleistocene. This hypothesis evokes both positive and negative comment throughout the literature. Bateman (2005) in Horsman (2005) stated that the degree of molecular divergence shown by *S. romanzoffiana* is far too little to have allowed it to exist prior to the opening of the North Atlantic. This research has been unable to determine what molecular evidence is being referred to in this comment. C. Sheviak (2004, *pers. comm.*) considers the refugial hypothesis unlikely, arguing that the species occurs commonly in Arctic America, growing in tundra. Thus he sees little reason why the species should be extirpated in the Pleistocene. Webb (1983) suggested that *Spiranthes romanzoffiana*, among other species (e.g. *Eriocaulon aquaticum*) could have survived the last glaciation in some sheltered nook, perhaps on land now submerged by the rise of sea level off the coasts of Scotland and Ireland. Webb goes on to say that *E. aquaticum*, another amphi-atlantic plant species with an uneven distribution very similar to that of *S. romanzoffiana*, is known as a post glacial species and very probably as an interglacial fossil in Irish deposits. Webb (1983) suggests that this evidence, along with the fact that Irish populations differ in chromosome number (2n=22 – Heslop-Harrison, 1968) from the North American plants (2n=30 - Sheviak, 1984) is enough to infer that glacial refugia hypothesis is the "only real alternative". However a difference in chromosome number does not necessarily imply a great period of separation as a change in chromosome number may occur relatively rapidly. This could arise if the chromosome number remains fixed and if there are no further colonists and if all the

plants with the previous number are extirpated. Currently there are no fossil records of *Spiranthes romanzoffiana* in Europe, though the lack of fossil evidence cannot conclusively prove the absence of the species.

Coxon and Waldren (1995) in their work on Ireland's floristic record during the Pleistocene report a number of instances in the floral record that may provide explanations for the current amphi-atlantic distribution of *Spiranthes romanzoffiana*. Two species in particular, *Eriocaulon aquaticum* and *Najas flexilis* are plants with uneven distributions, both far more widespread in America than in Europe. The long Pleistocene and Holocene records of *E. aquaticum* and *N. flexilis* suggests that a glacial refuge may have existed, somewhere to the south or south east of Ireland (Coxon and Waldren, 1995). There is clear evidence that *N. flexilis* has retreated westward, as fossil records extend to central Europe, where the species is currently absent (Hulten, 1953; Godwin, 1975). The existence of these two aquatic species and a number of other wetland/high moisture requiring species (e.g. *Trichomanes speciosum*, *Juncus effusus* and *Hymenophyllum wilsonii*) in glacial refugia may indicate the type of habitat present at the time, i.e. one of an open, wet habitat. In Ireland *Spiranthes romanzoffiana* is almost entirely restricted to this type of habitat, i.e. seasonally flooded, lakeshore habitats. It is possible that the current populations of *Spiranthes romanzoffiana* have evolved from an ecologically restricted, refugial population during the Pleistocene. The occurrence of contemporary populations of *S. romanzoffiana* in arctic North America suggests that the species, if present in Europe during the last glacial maximum could have survived in the wet, tundra-like conditions found at the edge of an ice mass. Without clear, irrefutable fossil evidence for the occurrence *S. romanzoffiana* during the early the Pleistocene the question of whether the species is a glacial relict will remain a matter of conjecture.

The amphi-atlantic distribution of Spiranthes romanzoffiana is a result of a single or multiple long distance dispersal events?

The hypothesis that *Spiranthes romanzoffiana* arose in Europe as a result of long distance transport of propagules across the Atlantic, either through wind currents or as passengers on

migratory bird species is mentioned in the literature (e.g. Horsman, 2005). Webb (1983) considered the hypothesis highly improbable. Others suggest that, if long distance dispersal by seed was possible, it would seem probable that the species would appear on Greenland or Iceland, where it is unknown (C. Sheviak 2004, *pers comm.*). The maximal detected dispersal distance varies between species, however the general rule is that long-distance dispersal is very rare (Ouberg *et al.*, 1999).

Despite the perceived rarity of these effects, gene flow between relatively widespread populations is possible. Furthermore infrequent long distance dispersal and establishment by plant propagules can have marked effect on the genetic structure a given population. The disjunct plant species, *Cerastium arcticum,* showed many similar and even some identical multilocus genotypes in two different populations separated by > 1800 km. Populations of *C. arcticum* from different sides of the Atlantic were to a large extent intermingled in the UPGMA and PCO analysis of the data, which suggested the different Atlantic regions are poorly differentiated. The evidence from the *C. arcticum* study concluded that this was most likely caused by post-glacial dispersal (Hagen *et al.,* 2001).

Long distant dispersal and colonisation by orchids via their seed has been documented (Arditti and Ghani, 2000). Certain characteristics of orchid seeds make them adaptable to long distance dispersal. Because of their small size, shape and large air space orchid seeds can float in the atmosphere for long periods. Seed volume and the percentage of free air space seem to be important factors that affect and perhaps even determine flotation time. The mean percentage of free airspace in *Spiranthes* seeds, as determined by Arditti and Ghani (1999) in Tansley review (2000) was 69%. Orchid seeds have been recorded as traveling between Australia and New Zealand, with distances >2000 km being recorded (Close *et al.*, 1978). It has been generally been assumed that orchid seeds reached and re-colonised Krakatau on wind currents (Gandawijaja and Arditti, 1983).

Thus, it is possible that seeds of *Spiranthes romanzoffiana* reached the west coast of Ireland or Scotland from North America by long distance dispersal on air currents. The major issue with this theory is that there are 66 species of orchid growing in the North Eastern America.

Six of these species are also growing in the British Isles; *Coeloglossum viride, Corallorhiza trifida, Goodyera repens, Liparis loeselii, Listera cordata* and *S. romanzoffiana*. However, of these six species, only *S. romanzoffiana* has an asymmetric amphi-atlantic, distribution. Horsman (2005) posed the question, if the seed of *S. romanzoffiana* has indeed blown over from North Eastern America, why are none of the other 57 species present in the British Isles? Results obtained in this research highlight the fact that if seed were continually blowing over, resulting in establishment, there would be no genetic differentiation between Irish and North American material. Irish material would in fact be a subset of, but not distinct from, that of North America. The structuring observed, with some separation of Scottish, Irish and North American populations suggests that colonisation is very infrequent. Closer inspection of the flowering time, seed set and dispersal of these remaining 57 North American species may shed light on the characteristics of *S. romanzoffiana* that potentially enable it to disperse over great distances.

Another theory suggests that orchid seeds may also have arrived on the legs or feathers of birds. Transport by birds (exoornithochory), which is relatively quick, is a possible mode of long distance dispersal of orchid seed. Transport of orchid seed on the muddy feet of birds or their feathers could reduce desiccation and make possible wider dispersal (Gandawijaja and Arditti, 1983). John Heslop-Harrison (1953) postulated that the Greenland White Fronted goose (*Anser albifrons* ssp. *flavirostris*) could carry the seed of *S. romanzoffiana* from North America to the British Isles. Horsman (1998) examined this theory and suggested there was indeed a possible link that was difficult to ignore. He did, however later refute the theory, as the Greenland white fronted goose uses Iceland and to a lesser extent Greenland as a staging post. To date there are no records of *S. romanzoffiana* in either of these locations, though its presence in Greenland cannot be definitely ruled out (Horsman, 1998).

Horsman (1998) further investigated the idea that *S. romanzoffiana* was transported across the Atlantic via migratory birds. He postulated that the Pectoral Sandpiper (*Calidris melanotos*) is a potential candidate as a suitable seed carrier. In 1986 the Pectoral Sandpiper was taken off the rarity list of the Irish birds committee. In the period from 1950 to 2000 there were approximately 2976 records for the Pectoral Sandpiper in the British Isles (Cramp

et al, in Horsman, 1998). Horsman (1998) suggests that the total number of actual Atlantic crossings made by the Pectoral Sandpiper would be quite substantial. In eastern Canada, where there are large populations of *S. romanzoffiana* the Pectoral Sandpiper is mainly an Autumn transient en route to its over wintering grounds in South America, following the coastline of the Hudson and James Bays, then across Quebec to the Gulf of the St Lawrence. It is common in New Brunswick, Prince Edward Island and Nova Scotia. It feeds on small invertebrates, mainly in wet habitats, and uses staging posts along the way to refuel. Given that the seed of *S. romanzoffiana* is dust like, Horsman (2005) suggested it is not difficult to appreciate that feathers of the Pectoral Sandpiper could easily pick it up at a staging locality where the orchid was in seed. Again the possibility that seeds are inadvertently transported over vast areas by unsuspecting birds is a tantalising prospect. At best links between various circumstantial evidence can be used to build up cases for and against competing hypotheses.

Spiranthes romanzoffiana was introduced into Ireland via anthropogenic means?

The final theory as to the origin of the European populations proposes that European populations of *Spiranthes romanzoffiana* are of recent origin and were introduced by humans during historic times. The first known record of *Spiranthes romanzoffiana* in Europe was from west Cork in 1810, it was found in Galway in the west of Ireland in 1958. The species was not recorded in the North of Ireland until 1892. In Scotland the first recording was on Coll in the Outer Hebrides sometime in the early 1920s. At some period in the 1950s it was first recorded on the Scottish mainland though it appears to be absent now. The first discovery in England was in Devon in 1957. These dates, the geographic spread of the locations and the temporal succession in which they were discovered, does suggest that the species maybe a recent arrival in Europe and that it may have first arrived in the south west of Ireland, having subsequently spread in a predominantly northerly direction. However the evidence from the microsatellite data analysed in this research do not support this interpretation. The molecular data suggest a clear distinction between the southern and northern populations, which may imply multiple colonisation events.

The existence of numerous marine-trading routes between Ireland and North America during historical times may be responsible for the introduction of several taxa, which are now very local components of the Irish flora (T.F.G. Curtis 2002, *pers. comm.*). More notably the existence of trade and passenger ships between the west of Ireland, the west of Scotland and Northeastern Canada from the late 18th century until the middle of the 20th century was substantial. It is possible that the species was inadvertently introduced during this period. "Given the weediness of *Spiranthes romanzoffiana* in North America, this has always seemed the most likely explanation to me" (C. Sheviak 2004, *pers. comm.*).

Sisyrinchium bermudiana is another plant species with a similar uneven amphi-atlantic distribution. In Europe it is restricted to Ireland as a native but is widespread in North America (Curtis and Mc Gough, 1988). The species is a geologically recent arrival in North America from the south (Raven and Axelrod, 1974). Given the species restricted distribution to the west of Ireland it must have arrived from North America by natural or man made means (Preston and Hill, 1999). These comments do not appear to be based on hard evidence, or at least this research was not able determine the source. The results from the AFLP data (section 3.4) strongly suggest that a recent introduction of *Spiranthes romanzoffiana* into Ireland is unlikely. Given the evidence that the plant appears to set very little seed in Ireland there would no be enough time for the genetic distinction between Irish, American and Scottish material to have evolved. If *S. romanzoffiana* was a recent introduction the expectation would be for the Irish material to be nested within the North American material.

There is no conclusive data to clearly determine the likelihood of any the hypotheses mentioned. Without irrefutable evidence, for example the discovery of fossil evidence, these theories, will remain as points of debate. The ideas discussed above reveal the scope of the theories in the literature and the extent to which individuals agree and disagree as to how the amphi-atlantic distribution of *Spiranthes romanzoffiana* came about.

4.5.4 What does the molecular evidence indicate?

Despite the lack of agreement over how the amphi-atlantic distribution of *Spiranthes romanzoffiana* came about, it can now be agreed that there is some degree of genetic divergence between the European and North American samples analysed in this research. The question however remains; what does this genetic divergence reveal about the origin of the amphi-atlantic distribution of *S. romanzoffiana*?

The genetic pattern described by the data in chapter 3 suggest that the Irish populations of *Spiranthes romanzoffiana* contain marginally higher levels of genetic diversity than that found in the North American samples analysed. The total gene diversity (H_T) recorded in the Irish samples was (0.26), for the North American samples H_T = (0.17). Nei's (1978) gene diversity estimates for the Irish samples = (0.26), North America = (0.24). The partitioning of genetic diversity among and within populations revealed a similar pattern in both the North American and Irish populations. In both cases the majority of the variation was found within the populations (Ireland = 85%, North America = 77%. In the Irish populations 14% of the variation was accounted among populations, in the North American populations this figure = 22%. These results are however based on a small sample size and may therefore be skewed.

Yet still there are two genetic characteristics evident in these results that would suggest the Irish specimens of *Spiranthes romanzoffiana* are not representative of a typical recently colonizing plant species. Colonization often involves severe founder effects, which have an important genetic impact (Barret and Husband, 1990). Particularly in the case of anthropogenic introductions into new areas, species populations are often founded from only a few individuals that are completely isolated from the source population and thus go through a period of substantial genetic drift (Parisod *et al.*, 2005). Introductions from one region to another will almost certainly involve a population bottleneck, as the introduced individuals will only represent a subset of the total native population (Barrett and Husband, 1990). Theory predicts that introduced species will show lower levels of intrapopulation diversity and higher levels of population differentiation than their native counterparts

(Brown and Marshall, 1981). The high level of genetic diversity detected within the Irish populations indicates that the species has not gone through a significant recent bottleneck, however the reduced microsatellite allelic diversity does suggest some sort of bottleneck. Caution is required with this interpretation as the data are based on only one locus with a small number of alleles. Further investigation with a larger number of loci may help to answer this question.

The mating system of an introduced plant species is thought to be of fundamental importance in colonising success (Brown and Burdon, 1987). Many colonising plant species appear to be predominantly self-fertilising or apomictic species, and nearly all out-crossing colonisers are self-compatible (Price and Jain, 1981). Sun (1997) found that the self-pollinating species *Spiranthes hongkongensis* was a more frequent coloniser of newly formed habitats than its out-crossing relative, *S. sinensis*. Catling (1983 and 1990) found a similar pattern in North American *Spiranthes*. The autogamous *S. ovalis* var. *erostellata* and the agamospermic *S. cernua* and *S. casei*, frequently colonise disturbed or newly created habitats (Catling 1983, 1990). The genetic patterns and mode of sexual reproduction of Irish samples of *Spiranthes romanzoffiana* observed in this research suggest it is a sexually out-crossing species and, therefore it does not fit the model described here. Caution however is required as the range of genetic attributes, evolutionary outcomes or modes of reproduction found in plant colonisers suggests they are not a homogenous group and species should be looked at on an individual basis (Sun, 1997).

4.6 Conclusion

The results from this research appear to be congruent with those described by Forrest *et al.* (2004) and point to a genetic-geographic split between Northern Scottish populations and Southern Scottish and Irish populations. This may indicate that multiple introduction events from different source populations have occurred in the past or are still taking place. The occurrence of haplotype 2 in Irish and North American samples and its absence from Scottish samples, along with the occurrence of haplotype 4 in North America and Scotland

and its absence from the Irish samples give credence to this idea. The genetic data from the AFLP and the microsatellite analysis indicate that the populations in the west of Ireland, particularly at Lough Cuilin and Lough Mask have relatively high levels of genetic diversity. These data suggest that this area may well be the location of a number of these putative introduction and colonisation events. It has been argued that *S. romanzoffiana* survived in glacial refugia during the last glacial maximum. Expanding on this, Coxon and Waldren (1995) suggest that *Spiranthes romanzoffiana* may have re-colonised Scotland via the narrow Northern channel separating Scotland and Northern Ireland in early Holocene times. The persistence of refugial populations of *S. romanzoffiana* during the last glacial maximum is possible. However the levels of genetic divergence detected between the European and North American populations appears too low for this to have been the case and the microsatellite data suggest the northern Scottish population has not evolved from the southern population but is more likely from North America.

Based on the levels of genetic divergence between the North American and Irish samples and low levels of population differentiation found in Irish populations this research suggests the Irish populations of *Spiranthes romanzoffiana* are of relatively recent origin, perhaps during the Holocene most probably via multiple long distance dispersal events or through inadvertent human introductions. The results from the PCO, Cluster and UPGMA analyses and the distribution pattern of the length variable, single locus allele, suggest that the source of the European plants may well be from eastern Canada and possibly Newfoundland.

Based on the levels of genetic divergence between the North American and Irish samples and low levels of population differentiation, this research suggests two possible hypotheses to explain the occurrence of *S. romanzoffiana* in Ireland. Irish populations may be of recent geological origin and may have originated from populations in North Eastern Canada, possibly Newfoundland or Irish populations represent a remnant of a formerly more widespread pre-glacial, European distribution. A more extensive sampling regime and the development and utilisation of novel microsatellites would contribute greatly to clarifying this hypothesis. It is perhaps unwise to make such large, sweeping assumptions based on what is effectively a limited data set. However it is hoped that these generalisations can act

as a polemic to stimulate further discussion and research into this issue. These results have important implications for the future conservation status of *Spiranthes romanzoffiana* in Ireland.

Chapter 5

Population and Reproductive Biology of *Spiranthes romanzoffiana* in Ireland.

5.1 Introduction

To assess the status of rare plant species and to prioritise conservation approaches, an understanding of the factors affecting the numbers of individuals within a species is imperative (Schemske *et al.*, 1994). The study of population demographics is thought to be an essential component of conservation research and practice (Bradshaw 1978). Demographic studies in isolation are however not sufficient to ensure the successful conservation of rare plant species. Information on the species genetic diversity is considered by some authors to be the key to a species long-term survival, since genetic variation is a requisite for evolutionary adaptation (Berry, 1971; Lande and Barrowclough, 1987; Vrijenhoek, 1987; Hamrick *et al.*, 1991). Furthermore, there are those who consider autecological research, i.e. characterisation of the biotic interactions and habitat requirements of a species is critical to sound conservation science (Brussard, 1991).

In addition to these requirements, knowledge of a rare plant's reproductive biology is essential to its conservation. It is also important to know whether a species reproduces sexually and whether it requires the aid of pollinators to do so. Sexual reproduction is the primary way in which organisms maintain genetic variability and novelty in their progeny. If this is only achieved with the aid of animal pollen vectors then these vectors also need careful study (Sipes and Tependino, 1994). Thus for many rare plant species, conservation plans for the maintenance of isolated populations will depend on detailed study of demographic history, ecological requirements, the genetic structure and the breeding systems of a given species (Holsinger and Gottlieb, 1991).

5.1.2 Reproduction and pollination in the genus Spiranthes.

Several different breeding systems are known to occur in *Spiranthes*, from entirely sexual to partially or exclusively self-pollinating or apomictic (Catling, 1982; Sheviak, 1982). For instance, *S. lacera, S. romanzoffiana* and *S. vernalis* are pollinator-dependent outbreeders (Catling, 1982; Larson and Larson, 1987). *Spiranthes odorata*, *S. magnicampourm* and *S. ochroleuca* include both sexual and agamospermous races and *S. casei* and *S. cernua* var. *cernua* appear to be totally agamospermous. Some species include self-pollinating local variants, such as the widespread Asian-Oceanic *S. sinensis*, the European-Meditteranean *S. spiralis*, and the Northeastern North American *S. ovalis* var. *erostellata* (Catling, 1982).

In *Spiranthes,* automatic self-pollination usually results from incomplete development or absence of the rostellum (Catling, 1982). The ability to produce sexual seeds without relying on a pollinator may have contributed to the successful spread of *S. sinensis* throughout the islands of the western Pacific Ocean (Sun and Wong, 1992). Apomixis, on the other hand, results from proliferation and adventitious polyembryony of the internal integument of the seed, mostly near the micropyle. Sometimes the integuments and its derivative cells produce embryos extruding through the micropyle or ruptured embryo sacs (Catling, 1982). These features allow the detection of adventitious embryony in *Spiranthes*, even in herbarium material (Catling, 1982; Sheviak, 1982). Sheviak (1982) suggested an association between apomixis and polyploidy and polyembryony, although some obligate apomictic plants are able to produce "normal" monoembryonic seeds (Catling, 1982; Sheviak, 1982).

All documented accounts of pollination in *Spiranthes* indicate that bees are the pollinating agent, most commonly bumble-bees (*Bombus* spp.) but also honey bees (*Apis mellifera*) and members of the families Halictidae, Megachlidae and Andrenidae (Larson and Larson, 1987). Other visitors to the flowers, which have not been seen carrying or removing pollinia, include various Lepidopterans, Hymenopterans and Dipterans (Godfrey, 1933; Catling, 1982).

Catling (1982) provided a detailed account of the pollination mechanism of several North American species of *Spiranthes*. In species adapted to pollination by bumblebees and Megachilidae, such as *S. lacera* and *S. romanzoffiana*, nectar accumulates at the bottom of the floral tube. The bees land on the lower most, open flowers and crawl upward on the raceme while probing other flowers for nectar. The viscidium in these species is comparatively long and rigid and adheres to the dorsal surface of the bee's galea. In *S. lucida*, pollinated by halictid bees, the bees visit many flowers per plant. Their approach to the flower always appears to involve flight. In this case the nectar accumulates on the ventral surface of the column, and the oval viscidium is attached to the clypeus (Catling, 1983).

In all members of *Spiranthes* the flowers open sequentially, with the more basal flowers opening first (acropetaly). The flowers of most of the species within this genus are protandrous (Catling, 1982). In *Spiranthes*, protandry occurs as the column lies close to the lip in newly open flowers, and the pollinia carried out by the bee probing at this stage cannot be deposited on the stigma. However, the pollinarium from that flower can be attached to the mouth parts of the bee. Later, the gap between the column and the lip enlarges considerably, allowing deposition of the pollen on the stigma (Catling, 1983). From the data available, it is apparent that protandry is associated with pollination by Apidae (*Apis, Bombus*) and Megachilidae, being less marked or absent in species pollinated by Halictidae (Salazar *et al.*, 2003).

5.1.3 Vegetative reproduction

Persistence of European populations of *Spiranthes romanzoffiana* has often been attributed to vegetative reproduction (Horsman, 1994). The evidence for this relies largely on the fact that to date no reliable seed set has been documented in Europe. Plants produce lateral buds in the summer, which over winter just above the ground and develop into the following year's aerial parts. The only documented evidence for vegetative reproduction is the occasional production of twin (rarely three or four) lateral buds, which can lead to the formation of multiple aerial parts. These may eventually divide to form different ramets (Summerhayes, 1968).

5.1.4 Potential problems associated with Spiranthes romanzoffiana Irish census

The census of Irish populations of *Spiranthes romanzoffiana* posed a number of problems. Firstly the records for the species in Ireland, since it was first recorded in 1810, are extremely poor and sporadic. Therefore there are large gaps in time between accurate recordings. Furthermore, there is no population size data pre-1990. The records simply state that the species is present. Thus it is difficult to piece together a historical record of the species numbers and distribution. As a result, inferences regarding the current status of the species in Ireland, i.e. whether populations are increasing, decreasing or stable, require a great deal of caution.

A further challenge was faced in detection of the plant in the absence of a flowering spike. The leaves of *Spiranthes romanzoffiana* are narrow and superficially resemble grass leaves, making it extremely difficult to detect the plant in its vegetative state (Gulliver, 2000). Adding to this dilemma, orchids in general have a propensity to remain underground for several years. Using permanently marked plants, Robarts (in Gulliver *et al.*, 2000) determined that a single specimen of *S. romanzoffiana* had remained below ground for six years. These issues again make an accurate population census difficult. The apparent disappearance of plants may cause an incorrect assumption of mortality, when the total length of time of a census is less then six years.

Hutchings (1987) in his work on *Ophrys sphegodes* considered plants absent for more than three years to be dead, though he did record a single individual reappearing after a five year absence. Nevertheless he concluded that the absence of previously recorded plants cannot be interpreted as evidence that they have died until three years have passed. In addition, Hutchings (1987) concluded that the appearance of new orchids within three years of the start of the study might not indicate recruitment, but merely the reappearance of plants that had been below ground for a number of years. This is a major issue with orchid population censuses and one that is often ignored, largely due to the time commitments and financial support required to maintain such long-term studies.

A further issue, which was considered during this population census, relates to the clonal nature of *S. romanzoffiana* (Gulliver, 2004). Clonal species can complicate demographic analysis through the uncertainty of what constitutes a genetic individual, or genet. Many studies focus on the ramet demography. To help counteract this issue some authors (Fielder, 1987; Ehrlen, 1995) have classified plants according to their size classes. This method was adopted during the field season in 2005 and met with a reasonable amount of success. However, in the long-term this method may still be inappropriate for long-lived plant species where long survey periods are required to detect transitions between size classes.

Terrestrial orchids have associations with mycorrhizal fungi that are considered necessary for seed germination and growth of the orchid plant (Clements, 1986; Rasmussen, 1995). The nutritional role of orchid mycorrhiza in the early development of seedlings and in the entire lifestyle of chlorophyll-deficient species is well established (Rasmussen, 1995). However the role of mycorrhizal associations throughout all stages an orchid life cycle is not so clear. Species belonging to *Corallorhiza* and *Galeola,* for example, have low photosynthetic capacity as adults; as indicated by small leaf area, brief aboveground growing season, low chlorophyll content or apparent lack of chlorophyll (Leake, 1994). These plants are assumed to be entirely or predominantly mycotrophic (Rasmussen, 1995). In species that do develop green leaves, the adult stages are often assumed to be entirely phototrophic, but little is known about the transition from the mycotrophic seedling stage.

The ecological specificity of these mycorrhizal species and the associations between specific mycorrhizal species and their host orchid in natural habitats is poorly understood (Batty *et al*, 2001). To fully understand population demographics, reproductive biology, seed germination and recruitment, a clear understanding of the mycorrhizal associations between fungi and their hosts is required. The overemphasis of research on the above ground stages may in fact lead to incorrect conclusions and ultimately to misguided conservation initiatives.

However with limited time, resources and the relevant expertise to study these fungal associations many rare plant conservation initiatives cannot afford such focused endeavours.

More typically, as is the case with *Spiranthes romanzoffiana* in Ireland, the basic population, reproductive and ecological data do not exist. Thus initial research activities must focus on gathering this essential baseline data.

This section of the research aims to gather baseline data on the number and distribution of populations of *Spiranthes romanzoffiana* in Ireland. The floral biology and the species morphological and phenological characteristics are examined. The occurrence of vegetative and sexual reproduction in the species Irish range is examined. Finally existing and potential threats to Irish populations of *Spiranthes romanzoffiana* are investigated.

5.2 Aims

This section of the research attempts to accurately determine the current number of populations of *Spiranthes romanzoffiana* in Ireland. It further aims to describe how these populations are distributed and to determine whether the distribution has changed since the species was first recorded. Traditional census methods based solely on the number of flowering spikes observed are investigated to assess whether results from this method reflect the true extent of population sizes and distribution in Ireland. The question as to whether current populations are declining, increasing or stable is addressed. This is a very difficult question to accurately determine in the time available; however the results from this research will act as a baseline for future monitoring initiatives.

The reproductive biology of *Spiranthes romazoffiana* is examined and a number of key questions are tackled. What is the dominant mode of reproduction in Irish populations of *Spiranthes romanzoffiana*. Secondly, what influence do the floral dynamics have on the reproductive potential of the species in Ireland? The project aims to explore any evidence of cross-pollination in Irish populations. Finally the data gathered and the observations made throughout the course of this research will be used to quantify potential threats to *Spiranthes romanzoffiana* in Ireland.

5.3 Methods

5.3.1 Population census

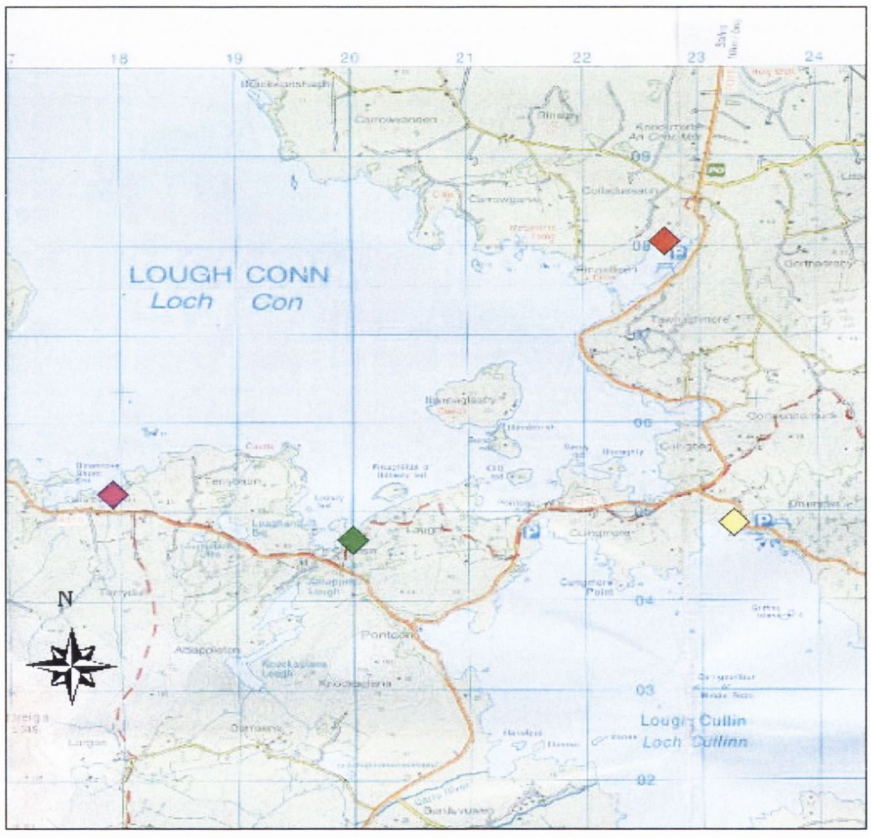
Survey Area

Fieldwork commenced in April of 2003. The initial objective was to gather all available data on the recording and distribution of *Spiranthes romanzoffiana* in Ireland. Historical data were gathered from the herbaria at Trinity College Dublin and the National Botanic Gardens, Glasnevin. Distribution maps and contemporary data were supplied through the National Parks and Wildlife Service (NPWS) and the Environmental Heritage Service Northern Ireland (E.H.S.). By cross-referencing published information with historical maps it was possible to produce a series of maps depicting the general distribution of *S. romanzoffiana* since the plant was first recorded in Co. Cork in 1810. The records for the species are less than satisfactory, nonetheless it was important to collate all the available data so that a rudimentary record of the species distribution and possible range contraction or expansion could be observed.

Due to the elusive nature of the species and the potential for time wasting with regards to locating plants, it was decided that four sample sites would be selected to carry out more in-depth population surveys (Table 2.3.1). Contact was initiated with NPWS conservation rangers, who proved to be a valuable source of information. Contact made with members of the Botanical Survey of the British Isles (B.S.B.I) also provided a useful source of population information. Once plants were located, their positions were marked using GPS (Magellan GPS 315). Accuracy of each position was within 2.5m to 3m. Each population was demarcated using measuring tapes and GPS coordinates. The distributions of historic and contemporary populations were mapped using GIS (Arcview version 3.2).

Sites were chosen based on a number of criteria:

1. Reliable information on exact locations
2. Population size
3. Time since the population was last observed (2 years in all cases)
4. Potential of site representing putative habitat for the species in Ireland.



Location	
Knockmore	
Corysola Bridge	
Carraig-a-Moiltin	
Drummin wood	

Figure 5.3.1 Map of the four sample populations

In addition to the four survey sites it was decided that eight more sites would be surveyed. Four of these sites were historic sites, i.e. sites where plants had once been recorded but not in the last 20 years. The other four locations were potential sites, i.e. sites where there were no known records but the habitat looked appropriate for the species to persist (Table 2.3.1). Information on these sites was acquired through the N.P.W.S.

Survey technique

During the first year of the survey (2003) finding the plant proved difficult and time consuming. All survey work at this point was based on the presence of flowering spikes. The population census was carried out between late July and mid September from 2003 to 2006. Natural fluctuations in flowering numbers in orchid populations are well documented. An understanding of this phenomenon is essential to the understanding of an orchid's life cycle and ultimately to the conservation of orchid species. Data on the number of flowering spikes from each of the four sample populations was gathered between 2003 and 2006. During each year of the survey, each site (Figure 5.3.1) was visited on three separate occasions during the flowering season. At each visit a flower spike count was undertaken. To avoid counting the same flowering spike twice, each plant was tagged with a coloured piece of nylon sewing thread, once it was counted.

Survey of vegetative plants per sample population

In 2004 and 2005 the focus shifted to determining a more accurate estimation of the number of plants in sample populations i.e. flowering and vegetative plants. All the plants in a given population were still not accounted for, as this method does not include plants in the below ground phase of the life cycle. Nor does it account for a possible soil seed bank. Nevertheless given the time and resources available it was decided that gathering baseline data on the ratio of vegetative plants to flowering plants was a useful and manageable way to determine more precise population census data.

Rather than attempting to count all of the vegetative plants in all of the known populations, it was decided that a number of suitable sample sites would be selected for the study (Table 2.3.1). Within these sites a $40m^2$ quadrat was demarcated and a sample of each population was surveyed. Within each large quadrat, smaller $1m^2$ quadrats were selected using random numbers. When a random point was chosen, the $1m^2$ quadrat was placed around the nearest flowering spike or spikes. This may of course be non random and biased but it was decided that given the rarity and sparse distribution of *S. romanzoffiana,* too many vegetative plants could have been over looked if some bias was not employed.

Selecting flowering plants as the central point of each quadrat in theory should enable the surveyor to accurately determine the number of actual plants in given area. Detailed observations were carried out by carefully sifting through the vegetation in each $1m^2$ quadrat. Vigilance was observed at all times, as it is very easy to overlook the grass like leaves. The leaf has a waxy feel to it and is slightly thicker than the leaf of *Molinia cerulea.* It is folded at approximately 45 to 60 degrees along the slightly raised mid-rib, the angle of the fold appearing to become more acute as the leaf ages. The leaf is linear to lanceolate with a pointed tip that folds back on itself forming a small cup with an acuminate to mucronate tip. The leaf can be confused with the leaves of *Dactylorhiza* species. However *Dactylorhiza* spp. tends to have noticeably thicker, fleshier leaves and the midrib is more pronounced.

Table 5.3.1 Number of $1m^2$ quadrats surveyed for the presence of vegetative specimens of *Spiranthes romanzoffiana.*

Population	**No. of $1m^2$ Quadrats**
Drummin Wood	66
Carraig-a-Moiltin	33
Corrysola bridge	10
Knockmore	26

5.3.2 Spiranthes romanzoffiana: size class survey 2005.

During the field season in 2005, the focus of the research expanded to include the determination of age classes within four sample populations (Table 5.3.1). Plants were divided into size classes according to the length of the longest leaf. The size classes were divided into four categories (Table 5.3.3).The proportion of each size was determined by combining the data gathered from the four survey populations.

Table 5.3.2 Size class scale used during the

Size class (leaf length)
< 2cm
2 – 4 cm
4 – 8cm
> 8cm

5.3.3 Reproductive biology of *Spiranthes romanzoffiana*

Investigations into the reproductive biology of *Spiranthes romanzoffiana* began in July 2003. Observations and experimental tasks were carried out during between 2003 and 2005. In 2003 the aims of the project focused on locating populations, determining the number of flowering spikes within a population and gathering information on the floral dynamics of the species. Efforts were made to determine the possible pollinators of *S. romanzoffiana* during the first year of fieldwork. Some data were gathered but it became apparent that the time was not available to carry out all of the tasks required. It was decided that all pollinator observations would be incidental to the research and would be made on an ad hoc basis. Determining whether European populations of *S. romanzoffiana* have pollinators is integral to the understanding of the species biology and distribution. It was therefore decided that another PhD project looking at the pollination biology of several rare Irish orchids would include *S. romanzoffiana.* This project (Karl Duffy, PhD thesis, unpublished) commenced in 2004 and has made a number of important discoveries. See section 5.5.2 for further details.

5.3.4 Reproductive experiments

In 2004 a range of hand pollination experiments were carried, in an effort to determine the dominant mode of sexual reproduction in Irish populations (Knockmore and Drummin Wood) of *S. romanzoffiana*. Four treatments were chosen, involving removal of pollinia from the rostellum on the dorsal side of the column and transferal to the appropriate stigmatic surface. During each of the crossing treatments, observations were made within each flower to check for the natural removal or deposition of pollinia. The values for the 'natural' removal of pollinia were expressed as a percentage of the total number of plants examined.

A total of 48 pollinator exclusion tests were set up, with 12 replicates of each treatment. In total 463 individual flowers were examined. Each plant was tagged with a waterproof label. Each label was colour coded according to the treatment type. To prevent any pollen transfer between plants a wire frame was constructed and placed over the inflorescence of each plant being treated. Fine (1mm diameter) white nylon wedding veil netting was tied around the top of each wire frame so as to exclude any potential pollinators from the experiment (Plate 5.3.1). As new flowers opened the netting was removed and the treatment specific to the particular plant was carried out. All plants required repeat visits to ensure all individual flowers were treated. Each treatment had a corresponding control plant. To minimise potential complications arising from for example, resource allocation, a maximum of 25% of flowers per inflorescence were treated. The control plant was left untouched and no exclusion netting was applied. Experiments were set up between the 22/7/04 and 12/8/04. Capsule formation and seed set were examined in the first week of September. This time coincides with the peak flowering period of the species in Ireland. Plants were selected randomly and individual flowers were selected based on the age (flowers opened <3 days) and condition of the flower.

Table 5.3.3 Reproductive treatments carried out in 2004.

Treament	Discription
Autogamy	Pollinia transferred to stigma on the same flower.
Xenogamy	Pollinia transferred to stigma on another plant.
Apomixis	Pollinia removed before anthesis.
Geitonogamy	Pollinia transferred to the stigma on another flower on the same inflorescence.
Control	Plant left untouched

Plate 5.3.1 Example of an exclusion treatment at the Knockmore site 2004. Wedding veil netting is secured over a wire frame.

Removal and transfer of pollinia

In *Spiranthes romanzoffiana* the anther is dorsal and the pollinia are attached to a distinct "wedge shaped" viscidium, which terminates the rostellum (Greenwood, 1982). In *Spiranthes sensu stricto* to which *S. romanzoffiana* belongs, the rostellum is two cleft and the stigma is ventrally located on the column behind the rostellum (Garay, 1982;

Greenwood, 1982). The pollinia are relatively easy to remove from a newly opened flower (2-3 days). The long viscidium protrudes horizontally from the rostellum. The sticky surface of the viscidium, which in normal circumstances allows the pollinia to attach to a suitable pollinator, readily adheres to a dissection needle. Once the pollinia are removed they can be transferred to the stigmatic surface of another flower without difficulty. Studies in North America show that bumblebees arrive at the bottom of the spike, or on the lower most non-wilted flowers and move progressively upwards (Catling, 1983a). In order to mimic the natural environment all hand pollination carried out during these experiments replicated this upward direction (acropetally).

Capsule development and seed production.

Potential capsule formation was observed continuously throughout the flowering season. Observations in 2004, during the first week of September, suggested that many of the capsules were failing to swell and in fact many had already begun to deteriorate. Research carried out by Tatarenko and Kondo (2003) showed that seed maturation in *Spiranthes sinensis* took less than one month. To allow for variation in capsule development and to mitigate against environmental influences, capsule observations in 2004 were carried out over a 6 week period. In 2004, 30 individual capsules were collected from 10 individual, unmodified plants during the second and third week of September. All harvested capsules were opened in the laboratory and examined for seed content. Capsules were inspected using a stereo microscope at x 20 magnification.

Assessment of seed development (2005)

A presence/absence matrix was constructed for each capsule examined. Capsules were opened using a scalpel and the contents removed using a mounting needle onto a plain sheet of paper on which a grid of 100, $1cm^2$ squares was marked out. Using a small paintbrush, the seeds were evenly distributed among these squares. The seed content of each of the squares

was counted with the aid of a dissection microscope. The mean seed number per cm^2 was then determined and multiplied by the total number of squares the seeds occupied on the page. The paintbrush was then used to place a sub-sample of the seeds on a microscope slide. These were covered with a cover slip and placed under a stereo microscope. Under x 20 magnification, ten fields of view were used to determine the percentage of seeds that contained an embryo. These data were collated and the mean number of seeds produced per capsule and the mean number of embryonated-seeds per capsule were calculated. In order to determine their viability, a fluorescein diacetate test (Pritchard, 1985) was performed on the seeds collected in 2005. This test stains the seeds, so that viable embryos will appear bright yellow.

5.3.4 Vegetative reproduction.

The occurrence of lateral buds and production of twin plants was investigated between 2003-2005. A total of 100 randomly sampled plants from four populations (Table 5.3.1) were checked for lateral bud development. This was achieved by removing a shallow layer of soil (1cm-2cm) deep from around the base of the specimen. In many cases the vegetative bud is visible above the soil surface. The occurrence of twin plants was determined by counting the number of flowering spikes per plant. Values for production of lateral buds and two or more flower spikes per plant were expressed as a percentage of the total number of flowering plants.

5.3.5 Assessment of threats to Spiranthes romanzoffiana

The lack of knowledge of the basic biological requirements of *Spiranthes romanzoffiana* is perhaps, one of the most serious threats to the species existence in Europe. In addition, the paucity of information in relation to potential threats to the species and its habitat can also be considered a serious impediment to current and future conservation initiatives. In conjunction with the population and reproductive parameters assessed in this chapter a

number of potential threats to populations and habitats have been examined. All threat assessments were determined with the help of local residents, landowners and anglers.

The assessment of threats is largely qualitative and in many cases is based on personal observations made during the course of the research. In the case of threats posed by grazing, grazing was either by invertebrates or by livestock, in the case of livestock the grazing was classified as either light or heavy grazing. Light grazing constituted infrequent visits by cattle. Heavy grazing describes complete removal the flowering spike on >50% of the plants in a given population. A number of other potential threats to *S. romanzoffiana* and its habitat were investigated. 1) Damage caused by vehicular traffic through the *S. romanzoffiana* populations, 2) Damage caused by pedestrians trampling or picking the flowering spikes, 3) potential threat of competition or heavy shading caused by encroaching vegetation and 4) assessment of whether action was being taken to protect the plants on the four sites surveyed.

Table 5.3.4 Summary of variables measured at the four survey-sites between 2003 – 2005

Variables measured	How and when measured	Variable type
Vegetation identification	In field and lab.	Categorical
Total flowering spikes per site	In field to nearest	Quantitative
Number of vegetative plants (2004-2005)	In field	Quantitative
Height of flowering spike (cm)	In field to nearest 0.5cm	Quantitative
Number of leaves per plant	In field	Quantitative
Number of flowers per inflorescence	In field	Quantitative
Height of tallest plant sp.	In field to nearest 0.5cm	Quantitative
Identification of pollinators	Field and lab	Categorical
Capsule formation	In field	Quantitative
Seed counts	Lab.	Quantitative
Land use	In field	Categorical
Extent of flooding	In field	Categorical
Extent of grazing	In field	Categorical

5.4 Results

5.4.1 Population census

The population census began in July 2003. The initial objective was to visit known sites to gain experience with identifying *Spiranthes romanzoffiana.* Identification was at first carried out through identifying the flowering spike. By 2004 a high degree of proficiency in the identification of the species was attained, this included detecting the species in both the flowering and vegetative state. Twelve sites were visited between 2003 and 2005; plants were recorded at all of the known sites however no plants were recorded at the historic sites (Table 5.4.1). One new population was discovered in 2004 at Lough Levally in County Mayo, no plants were recorded at the 3 remaining potential sites (Table 5.4.1). In addition to the twelve sites originally selected a number of other sites were visited. Several National Park rangers and local farmers reported sighting the species during the flowering season in 2004 and 2005. Visits were made to these sites and the presence of the species was verified. In 2004 and 2005 the research expanded its range to include populations from the north of Ireland. 11 populations were visited in Northern Ireland (Table 5.4.2) and the presence of *S. romanzoffiana* at each site was confirmed. In total, throughout the duration of this research, 23 populations were verified on the island of Ireland (Table 5.4.2). The range of flowering plants recorded at the various populations was from 1 - >280. This figure does not include vegetative plants. Four of the 23 populations are new records for Ireland (Table 5.4.2).

Table 5.4.1 The 12 sites surveyed for the presence of *Spiranthes romanzoffiana* during 2003 and 2005. Plants were recorded as present based on the presence of a flowering spike. Repeat visits to all sites were carried out over the three years to reduce the likelihood of recording error.

County	Location	Grid ref	Plants recorded
Known sites			
Mayo	Knockmore	G2284 / 0815	Yes
Mayo	Corysola bridge	G1980 / 0453	Yes
Mayo	Carraig-a-Moiltin	G1764 / 0511	Yes
Mayo	Drummin wood	G2321 / 0481	Yes
Historic sites			
Cork	Gouganbarra	W080 / 660	No
Cork	Lough Glenbeg	V747 / 545	No
Kerry	Lough Carragh	V7274 / 9297	No
Kerry	Lough Glanmore	V7720 / 5543	No
Potential sites			
Mayo	Lough Levally	15026 / 04357	Yes
Galway	L. Corrib	M4880 / 0885	No
Mayo	L. Levalliree	M2065 / 9751	No
Mayo	L. Beltra	G0745 / 97541	No

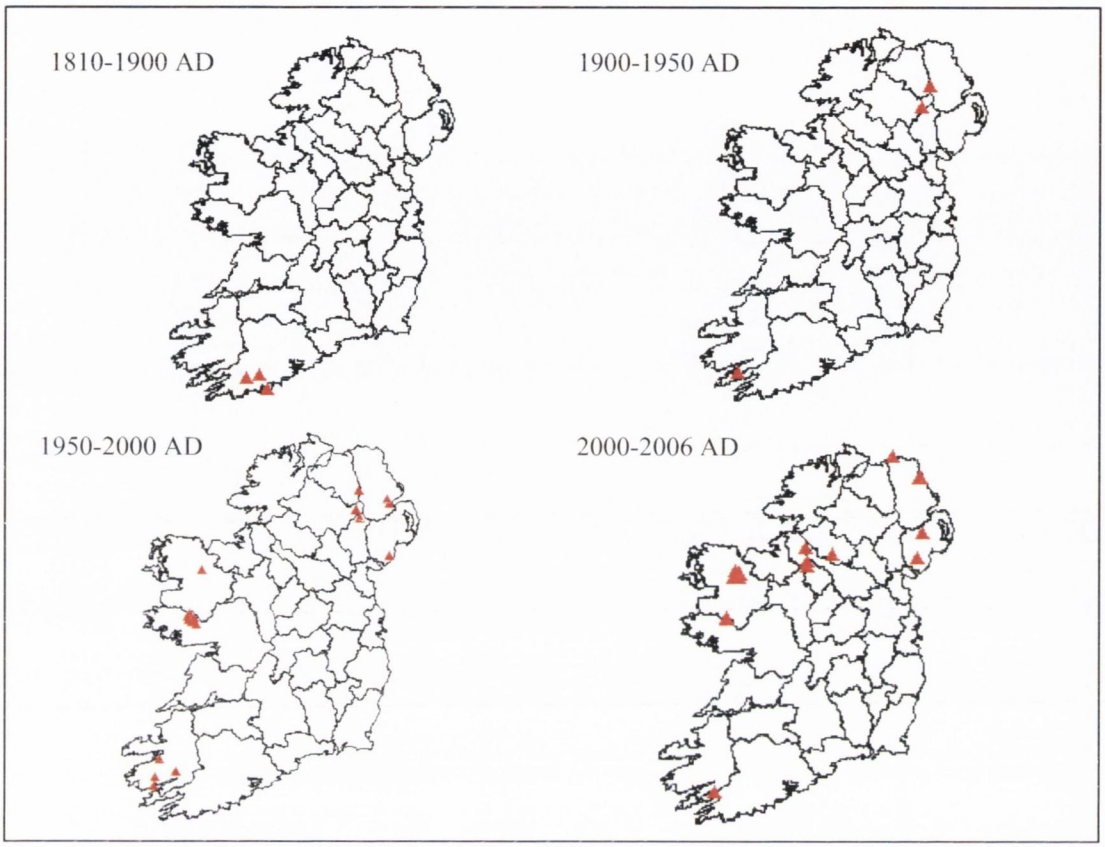


Plate 5.4.1 Shows the approximate distribution of *Spiranthes romanzoffiana* in Ireland since its discovery in 1810 AD. The red triangles denote populations where reliable data on their location exists. Records predating the 1990's do not contain reliable data on the number of individuals within in population. The maps suggest a northerly shift in the distribution of the species.

Table 5.4.2 The county, population name and location name of each site in which *Spiranthes romanzoffiana* was recorded between 2003 and 2006. All site visits were undertaken between July and September. A green tick mark indicates that flowering plants were recorded at the corresponding site. A red X indicates that no flowering plants were recorded that year. The wavy line means no site visit was carried that year. Populations in blue font are new records for *S. romanzoffiana* in Ireland. For conservation reasons the grid references have not been included.

County	Population title	Location	2003	2004	2005	2006
Mayo	L. Conn	Knockmore	✓	✓	✓	✓
Mayo	L. Conn	Corrysola bridge	✓	✓	✓	✓
Mayo	L. Conn	Carraig a Moiltin	✓	✓	✓	✓
Mayo	L. Cuilin	Cuingmore	X	X	✓	✓
Mayo	L. Cuilin	Drummin wood	✓	✓	✓	✓
Mayo	L. Levally	Bofeenaun	X	✓	✓	✓
Galway	L. Mask	Ferrybridge	✓	✓	✓	✓
Galway	L. Corrib	Opp. Duck rock	X	✓	✓	✓
Roscommon	L.Allen	Maguires shore	X	✓	✓	✓
Leitrim	L. Allen	Kilgarriff lake	X	✓	✓	✓
Cork	Co. Cork	Glengarriff	✓	X	X	X
Cork	Skibbereen	Kilclashna	~	X	X	~
Antrim	Antrim	Montiaghs Moss	~	X	✓	~
Antrim	Carnlough	Gortnagory	~	✓	✓	✓
Antrim	Aird Snout	Giant's Causeway	~	~	X	~
Antrim	Carnlough	Little Trosk	~	✓	✓	~
Antrim	Three Islands Bay	Lough Neagh	~	✓	✓	~
Armagh	Drumilly Mountain	Armagh	~	X	X	~
Down	New Bridge	Upper Bann	✓	X	X	~
Fermanagh	Upper Lough Erne	Fields N of Corraslough Point.	~	✓	✓	~
Fermanagh	Upper Lough Erne	Corraslough Point	~	✓	✓	~
Tyrone	NE Quadrant	Rousky	~	✓	X	~

5.4.2 Population Demographics

Proportion of vegetative to flowering plants in sample populations.

During fieldwork in 2005 attempts were made to ascertain the number of vegetative plants growing within a series of sample sites. In total, 135, $1m^2$ quadrats at the four study

populations were sampled; 283 plants were recorded, 200 of which were flowering plants and 83 were vegetative. The proportion of vegetative to flowering plants for each sample population ranged from 0.24 to 0.46 with an overall mean value of 0.37 (table 5.4.3). There was a strong positive correlation (R^2 = 99.9, df = 2, $p<0.0005$) between the increase in flowering plants and vegetative plants (figure 5.4.1).

Table 5.4.3 Numbers of vegetative plants and flowering plants within four sample populations of *Spiranthes romanzoffiana* during 2005.

	No. of $1m^2$ Quadrats	Total plants	Vegetative	Flowering	Proportion of veg plants to flowering plants.
Drummin Wood	66	161	51	110	0.46
Carraig – a Moiltin	33	64	18	46	0.39
Corrysola bridge	10	18	4	17	0.24
Knockmore	26	37	10	27	0.37
Total	135	283	83	200	
Mean proportion					**0.37**

Plate 5.4.1 Vegetative growth of *S. romanzoffiana*. Photograph taken at the Knockmore population in 2005.

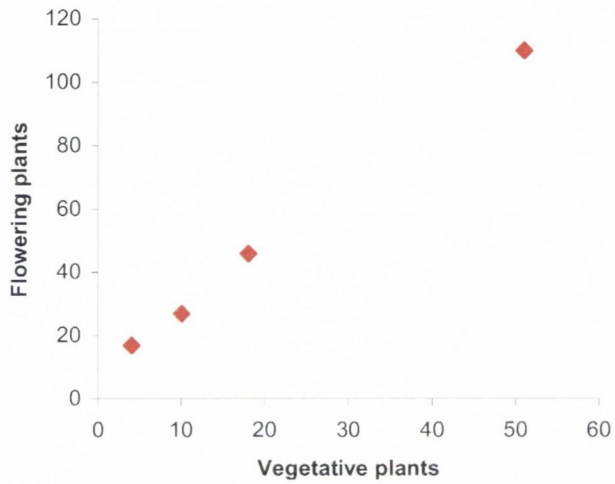


Figure 5.4.1 Correlation between the increase in flowering plant number and numbers of vegetative plants in the four sites of *S. romanzoffiana* . The R^2 value for the correlation for the two variables was 99.9(df = 2. $p<0.0005$).

Annual fluctuations in flower spike production in sample populations

The number of flowering spikes at each of the four populations was counted each year during the flowering season from 2003 to 2006. In 2003 the flowering numbers at all four sites were relatively low. The low figures for 2003 may be a true reflection of the actual flowering plant numbers in that year. However due to the difficulty encountered locating populations and developing an eye for detecting the species, the under recording of flower spikes in 2003 cannot be discounted. However, given the substantial rise in flower spike recordings in 2004 and the subsequent decline, it seems likely that the 2003 count was reasonably accurate (Figure 5.4.2). The Drummin Wood population had consistenly the highest number of flowering plants over the four-year period. Knockmore appears to be the next largest population followed by Carraig-a-Moiltin and Corysola Bridge respectively. With regards to the fluctuation in flowering-spike numbers, all four populations followed the same annual trends (Figure 5.4.2).

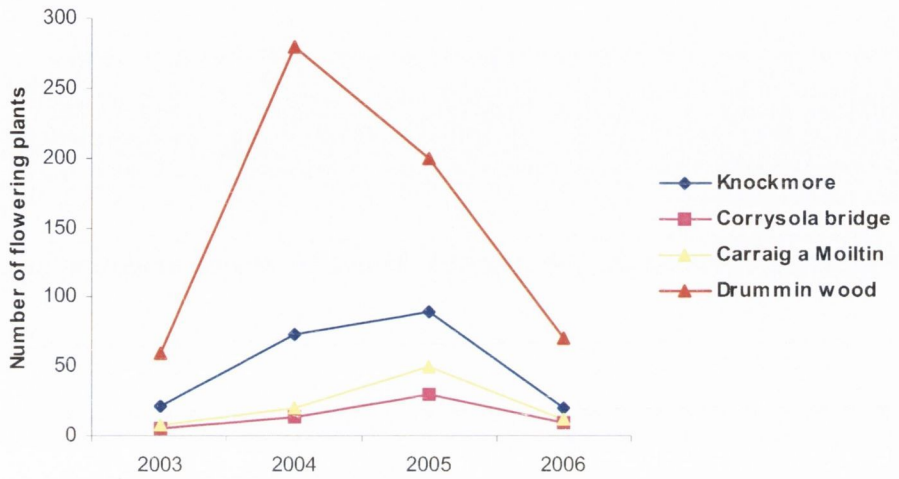


Figure 5.4.2 Annual variation in flowering plant numbers of *S. romanzoffiana* at the four survey site between 2003 and 2006.

5.4.3 Reproductive biology of *Spiranthes romanzoffiana*

Floral and morphological characteristics

Investigations into the reproductive biology *Spiranthes romanzoffiana* initially focused on the assemblage of data on the floral and morphological characteristics. The survey work was undertaken on 11 populations (Table 5.4.5) between 2003 and 2005. The number of plants surveyed per population was generally related to the total number of flowering plants in a given population. The range of values for each of the four parameters measured are as follows; the number of flowers per inflorescence ranged from 9 to 36; the number leaves per plant ranged from 1 to 6; and the range of height of inflorescence was 6cm to 38cm; percentage of flowering plants with lateral buds was 100%. The bud was visible from mid June onwards. By mid September the bud was emerging above the soil surface. The bud

remains in this position until the following May at which point it begins to develop to form that years flowering stem.

Table 5.4.4. Results from the investigation into the floral and morphological characteristics of *Spiranthes romanzoffiana*. Values in the columns represent the mean values for each of the populations surveyed between 2003-2005.

Population	Flowering plants	%twin plants	% with lateral buds	% twin lateral buds	Flrs/plant	Lvs/plant	Hgt of inflorescence cm
Knockmore	30	15.7	100	2.9	21	3	17
Corrysola bridge	34	10.8	100	1.7	18	2	11
Carraig a Moiltin	60	7.8	100	0	18	2	14
Cuingmore	5	20.5	100	5.8	21	1	8
Drummin wood	200	18.9	100	10.4	24	3	19
Bofeenaun	5	0	100	0	15	2	9
Ferrybridge	65	15.9	100	4.8	12	2	7
Opp. Duck rock	44	8.6	100	9.8	15	2	9
Maguires shore	30	4.6	100	6.2	18	2	12
Kilgarriff lake	87	12.8	100	12.8	15	2	14
Glengarriff	1	0	100	0	15	3	18

By combining data gathered from the ecological and population surveys it was possible to investigate potential correlations between the variables measured. Coefficients were determined using Spearman's rank correlation (Table 5.4.5) and these were tested for significance using the t-statistic. A t-value of 2.46 in a test at the level 0.01 must be exceeded for the correlation to be significant. A number of significant values were determined; the correlation between the increasing height of associated plants with the increasing height of *Spiranthes romanzoffiana* recorded was **t = 2.52**. The value for the correlation between increasing numbers of flowers per inflorescence and increase height of individuals of *S. romanzoffiana* was **t = 3.66.** The coefficient for the correlation between increase number of leaves and increased flower number was **t = 3.98.** The positive correlation between an increasing pH value and the tallest associated vegetation had a t-test value of **t = 1.97**, which is below the 2.46 value required. Thus the correlation was not significant.

Table 5.4.5 Spearman's rank correlation table of ecological and population variables measured in 2003. These data were gathered from 11 populations in Counties, Mayo, Galway, Leitrim and Cork. The values in yellow - significant positive correlations, values in red - significant negative correlations.

	Tallest Spiranthes	Tallest associated plant	No. of Flrs	No. of Lvs	pH	O.G.M%
Tallest Spiranthes	1					
Tallest associated plant	0.418	1				
No. of Flrs	0.55	0.146	1			
No. of Lvs	-0.055	0.236	0.577	1		
pH	-0.078	0.368	-0.086	0.161	1	
O.G.M%	-0.229	-0.366	-0.09	-0.145	-0.369	1

These data begin to reveal a possible trend in the production of flowers within the populations of *S. romanzoffiana* studied. The height of the vegetation associated with *S. romanzoffiana* may influence the height of the individual plants. The tallest of the *S. romanzoffiana* plants sampled appear to produce the highest number of flowers per inflorescence. It has not been ascertained in this research but the increase in height may be associated with competition for light with taller possibly more aggressive plant competitors. In terms of resource allocation this may be costly to *S. romanzoffiana*, however the Spearman's rank correlation does appear to indicate that the cost of increased flower production is mitigated by an increase in leaf production and therefore presumably an increase in photosynthetically derived resources.

Plates 5.4.2 and 5.4.3 Two examples of *S. romanzoffiana* growing along the shores of Lough Cuilin, Co. Mayo. The plant on the left is > 32cm in height, the plant on the right is < 4cm. Similar variation was observed in all populations visited in Ireland and Canada.

Pollination biology observations

No pollinators were observed at the Carriag-a-Moiltin site. A bumble bee species (*Bombus terrestris*) was observed on a number of flower spikes at the Knockmore site, though foraging within individual flowers was not recorded. Bumblebees were observed flying around the *S. romanzoffiana* population at Corrysola Bridge. However they appeared to be more interested in foraging on the flowers of *Calluna vulgaris*. One bumblebee (*Bombus terrestris*) was observed landing on the inflorescence of *Spiranthes* at this site. After approximately 10 seconds the bumblebee flew off, without attempting to forage for pollen or nectar.

Up to 50% of flowers examined at all four survey sites contained between 1 to 10 individuals of an unidentified species of Hymenoptera (Plate 5.4.4). Flowers containing these Hymenoptera were observed for 10 minutes at a time, these observations were repeated

throughout the day to check for insect movement. The Hymenoptera species tended to be inactive and static throughout the day. It was noted that *Prunella vulgaris* attracted a large number of pollinators, particularly *Bombus* spp. throughout the day. Every flower of *S. romanzoffiana* surveyed was inspected for the presence or absence of pollinia. It was noted that approximately and average of 6.15% of the flowers surveyed had pollinia removed, indicating the pollinia were being extracted by natural means (Plate 5.4.5). However no flowers surveyed contained deposited pollinia, i.e. pollinia from another flower or inflorescence. All flowers inspected contained a quantity of nectar at the base of the floral tube. The amount or concentration of the nectar was not quantified, however it can now be confirmed that Irish populations do offer a reward to potential pollinators. Research into this aspect of the species was conducted by a colleague as a part of his PhD research in to the pollination biology of *Spiranthes romanzoffiana*. Results of this work are referred to in section 5.5.2.

Table 5.4.6 Observations made into the 'natural' removal of pollinia from randomly chosen flowers in four sample populations of *Spiranthes romanzoffiana*.

	Knockmore	Carraig-a- Moiltin	Corrysola bridge	Drummin wood
Total number of flowers examined / site	178	189	87	220
Percentage of pollinia removed	3.30%	6.30%	2.20%	12.8%

Plate 5.4.4 *S. romanzoffiana* showing the presence of a hymenoptera species within an individual flower.

Plate 5.4.5 Photograph of pollinia stuck to the petal of *S. romanzoffiana* at Knockmore 2004. The pollinia was absent from the flower in question, however it is not known if this is the source of the pollinia in the photograph.

Reproductive experiments

In total 463 individual flowers were examined, 12 replicates of each treatment (total 48) were set up (Table 5.4.8). Seed set was determined by observing and recording capsule formation. In all 463 individual flowers, no single capsule was observed as being ripe. All capsules had withered and split by mid to late September. It was assumed that no seed had been set and that species was suffering from some post-pollination problem.

Table 5.4.7 Results of the four reproductive treatments carried out in 2004. Using swollen capsules as an indicator of seed set, no seed set was observed during these tests.

Treatment	Knockmore	Corrysola bridge	Carraig-a-moiltin	Drummin wood	Total	Ripe capsule set
Autogamy.	67	18	16	4	105	0
Xenogamy.	75	18	24	4	121	0
Apomixis.	75	18	24	4	121	0
Geitonogamy.	74	18	20	4	116	0
Open control plants	4	4	4	4		
Total No. of flowers.	291	72	84	16	463	

Seed survey 2005

Following on from the negative results obtained from the cross-pollination experiments in 2004, it was decided that a repeat of the experiments would be too time consuming. It was instead felt that samples of the capsules at different developmental stages should be removed in the field and brought back to the laboratory at Trinity College for closer inspection.

Observations of the capsule at 1) Pre-anthesis; 2) Post-anthesis and 3) dehiscence reveal a change in ovule morphology. The white ovules begin to expand at post-anthesis but quickly become elongated, withered and turn a mottled brown colour (Plate 5.4.7). The capsule during this stage appears to swell and in some cases feels like seed may be forming (Plate 5.4.6). In the majority of cases the observed capsule eventually splits, revealing the withered ovules. However a more careful and detailed look at the structures, using a stereo-

microscope revealed the presence of large quantities of testae (Plate 5.4.8). Preliminary counts show that an individual capsule can contain between 1,800 to 4,000 testae. However samples of putative seeds within each capsule using the technique described in section 5.3.3 reveal a low occurrence of embryos within the seeds (Plate 5.4.9). This may indicate that there is an issue with resource allocation in the sampled plants, though given the individual seeds are so small this does seem unlikely. It may be an issue with insufficient penetration of the ovary by the pollen tube, this may result in low levels of fertilization.

Plate 5.4.6 *S. romanzoffiana* seed capsule post cross-pollination. Capsule begins to swell giving the impression of possible seed production

Plate 5.4.7 Seed capsule 18 days after plate 5.4.6 was taken. Capsules have withered and split open. No seed was produced. The capsules still contain the ovules attached to the ovary wall (parietal placentation).

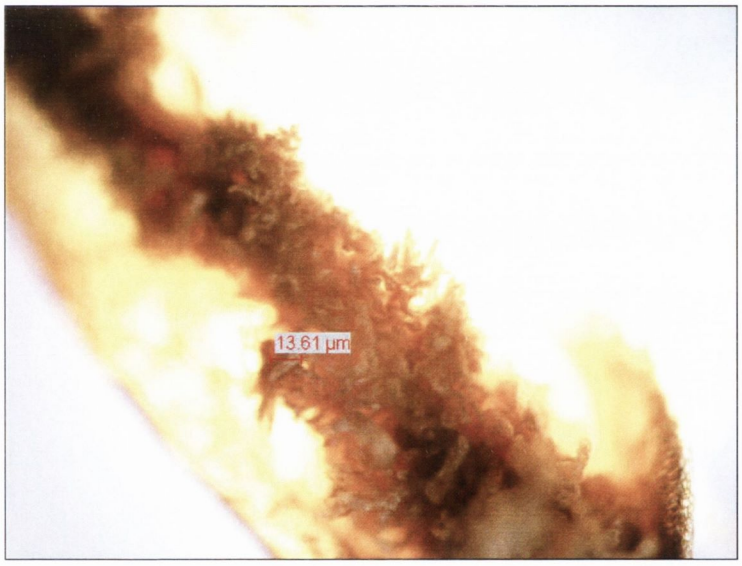


Plate 5.4.8 Photograph of an opened capsule, the brownish- yellow structures are the testae. These ranged in number from 1,800 – 4,000 per capsule.

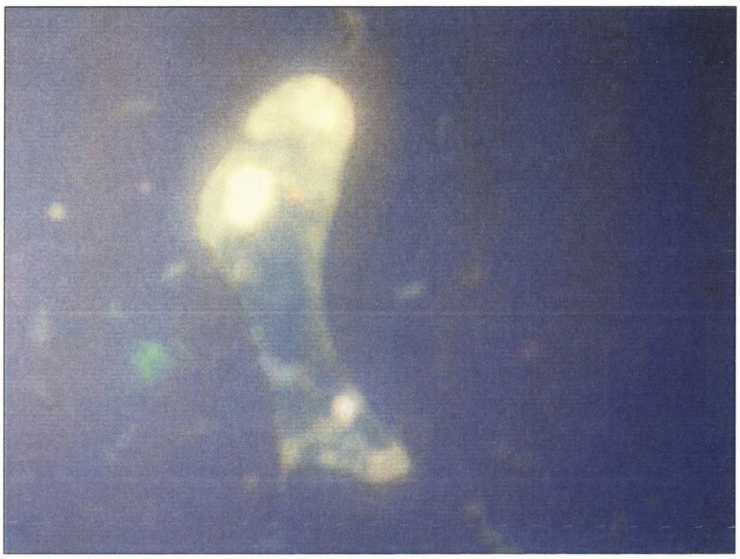


Plate 5.4.9 Stained seed of *Spiranthes romanzoffiana*. The fluorescein diacetate causes the intact embryo to fluoresce a bright yellow.

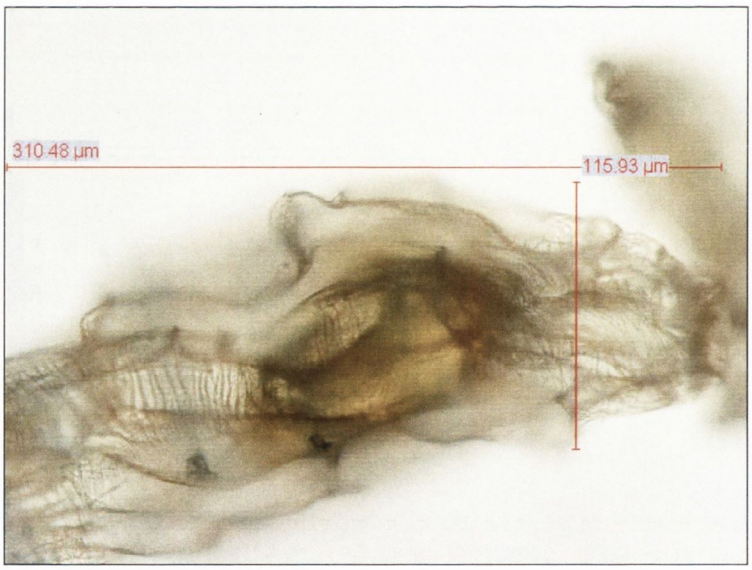


Plate 5.4.10 Seed of *S. romanzoffiana* containing a large intact embryo. Seed was collected at the Drummin wood site in 2005.

5.4.4 What are the major threats to Spiranthes romanzoffiana?

Investigations into possible threats to *Spiranthes romanzoffiana* and its habitat began in 2003. Grazing by both invertebrates and cattle was observed at all four of the field sites. The extent to which invertebrate grazing effects the species was not quantified, though some negative effect is expected. Timing of grazing by cattle at populations in Knockmore and Carraig-a Moiltin poses a major threat to the species ability to produce seeds. In 2003 and 2004 80% of the flowering spikes at Carraig-a-Moiltin were completely grazed thus only 20% of the total flower number remained. Combine the 6.15 % natural pollinia removal and high levels of grazing and it can be assumed that reproductive potential in grazed populations is severely hampered. Trampling by cattle, as witnessed by the presence of hoof marks, is also considered detrimental to the species ability to reproduce sexually.

A level of human disturbance was observed at all the sites surveyed during the research. The lakeshores are used as makeshift marinas for anglers. Damage caused by vehicles was evident e.g. large mechanical diggers seriously damaged the Knockmore site in late August 2003. Large track marks were observed through the middle of the population. Workers from Mayo County Council who were conducting work along the lakeshore operated these vehicles. It was clear that these individuals had no knowledge of the presence of a protected plant at the site. This lack of communication between all relevant parties and the apparent poor enforcement of the law must to be considered a serious threat to future conservation initiatives.

Table 5.4.8 Threats posed to the species are listed on the left side of the table, the four populations investigated are listed along the top of the four columns. The presence of each threat is denoted by a tick mark.

	Knockmore	Carraig-a-Moiltin	Corysola bridge	Drummin wood
Grazed by invertebrates.	✓	✓	✓	✓
Lightly grazed by livestock (Cattle).	✓			
Heavily grazed by livestock.		✓		
Damage caused by vehicles	✓	✓	✓	
Damage caused by pedestrians	✓	✓	✓	
Potential threat from encroaching vegetation	✓			

5.5 Discussion

5.5.1 Distribution of Spiranthes romanzoffiana in Ireland

Imperative to any active policy of conservation is the knowledge of the size, distribution and stability of the populations of the species concerned (Bradshaw and Doody, 1978). Research was carried out by (Jacquemyn *et al.,* 2005) into the possible change in distribution of 69 orchid species in the Netherlands and Belgium. They gathered data from historical records and compared them to present distribution patterns. 81% of the Belgian species had reduced their distributional range and 78% of species showed a similar decline in the Netherlands. The study concluded that habitat loss and fragmentation were the most deterministic causes of decline. The key aim of this research was to gather similar data on population sizes and possible changes in the distribution of Irish populations of *Spiranthes romanzoffiana.*

There are no reliable data on the number of flowering plants occurring in the historical records for *S. romanzoffiana* in Ireland. However, anecdotally, population numbers in the south west of Ireland appear to have dwindled to one or two individual plants in the west of Cork, whilst population numbers in the west and north of the country have increased. (Plate 5.4.1). Results indicate an increasing number of more northerly records in Ireland with time.

This may indicate a shift in distribution but could equally indicate a shifting bias in recording efforts with time

At best, the records for the species in Ireland are scant and lacking a definitive baseline. Therefore it is possible that the perceived disappearance of plants from previously known locations may simply be reflective of gaps in the recording. Survey work carried out in 2003 and 2004 on a number of historic sites in Cork and Kerry (Table 2.3.1) recorded no plants, although a cursory examination of the habitat did suggest that the sites were suitable for the growth of the species. However the eutrophication levels of the lakes in question was not assessed during the research. The pollution and eutrophication of lakes due largely to the intensification of agriculture in Ireland is a serious problem. This had resulted in the government incurring heavy fines by the European parliament for ignoring guidelines set out to reduce this problem. It is conceivable that the intensification of agricultural practices in Ireland has contaminated the habitats of *S. romanzoffiana* in the South west of Ireland. A more detailed study of the changes in farming techniques and changes in the edaphic characteristics of the historic sites may yield some insight into this apparent severe decline in numbers.

Another potential explanation for the change in the distribution of *S. romanzoffiana* in Ireland is related to the theory that ultimately the distribution of a species is limited to its physiological response to climate. Carey (1999) on his work on *Himantoglossum hircinum* suggested that the increase in distribution and the number of records was due to climate change, more specifically to amelioration of winter and spring temperatures and in a slight preponderance of winter rain'. In contrast to this, Wells (1967) in his work on *Spiranthes spiralis* found there was no evidence that even extremes of climate had any effect on the survival of the species. If it assumed that there is a shift in distribution of *S. romanzoffiana* towards to the north of Ireland, then perhaps it is possible to explain this using the Good (1936) theory that the population expansion is due to a more favourable change in climatic conditions. This theory however does not explain the virtual disappearance of the species from the South West of Ireland, as the climate conditions in this part of the country would seem favourable to the species persistence. This research suggests that the species is most

likely present in the south west of Ireland in larger numbers than the current data suggests. The discovery of new populations at Lough Levally (5 flowering plants) in Co. Mayo (2004), Lough Allen (<100 plants) in Co. Roscommon (2004) and Skibbereen (1 plant) in Co. Cork (2006) are encouraging and may suggest that the species is under recorded in Ireland. A concerted survey effort over a period of, for example, five years, would in all probability confirm this.

It appears that all surveys of *Spiranthes romanzoffiana* in Ireland to date have been based on the presence of flowering spikes. This study found no record of prior population surveys, which included plants in the vegetative stage of their life cycle. All surveys have been conducted on an *ad hoc* basis and there have been no consecutive surveys over periods longer than 2 years. Thus the influence of below ground dormancy and natural population fluctuations has not to date been considered.

Results gathered during this research found that the proportion of vegetative to flowering plants for each of the four sample populations ranged from 0.24 to 0.46 with an overall mean value of 0.37 (37%), this leaves a mean value of 67% flower spike production in the sample populations. It is possible to extrapolate that a more accurate estimate of population size can be determined by multiplying the number of flowering plants by 1.37. Hutchings (1987) found that on average 83% of the recorded population of *Ophrys sphegodes* flowered in a given year. Comparable mean figures for *Aceras anthropophorum, Spiranthes spiralis* and *Herminium monorchis* over a 14-year period were 41.1%, 32.8% and 16.9% respectively (Wells, 1981). Although there is some considerable differences in the range of these data, it is clear that population counts based on flower numbers can grossly underestimate the true population size.

Analyses of the relationship between the increase in flowering spike number and the increase in vegetative plant numbers showed a strong positive correlation ($R^2 = 99.7$). In other words, populations with high flowering plant numbers are probably much larger than is indicated by the number of flowering plants recorded, using traditional surveying methods. For example,

the population at Drummin wood at Lough Cuilin in Co. Mayo, recorded 283 flowering spikes in 2004. Using the proportions above, this could in fact represent over 400 plants.

Fluctuation in flowering plant numbers between the four survey sites was observed between 2003 and 2006. The lowest number of flowering spikes was recorded in 2003. This was followed by a sharp increase in flower number at all of the four sites in 2004, with the Drummin wood population peaking that year. In 2005 Knockmore, Corysola Bridge and Carraig-a-Moiltin peaked. Flowering spike numbers declined at all four populations in 2006. The reason for this fluctuation in flowering numbers from year to year is not fully understood. There are a number of hypotheses in the literature that go some way to explaining this anomaly.

It is thought that flower production reproduction and seed set has a significant cost on subsequent growth or flower production, or both (Ackerman and Montalvo, 1990). Resource limitations following high levels of flower and fruit production may result in the absence of flower production in subsequent year(s) (Montalvo and Ackerman, 1987; Primack and Hall, 1990). Another school of thought however suggests that the cost of flowering and reproduction is mitigated by the energy supplied by below ground structures (Antlfinger and Wendel, 1997). Wells (1981) suggested that the tubers of *Spiranthes spiralis* contributed to the carbon needs of reproduction and Antlfinger and Wendel (1997) showed that the number of flowers per inflorescence in *S. cernua* was related to tuber biomass. This latter hypothesis is bolstered by reports in the literature of 'record' producers, i.e. individuals that flower nearly every year. For example Willems and Bik (1991) reported that the founding individual of a population of *Orchis simia* flowered for 19 years. Other examples of long flowering periods include, *Aceras anthroporphorum* (13yrs; Wells, 1981), *Cypripedium acaule* (9yrs; Gill, 1989) and *Spiranthes cernua* (9yrs; Antlfinger and Wendel, 1997).

It is clear that different species have varying mechanisms to deal with the cost of flowering and reproduction. Work on *S. spiralis* and *S. cernua* indicates that the genus has the ability to compensate for reproduction by storing nutrients in their root system. It may also be the case that resource levels are sustained by organic carbon from its associated mycorrhizal

partner, which provides a nutritional source in addition to photosynthesis. There may be other factors influencing the annual fluctuations in flower numbers, for example the influence of variations in precipitation and temperature must be considered.

Due to time and resource constraints it was impossible to investigate the dormant phase of *S. romanzoffiana*. However as a way of highlighting the problems in conducting orchid population surveys, it is worth mentioning the results from some similar work carried out on a number of orchid species. Waite and Farrell (1998) determined that on average the dormant fraction of a population of *Orchis militaris* accounted for 14% of the plants present. The length of this apparent dormant period was from 1-8 years, though in most cases plants remained below ground for one year only. Tamm (1972) found that plants of *O. mascula* remained dormant for 1-12 years and the average proportion of a population dormant at anytime was 17%. All of these factors - difficulty in the detection of vegetative plants, fluctuation in annual flowering plants and the occurrence of dormancy - further emphasise the need for surveys to be conducted over much greater time periods than the 3-4 years represented by this study.

5.5.2 Reproductive biology of Spiranthes romanzoffiana

Vegetative reproduction

To date vegetative reproduction has been considered the primary mode of reproduction for Irish and indeed European populations of *S. romanzoffiana*. One way in which the plant can reproduce vegetatively is twin lateral bud production (Gulliver, 2004). Lateral buds arise at the tip of the root system and can be in groups of one, two or in some cases three. The separation of these buds varies from 2mm to 8mm as was observed at the Knockmore site. Gulliver (2004) recorded similar distances (1mm – 5mm) in Scottish populations. On Colonsay (Figure 4.4.2) in 2000 and 2001, Gulliver (2004) found that rates of twin plants in the population were 4.3% and 3.0% respectively. On Coll (Figure 4.4.2) in 2000 and 2001,

Gulliver (2004) recorded rates of twin plants was 26.3% and 22.0%. He concluded that the importance of this mechanism might vary from island to island.

Gulliver's results are comparable to the results obtained during this research. The occurrence of twin plants in the populations at Knockmore, Carraig-a-Moiltin, Corrysola Bridge and Drummin Wood, ranged from 4.5% to 20.5% (Table 5.4.5). It is conceivable that these lateral buds could break off the parent plant with a fragment of root attached and be dispersed in the soil. Rasmussen (1995) has suggested that root fragments in the Orchidaceae may be capable of a period of mycotrophic existence and subsequent development into new plants. Wilcock and Neiland (1998) have suggested that some species, e.g. *Dactylorhiza lapponica* persist by continuously regenerating from protected underground buds.

Scottish populations of *Spiranthes romanzoffiana* occur in habitats where there is cattle and sheep activity and slug grazing so there is a strong possibility of periodic root severance by the hooves of the livestock or by slugs grazing underground (Gulliver, 2004). In Ireland *S. romanzoffiana* also grows in habitats grazed by a variety livestock and invertebrates, so it is also conceivable that the same root fragmentation can occur in Irish populations. An interesting fact is that the majority of Irish populations of *S. romanzoffiana* are confined to seasonally flooded lakeshores. It may be that root fragments, separated from the parent plant by hooves or grazing, could be dispersed through the lake waters and eventually be deposited on a lakeshore a few centimetres, or indeed a few kilometres from its origin. This would somewhat explain why the species appears to be confined to lakeshores, as opposed to a wider variety of habitats. It would be very interesting to test the survivability and subsequent growth of root fragment in water bodies. This would be possible in a controlled environment, where the potential shoot and root development could be monitored.

In conclusion, vegetative reproduction by lateral buds does occur in the populations studied. The effectiveness of vegetative as a means of dispersal is unknown. Without further examination it can only be presumed that dispersal distances are at a scale of millimetres rather than metres or kilometres. The high levels of genetic variation detected within and

among the Irish populations surveyed (Section 3.4) suggest that the populations are not entirely vegetative and are more likely to be dispersing by seed.

Sexual reproduction

As mentioned in the previous section, vegetative reproduction to date has been considered the primary method of reproduction in Irish and European populations of *S. romanzoffiana.* Much of this idea has come from the lack of evidence of seed set in the species European range. High levels of genotypic diversity (P_D =0.98) detected in Northern Scottish populations of *S. romanzoffiana* are thought to be evidence of sexual reproduction (Forrest *et al.*, 2004). Whether or not this diversity is resultant of historic or contemporary gene flow has yet to be resolved. Observations of capsule development have been carried out in Scotland since 1995 (Gulliver, 2004). Most of these observations have been based purely on the development of the capsule, though in some instances the capsules have been examined for seeds. Mature fully developed capsules, such as in *Spiranthes spiralis,* have never been observed in Scottish populations of *S. romanzoffiana* (Gulliver, 2004).

Floral biology

Results from this research show that the flowers of *Spiranthes romanzoffiana* are nectar rewarding and are strongly scented, the scent being reminiscent of vanilla. The nectar is stored at the base of the flower in a small, elongated spur, 1-3mm long. The inflorescence consists of white to off-white flowers, 10-15mm long (excluding the ovary) in three spiral ranks. The height of the inflorescence ranged from 6cm to 38cm, the number of flowers per inflorescence ranged from 9 to 36 and the number leaves per plant ranged from 1 to 6. Results indicate a correlation between the height of surrounding vegetation and the height of *S. romanzoffiana*, i.e. in tall vegetation *S. romanzoffiana* appears to compete with the vegetation by growing taller, perhaps to avail of light. The number of flowers per inflorescence appears to be positively correlated with the increase in height of the flowering spike and the also the number of leaves per plant. This may suggest that flower production in *S. romanzoffiana* is influenced by rates of photosynthesis and subsequent resource

allocation. It may also be that the number of flowers per inflorescence is simply a function of the physical height of the flower spike, i.e. smaller flower spikes can only accommodate a small number of flowers. Height of the flower spike is only one indicator of plant vigour. Further surveys of, for example, the below ground biomass, would perhaps be a useful next step in the investigation of possible factors influencing the rates of flower production.

Investigations into the pollination biology of *Spiranthes romanzoffiana* were conducted during the first year of the investigation. During this time only two potential pollinator visits by a single species (*Bombus terrestris*) were observed in the vicinity of the inflorescence. There were no observations made of pollinators actually alighting on a flower. However, a species of Hymenoptera was recorded within >50% of the flowers studied at all four survey sites. Observations suggest that this species is not very mobile diurnally. Nocturnal surveys were carried out but no movement of the species was observed.

However, the possible disturbance, that is the mimicking of diurnal conditions caused by the use of a flashlight may have resulted in cessation of movement by the hymenopteran. This Hymenoptera species cannot be discounted as a potential cross-pollinator of *Spiranthes romanzoffiana*. Given the size of this Hymenoptera species (<3mm long – Plate 5.4.4) the transport of a whole pollinarium or single pollinia would be improbable. Nevertheless it was observed that on older flowers (>10 days old) the pollinia began to break up, resulting in the shedding of small amounts of pollen grains within the flower. Thus the Hymenoptera species could inadvertently transfer pollen grains from one flower to another. A closer look at the head, thorax and wing surface of this species may reveal the presence of small amounts of *S. romanzoffiana*. Due to time constraints it was decided that the research into this aspect of the species biology would be passed on to a colleague who is conducting pollination research into 5 rare Irish orchid species.

Duffy and Stout (2006, under review) recorded visitation and pollinia removal in *S. romanzoffiana* by a number of bumblebee species (*Bombus pascuorum* and *Bombus hortorum*). Duffy and Stout (2006) found that visitation occurred mainly between 10.30 and 17.00h. Foraging lasted for up to 60 seconds and a maximum of 10 flowers were visited in

sequence per inflorescence. 19 pollinia were observed on *Bombus pascuorum* in flight and four individuals of *B. hortorum*. Duffy and Stout (2006, under review) concluded that visitation rates appear to be density dependent, with plants at lower densities receiving higher visitation rates per hour. This is attributed to competition for pollinators amongst highly dense patches of individuals (Duffy and Stout 2006, under review). To date Duffy and Stout (2006, under review) have not recorded a pollinator successfully transferring pollen between plants or flowers. This research has recorded the presence of *S. romanzoffiana* pollen on the stigmas of individual flowers of *S. romanzoffiana* within the same population. These data in conjunction with the evidence that at least 6% of flowers surveyed in this research (Table 5.4.7) had pollinia removed by natural means. Evidence has now emerged that pollinator visitation in Scottish populations is also occurring, though the data for Scottish visitation rates are low relative to Irish populations (C. Wilcock and A. Scobie, 2006, *pers comm.*). While this is an important discovery more evidence is required to determine the rate of successful pollen transfer between flowers as the removal of pollinia must be followed by successful cross pollination for the process to have real significance.

The original hand pollination experiments conducted in 2004 yielded no seeds. These data were however based on the presence of a swollen capsule. As orchids contain many thousands of dust like seeds, fruit set is the conventional method to measure reproductive success (Neiland and Wilcock, 1998). Neiland and Wilcock (1998) also reported that fruit set for nectariferous orchids in Europe is approximately 63%. Greenhouse experiments on Canadian populations of *S. romanzoffiana* resulted in higher levels of fruit production in xenogamous crosses (100%) compared to those found in geitonogamous crosses (64%) (Catling, 1982). Flower to fruit set was reported at levels >75% in North American populations of *S. romanzoffiana* (Larson and Larson, 1987).

Given these reported values it was decided in 2005 to take a closer look at the contents of the capsules. Capsules that would traditionally have been overlooked or regarded as barren were collected and examined in the laboratory. In September 2005 seed was detected in the harvested eight capsules. The mean number of viable embryonated seed per capsule was just

4.5%, however the discovery of even these relatively small amounts of seed in Irish populations was very encouraging.

In 2006 a one year Masters project at Trinity College Dublin endeavoured to re-examine the hand pollination experiments carried out by this research in 2004. The results for rates of seed set and the percentage of embryonated seed from this experiment, which examined geitonogamous and xenogamous reproduction, revealed data comparable to those recorded by this research in 2005 (O'Connor 2006, unpublished). In the 2006 study, 36% of capsules contained testae as compared to 46% in the 2005 study. The 2006 study revealed a difference in the number of seeds per capsule per treatment. The study showed that the geitonongamous treatments produced the highest amount of seed when compared to the xenogamous study. However Analysis of variance (ANOVA) revealed no significance between treatments ($p = 0.212$). In contrast to this only 3.88% of geitonogamous testae contained embryos, this result is again comparable to the 4.5% of viable embryonated seed detected during this research. The study revealed that 12% of xenogamous treatments contained embryos. The 2006 unpublished thesis concluded that xenogamous reproduction, particularly between study sites improved embryonated seed production.

The quantity of embryonated seed appears to be low and thus suggests that there may be a problem with the one or a number of the stages of reproduction in *Spiranthes romanzoffiana.* There are a number of factors that affect seed set such as pollen quantity and quality (Tremblay *et al*, 2005), stigma receptivity, seed parent influences (Silverton, 1984) and pre-dehiscence factors including resource limitations during seed development, infection or infestation and temperature (Light and MacConaill, 1998). Duffy and Stout (2006 in press) suggest, that the lack of fruit development in Irish populations of *S. romanzoffiana* was not attributed to pollinator limitation. If successful transfer of pollen is occurring in Irish populations then there is possibly an issue with pollen quantity and or quality.

Orchid pollen is not always deposited as indivisible pollinia (Tremblay *et al.*, 2005). Experiments by Greg (1991) investigated the effect of variable deposition on seed development in *Cleistes divaricata* and found that seed production declined with reduced

deposition while seed fertility (percentage of seeds with embryos) was unaffected. Observations made during this research suggest that the pollinia of *S. romanzoffiana* are soft and are easily broken apart or crumbled. Therefore it is possible that only very small amounts are being successfully transferred between flowers. Work by Catling (1982) on the pollination of North American populations *Spiranthes romanzoffiana* reported that seed produced in the capsules only developed in the upper portions of the ovary, this was suggested to be a result of a restriction in the development of pollen tubes. However, Wilcock (2001) reported good pollen tube growth in two intra-population (36% and 73% respectively) and one inter-population (75%) cross-pollination experiments, in three Scottish populations of *S. romanzoffiana.* He concluded that reproductive failure in *S. romanzoffiana* was not a result of non-viable pollen or the failure of pollen tubes to grow on the stigma. Duffy and Stout (2006 in press) checked pollinia viability on randomly sampled flowers of *S. romanzoffiana* using Alexander's (1980) stain on 'young' (<1 day old) and 'old' (>7 days old). All pollinia examined were found to be viable (Duffy, 2006 in press). These data suggest that pollen quantity and quality have an affect on the reproductive success of *S. romanzoffiana*. Though the complete ramifications of this influence given the very low numbers of viable seed being produced are not fully understood at present.

A comparative study into the rates of pollen germination and pollen tube growth between European and North American plants could potentially highlight differences in two stages. Such a study would enable researchers to determine whether the perceived pollination problems of *S. romanzoffiana* are inherent in the species or are due associated factors e.g. differences in geographic location, temperature variations or ecological incompatibility within the Irish populations.

Assuming there is no problem with pollen quality or quantity, another potential cause of low seed set may be associated with predehiscence factors. These would include resource limitations, unsuitable temperatures during the seed maturation phase, fungal infections in the seed capsule or the grazing of flowers and capsules by livestock or invertebrates. Reproductive failure in plant species can often be attributed to resource limitation where insufficient resources, such water or nutrients are available to allow maximum fruit set to

take place (Bierzychudek, 1981). Among the Orchidaceae some studies have suggested that fruit production in one season may incur a cost to reproduction such that reproductive output and or vegetative growth is lower in future seasons (Primack and Hall, 1990). The issue of temperature and its affect on fruit maturation and seed production was not investigated thoroughly.

The discovery of seed in Irish populations of *S. romanzoffiana* in 2005 and 2006 has been a very important breakthrough in the research into the species' enigmatic existence in Ireland and Europe. The level of embryonated seed production appears to be at an insufficient quantity to maintain to genetic diversity within populations. However, as previously mentioned, orchid species are capable of producing many thousands of testae per capsule. For example in the 2005 data, the median number of total seeds per capsule is somewhere in the region of 2,900 and of these 4.5% contained potentially viable embryos. We can thus extrapolate, that on average each capsule examined contained approximately 130 viable seeds. In relation to other orchid genera, 130 embryonated seeds per capsule is extremely low (Arditti, 1992). This would help explain why *S. romanzoffiana* is so limited in its Irish distribution. The specific requirements with orchid seed germination and subsequent recruitment (Section 1.1.5), coupled with such low seed set make it not surprising that *S. romanzoffiana* exists in Ireland in relatively low numbers. This does not however explain why populations in Ireland are predominantly confined to the West and North-East of Ireland. Extensive surveying is required across the whole of Ireland to see whether this distribution is accurate or whether it is a result of insufficient surveying.

5.6 Conclusion

During this research 23 populations of *Spiranthes romanzoffiana* were visited in Ireland, four of these are new records for the Republic of Ireland. Four historic sites in the south west of the country were surveyed and no plants were recorded. The data suggest that Irish populations of *S. romanzoffiana* have undergone or are currently experiencing a northerly shift in their distribution.

The population demographic data suggest that current population census methods are under-recording the true extent of population sizes. The proportion of vegetative to flowering plants was 0.37, which suggests that populations sizes may be underestimated The fluctuation in annual flower spike production (Figure 5.4.2) detected in this research suggest that population monitoring should be conducted over a minimum of five consecutive years. This would compensate for natural fluctuations, which could lead to erroneous population size estimates if surveys were conducted over shorter periods.

Irish populations of *S. romanzoffiana* appear to reproduce vegetatively by the production of twin lateral buds during the summer months. However the high levels of genetic diversity typically associated with a sexually out-crossing species, the discovery of natural pollinia removal and the detection of potential pollinator species (Duffy and Stout, 2006, in press) suggest that Irish populations are not totally reliant on vegetative reproduction for their persistence and distribution. The dominance of mature plants in the size and age class survey coupled with the small quantities of viable seed found in the capsules suggests that recruitment by seed is low in Irish populations.

Chapter 6

Overall discussion

6.1 Aims of the discussion.

The layout of this thesis is such that each chapter has focused on a particular biological, ecological or genetic aspect of Irish populations of *Spiranthes romanzoffiana*. In most cases these characteristics have been examined in the overall context of the species Irish, European and global distribution. Where appropriate each chapter has concluded with a discussion on the results obtained. These individual discussions have so far been constructed mostly in isolation from the other chapter's subject matter. It is not the intention of this chapter to rehash the previous chapter's conclusions; rather the intention is to combine the ideas in succinct format.

In this concluding chapter there are a three aims, 1) to review some of the molecular methods used in this research and discuss briefly their suitability to the project, 2) to bring all of the facts obtained throughout this research into a concise and clear overall conclusion, 3) to highlight the implications of the results and contribute realistic recommendations for the conservation of *S. romanzoffiana* in Ireland.

The project set out to gather baseline data on the *S. romanzoffiana* in Ireland. Basic data on population numbers, population distribution and the numbers of individuals within populations were virtually non-existent at the time this research commenced. More in-depth data on the species ecology, habitat preferences, population demographics, floral dynamics, pollination biology and reproductive output did not exist at all. It was therefore imperative that these data were gathered from the outset. It was decided that without a basic understanding of these fundamental characteristics the more involved genetic analysis of the populations would have been too great a leap forward as genetic analysis requires at least a basic understanding of the species biology so that the results can be interpreted in an overall

context. The project therefore has endeavoured to collect and compile the data required and view the results in a holistic manner. The ultimate goal is to contribute to the successful conservation of *S. romanzoffiana* in Ireland and to contribute to furthering the knowledge of *S. romanzoffiana* in a European and global context.

6.2 A critique of the methods used in this research to assess the levels of genetic diversity in Irish populations of Spiranthes romanzoffiana.

There have been a number of important results obtained during this research project, a number of these have been on a very basic level, e.g. population numbers or the putative change in distribution of past and present populations. However there have also been a number of results obtained through the molecular analysis of the sampled populations, which make large and important statements about the status of *Spiranthes romanzoffiana* in Ireland. In following section some of the assumptions made by the molecular techniques are discussed in an attempt to put the conclusions gained from these analyses into context.

The estimation of genetic diversity statistics are based on a number of assumptions which impose limitations on the accuracy on which the results can be relied upon to make effective conservation decisions. In the case of AFLPs the main source of error is involves estimation of allele frequencies. The absence of a particular fragment gives the proportion of the null homozygotes, but the presence of the fragment does not allow the 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.*, 1990). However the reliability of this method has not been stringently tested and is subject to significant error, as a weakly concentrated or impure DNA may also result in reduced intensities of the bands in some samples. The estimation of allele frequencies remains the only viable option and is used universally in plant population genetic studies.

Apart from these issues the AFLP technique also makes assumptions that may not reflect the true genetic structure of the populations being studied. In particular the assumption of Hardy- Weinberg equilibrium may not apply to all populations, and deviations from this may occur, particularly in small populations of *Spiranthes romanzoffiana* were clonal spread is potentially high.

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, similar to the one used in this research yield genotypic data and analysis of genetic structure does not therefore require the estimation of allele frequency. Lynch and Milligan (1994) 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 a study by Ribeiro *et al.* (2000), on chloroplast microsatellite and AFLP diversity in maritime pine (*Pinus pinaster*), showed that the genetic distance matrices calculated from the two different sets of markers were significantly correlated. The two techniques may therefore be significantly correlated.

Aside from the inaccuracies that arise from inherent assumptions in the use of dominant markers for the estimation of population genetic parameters, there are a number of other sources of error. These are mainly related to the technique itself and include contamination of the DNA, partial digestion of the template DNA, or fragments not being fully amplified during the PCR reactions. Of course these errors are not unique to AFLPs techniques. In this study every precaution was made to guard against contamination but there is always the chance 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.

Another major criticism of the AFLP method relates 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. 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 fragments to whole numbers of base pairs. As discussed in section 3.2 the data in this research were scored by eye to ensure accuracy.

The advantages of the AFLP technique far outweigh the shortcomings and one of the major benefits in their use in the study of rare or threatened species is the small amount of DNA required to obtain results, this enables widespread sampling of populations without the destruction of plants. AFLP is a highly reproducible method of obtaining genetic fingerprints from small amounts of DNA (Vos *et al.*, 1995; Jones *et al.*, 1998). The large number of bands gives a realistic and reproducible measure of variation across the entire genome, thus providing a good estimate of the overall level of genetic variation (Tali *et al.*, 2005). Also, the AFLP method provides 10-100 times more markers and thus is more sensitive than most

other fingerprinting techniques; small genetic differences can easily be detected (Matthes *et al.*, 1998). The precision of genetic distance estimates increases with increasing numbers of markers, and Klein *et al.* (1992) have suggested that if more than 90 markers are used, error is reduced to acceptable levels. The number of markers used in this research (250) greatly exceeds this number so the error due to variation among loci should be reduced.

Although AFLPs may not give exact estimates of the 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. Although this research has had its problems, particularly with achieving adequate sampling sizes for genetic analysis it is felt that some understanding of the genetic diversity of Irish populations of *Spiranthes romanzoffiana* has been attained.

6.3 Spiranthes romanzoffiana in the context of the Irish flora.

Ireland's flora is considered to be an impoverished sample of the flora of north-west Europe (Webb, 1983). The total flora, according to Curtis and McGough (1988) is 1,000 vascular plants. About one half of the species are widespread in Europe and the rest of the species are said to represent groups made up of a number of distinct distributional elements (Curtis and McGough, 1988). Within these are the reputed elements of Atlantic-Mediterranean and Arctic-alpine affinities, 73 species belonging to the former category and about 16 to the latter (Webb, 1983). Ireland also contains a number of species within what is termed as the Lusitanian flora (e.g. *Arbutus unedo, Erica mackaiana*). The floristic assemblage this research is concerned with is the Amphi-Atlantic element of which there are a number key species, including *Spiranthes romanzoffiana*.

Endemism is not widely expressed amongst the Irish flora, though is notable in some apomictic groups such as *Sorbus* and *Hieracium* (Webb, 1983). Future molecular evidence may well begin to pick up differences at the species level, such work may reveal that Ireland contains a higher number endemic species than are currently known, e.g. molecular work on

Dactylorhiza occidentalis, suggests this species is endemic to Ireland (R. Bateman, 2007, *pers comm.*). At the sub-species level there is a higher frequency of endemism, e.g. *Dactylorhiza fuchsii* subsp *O' Kellyii.*

Irelands botanical uniqueness is not strictly related to its species composition but rather on the ecological groupings in which the associate themselves, e.g. the Burren in Co. Clare or Cork and Kerry. Ireland is also rich in bogs and wetlands. Many wetland species are still commoner in Ireland than on continental Europe or in Britain. This is an interesting point as virtually all of the North American, amphi-Atlantic element of the Irish flora are wetland species or species of flushed habitats, e.g. *Eriocaulon aquaticum* and *Najas flexilis*.

Given the relatively low numbers of vascular plants found within Ireland and the low levels of endemism, Ireland has a duty to conserve the unique species assemblages found on the island. In addition to this we have a responsibility to conserve and protect species with a European or global significance. *Spiranthes romanzoffiana* by virtue of its unusual distribution, enigmatic existence and importance in the European flora is a prime candidate for novel conservation research. It is hoped that the following conclusions and recommendations derived from this study will contribute significantly to the understanding of *Spiranthes romanzoffiana* in Ireland. It is also crucial that the outcome of this work does not remain purely aspirational but actually stimulates impetus for future work on this and other species in the Irish flora.

6.4 The conservation biology of Spiranthes romanzoffiana in Ireland

Over the three years of fieldwork a total of 23 populations were visited on the island of Ireland, this includes four new records for the Republic of Ireland. In tandem with this research data were collected on the potential shift in the species distribution since it was first recorded in Co. Cork in 1810. No reliable data exists on the on the size of the populations accounted for in the early Irish records. However the absence of any current evidence for the existence of plants at these locations suggests that the populations have dramatically reduced in size or have become extinct. Currently *S. romanzoffiana* appears to be largely confined to

a few counties on the Atlantic coast of the Republic of Ireland. (Cork, Galway, Mayo), there are however three substantial populations on the shores of Lough Allen in Co. Leitrim and Co. Roscommon, these represent the most inland of the Irish populations. In the North of Ireland the species is again predominantly confined to the coastal counties, though exceptions to this are found in Co. Fermanagh and Co. Tyrone.

If the perceived northerly shift is correct and not as a result of recording bias or poor records, then what is causing this shift? Before explanations for this possible northerly shift are discussed it is worth mentioning the potential occurrence of error in traditional population surveys. One of the significant results of the study of *Spiranthes romanzoffiana* is the identification of a relatively high proportion of vegetative plants growing within flowering plant populations. This has a number of important implications for future census initiatives and conservation programmes. Traditional census methods have relied on the presence of flowering plants to determine the location and size of populations. The results suggest that this is an unsatisfactory method as it has a high potential for under estimating actual population size. It is possible that *S. romanzoffiana* is under recorded in Ireland due to the problems inherent in the species' often elusive nature. Future census work should focus on fine scale surveys for sample populations. Figures for the true extent of Irish population sizes could be extrapolated from fine scale surveys conducted over a number of consecutive years.

Climate change is a popular explanation for rapid changes in distribution of taxa around the globe. In the case of Irish populations of *Spiranthes romanzoffiana* changes in climate over the last 100 years seems an unlikely cause for the species to become extinct or at least severely reduced from the south of the country. Any slight change in the temperature or rainfall etc over the last 100 years in the south of Ireland would still be well within the range of the species climatic tolerances in North America. However the environmental results gathered during this research may suggest the Irish populations have a limited range of ecological tolerances.

Apart from the population in Gortnagory in Co. Antrim which is located at 280m above sea level, the rest of the Irish populations are confined to seasonally flooded, lakeshore habitats, in a substrate with a pH range between 5.8 and 6.8, and a organic matter content of <2%. The vegetation associated with *S. romanzoffiana* in Ireland occupies a narrow community range (section 2.3). These two results suggest that the species is ecologically restricted in Ireland. The molecular results (section 4.3) indicate that the Irish samples are somewhat genetically distinct from the North American samples. It is possible that the perceived ecological restriction is based on a narrow gene pool within the Irish populations. It seems conceivable that the Irish samples do not have the genetic variation to cope with climatic or environmental perturbations.

It is feasible that shifts in the species distribution may be a result of changes in land use and the intensification of farming practices in the south of the country. This has lead to massive issues with lake-water quality and the eutrophication of the lake habitats. It has not been proven in this research but it may be no coincidence that the largest populations of *S. romanzoffiana* in Ireland occur in Galway and Mayo (+250 flowering plants per population in some cases e.g Lough Cuilin, Co. Mayo) on poor land, unsuitable for agriculture. In some instances low level stocking of cattle and sheep has been observed adjacent to a number of the sites; however there is no arable farming in the vicinity of these large populations. Arable farming arguably contributes more to the pollution of lake-waters as this practice uses large volumes of nitrogen and phosphorous. Understanding how *S. romanzoffiana* responds to, for example, climate change, habitat loss and grazing pressure is vital if the species is to be effectively conserved within Ireland and Europe.

Determining the pattern of genetic diversity of *S. romanzoffiana* is crucial to future conservation initiatives. 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 common assumption that larger populations are more diverse and contain greater evolutionary potential and conservation value than smaller populations is not always correct, e.g. genetic data on the endangered Californian plant *Cordylanthus palmatus* showed that contrary to many other similar studies,

genetic diversity was not significantly correlated with population size (Fleishman *et al.*, 2001). Molecular methods allow the identification of populations most at risk from genetic erosion; this approach can lead to specific management recommendation that can minimise the deleterious effects genetic deterioration. Godt *et al.* (1996) analysed the genetic diversity in *Geum radiatum* and *Calamagrostis cainii* and identified several 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. RAPD analysis of *Acacia raddiana* in the Negev desert revealed that the Negev and the Arava valley populations are significantly differentiated from each other (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 out-breeding 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 two genetically differentiated populations are mixed (Templeton, 1986; Fenster and Dudash, 1994).

The benefits of genetic information on the successful conservation of species are enormous. The examples mentioned above are a small sample of the input such data can have on the decision-making processes involved in designing and implementing conservation plans. Values for genetic diversity are meaningless unless they are 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. If populations of *S. romanzoffiana* were examined in isolation and low levels of variation were found, there would be no indication of whether this was a trait of the species or just a characteristic of the Irish populations. The level of population differentiation should also always be assessed in the broader context, as if populations in one locality have differentiated significantly enough to be regarded as genetically distinct they may have a high conservation priority. Genetic data for *S. romanzoffiana* show a degree of genetic differentiation between the North American and Irish samples. The levels of genetic divergence detected in this thesis are high enough to indicate that the Irish populations are probably not of recent origin, that is, not in the last few hundred years. These data have two

important implications for the conservation of *S. romanzoffiana* in Ireland. Given the evidence discussed so far this research concludes that *S. romanzoffiana* is native to the Irish flora. The high levels of diversity detected within the Irish samples and the low levels of genetic differentiation identified between the populations were unexpected (section 3.3). For example, using the data analysed for the chloroplast microsatellite marker the populations at Lough Mask and Lough Allen showed the highest levels of allelic diversity among the Irish populations and the presence of a unique allele in the case of Lough Mask (section 4.3). It would be a logical step to target these genetically diverse populations in future conservation initiatives. The divergence of Irish from North American and to some extent Scottish populations means the Irish plants form an important genetic component of the wider European and global *S. romanzoffiana* gene pool. The Lough Mask population contains high levels of genetic diversity and is therefore very important for conservation. The large populations at Drummin Wood and Knockmore also have a high conservation priority as they represent large, relatively stable and sexually reproducing populations. The role of sexual reproduction in maintaining genetic diversity within populations is well recognised (Brown, 1979; Hamrick, *et al.*, 1979; Gottlieb 1981; Loveless and Hamrick 1984; Hamrick and Godt, 1990). The design of any conservation strategy for a rare plant species requires information on the mating system of the species (Brown 1979; Hamrick *et al.*, 1991; Demauro 1993). These data will assist in informing future conservation plans that seek to target genetically diverse populations as sources of living material for *in-situ* or *ex-situ* initiatives. These populations are also ideal for further in depth reproductive studies that may elucidate an explanation for these relatively high levels of genetic diversity.

The chloroplast microsatellite data indicate a close relationship among Irish populations this may be due to a low number of colonisation events from North America with subsequent local spread by seed resulting in the high levels of within population diversity estimates. The northern Scottish populations possibly arose through separate colonisation events. However there is some evidence from this research suggesting the spread of northern Irish Irish plants to the Southern Hebrides, again this would seem to have come about through seed dispersal rather than vegetative means. The data suggest that seed dispersal has occurred, however the

low level of seed set detected in Irish populations indicate that this is likely to be limited to occasional events.

The dispersal of vegetative propagules however cannot be discounted at a local level. The perceived confinement of the species to lakeshore habitats may have an ecological explanation but equally the source of this narrow distribution could be related to the dispersal of vegetative fragments through the lake-waters. Fragments of root material severed from parent-plants by, for example cattle could conceivably be transported in the lake water and deposited on a lakeshore some distance away. It is currently unknown if the species can propagate itself using this method. The data gathered during this research do however confirm that *S. romanzoffiana* reproduces asexually by means of the annual production of lateral buds at the base of the flowering stem. This method of reproduction undoubtedly is important for the maintenance of local populations but is unlikely to be responsible for long-range dispersal of the species.

Until 2005 no seed or potential pollinator species had been reliably recorded in Irish or European populations of *S. romanzoffiana.* These two discoveries greatly enhanced the interpretation of the genetic data and have important consequences for the conservation of the species in Ireland. Although the quantity of seed produced is very low and nothing is known about the levels of recruitment within populations the detection of seed is nonetheless significant. The discovery of seed and the detection of nectar within each flower were bolstered in 2004 and 2005 by the uncovering of a number of pollination issues. Firstly it was noted by this research that a small number of flowers across a range of populations have their pollinia removed, this indicates that there is a potential pollinator available in Ireland. Duffy and Stout (under review) recorded a number of bumblebee species successfully removing pollinia from flowers of *S. romanzoffiana*. To date however the transfer of pollinia from one flower to another has not been recorded. The presence of large quantities of a hymenopteran species in the individual flowers may also have an influence of the transfer of pollen from one flower to another. Very little is known about the role this species plays in the life cycle of *S. romanzoffiana*, a closer look at its impact would be desirable in future research. The presence of pollinators potentially relies on the occurrence of suitable co-

flowering species to attract the pollinators to *S. romanzoffiana*. The narrow ecological niche occupied by *S. romanzoffiana* in Ireland and the importance of co-flowering species highlights the necessity for conservation efforts to not only focus on the species but on its entire habitat and community structure.

The genetic study into of the sample of Irish populations of *Spiranthes romanzoffiana* using AFLP markers identified a high level of genetic diversity and a weak population differentiation. The results suggest that *S. romanzoffiana* is an out-crossing species. Consequently, conservation and potential restoration genetic should focus on the maintenance of historical processes such as high levels of out-breeding and gene flow.

6.5 Potential threats to Spiranthes romanzoffiana in Ireland.

Deterministic threats to *Spiranthes romanzoffiana* in Ireland include grazing of the flowering by livestock, the cessation of grazing in densely vegetated sites, encroachment of shade causing tree species (e.g. *Alnus glutinosa* and *Salix caprea*), the destruction of plants by pedestrian and vehicular traffic, eutrophication of lakes and the erosion of lakeshore habitats. *S. romanzoffiana* is protected under the Flora Protection Order and by the designation of some of its sites as Special Areas of Conservation under the EU Habitats Directive. Under this directive, landowners have a legal obligation to protect the populations and to manage the sites under guidance from the Irish National Parks and Wildlife Service. Unfortunately this does not appear to be the case as many of the sites exist unprotected from some or all of the threats mentioned above. The reality is that legally enforced conservation measures are often met with suspicion and conflict between landowners and the conservation body in question. Often this is due to a lack of communication and consultation between the parties involved.

During the course of this research a number of landowners, including farmers, anglers and managers of private estates were consulted on the significance of the rare plant species growing on their land. In most cases the response was positive and in a number of cases the response was pro-active. In one instance a farmer on the shores of Lough Allen in Co.

Roscommon agreed to facilitate the flowering and potential seed production of *Spiranthes romanzoffiana* on his land. With a little investigative research it was discovered that the farmer in question was entitled to compensatory payments under the EU Habitats Directive. This one case highlights the advantages of communication, education and awareness on the values of conserving biodiversity. One of the positive outcomes from this research is the establishment of genial working relationships with local landowners. Without co-operation between the landowners, the statutory bodies and the conservationist's methods to tackle potential threats to *Spiranthes romanzoffiana* will remain purely aspirational.

6.6 Further Study and Management Recommendations

In 2003, in response to action 26 of the 2002 National Biodiversity Action Plan, the National Parks and Wildlife Service implemented a number of Species Action Plans (SAP) for species of conservation concern. *Spiranthes romanzoffiana* was selected as an appropriate vascular plant species. It was agreed that the SAP's should be completed jointly between the NPWS and the Environment and Heritage Service Northern Ireland. In November 2005 following on from a number of steering group meetings a final draft of the All Ireland SAP for *Spiranthes romanzoffiana* was published. The SAP lists a number of Action Plan Targets, including, 1) maintenance of all viable populations of *S. romanzoffiana* throughout Ireland, 2) the restoration of *S. romanzoffiana* to at least five historical sites where it was previously recorded during the last 60 years, by 2025, 3) achieve or maintain favourable conservation status of *S. romanzoffiana* in Ireland by 2030.

While these targets are certainly desirable in the long term this research suggests that more immediate short-term goals would be advantageous to the species persistence. From the results discussed in earlier chapters it is evident that the demographics of *S. romanzoffiana* in Ireland are poorly understood. Before the long-term aims are achieved, more in depth analysis into natural fluctuations in the number of individuals per population should be conducted. Populations should be studied and monitored over long periods of time (> 6 years) to determine whether these fluctuations are caused by an increase in dormancy or by internal demographic stochasticity or whether there is an external deterministic factor, such

as climate change for example. This could be easily achieved by continuing to monitor the four survey sites studied in this research. The patterns detected over this time period would pin point what type of fine-scale conservation action is required for the species.

In conjunction with this, efforts should be made to educate interested parties, such as the BSBI recorders and the NPWS rangers, on the appearance of the *S. romanzoffiana* in the absence of a flowering spike; this should include mature and juvenile plants. Without this knowledge future population surveys will undoubtedly under-record the true extent of population sizes and distribution. As species are assigned conservation categories according to the number of individuals within a population and the extent to which those populations are represented in 10km square grids, it would seem imperative to accurately determine population data.

Using the AFLP data the degree of genetic variation detected within Irish populations of *Spiranthes romanzoffiana* suggests that the genetic diversity of these populations is high and could be maintained by managing populations at the current level. However the microsatellite data suggest that this genetic diversity is not evenly distributed between all the populations surveyed. The majority of populations surveyed were fixed for a single allele, this is in stark contrast the Lough Mask population, which contains four alleles. This is important to know as future management may require the use of this population as a source, for example in reintroduction programmes. One of the targets set out in the SAP aims to reintroduce plants back in to historic sites. Reintroductions are generally reserved for situations were the species is likely to disappear and all other options have been exhausted in many cases and it therefore often relies on the utilisation of *ex-situ* resources. To capture the maximum amount of genetic variability in an ex-situ collection knowledge of target populations is essential. The high allelic diversity recorded at Lough Mask make this population a suitable source of living material for reintroduction or translocation initiatives. This would also include the collection of seed for storage in the Irish Threatened Gene Bank Project at Trinity College Botanic Gardens. To date no seed collected during this project has been germinated. In 2007 efforts will be made to collect and germinate seed from at least three populations in Ireland. The National Botanic Gardens at Glasnevin are in the process of

developing an orchid propagation unit. Once the facility is operational, germination trials on *S. romanzoffiana* will commence. Failing this, seed will be sent to the Royal Botanic Gardens, Kew, England for similar germination trials. It is important that efforts to collect seed are continued each year from as many populations as possible, this will help to capture a high level of genetic diversity and will mitigate against excessive loss of seed during future germination trials. It is recommended that soil surveys are conducted to determine whether a soil seed bank exists within close proximity to flowering plants. The detection of a soil seed bank and the absence of recruitment may highlight potential problems with soil mycorrhiza availibilty. This issue was not been explored during this research, however it is potentially a limiting factor to the distribution of the species in Ireland and Europe.

In 2004 and 2005 ten mature, flowering plants were collected from three populations (Lough Cuilin, Lough Conn (Knockmore) and Lough Mask) and translocated to the National Botanic Gardens at Glasnevin, Dublin (plate 6.1). In contrast to the wild populations these plants were planted in a soil medium rich in organic matter. The plants have been monitored closely over the last number of years and appear to be thriving. As orchids are notoriously difficult to successfully introduce into cultivation this achievement should be commended and utilised to promote the conservation the species and other threatened or rare species. It is hoped that the plants at Glasnevin can be used to help test two hypotheses suggested as a result of this research. These are, low seed set is a result of resource limitation in a low nutrient soil and seed development and maturation is limited by low mean Autumn temperatures in Ireland. The *ex-situ* plants by their nature are easier to manage and manipulate relative to their wild counterparts. This affords the opportunity to carry out trials on the effects of resource limitations and temperature effects on seed development within Irish populations. Results from these experiments will contribute to work being carried out on Scottish populations and will feed into both the Irish and Scottish conservation initiatives for the species. It is important that conservation issues are diffused out from the institutions that carry out this kind of work. Enigmatic and aesthetically pleasing species like *Spiranthes romanzoffiana* are ideal as flagship species for conservation and biodiversity education. The *ex-situ* collection at the NBG Glasnevin could be used to promote the issues facing, habitat loss, species extinction, global warming and the biogeography of species.

Plate 6.1 *Spiranthes romanzoffiana* growing at the National Botanic Gardens, Glasnevin, Dublin.

Table 6.1 Short and long-term recommendations for the conservation of *Spiranthes romanzoffiana* in Ireland.

Conservation recommendations
Short-term
• Enforcement of the laws protecting the species in Ireland.
• Increase communication with NPWS rangers, BSBI recorders and land owners regarding the presence and requirements of *S. romanzoffiana*
• Encourage livestock grazing regimes beneficial to the persistence of *S. romanzoffiana*
• Set up four permanent monitoring sites. (Minimum of 6 years)
• In depth, long-term study of natural population fluctuations
• Continue pollination observations
• Record annual rates of seed production
• Carry out seed germination trials (Autumn 2007)
• Maintain and propagate the *ex-situ* collection at the NBG Glasnevin
• Develop and publish a protocol for the cultivation of *Spiranthes romanzoffiana*
Long-term
• Maintenance of all viable populations of *S. romanzoffiana* throughout Ireland
• The restoration of *S. romanzoffiana* to at least five historical sites where it was previously recorded during the last 60 years by 2025
• Achieve and maintain favourable conservation status for *S. romanzoffiana* by 2030

6.7 Concluding remarks

This study set out to gather baseline data on Irish populations of *Spiranthes romanzoffiana*. The thesis aimed to assess the distribution, habitat requirements and vegetation associated with *S. romanzoffiana* in Ireland. In conjunction with these endeavours, efforts to determine the genetic variability within and among Irish populations were undertaken and placed within the context of North American and Scottish variation. The study attempted to gather data on the reproductive and pollination biology of the species and determine whether seed production was possible. The goal of these data was to contribute to the understanding of the species and to input to the meaningful conservation of the species within Ireland and Europe.

These aims have largely been achieved and in some cases they have surpassed expectations. As a result of this study more precise data now exist on the distribution of Irish populations, habitat requirements and vegetation association. Genetic variation within Irish populations is higher than expected and is comparable to levels detected in the North American samples. The Irish plants appear to be somewhat divergent from the North American plants, suggesting a relatively long period of separation; this has lead to the important conclusion that *Spiranthes romanzoffiana* is probably native to Ireland. The discovery of seed in the West of Ireland, the recording of pollinia removal and pollinator visitation coupled with the patterns of genetic variation strongly suggests that *S. romanzoffiana* in Ireland is predominantly sexually out-crossing, animal pollinated species.

This study has shown that molecular data are more informative when used in conjunction with other types of data. These data highlight the importance of this kind of study and demonstrate its value to conservation and conservation planners. In the course of this research numerous personal and intellectual discoveries have been made. New initiatives have been instigated, for example the publication of Ireland's first All Ireland Species Action Plan and the formation of the UK and Ireland *Spiranthes romanzoffiana* steering group. These achievements have brought together a number of experts on the species and have resulted in the development of a coherent and achievable set of conservation goals for European populations of *Spiranthes romanzoffiana*.

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Appendix 1. List of abbreviations and corresponding species names (chapter 2)

Abbreviation	Botanical name	Abbreviation	Botanical name
Acer p.	*Acer platanus*	Leontodon t.	*Leontodon taraxacoides*
Achillea p.	*Achillea ptarmica*	Lotus c.	*Lotus corniculatus*
Agros g.	*Agrostostemma githago*	Lotus p.	*Lotuspedunculatus*
Alnus g.	*Alnus glutinosa*	Lycopus e.	*Lycopus europaeus*
Anagallis t.	*Anagallis tenella*	Lysimachia n.	*Lysimachia numellaria*
Anthoxanthum o.	*Anthoxanthum odoratum*	Lysimachia v.	*Lysimachia vulgaris*
Betula p.	*Betula pendula*	Mentha a.	*Mentha aquatica*
Brachythecium r.	*Brachythecium*	Molina c.	*Molinea cearulea*
Calliegron c	*Calliegron cordifolium*	Myosotis s.	*Myosostis sylvatica*
Cardimine p.	*Cardimine pratensis*	Odonites v.	*Odonites verna*
Carex d.	*Carex dioica*	Parnassia p.	*Parnassia palustris*
Carex dis.	*Carex distichia*	Plantago l.	*Plantago lanceolata*
Carex s.	*Carex sylvatica*	Poa a.	*Poa annua*
Carex p.	*Carex panacea*	Potentilla a.	*Potentilla anserina*
Crepis c.	*Crepis capillaris*	Potentilla p.	*Potentilla palustris*
Cynosorus c.	*Cynosorus cristatus*	Prunella v.	*Prunella vulgaris*
Danthonia d.	*Danthonia decumbens*	Ranunculus f.	*Ranuculus flammula*
Drosera l.	*Drosera intermedia*	Ranunculus r.	*Ranuculus repens*
Eleochoris p.	*Eleochoris pauciflora*	Sagina n.	*Sagina nodosa*
Eriophorum a.	*Eriophorum angustifolium*	Rhinanthus m.	*Rhinanthus major*
Festuca r.	*Festucs rubra*	Salix c.	*Salix caprea*
Filipendula u,	*Filipendula ulmaria*	Salix r.	*Salix repens*
Galium p.	*Galium pumilum*	Senecio a.	*Senecio aquaticus*
Holcus l.	*Holcus lanatus*	Taraxacum o.	*Taraxacum officianalis*
Hydrocotyle v.	*Hydrocotyle vulgaris*	Taraxacum sp.	*Taraxacum sp.*
Juncus acu.	*Juncus acutiflorus*	Trifolium r.	*Trifolium repens*
Juncus art.	*juncus articulatus*	Triglochin p.	*Triglochin palustris*
Juncus b.	*Juncus bulbosus*	Vicia s.	*Vicia sepium*
Juncus e.	*Juncus effusus*		