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The reproductive biology and conservation of rare orchid species in Ireland

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Thesis submitted for the degree of Doctor of Philosophy

March 2008

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Summary

This thesis investigates the factors that affect the conservation and reproductive biology of rare Irish orchids. Research focused on various aspects of the ecology of six species that occur throughout Ireland. Four endangered, red-listed species were examined: *Cephalanthera longifolia*, *Hammarbya paludosa*, *Pseudorchis albida*, and *Spiranthes romanzoffiana*. In addition, one rare species *Neotinea maculata* and one relatively abundant species *Spiranthes spiralis* were also examined. I employed probabilistic models to infer threat of four species (*C. longifolia*, *H. paludosa*, *N. maculata*, and *P. albida*). These models have various assumptions regarding the sighting process, however little is known about how sighting records were generated, therefore it is imperative to use different models. Using five models, a optimal linear estimate to infer extinction time, and a rarefaction analysis, I found that there have been declines in the three endangered species (*C. longifolia*, *H. paludosa*, and *P. albida*). However the models erroneously inferred that *N. maculata* was threatened. Populations of all four species flowered in low numbers at all populations examined. To test for the effects of rarity on pollination of an endangered orchid, I examined the pollination of *S. romanzoffiana*. I found that bees visited *S. romanzoffiana* and observations made in replicate patches of varying density showed that *S. romanzoffiana* competes for pollinator attention at high densities. In addition, I found that *S. romanzoffiana* offers a nectar reward that varies over the flowering season, with more nectar produced at the middle of the season. Morphologically similar, nectar rewarding co-flowering species may also facilitate visitation to inflorescences of *S. romanzoffiana*. To compare the differences between the endangered *S. romanzoffiana* and the more common *S. spiralis*, I measured different components of pollination success (pollen export, pollen import, fruit and seed set) at different spatial scales (population size and varying densities within populations), as well as interactions with co-flowering species. I found that population size only affected pollinator visitation in very small populations of *S. romanzoffiana*. However, the proportion of pollen export and import did not vary with population size in either species. The density of co-flowering species had a positive effect on pollen export in *S. romanzoffiana*, while *S. romanzoffiana* density and visitation rate positively affected pollen import. Although co-flowering species had no effect on *S. spiralis* pollination, its own intraspecific density increased fruit set and decreased pollen loss from patches. In addition, I found that visitation rate to *S. spiralis* increased its pollen export. Both species are self-compatible but rely on pollinators for fruit set. Fruit set was found to be abundant in *S. spiralis*, however, despite evidence for pollination, fruit does not mature in *S. romanzoffiana*, yet some seeds are present in the

immature fruits. To examine the effects of rarity across a species range, I compared the ecology and population genetics of *N. maculata* at the centre (Italy) and edge (Ireland) of its European range. For this I used vegetation analysis, breeding system experiments, and a DNA fingerprinting technique, AFLP. I found that *N. maculata* occurs in a wide variety of habitats and is highly self-compatible in both Ireland and Italy, yet has high levels of genetic polymorphism, according to AFLP markers. It also has high levels of population genetic differentiation, with Irish populations significantly different from Italy populations. I examined the effects of rarity on the population genetic structure of two endangered Irish orchid species: *C. longifolia* and *P. albida*. Three plastid microsatellite markers showed low genetic diversity in Irish populations of *C. longifolia*. However one unique cytotype was present in the smallest population. I used four microsatellite marker and AFLP markers to examine *P. albida*. No genetic variation was found at the microsatellite loci, however the AFLP markers showed *P. albida* had high levels of polymorphism and significant population genetic differentiation, indicating population isolation through distance. The findings are discussed in the broader context of conservation biology and the effects of rarity on reproductive biology, with reference to effective population management to prevent the extinction of these species in Ireland.

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"*Biodiversity losses of small inedible species often provoke the response; "who cares? Do you really care less for humans than some lousy fish or weed...?" This response misses the point that the entire natural world is made up of wild species providing us for free with services that can be very expensive, and in many cases impossible, for us to supply ourselves. Elimination of lots of little lousy species regularly causes big harmful consequences for humans, just as does randomly knocking out many of the lousy little rivets holding together an airplane.*" **- Jared Diamond, Collapse: How societies choose to fail or survive, 2005**

"*In the end, we conserve only what we love. We will love only what we understand. We will understand only what we are taught*" **- Baba Dioum, Senegalese poet**

"Conservation is a state of harmony between men and land." **- Aldo Leopold, A Sand County Almanac, 1949**

1.1 Conservation biology

Habitat destruction and fragmentation are recognised as immediate threats to biodiversity (Wilcove *et al.*, 1986; Tilman, 1994; Kearns *et al.*, 1998; Fahrig, 2003), in addition to other factors such as invasive species and climate change (Schroter *et al.*, 2005; Araujo and Rahbek, 2006). Natural habitats are currently being destroyed at an unprecedented rate and extinction of many species is a likely result (Sala *et al.*, 2000). This is due to pressures of an expanding human population and consequent intensification of agriculture, industrial development, and large-scale destruction of vegetation, leaving only isolated remnants of natural habitat surrounded by a transformed matrix of alien vegetation or development (Wilcove *et al.*, 1986; Ehrlich, 1994; Fahrig, 2003). Therefore, there is an urgent need to understand how ecological processes are affected by habitat fragmentation in order to make useful attempts at halting and preventing further species declines.

1.1.1 The Equilibrium Theory of Island Biogeography

Due to accelerating habitat destruction, there is increasing need for the study of conservation biology. The theoretical underpinning of conservation biology stems from MacArthur and Wilson's 'Equilibrium Theory of Island Biogeography' (1967), though this was influenced by earlier works, which addressed topics such as, species-area relationships (Arrhenius, 1921; Gleason, 1922), and the relative rarity and abundance of a species (Preston, 1948; 1962a; 1962b). The equilibrium theory of island biogeography addresses the relationship between the equilibrium number of species (a constant number of species, with extinction or colonisation of individual species over time) and area of an island. Theoretically, the relationship between species and area is logarithmic and can be summarised by the formula:

$S = {}_{C}A^{Z}$,

where, S is the number of species, A is the area of the island, and c and z are constants that define the relationship depending on the island and species (MacArthur and Wilson, 1963; 1967). The equilibrium number of species found on an island can be influenced by a number of factors including; (i) degree of isolation (distance to nearest neighbour and mainland), (ii) length of isolation (time), (iii) size of island (larger area usually facilitates greater diversity), (iv) climate, (v) location relative to wind and ocean currents (influences nutrient, fish, bird, and seed flow patterns), (vi) initial plant and animal composition if previously attached to a larger land mass, and (vii) human activity (MacArthur and Wilson, 1963; 1967).

The practical application of the theory of island biogeography in conservation is based on the assumption that nature reserves, national parks, and fragmented natural populations form

'islands' in human-altered, fragmented landscapes. These areas are usually limited in size and of lower quality than the original ecosystem (Simberloff, 1988). Recently fragmented areas tend to lose species as they 'relax towards equilibrium' (Diamond, 1972) i.e. they lose species as they achieve a new equilibrium number. This is also known as 'ecosystem decay' (e.g. Laurance *et al.*, 2002). How many species are lost can depend on the degree of isolation, size or quality of the remaining fragment (Fisher and Lindenmayer, 2007). Indeed, Newmark (1987) showed that larger species tend to have larger ranges, and there was a strong correlation between the size of a protected National Park in the U.S. and the number of species of mammals. In addition, Bascompte *et al.* (2002) predicted a negative effect of habitat loss on population growth rate. The quality of the remaining fragment can be affected by loss of such factors as, optimal soil conditions, mutualists, climate, and a subsequent increase in edge effects (Jennersten, 1988; Murcia, 1995; Fahrig, 2003). Edge effects are the result of the interaction between two adjacent habitats, when the two are separated by an abrupt transition i.e. the edge. Edge effects may affect organisms by causing changes in the biotic and abiotic conditions. For example, there may be increased growth of certain species due to more light available at the edge of a fragment, which leads to an increase in photosynthesis or there may be increased herbivory at the edge (Murcia, 1995). Therefore, the study of conservation biology has been mostly concerned with investigating the causes of the loss of biodiversity and preventing its loss.

1.1.2 Stochastic processes

The study of fragmented populations is important because organisms that occur in small and isolated populations experience increased vulnerability to stochastic processes (e.g. Barrett and Kohn, 1991; Schemske *et al.*, 1994). There are three types of stochasticity: demographic, environmental and genetic. Demographic stochasticity is concerned with random variation in demographic parameters, like birth and death of individuals (Lande, 1988). Environmental stochasticity includes catastrophes, sudden extinctions or severe reductions in population size, due to extreme conditions like flooding, volcanic eruptions or epidemic diseases. However, catastrophes will result in the eventual extinction of any population (Mangel and Tier, 1994). For rare species in fragmented habitats, such local population extinctions may result in complete extinction of the entire species. Genetic stochasticity is a result of increased random genetic drift and inbreeding in small and isolated populations, which might result in a lower fitness and increased susceptibility to demographic and environmental stochasticity (Ellstrand and Elam, 1993; van Treuren *et al.*, 1993; Oostermeijer *et al.*, 1994). Together, the negative effects of isolation and small population size on fitness are referred to as genetic erosion, which can enhance the extinction risk of small populations (Ellstrand and Elam, 1993). The impact of

demographic and environmental stochasticity for population extinctions is widely accepted (Lande, 1988). In contrast, empirical evidence for population extinctions due to genetic stochasticity is scarce and its importance for population persistence has been debated (e.g. Lande, 1988; May, 1995). However, recently Spielman *et al.* (2004) showed, using a meta-analysis of population genetic studies of endangered species that genetic studies reveal much about the conservation value and potential of endangered species and are of critical importance.

1.1.3 Scale dependency of rarity

Although we know that there are negative consequences for a species being rare, defining rarity can be subjective and difficult (e.g. Kunin and Gaston, 1993). However for conservation purposes it is important to have an accurate scale by which to measure rarity. For example, in developing red data books of threatened species, the World Conservation Union (IUCN - International Union for the Conservation of Nature) uses measures of rarity, rates of decline, and population fragmentation to categorise species according to their risk of extinction. However, most quantitative measures of these three concepts are sensitive to the scale at which they are made (Hartley and Kunin, 2003). For instance, rarity can be a low abundance of populations, a low number of individuals within population, a narrow geographic range, or a combination of these. Rabinowitz (1986) defined seven forms of species rarity: (i) sparse, (ii) habitat specific, (iii) habitat specific and sparse, (iv) localised, (v) sparse and localised, (vi) localised and habitat specific, (vii) sparse, localised, and habitat specific. However, although useful, the practical value of these definitions is limited. For instance, some species are naturally rare; indeed most species are rare somewhere in their range (Gaston and Kunin, 1997). If a species is naturally rare, it likely has evolved to cope with the consequences of rarity, for example it may have purged its genetic load and reproduce autogamously due to low pollinator attention (Charlesworth and Charlesworth, 1987). However, if a species has become rare recently and requires specific conditions for example pollinators for reproduction, then the species will suffer more severe effects of habitat fragmentation (Huenneke, 1991).

Some rare species have congeners that are relatively common. The rare and the common congeners may only differ in a small number of traits, for example breeding system and reliance on pollinators, but these may be enough to cause rarity (Karron, 1997). However, Bevill and Louda (1999) highlight that comparative studies of common and rare congeners are lacking, yet they yield important theoretical and practical information as to the causes and consequences of rarity. For instance Karron (1987) showed that a restricted species of *Astragalus* had less pollinator attention than a more widespread congener. Rare species tend to have higher rates of

self-compatibility, lower reproductive investment, less dispersal ability, higher homozygosity, and use less common resources than common species (Gaston and Kunin, 1997). Studies on rare species can be hampered by the scale they occur at hence a comparison with a more common congeneric species can yield useful data as to the causes and effects of rarity (Bevill and Louda, 1999).

1.1.4 Rarity and conservation biology

Combined with stochastic factors, deterministic processes such as habitat loss and fragmentation can lead to species becoming rare at accelerating rates. This, in turn, puts increasing pressure on monitoring programmes, which conservation bodies such as the IUCN depend on for inferring the level of threat species face (Rodrigues *et al.*, 2006). Monitoring programmes can involve estimating the effects of demographic, environmental, and genetic stochasticity of a rare species, but require considerable resources in terms of experts, time and funding. As fragmentation accelerates, there is increased pressure on experts with limited resources. Often the only information we have on a species is that it is rare (Gaston, 1997). We know little of the biology of most rare species, and in many cases it is difficult to know if a species is in decline or extinct (Diamond, 1987) or if a species are naturally rare (e.g. Kunin and Gaston, 1993). In such situations the sighting record, herbarium specimens, and museum collections of a species can provide data of when and where a species has occurred, though for rare species these data can be scarce and piecemeal. Recently, numerous statistical models have been developed to infer threat of species based on sparse herbarium specimens, museum collections and sighting records (e.g. Solow, 1993a; McCarthy, 1998; Roberts and Solow, 2003). Although these models are dependent on collection or sighting effort, they can reveal much on the rarity and threat level of a species (e.g. Roberts and Kitchener, 2006). Such methods can highlight species and regions for conservation concern, and can focus conservation efforts, in addition to genetic and demographic analysis.

Genetic variation within populations of endangered species has long been recognised to be an important factor in determining whether species go extinct (e.g. Barrett and Kohn, 1991; Ellstrand and Elam, 1993; Young *et al.*, 1996). There have been many recent advances in genetic techniques to address conservation questions and to assess genetic variation of rare species, (Sunnucks, 2000), particularly the development of reliable multilocus markers such as amplified fragment length polymorphisms (AFLPs). These markers can reveal much regarding the genetic variation of a rare species without prior knowledge of the organism's genome (Meudt and Clarke, 2007). There have also been advances in sequencing and the development of co-

dominant variable microsatellite markers (SSRs – simple sequence repeats) (e.g. Selkoe and Toonen, 2006). SSRs tend to take a longer time to develop and tend to be species specific (e.g. Peakall *et al.*, 1998), while AFLPs generate data faster, are very useful when genetic variability is low as they amplify many loci, and are useful in intra-specific studies where there is no prior sequence information (Meudt and Clarke, 2007). However, SSRs are more useful in answering fine-scale ecological questions such as whether late flowering individuals are genetically distinct from early-flowering individuals in a population (Selkoe and Toonen, 2006). The power of SSRs is derived from their high mutation rates, which results in high allelic diversity. They also detect the frequency of different alleles at each locus, while AFLP does not (Sunnucks, 2000; Selkoe and Toonen, 2006). The choice a particular technique will depend on the costs, the quantity and quality of the DNA available. For instance, protein markers such as allozymes tend not to work on older material (Sunnucks, 2000). The application of such population genetic techniques can help conservation assessment in conjunction with analysis of the demographic trends of a population.

Demographic stochasticity is a key component in rare species survival, as the birth, death and survival of individuals within a population is crucial for species survival (Lande, 1988). However, there are problems estimating population demographic parameters in rare species (Beissinger and Westphal, 1998). The analysis of population demographic data has advanced in recent years (e.g. Lebreton *et al.*, 1992, Alexander *et al.*, 1997) with sophisticated modelling programs such as MARK (White and Burnham, 1999). Recent methods allow great sensitivity of estimating population parameters, such as estimating the birth and death of individuals, which may be confounded by sampling effects and are known to be problematic (e.g. Beissinger and Westphal, 1998). For instance, some perennial plants do not produce annual shoots and return to dormancy after flowering the previous year ('adult whole-plant dormancy'), which make it difficult to ascertain if the individual is dead or dormant. Such techniques have been used to estimate survival rates in a rare orchid species (e.g. Shefferson *et al.*, 2001). Many studies do not accurately account for dormancy when estimating population dynamics. When dormant, a herbaceous perennial produces no photosynthetic material during the growing season. This results in an inherent bias in studies of population dynamics, since the demographic methods require the organism to be observed to know it is alive, which can result in an underestimation of survival (Shefferson *et al.*, 2001) or inaccurate estimation of population size (Alexander *et al.*, 1997). Shefferson *et al.* (2001) proposed a method of open-population mark-recapture models in the style of Cormack (1964), Jolly (1965), and Seber (1965) to estimate annual and average likelihoods of survival and dormancy for plants that exhibit dormancy. This approach requires

individuals to be tracked by marking and re-detecting them over a number of years. Binary resighting histories are constructed for each individual organism in a population. As such, the application of mark–recapture models allows for greater sensitivity in estimating the population size of plants (Alexander *et al.*, 1997; Kéry and Gregg, 2004; Shefferson *et al.*, 2005). Therefore, conservation biology is viewed as a 'crisis discipline', analogous to emergency surgery, as the discipline takes its methods and techniques from a broad range of fields (e.g. statistics, genetics, evolutionary biology) to make assessments on the threat status of a species (Soulé, 1985).

1.2 Plant-pollinator interactions

In addition to pure species loss, the loss of species interactions within communities is affected by fragmentation (Kearns *et al.*, 1998; Spira, 2001). Mutualisms, such as those between plants, pollinators and seed dispersers, can be affected if there is a decline in one of the mutualists due to fragmentation (Bond, 1994). This is because, due to their sessile habit, plants cannot actively migrate to patches of suitable habitat. Angiosperms (flowering plants) comprise an estimated 250,000 species, and by some estimates, 90% of angiosperms are pollinated by animals, particularly insects (Buchmann and Nabhan, 1996). Therefore, we need to understand the extent to which plants are affected by the loss of animal pollinators and vice versa. One measure of the value of the ecosystem services provided by pollinators is monetary, with the component due to pollination services estimated at $112 billion dollars per year (Costanza *et al.*, 1997). The diversity of wild pollinators can improve crop production (e.g. coffee; Klein *et al.*, 2003), although the effect of pollinator declines on crop production has been debated (Ghazoul, 2005), there is still much evidence to suggest that crops benefit from pollinator diversity (e.g. Klein *et al.*, 2007). Therefore, in addition to the aesthetic and ethical values of conserving wild species (Soulé, 1986), the conservation of pollination systems is therefore an urgent priority both ecologically and economically. Furthermore, the study of pollination ecology is an important component of understanding the effects of rarity on species conservation (Spira, 2001).

1.2.1 Pollinator behaviour and pollination in fragmented habitats

Pollination of animal-pollinated plants depends strongly on local conditions; for instance, the presence of nesting sites and additional food plants for pollinators (Spira, 2001). Such conditions may determine to a large extent which pollinator species are available, together with their abundance and foraging behaviour. Due to habitat fragmentation, the surrounding vegetation, spatial population structure and pollinator species composition will often change, resulting in different patterns of pollen flow (Thomson, 1978; Sowig, 1989; Conner and Neumeier, 1995;

Kwak *et al.*, 1998). For instance, floral resources in fragments have been found to be important in determining the number and diversity of colonising *Bombus* queens in early summer (Bower, 1995). In addition, specialised pollinator nesting requirements can include specific soil attributes, spaces under rocks, old rodent burrows, as well as mud, resins, or plant hairs to line and plug nests (Kearns and Inouye, 1997). The availability of such resources may limit the abundance and diversity of pollinators a habitat fragment can support.

In specialised pollination systems (where one pollinator species visits one plant species), habitat fragmentation may lead to the extinction of either the plant or the pollinator species. Extinction of one of the partners may induce the extinction of the second, if other species are unable to take over the ecological function of the former species. Plants that are pollinated by a single pollinator species are rare, although notable exceptions include figs, yuccas, and some orchids (Nilsson, 1992; Pellmyr *et al.*, 1996; Anstett *et al.*, 1997; Rønsted *et al.*, 2005). Most ecological interactions are generalised and vary through both space and time (Nielsen and Bascompte, 2007). Indeed, most pollination systems are generalist, with plants pollinated by several pollinator species and pollinators foraging on many food plants (Waser *et al.*, 1996). As a result, pollination systems typically represent interaction webs involving multiple species assemblages that vary in space and time depending on the landscape (Bronstein, 1995; Memmott and Waser, 2002). Nevertheless, a shift in pollinator species composition might still be accompanied by changes in patterns of pollen flow, if the behaviour and pollination efficiency of the new pollinator differ from those of the original species. For example, Tepedino *et al.* (1999) showed that not all pollinators were equal, and species varied in their pollination efficiency of *Penstemon penlandii.*

In addition to the effects of insect species composition, pollinator behaviour depends on the presence of other flowering plants individuals, or species, in the case of polylectic foragers such as bumblebees. Habitat fragmentation will frequently result in lower floral diversity and abundance, reducing the diversity and numbers of pollinators that can be sustained in an area (Kwak *et al.*, 1998). As a consequence, the negative effects of fragmentation on pollination on seed production and gene flow might happen regularly. Reductions in population size imply an increase in the relative importance of the flowering surroundings, which is often accompanied by increased competition for visitation. The number of pollinator visits to *Blandfordia grandiflora* was lower when *Banksia serrata* flowered simultaneously (Ramsey, 1995). Similarly, the orchid *Spiranthes spiralis* suffered a reduced fruit set, due to co-flowering plants (Willems and Lahtinen, 1997). In other plant species, more pollen was wasted on co-flowering plants (Murcia

and Feinsinger, 1996) or seed set declined, due to deposition of heterospecific pollen (e.g. Galen and Gregory, 1989; Murphy and Aarssen, 1995). Even patterns of pollen flow may depend on the presence of co-flowering plants. Pollen dispersal distance in *Stellaria pubera* was found to be less in mixed plots with *Claytonia virginica*, compared to pure stands of *Stellaria* alone (Campbell and Motten, 1985). Alternatively, facilitation may occur where the pollinators of one species may also visit another species, which may enhance the pollination success of each individual species (Feinsinger *et al.*, 1986; Ghazoul, 2006). In this case, a loss of plant species could have negative effects on the pollination of the remaining species.

Pollination facilitation occurs when co-flowering plant species provide nectar, pollen, and occasionally other rewards that maintain pollinators over time (Ratchke, 1983). Waser and Real (1979) showed that there can be a 'sequential mutualism' among plant species that flower early in the season, which support initial pollinators populations and survive and reproduce and are available to other plant species that flower later in the season. When early flowering *Delphinium* spp. flowers are sparse, migrating hummingbirds do not stay in the area and are unavailable to pollinate the later flowering *Ipomosis* spp. As a result, *Ipomosis* spp. has lower visitation rates and sets fewer seeds. Facilitation can also occur in species that depend on pollinator visits but lack a floral reward (Nilsson, 1980). Although such rewardless species have pollen-limited fruit and seed set (Neiland and Wilcock, 1998), female reproductive success can increase significantly in areas where plants associate with a "magnet" species that are highly attractive to pollinators (Thomson, 1978; Laverty, 1992). This has been demonstrated in natural populations of a nectarless orchid, *Traunsteinera globosa*, where fruit set was higher in the presence of co-flowering species, specifically the presence of the morphologically similar co-flowering species *Trifolium pratense*, was significantly correlated with the orchid's fruit set (Juillet *et al.*, 2007). Such a phenomenon has also been documented in the nectarless *Podophyllum peltatum* experiences higher fruit set in the presence of *Pedicularis canadensis*, a nectar rewarding species (Laverty, 1992).

Facilitation among co-flowering plant species may have implications for rare plants that offer floral rewards and depend on pollinator visits. For example, reproductive success in rare or sparsely distributed plant species can increase if there are nearby species with similar flowers, and if pollinators do not distinguish between the two (Bobisud and Neuhaus, 1975; Schemske, 1981). Simply the presence of other flowering plants with overlapping pollinators enhances the reproductive success of rare species (Kwak *et al.*, 1991). However, there may be negative effects for rare species flowering with more common co-flowering species. For instance, rare flowers

can have a minority disadvantage in attracting pollinators (Levin and Anderson, 1970) and, if pollinators visit two or more species on foraging bouts, there may be a reduction in pollen quality due to interspecific pollen flow (Thomson *et al.*, 1981).

To predict the risk of plant extinction due to loss of pollinator or seed disperser mutualisms, Bond (1994) developed a vulnerability index based on three criteria: degree of pollinator specialisation, breeding system, and demographic dependence on seeds. Plants of high risk are species that depend on a single pollinator species, whereas plants that are relatively generalised are likely to be resilient to pollinator loss (Memmott *et al.*, 2004). Plant species may also lower their extinction risk by being facultatively autogamous, whereby self-pollination occurs in the absence of pollinators e.g. (Fausto *et al.*, 2001). Finally, a species may reduce its dependence on seeds (and therefore pollinators and dispersers) by vegetative propagation or by possessing a long life span (Honnay and Bossuyt, 2005).

1.2.2 Density and population effects of fragmentation

The spatial structure of a plant population includes several factors, such as the size, shape, the density of patches, and the distance by which patches are separated from each other (e.g. Jennersten, 1988; Steffan-Dewenter and Tscharntke, 1999). Although fragmentation does not directly affect plant population density, the population may decline over the long term, leading to a lower density within a habitat fragment. For instance, isolated patches of lower density of *Nepeta cataria* received fewer visits and showed higher degree of pollinator limitation (Sih and Baltus, 1987). Small populations often exhibit a reduction in visitation, reproductive success and genetic variation compared to large and continuous populations (e.g. Jennersten, 1988; Sowig, 1989). In fragmented natural populations, such negative effects of reduced population size are difficult to separate from similarly deleterious consequences of a simultaneous decline in density. Low plant densities frequently result in a lower number of visits per plant, more flower visits within the same plant, increased selfing rates and reduced seed production (e.g. Dreisig, 1995; Karron *et al.*, 1995a). The negative effects of low density on seed set were especially clear when other flowering plant species competed for pollination (Kunin, 1993). Several experimental studies suggested that the harmful effects of low density are even stronger than those of small population size (van Treuren *et al.*, 1993; Kunin, 1997). A combination of effects of size and density might be found for patchy distributions of plants. For instance, small patches of a tropical tree were found to be less visited by pollinators than large patches (Stacy *et al.*, 1996). However, when pollinators did visit patches, almost all trees within a patch were visited, inducing a high frequency of local mating (Stacy *et al.*, 1996). In addition, plants in small

patches received pollen from a further distance, compared to plants in either large patches or continuously distributed populations (Stacy *et al.*, 1996).

In experimental populations, the absence of floral resources in between isolated clumps of plants forced the pollinators to fly larger distances between flower visits, resulting in lower reproductive success in isolated plants (e.g. Steffan-Dewenter and Tscharntke, 1999). However, many pollinators might stay longer within the same group of plants and visit more flowers per plant, thus increasing the frequency of within-patch pollination in relatively isolated groups of plants (Snow *et al.*, 1996; Richards *et al.*, 1999). Pollinators usually fly only limited distances and isolation by a few hundred meters already seriously reduces the import of pollen (e.g. Ellstrand and Marshall, 1985; Kwak *et al.*, 1998). Other aspects of population structure might influence the effects of isolation. Large populations at large distances were found to contribute more to pollen import into small populations of wild radish, than nearby small populations (Ellstrand *et al.*, 1989). In *Silene alba*, patch size and isolation distance interacted in a complex manner with each other (Richards *et al.*, 1999). A distance of only 80m severely diminished pollen import. Almost all pollination was between plants from the same patch. Extremely small patches received no foreign pollen at all, pollen import into medium sized and large patches occurred rarely at this interpatch distance. In contrast, with isolation distances of 20m, pollen dispersed almost freely and the array of patches could be viewed as a panmictic population (Richards *et al.*, 1999). Larger patches contributed more to their own pollination success than relatively small patches, resulting in a negative correlation between pollination by foreign pollen and patch size for these small interpatch distances. Interactions between several components of population structure and flowering environment will often be complex and their consequences therefore difficult to predict (Ratchke and Jules, 1993). More research is needed to gain insights into these interactions, since habitat fragmentation will regularly induce simultaneous changes in factors that influence pollen flow and reproductive success of plant species.

1.2.3 Genetic consequences of disruption to plant-pollinator mutualism

Reductions in population size can create genetic bottlenecks because remaining individuals contain only a small sample of the original gene pool (Barrett and Kohn, 1991; Young *et al.*, 1996). Previous studies suggest a positive association between population size and genetic variation (e.g. Ellstrand and Elam, 1993; Leimu *et al.*, 2006). Random genetic drift may occur in remnant populations that stay small and isolated for several generations (Young *et al.*, 2006). Isolation of small fragmented populations plays a role in reduced genetic diversity in the tropical tree *Pithecellobium elegans* (Hall *et al.*, 1996). In addition, a decline in variability can affect the

fitness levels of a species, for example *Gentiana lutea* and *Primula veris* (Kéry *et al.*, 2000). Recently, Jacquemyn *et al.* (2007c) investigated the rare orchid, *Orchis purpurea*, and found that despite small population size, genetic diversity within populations was high and genetic differentiation among populations was similar to that typically observed in deceptive orchids. Fruit set was very low (average fruit set: 5.5%), suggesting strong pollinator limitation, which resulted in very low recruitment rates (average seedling recruitment per flowering individual: 0.12). Both measures significantly increased with increasing population size, indicating that a certain threshold value (> 50 flowering individuals) has to be reached to produce a sizeable number of fruits and seedlings in this species.

In addition, changes in pollinator movement or type can alter the degree of relatedness between pollen donors and receptors, and potentially decrease the genetic variability and fitness via inbreeding or outbreeding depression (Oostermeijer *et al.*, 1994; Fischer and Matthies, 1997). This is exacerbated as plant populations have a spatial genetic structure as a result of limited dispersal of pollen and seeds, resulting in greater pollination between related individuals (biparental inbreeding) (Ellstrand and Elam, 1993; Bosch and Waser, 1999). Smaller populations overall may suffer greater inbreeding depression than larger ones because of reduced effectiveness of selection relative to genetic drift, deleterious recessive alleles could become fixed by chance instead of being eliminated by selection (Ellstrand and Elam, 1993). In plant populations that naturally experience high levels of inbreeding, the frequency of deleterious recessive alleles may decline as they become homozygous and are purged by selection (Young *et al.*, 1996). Naturally inbreeding populations should thus be less vulnerable to inbreeding depression than typically outbreeding populations. One group of plants that may be particularly sensitive to the negative effects of fragmentation on pollinator interactions are the Orchidaceae.

1.3 Orchid biology and pollination

1.3.1 Features of orchids

Members of the Orchidaceae display a diverse array of strategies to attract pollinators, ranging from rewarding species, with rewards such as, nectar, oils, and resins, to non-rewarding deceptive species that offer no reward to pollinators (Nilsson, 1992; Cozzolino and Widmer, 2005). Orchids are distinguished by floral characteristics such as: (i) pollen amassed into a unit termed 'pollinia', which has a glue ('viscidium') to adhere the pollinia to the pollinator (the entire unit is called the 'pollinarium'), (ii) fusion of the male (androecium) and female (gynoecium) reproductive organs into one structure termed the 'gynostemium' (also called the 'column'), and (iii) zygomorphic flowers with a modified petal called a 'labellum' that facilitates

pollinator visitation. The variation and diversity of floral shapes found across the family are largely a result of variation in these three features (van der Pijl and Dodson, 1966; van der Cingel, 1995). In addition, their seeds are dust-like and contain no endosperm; hence they depend on soil mycorrhizae in order to germinate and seedling establishment (Rasmussen, 2002). However, the precise role of mycorrhizae in seed establishment in orchids is an area about which we know little (Rasmussen and Whigham, 1998), although recent studies show it may be a specific mutualism (e.g. Shefferson *et al.*, 2007). There is an estimated 25,000 species within the Orchidaceae (although this figure is debated: Dressler, 2005), rivalled only by the Asteraceae (approx. 23,000 species: Bremer, 1994). This taxonomic diversity is often attributed to adaptive radiation for specific pollinators by selection for outcrossing (van der Pijl and Dodson, 1966). Indeed, Darwin (1862) argued that orchids provide strong evidence for both natural selection and the advantages of cross-pollination. Since then much effort has been devoted to describe orchid pollination (e.g. van der Pijl and Dodson, 1966; van der Cingel, 1995), suggesting the link between orchid diversity and pollination systems. However, in a recent review, Tremblay *et al.* (2005) examined the evidence for such pollinator-mediated selection and found little evidence to support this hypothesis. They suggest that species diversity in orchids is a product of the interplay between drift and natural selection.

1.3.2 The age of the Orchidaceae

Due to their taxonomic diversity orchids are viewed as young, actively speciating family, however the age of the orchid family has been debated (Dressler, 1981; Chase *et al.*, 1994), with molecular data suggesting the most recent common ancestor at the end of the Cretaceous 65 million years ago (Chase *et al.*, 1994). Recently Ramirez *et al.* (2007) described a fossil orchid (*Meliorchis caribea*) from a pollinarium (pollinia and viscidium) attached to a stingless bee preserved in 15-20 Myr old Dominican Amber. This is the first unambiguous fossil orchid and also an observation of an early plant-pollinator interaction in the fossil record. They show that the most recent common ancestor of extant orchids lived in the late Cretaceous ~76-84 million years ago. Orchids likely evolved at a time when most angiosperm flowers would have been visited by unspecialised beetles and flies, which may have pollinated them while feeding on floral and vegetative parts (Chase, 2005). Therefore, the orchid family is old enough to have had its distribution affected by the movement of the continents. Some of the biogeographic history of Orchidaceae is similar to that of other old plant families that were widely distributed before the break up of the old super-continents, such as Gondwana (Chase, 2005).

1.3.3 Resource vs. pollination limitation in orchids

Since the Orchidaceae comprises many species and have evolved from the Cretaceous, selection should favour levels of reproductive effort that yield optimal fruit and seed set. However, orchids provide a good example of a hermaphroditic species that have low flower-to-fruit ratios. This is believed to be a result of a paucity of resources available for fruit development, which forms the basis of the resource-limitation hypothesis (Stephenson, 1981). This hypothesis is believed to be the limiting factor in angiosperm reproduction because experimental reduction of resource availability causes elevated fruit abortion (Stephenson, 1981). Therefore, variation in reproductive success should be closely tied to variation in resource constraints. Moreover, flowers that fail to become fruits may not be wasted as they may function to enhance male fitness through pollen donation (Willson and Ratchke, 1974). For example, in members of the Apocynaceae (milkweeds), fruit production in poorly correlated with the number of flowers on an inflorescence, though the amount of pollen removed is strongly correlated with inflorescence size (Bell, 1985). However, in the orchid *Dendrobium monophyllum*, fruit set was correlated with number of flowers available on an inflorescence, but supplemental pollination experiments showed that a fruit-to-flower ratio above 1:10 in naturally pollinated plants decreased flowering in the subsequent year (Bartareau, 1995).

Tacit in these arguments is Bateman's Principle (Bateman, 1948), which examines the asymmetry in resource investments in offspring between males and females. For males, reproduction is limited by access to mates and for females, reproduction is limited by resources and they should therefore exercise a choice of mates to sire costly offspring. However, many studies have found that the reproductive success of many species is caused by low pollination rates (Bierzychudek, 1981; Burd, 1994). If reproduction is pollen limited, then Bateman's Principle cannot be applied. It may be that females are limited by access to mates and the potential for selective mate choice is reduced under these conditions. While males may still compete for mates under pollen-limited reproduction, the intensity of male-male competition is restricted (Tremblay, 1994).

Ultimately the dichotomy of resource vs. pollination limitation may be an oversimplification, for instance, pollination limited plants often show resource constraints (Montalvo and Ackerman, 1987). Indeed, Calvo and Horvitz (1990) showed, using a theoretical transition-matrix model, that the degree of pollination limitation is affected by the severity of resource constraints. In addition, Melendez-Ackerman *et al.* (2000) noted that plants may be pollination limited within a season, but resource limited over their lifetime. However, when estimating pollination versus resource limitation in herbaceous perennial species, it is important

to take into account of the population dynamics of the orchid species. For instance, orchids can return to dormancy from the adult stage, this may affect their detection in the field (Kéry and Gregg, 2003; Kéry, 2004) i.e. it will not be known if a dormant individual is dead or merely undetected. When dormant, the plant lacks resources to sprout new photosynthetic tissue and loses the opportunity for photosynthesis for the rest of the season. Hutchings (1987) showed that *Ophrys sphegodes* individuals had higher mortality when they were dormant than when sprouted. However, there was no evidence that current flowering reduced future flowering in *O. sphegodes* (Hutchings, 1987). Alternatively, dormancy could benefit plants by allowing them to conserve energy and survive harsh periods. It could be a bet-hedging strategy to minimise temporal variance in fitness caused by suboptimal environmental factors (Shefferson *et al.*, 2003).

1.3.4 Reward vs. rewardless orchid species

There are three types of reward offered by orchids. (i) Nutritional rewards that are consumed by pollinators. The most common nutritional reward an orchid provides is nectar, however some produce oils (Steiner, 1989) and occasionally pollen (Kocyan and Endress, 2001). (ii) Floral fragrances, which are particular to orchids pollinated by male euglossine bees. The bees are attracted by floral fragrances, which they collect from the orchid flower and use for courtship and perhaps territorial defence, though their precise function is not known (Dressler, 1982). (iii) Waxes and resins which are uncommon as a reward in orchids (Tremblay *et al.*, 2005). Interestingly, one-third of orchid species (8,000 – 10,000) offer no reward to pollinators. Non-rewarding orchids present a major puzzle for evolutionary biologists because these orchids deceive pollinators that are presumably beneficial to the orchid (Ackerman *et al.*, 1994). Indeed, rewarding orchid species have greater reproductive success than non-rewarding species (Neiland and Wilcock, 1998). Much work has addressed the evolution of floral deception in orchids (e.g. Peakall and Beattie, 1996; Schiestl *et al.*, 1999; Cozzolino *et al.* 2001; Smithson, 2002; Jersakova *et al.*, 2006).

There are two major types of floral deception in orchids (although there are others; e.g. brood site deceptive species: Jersakova *et al.*, 2006): reward (food) deception and sexual deception (Cozzolino and Widmer, 2005; Schiestl, 2005). Orchids have several mechanisms to promote outcrossing, and deception is believed to be one of them. Generalised food deception is common where the orchid plants have a general resemblance to nectar-rewarding species, though without providing a reward. A special case is Batesian floral mimicry, where non-rewarding species strictly resemble a specific co-occurring rewarding species (e.g. *Cephalanthera rubra*

and *Campanula* species: Nilsson, 1983). Sexual deception occurs when flowers mimic mating signals of female insects and are pollinated by deceived males while they attempt to copulate with the flower (Schiestl, 2005). In this case, floral odour signals are more important than visual or tactile signals to successfully attract pollinators. There are two hypotheses as to how deception could increase the fitness in plants: (i) reallocation of resources that would normally be associated with reward, however resource limitation is less than the effects of pollen limitation in most orchids (Tremblay et al., 2005). (ii) Higher levels of cross-pollination due to pollinators visiting fewer flowers on non-rewarding plants, resulting in more outcrossed progeny and more efficient pollen export. For instance, Johnson *et al.* (2004) found significant increases in self-pollination when artificial nectar was added to flowers of the nectarless *Anacamptis morio*. A recent review of the literature showed that although deceptive orchids do not have higher levels of genetic load (an indirect measure of outcrossing) than rewarding species, their cross pollination does result in higher quality seeds (Jersakova *et al.*, 2006). Theoretically, selection should favour floral deception when pollinators are abundant, because of its promotion of outcrossing. When pollinators are consistently rare, selection may favour a nectar reward or shift to auto pollination (Cozzolino and Widmer, 2005). In addition to not providing a reward, Darwin (1862) discovered pollinarium bending in some orchids as a contrivance to reduce or avoid self-pollination. Experiments have shown that this reduces geitonogamous pollination thus promotes outcrossing (Johnson and Nilsson, 1999; Johnson *et al.*, 2004). Pollinarium bending is particularly important in rewarding orchids. In rewarding species, the pollinators spend more time per flower and visit more flowers per inflorescence compared with rewardless flowers, which can lead to increased rates of geitonogamy (Johnson *et al.*, 2004). Peter and Johnson (2006) showed using 19 orchid species that there was a correlation between reconfiguration time of the pollinaria and duration of the insect visit. Reconfiguration times were also consistently longer than pollinator visit times. These results provide strong support for Darwin's idea that this mechanism promotes cross-pollination. More research is required to understand why deception remains a stable strategy in orchids.

1.3.5 Population genetic structure of orchids

The Orchidaceae is a large, heterogeneous, and diverse group, with a wide range of reproductive strategies and generation time. This should affect estimates of their population genetic structure. In their review of orchid population differentiation, Hamrick and Godt (1996) examined allozyme data from 16 orchid population genetic studies and obtained a mean estimate of G_{ST} for orchids of 0.087. They considered this an exceptionally low G_{ST} value and believed it was due to species-specific interactions with pollinators and their wind-borne seeds. These traits may have

produced high rates of gene flow among populations. This has been debated, as some authors believe that gene flow is more restricted in wild orchids than in other plants (Forrest *et al.*, 2004). Forrest *et al.* (2004) found a mean G_{ST} value of 0.187 (range: 0.012 - 0.924) among all orchid species analysed, indicating relatively low population differentiation in orchids though higher than the value obtained by Hamrick and Godt (1996). Cozzolino and Widmer (2005) showed that population differentiation values differ between rewarding and non-rewarding orchids. Non-rewarding species tend to have higher genetic diversity and lower population differentiation (Cozzolino and Widmer, 2005). They show that G_{ST} values range from 0.2 – 0.3 in rewarding species, and 0.1 – 0.15 in non-rewarding species. This indicates that gene flow is higher among populations of deceptive orchids. There have been studies on the population differentiation of rare orchid species, but this depends on the definition of rarity (see section 1.1.4). For example, Pillon *et al.* (2007a) compared fen and dune populations for the rare *Liparis loeselii* and found an overall significant F_{ST} value of 0.382. For fen populations there was significant population differentiation (F_{ST}: 0.370), however, for dune populations there was no differentiation (F_{ST}: 0.146). Tali *et al.* (2006) examined early and late flowering populations of the declining *Neotinea ustulata* and found little population genetic differentiation. In addition, Gustafsson and Sjogren-Gulve (2002) found higher levels of genetic differentiation of the rare *Gymnadenia odoratissima* when compared with the more common *G. conopsea.*

1.3.6 Rare orchids and conservation

The Orchidaceae are protected under CITES (Convention of International Trade of Endangered Species of Wild Flora and Fauna). This is because orchids suffer from over-harvesting and are sensitive to environmental damage and change. Even though many members of the family are not threatened, protecting the entire family under CITES makes it easier for border inspectors without taxonomic training to identify taxa (Walden-Walsh, 2005). As discussed, orchids are adapted for insect-mediated outcrossing, can have complex life histories and depend of mycorrhizae for establishment from seed. Therefore, precise data on orchid conservation biology can be difficult to obtain. Pollination success is believed to affect be a primary factor that affects orchid rarity. Indeed, Neiland and Wilcock (1998) found that there was a positive correlation between a lack of nectar reward and rarity of orchids in Britain and Ireland. However, Jacquemyn *et al.* (2005) found no relationship between the presence of nectar reward and extinction of orchids in Belgium and the Netherlands. A recent review of scientific approaches to Australian temperate orchid conservation (Brundrett, 2007) highlighted the use of long-term demographic studies to estimate rates of transition between life-history states and reveal factors, such as grazing and competition, which results in declining populations. Also, the effects of

mycorrhizae in rare orchid establishment need to be further investigated. Recently, Jacquemyn *et al.* (2007) show that local microsite conditions are important for the recruitment of new individuals from seed in the rare *Orchis purpurea*, possibly due to local soil conditions or the availability of mycorrhizae. There is still a lot of work to do to understand the factors behind the decline of many orchid species.

1.4 Study species

Twenty-eight orchid species occur in Ireland, representing thirteen genera. We know little of their reproductive biology. Four species are protected by the Irish Flora Protection Order (1999) under the Wildlife Act (1976) and the Wildlife Amendment Act (2000): *Cephalanthera longifolia*, *Hammarbya paludosa*, *Pseudorchis albida*, and *Spiranthes romanzoffiana*. One other species is considered rare and is restricted to the west of the country, *Neotinea maculata*. One other species, *Spiranthes spiralis*, is not considered rare in Ireland, however it is the only congener to *S. romanzoffiana* in Ireland and its reproductive biology is investigated for comparison with *S. romanzoffiana*. Here, I summarise what is known about the biology of the focal species of this study.

1.4.1 Cephalanthera longifolia (L.) Fritsch. (Plate 1.1)

Common name: Narrow-leaved Helleborine.

Global distribution: Throughout Europe, southern Scandinavia, North Africa, western Asia to China.

Flowering time in Ireland: May-June

Plant habit and floral structure: Perennial, single-stemmed, sometimes clumped, stem to 60cm, leaves bright green, alternate, two-ranked, to approximately 20 x 25cm, linear-lanceolate, acute tipped. Inflorescence a lax spike; bracts small, pointed, larger below. Flowers 5-15 (-20), large, pure white ± sessile, partially opening, scentless. Outer perianth segments 10-15mm, lanceolate, acute-tipped. Inner perianth segments shorter, broader, more obtuse, labellum shorter still; hypochile white, blotched with orange at the base; epichile cordate distally, wider than long, with a few orange basal ridges, tip downturned. Column erect; anher whitish with stigma below; pollinia two; ovary to 15mm, cylindrical, twisted, glabrous; 2n = 32.

Reproductive biology: It is a non-rewarding, food deceptive orchid, pollinated mainly by solitary bees (Dafni and Ivri, 1981). It is not capable of spontaneous self-pollination (Dafni and Ivri, 1981). There has only been one published study on its pollination biology (Dafni and Ivri, 1981). It has white flowers on which there is a bright orange spot on the labellum where solitary bees land when they visit. Dafni and Ivri (1981) showed it has higher fruit set in the presence of

a co-flowering rewarding species, *Cistus salvifolius*, and is believed to be a Batesian mimic. There are yellow papillae (pseudo pollen) on the labellum of flowers of *C. longifolia*, which resemble the pollen of *C. salvifolius*. However, its UV reflection is different to *C. salvifolius*, implying that UV reflection does not influence pollinator attraction (Dafni and Ivri, 1981). However, *C. longifolia* is pollinated in the absence of *C. salvifolius*, hence the mimicry system proposed by Dafni and Ivri is facultative. Batesian mimicry has also been demonstrated in the congeneric *Cephalanthera rubra*, which has higher fruit set in the presence of nearby *Campanula* species (Nilsson, 1983). Genetic analyses show that when compared with *C. rubra* and *C. damosonium*, *C. longifolia* is an obligate xenogamous (outcrossing) species, while *C. damosonium* is an auto pollinated species, and *C. rubra* has a mixed mating system (Scacchi *et al.*, 1991).

Population biology: Shefferson *et al.* (2005), used mark-recapture models (discussed in section 1.1.3), to show that shaded individuals of *C. longifolia* had increased survival and less dormancy. However, the major factor explaining this was not acclimation to low light, but initial plant size, which suggest that larger plants are more robust in dealing with shade than smaller plants. Dormancy might allow individuals to buffer stress in *C. longifolia* in the short term. In addition, models predicted that individuals can live >38 years in Estonian populations studied (Shefferson *et al.*, 2005).

Habitats: *C. longifolia* usually occurs in woodland where there is dappled light. It is a lowland species typically occupying *Fagus* woodland over lime-rich substrates, particularly chalk and limestone. It also thrives is *Quercus* and *Fraxinus* woods. Although it needs some light, too open a habitat allows competing vegetation to take over.

Conservation status in Ireland: Endangered. Red-listed, protected under the Wildlife Act (1976) and the Wildlife Amendment Act (2000) under the Flora Protection Order (1999).

1.4.2 Hammarbya paludosa (L.) Kuntze. (Plate 1.1)

Common name: Bog Orchid

Global distribution: North America, Scandinavia, Northern Europe, to Asia.

Flowering time in Ireland: July - August

Plant habit and floral structure: Perennial herb with two pseudobulbs connected by a rhizome; stem 3-8(-12)cm, erect, angled above, sheathed below. Leaves two to three, to 10 x 5mm, ovate to oblong, ± acute, pale yellow, often with the margin fringed with small bulbils. Inflorescence a spike-like raceme, dense at first, later elongating, with up to 20 flowers; bracts small, 2-3mm, lanceolate; flowers small approximately 5mm long, yellow-green. Outer perianth segments to approximately 3mm, ± ovate; inner perianth segments linear-lanceolate, with tips curved;

labellum upward-pointing, lanceolate, acute, yellow-green, with its base clasping the column; spur absent; column very short; anther united to the column; pollinia two, waxy; stigma borne on the front of the column; rostellum minute, ligulate, viscid; ovary small, stalked; 2n = 28.

Reproductive biology: The only published study of its pollination biology was by Reeves and Reeves (1984). They found it requires pollinators to set fruit, as individuals that were bagged to exclude pollinators set no fruit. Darwin (1862) noted that all flowers except, for one or two, had their pollinia removed, and that 13 out of 21 flowers set fruit on one inflorescence. Reeves and Reeves (1984), using an aspirator and night-lantern to catch nocturnal pollinators, found one individual fungus gnat, *Phronia digitata* carrying pollinia. The labellum has a blue-green strip, which is believed to be a nectar guide, and nectar is at the base of the lip adjacent to the column. They found fruit set was 20.5%. In addition, *H. paludosa* has bulbils on the edge of its leaves, which allow it to reproduce asexually. These bulbils blow off the leaf edges and establish new individuals

Population biology: Nothing is known about its population dynamics.

Habitats: Wet acidic *Sphagnum* bogs, often growing where there is a slight movement of the surface water; it can also occur in more open habitats in damp, peaty mud, as well as in flushes where the water is base-rich.

Conservation status in Ireland: Endangered. Red-listed, protected under the Wildlife Act (1976) and the Wildlife Amendment Act (2000) under the Flora Protection Order (1999).

1.4.3 Neotinea maculata (Desf.) Stearn. (Plate 1.2)

Common name: Dense-flowered Orchid

Global distribution: Western Ireland, south and south-west Europe where it ranges from the Canary Islands, Madeira, and Portugal, eastwards through much of the Mediterranean region to Cyprus, Turkey and Lebanon, to North Africa.

Flowering time in Ireland: April - May

Plant habit and floral structure: Perennial herb with two small ovoid root tubers; stem 10-30cm, bearing a few membranous basal leaf sheaths; leaves two to six, the basal elliptical-oblong, entire, green, sometimes spotted, the upper small, narrow and bract-like, pressed to and partially sheathing the stem; inflorescence a distinctly compact, cylindrical, somewhat one-sided spike, typically 2-6(-10)cm long; bracts small, ± lanceolate, acute; flowers very small, only partially opening, pinkish or white, occasionally spotted, faintly scented; outer perianth segments 3-4mm, connivent, lanceolate to ovate-lanceolate; inner perianth segments very narrow, linear; labellum very small, trilobed, the central lobe the longest, the latter linear and notched at the

apex; spur very short, conical, containing nectar; column very short, notched; anther placed at the top of the column and bearing two very small pale-green pollinia; stigmas two, large; rostellum in the form of a flat plate placed between two stigma lobes; ovary twisted, slightly ridged; 2n = 42.

Reproductive biology: Little is known of its reproductive biology. It is self-compatible, capable of autogamy, and is nectar rewarding (van der Cingel, 1995). Small flies are suggested to pollinate *N. maculata*, however no observations have confirmed this. It has been suggested *N. maculata* is cleistogamous i.e. its flowers are self-pollinated before the flowers open. It is the only member of the genus *Neotinea* that is rewarding and capable of self-pollinating. The other members of *Neotinea* (*N. ustulata*, *N. lactea*, and *N. tridentata*), formerly classified as members of *Orchis* (Bateman *et al.*, 2003), are food deceptive and obligate outcrossers

Population biology: Nothing is known of its population dynamics. However, in the related *N. ustulata*, dormancy has been shown to decrease adult survival (Shefferson and Tali, 2007) and has high levels of genetic population differentiation (Tali *et al.*, 2006).

Habitats: In Ireland, it occurs mainly in open grasslands on limestone substrate. However, can be found in mixed *Fraxinus* and *Corylus* woodlands. In the Mediterranean, it occurs in both calcareous and non-calcareous habitats. For example, it can occur in both *Castanea* and *Pinus* forests, on volcanic soils, and neutral to acidic soils.

Conservation status in Ireland: Not protected.

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1.4.4 Pseudorchis albida (L.) Á. Löve & D. Löve. (Plate 1.2)

Common name: Small-white Orchid

Global distribution: Circumpolar boreal distribution, ranging from temperate Europe and Asia in the east to Newfoundland in the west.

Flowering time in Ireland: June-July

Plant habit and floral structure: Perennial herb with divergent, palmate root tubers; stem 10-35cm, glabrous, erect, with two or three membranous, scaly leaf sheaths at the base; leaves three to five at the base of the stem, broadly lanceolate, pointed, entire, shiny green, those above ± bract-like; inflorescence a dense, cylindrical, somewhat one-sided spike; bracts lanceolate; flowers approximately 2-3mm in diameter, numerous, ± tubular, whitish to greenish white throughout, fairly scented; outer perianth segments ovate, connivant with the inner whorl, the segments of which are also similar in shape; labellum forward-pointing, trilobed, the lateral lobes shorter than the median; spur to 2.5 mm long, slightly thickened at the apex, downcurved; column erect; anther broad, greenish-white; pollinia two, very small; stigma ± reniform; rostellum long; ovary ridged, slightly twisted 2n = 42.

Reproductive biology: Little is known of its pollination biology, and is believed to have a mixed mating system (van der Cingel, 1995). Moths (of the genus *Crambus*) have been observed to pollinate *P. albida* in Austria (Baumann and Baumann, 2002). Darwin (1862) noted high levels of pollinia removal and fruit set may be over 90% (van der Cingel, 1995).

Population biology: Nothing is known of its population biology.

Habitats: Upland unimproved pastures, occurring either on soils that are neutral to acidic, though nutrient poor. Requires light grazing as will be out-competed if the habitat becomes overgrown. In Sweden, *P. albida* is distinguished from the close relative *P. straminea* based on morphological, habitat and genetic data (Reinhammar, 1995; Reinhammar and Hedrén, 1995; Reinhammar *et al.*, 1998). However, based on herbarium specimens, the distinction between the two species may not exist in central Europe (Reinhammar, 1998). *P. albida* has a declining distribution in Scandinavia, which is the result of a decline in semi-anthropogenic grassland habitats (Reinhammar *et al.*, 2002).

Conservation status in Ireland: Endangered. Red-listed, protected under the Wildlife Act (1976) and the Wildlife Amendment Act (2000) under the Flora Protection Order (1999).

1.4.5 Spiranthes romanzoffiana Cham. (Plate 1.3)

Common name: Irish Ladies' Tresses

Global distribution: Primarily North American, but known in Europe only from Britain and Ireland. Therefore, is a species with a very asymmetric amphi-Atlantic distribution.

Flowering time in Ireland: July-August

Plant habit and floral structure: Perennial herb with slightly thickened tuberous roots; stem 10-35cm, erect, pale green; leaves both basal and cauline on the current flowering stem, erect, linear-lanceolate, acute; inflorescence a spike, 2-5 cm, with flowers in a dense, somewhat twisted, three-ranked arrangement; bracts lanceolate to ovate, acute; flowers white, scented, 10-14mm, outer perianth and segments to 12 mm, lanceolate, white; inner perianth segments linear, white; labellum lingulate to pandurate, white green-veined, frilled and denticulate distally; column horizontal, beaked; anther cordate; pollinia two; stigma crescent-shaped; rostellum green; ovary cylindrical; subsessile; 2n = 60.

Reproductive biology: It is nectar rewarding and is pollinated by up to 13 species of bee in North America (Catling, 1983; Larson and Larson, 1987). These species range from medium-sized bumblebees such as *Bombus perplexus*, *B. vagans*, *B. borealis*, while Larson and Larson (1987) found that *B. bifarius* was the most common pollinator. In addition, the honeybee *Apis mellifera* and the solitary bee *Lasioglossum zonulum* were seen visiting flowers. *S. romanzoffiana* is self-compatible, but requires a pollinator for fruit set (Catling, 1983). It has a

protandrous mechanism for outcrossing, whereby the pollinator visits flowers sequentially on an inflorescence In North America, fruit set can be high, up to 75% (Larson and Larson, 1987). All previous reports show that fruit set does not occur in this species in Europe. However, recently it has been shown that seed is present in the un-ripened fruits (O'Conner, 2006; Lupton, 2008).

Population biology: It is noted for flowering fluctuations. Forrest *et al.* (2004) examined the population genetics of European *S. romanzoffiana* and found a genetic-geographic split between Irish and Scottish populations. They found that Irish populations had low genetic variation. However, Lupton (2008) found that Irish populations have relatively high genetic diversity and are different from North American populations.

Habitats: Valley bogs, and damp, peaty meadows, pastures and heaths, especially those that are susceptible to the inundation with water over winter.

Conservation status in Ireland: Endangered. Red-listed, protected under the Wildlife Act (1976) and the Wildlife Amendment Act (2000) under the Flora Protection Order (1999). *S. romanzoffiana* is a species of high conservation concern in Ireland, and is subject to an All Ireland Species Action Plan (2005). This action plan is aimed at recording and maintaining all currently known populations and establishing new populations to ensure the long-term survival of the species in Ireland.

1.4.6 Spiranthes spiralis (L.) Chevall. (Plate 1.3)

Common name: Autumn Ladies' Tresses

Global distribution: Widely distributed in southern Europe from North Africa through the Mediterranean north to Denmark and west to western Asia and Russia.

Flowering time in Ireland: August - September

Plant habit and floral structure: Perennial herb with elongated tubers; stem 5-20 cm, pale green; leaves developing laterally in late summer, simultaneously with or shortly after the development of the flowering spike from the previous years growing point, forming an over-wintering basal rosette from which the following years flowering spike will emerge, ovate-elliptic, acute, entire, patent, glossy green, withering in early summer; inflorescence a spike of 5-15(-20) flowers; bracts small, lanceolate; flowers very small, white, sweet-scented during the daytime, usually arranged on a single spiral rank; outer perianth segments 6-7mm, oblong, white green-nerved, faintly toothed; inner perianth segments slightly smaller than outer, lingulate, white; labellum 6-7mm, oblong, concave,, pale greenish white, with an indented margin; spur absent; column greenish; anther sessile; stigma on under surface of column; rostellum divided into narrow lobes; ovary narrow, green; 2n = 30.

Reproductive biology: Like *S. romanzoffiana*, it is a self-compatible species that depends on insects, mainly bees, for pollination and seed production (Willems and Lahtinen, 1997). Fruit set varies from 0 to 78% (mean: 35%). Although the seeds are wind-dispersed, the majority fall in the direct vicinity of the mother plant (Machon *et al.*, 2003).

Population biology: Individuals can survive dormant for many years and may consist of several rosettes (Wells, 1967), although dormancy occurs at a low rate (Jacquemyn *et al.*, 2007). It is capable of vegetative reproduction by the growth of a lateral bud on the underground stem, the new plant forms its own tubers, and eventually the connection between the mother plant and vegetatively produced plants withers away (Wells, 1967). It is a long-lived species, with some individuals living up to 50 years (Willems and Dorland, 2000).

Habitats: It occurs in open-grazed nutrient poor meadows and calcareous grassland. Ancient, undisturbed pastures and downs, rabbit-grazed cliff tops, coastal grasslands, and stabilised calcareous dunes are its most typical habitats.

Conservation status in Ireland: Not protected.

1.5 Aims of this study

As a result of a limited knowledge of orchid pollination and conservation in Ireland, and these species in particular, this study focuses on the effects of rarity on orchid reproduction and conservation. Particularly, we know little of where rare orchids are declining (if they are in decline), the reproductive biology of rare orchids and the effects of rarity on population their genetic structure. In this thesis I present the results of several investigations into aspects of threat status, reproductive biology, and population genetics of rare orchids in Ireland.

Specifically, the aims of this thesis are:

1. To investigate whether these species of orchids are declining in Ireland and to ascertain where declines are occurring (Chapter 2).
2. To investigate the pollination ecology of rare orchids. In particular, to examine:
    (a) Within population density, nectar reward and co-flowering species and how they affect visitation (Chapter 3).
    (b) Rare orchid pollination ecology compared with a more common congener and their co-flowering communities to see if there are similar traits in rarity and its effects on pollination (Chapter 4).
3. To investigate the population genetic structure of rare orchids. Specifically,
    (a) To compare differences in genetic structure where the species is common versus where it is rare, and if this can be explained by differences in the ecology and reliance on self-pollination (Chapter 5).

(b) To examine whether small population size and a small number of populations decreases genetic variation (Chapter 6).

Plate 1.1 (Photos K.J. Duffy)

Cephalanthera longifolia

Hammarbya paludosa

Plate 1.2 (Photos K.J. Duffy)

Neotinea maculata

Pseudorchis albida

Plate 1.3 (Photos K.J. Duffy)

Spiranthes romanzoffiana

Spiranthes spiralis

"*No precise estimate can be made on the numbers of species being extinguished... to witness the death of the last member of a parrot or orchid species is a near impossibility. With the exception of the showiest birds, mammals, or flowering plants, biologists are reluctant to say with finality when a species has finally come to an end. There is always the chance (and hope) that a few more individuals will turn up in some remote forest remnant or other. But the vast majority of species are not monitored at all*" – **E.O. Wilson, Biodiversity, 1986**

2. Inferring national and regional declines of rare orchid species with probabilistic models

In Press: Karl J. Duffy, Naomi E. Kingston, Brendan A. Sayers, David L. Roberts and Jane C. Stout. Inferring national and regional declines of rare orchid species with probabilistic models. *Conservation Biology*.

2.1 Abstract

Accelerating fragmentation of natural habitats can lead to increasing numbers of rare species. Conservation of rare species requires experts and resources, which may be lacking for many species. In the absence of regular surveys and expert knowledge, historical sighting records can provide data on the distribution of a species. Numerous models have been developed recently to make inferences regarding the threat status of a taxon based on variation in trends of sightings over time. Here I applied five such models to both national and regional (county) data of three red-listed orchid species (*Cephalanthera longifolia*, *Hammarbya paludosa*, and *Pseudorchis albida*) and one species which is a candidate for future red listing (*Neotinea maculata*) in the Republic of Ireland. In addition, I used an optimal linear estimate to calculate the time of extinction for each species both overall and within each county. To account for bias in recording effort over time I used rarefaction analysis. Results showed that based on sighting records, overall these species are not immediately threatened with extinction and, although there have been declines; no clear geographical pattern of decline was detected in any species. Most counties where focal species occurred had a low number of sightings; hence we were cautious in our interpretation of output from statistical models. I suggest the improvement of natural habitats and lack of appropriate management to be the main drivers of decline in these species in Ireland. This approach of regional scale analysis complements recent IUCN criteria for the 'Extinct' category and is useful for highlighting areas of under-recording and focusing conservation efforts of rare and endangered species.

2.2 Introduction

The rapid change and fragmentation of natural habitats due to anthropogenic activity has been well documented in recent years (e.g. Wilcove *et al.*, 1986; Saunders *et al.*, 1991; Young *et al.*, 1996; Fischer and Lindenmayer, 2007). Reduction and fragmentation of areas of natural habitat negatively affects population size and increases population isolation (Fahrig, 2003). Such a decrease in population size can adversely affect biological and ecological processes including interactions with mutualists (Kearns *et al.*, 1998). Certain groups of plant species, such as

orchids, can be particularly sensitive to such environmental change (Jacquemyn *et al.*, 2005; Kull and Hutchings, 2006) because of the reliance of many species on interactions with other species. For example, many orchids require pollinators for out-crossing (Nilsson, 1992; Tremblay *et al.*, 2005). The absence of pollinator attention (due to fragmentation or small population size) may cause reduced seed production in self-incompatible species or inbreeding in self-compatible species (Wilcock and Neiland, 2002). In addition, interactions with soil mycorrhizae are required for successful establishment from seed (Rasmussen, 2002). Anthropogenic activity such as the addition of artificial fertiliser to natural habitats can harm soil mycorrhizae, which will negatively affect recruitment of new orchid individuals (Silvertown *et al.*, 1994). In addition to fragmentation, orchids may be sensitive to habitat management and be out-competed for resources (e.g. light, nutrients) by co-occurring plant species if habitats are un-managed (Janečková *et al.*, 2006). Therefore, as habitat change increases, there is growing need for monitoring programmes to assess the conservation status of orchid species.

Monitoring programmes provide a valuable source of information for national red lists and the World Conservation Union (IUCN) (Rodrigues *et al.*, 2006). However, accelerating fragmentation of natural habitats negatively affects the monitoring of populations of rare and endangered species due to limited time, finances and resources available for conservation programmes and the increasing number of rare species (Reed, 1996; McCarthy, 1998). In the absence of annual monitoring, historical sighting records offer data on the occurrence of taxa through time and space, though these types of data are often sparse and inconsistent (Burgman *et al.*, 1995; Roberts and Solow, 2003). Numerous models are available for inferring threat using records of infrequent sightings (e.g. Burgman *et al.*, 1995; McCarthy, 1998; Solow, 2005). Recently these models have be applied to address various questions, such as the probability that endangered species are prematurely inferred as extinct (Roberts and Kitchener, 2006) and assess the conservation status of endemic species (McInerny *et al.*, 2006).

In the Republic of Ireland, several orchid species are considered rare, though basic data such as whether populations are still extant and how many individuals comprise populations are lacking in most regions of Ireland for most species. Several life history traits characteristic of terrestrial orchids including; (i) prolonged dormancy, (ii) erratic flowering between years (Shefferson *et al.*, 2003), (iii) inconspicuous morphology, and (iv) occurrence in remote habitats, may result in many species being under-recorded (Kull and Hutchings, 2006). This makes it difficult to ascertain whether populations of species are still extant or if a large proportion of individuals or populations are dormant (Shefferson *et al.*, 2005). As with much of North West

Europe, Ireland's habitats have undergone intense modification due to abandonment of traditional farming practices and rapid improvement of agricultural land (Wallis De Vries *et al.*, 2002). Kull and Hutchings (2006) highlight the need for local specific conservation action to prevent further decline of orchids, however we know little of where declines (if any) of rare orchids have occurred and where orchid species may be extinct. We studied four orchid species in the Republic of Ireland (*Cephalanthera longifolia*, *Hammarbya paludosa*, *Neotinea maculata*, and *Pseudorchis albida*) on the basis that three species, *C. longifolia*, *H. paludosa*, and *P. albida*, are red listed (Curtis and McGough, 1988) and protected in the Republic of Ireland by the Irish Flora Protection Order (1999) under the Wildlife Act of 1976, while one species, *N. maculata*, is a candidate species for future red listing and is considered locally rare as it is restricted chiefly to the west of Ireland.

Records of orchid species in the Republic of Ireland date from after 1800. These records were traditionally provided by botanists and orchid enthusiasts. In order to ascertain where declines are occurring in Ireland, I applied probabilistic methods to county records throughout the Republic of Ireland. I used county records because; (i) there are not enough sightings within 10 x 10 km units for the models to be applied, (ii) the majority of the plant records for Ireland were provided by members of the Botanical Society of the British Isles (BSBI) and vice-counties within counties were their survey unit (see Webb (1980) for a discussion of county versus vice-county boundaries in Ireland), and (iii) local conservation administration occurs at county level in Ireland.

By making field surveys and using statistical models on data from the rare plant records held by the National Parks and Wildlife Service (NPWS), the aims of this study were to investigate and highlight counties of decline and conservation priority for *C. longifolia*, *H. paludosa*, *N. maculata*, and *P. albida* in the Republic of Ireland. Specifically, based on records of previous sightings, I tested the following hypotheses:

1. Ranges of focal species in the Republic of Ireland have contracted since 1800.
2. Focal species are threatened with varying levels of extinction in different counties.

2.3 Methods

2.3.1 Field observations

Since little is currently known about the flowering population size and location of focal species, I visited 28 locations during the flowering periods in 2004 through 2006 where *C. longifolia*, *H.*

paludosa, *N. maculata*, and *P. albida* had been recorded previously within the past 20 to 30 years (although occasionally longer depending on species and how recent the last records were). At each location, I conducted intensive searches for orchids in all suitable habitats.

2.3.2 Historical records

A chronological record of sightings of *C. longifolia*, *H. paludosa*, *N. maculata*, and *P. albida* was constructed from historical records held by the NPWS of the Republic of Ireland. This data set contains sightings collated from published observations and records submitted from surveys and chance encounters with focal species. Chronological sightings are arranged for all species in the Republic of Ireland for the entire country and within each county in which each species has been recorded. I included only records that had a definite year of sighting and a named recorder. Multiple records within the same year were excluded (McCarthy, 1998). I mapped each county where the orchid species occurred in Ireland and highlighted counties for pre-1980 records, post-1980 records, and counties where a population of a focal species was recorded in my survey 2004 through 2006.

2.3.3 Threat-evaluation models

I used five probabilistic models (model 1, Solow, 1993a; 2, Solow, 1993b; 3, Solow and Roberts, 2003; 4, Weibull [Roberts & Solow, 2003]; 5, sighting rate [McInerny *et al.*, 2006]) to infer threat, with the records arranged as a binary series within the period, T, at ordered times, $t_1 < t_2 < \ldots < t_n$. I took the upper value of T to be 2004 (the year when this study began). These models detected various trends in the sighting process, and the use of the models simultaneously can highlight discrepancies in the sighting process (McCarthy, 1998).

Solow 1993a model

Given the time of the last sighting (t_n), the Solow equation (1993a) yields the probability (p) that n observations occurred within the period $0 < t < t_n$ given that sightings are equally likely to occur within the period T (Solow, 1993*a*):

$$p = \left(\frac{t_n}{T}\right)^n \qquad (1)$$

Since the first sighting (t_1) defines the start of the sighting period, n reduces to n-1 for all calculations.

Solow 1993b model

The Solow 1993*a* equation is suitable for small populations that are predisposed to rapid extinction because the sightings are assumed to follow a stationary Poisson process. However, in a declining population, sightings are less likely to occur toward the end of the period because sightings decrease as the population declines. Assuming that the sightings follow a Poisson process with decreasing rate function, Solow developed the following equation (Solow, 1993*b*):

$$p = \frac{Fs(t_n)}{Fs(T)}, \tag{2}$$

where, $s = \sum_{i=1}^{n} ti$, the sum of the observations and the function $Fs(t)$ is given by

$$Fs(t) = 1 - \sum_{i=1}^{[s/t]} (-1)^{i-1} \binom{n}{i} [1 - (it/s)]^{n-1}, \tag{3}$$

where, s/t denotes an integer, i is the iteration number, and n is the number of sightings.

Solow and Roberts 2003 model

The Solow and Roberts nonparametric equation (Solow and Roberts, 2003) does not require a complete sighting record because the number of sightings (n) is not required for its calculation. Using t_n, T, and t_{n-1} the equation generates the probability that another sighting will occur (p):

$$p = \left(\frac{t_n - t_{n-1}}{T - t_{n-1}} \right), \tag{4}$$

where t_n is the number of years since the first record to the most recent record, T is the total number of years from the first record to the present day, and t_{n-1} is the number of years from the first record to the penultimate record.

The Weibull model (Roberts & Solow, 2003)

A species may become increasingly rare before its final extinction and continue to exist unseen for many years. Therefore the time since last sighting may be a poor estimate of the time of extinction. Roberts and Solow (2003) propose the estimate of the shape parameter of the Weibull extreme-value distribution is given by

$$\hat{\nu} = \frac{1}{k-1}\sum_{i=1}^{k-2}\log\frac{T_1 - T_k}{T_1 - T_{i+1}}, \quad (5)$$

where k is the number of sightings, T_1 is the time of the most recent sighting, T_k is the time of the first sighting, and T_{i+1} is the iteration of the sighting records.

This equation can be used to produce an approximate p value for testing for extinction (Solow, 2005):

$$p = \exp\left[-k\left(\frac{T - t_n}{T - t_{n-k+1}}\right)\right]^{1/\hat{\nu}}, \quad (6)$$

where, k is the number of sightings, T is the present time, t_n is the time of the most recent sighting, and t_{n-k+1} is the time of the initial sighting. When calculating the shape-parameter estimate of the Weibull extreme-value distribution for the entire sighting record of each species, I reduced the total number of sightings, k, to the 10 most recent sightings for comparison among species. This is because if k is too large, the asymptotic argument leading to the Weibull model may not hold (Solow, 2005).

Sighting rate model (McInerny et al., 2006)

The probability that another sighting will occur can be generated by the previous sighting rate, n/t_n, and the time since last observation, T-t_n:

$$p = \left(1 - \frac{n}{t_n}\right)^{T - t_n}, \quad (7)$$

where, n is the number of sightings, t_n is the time from the initial sighting to the last, and T is the time from the initial sighting to the present. This probability generated by the sighting rate equation is correlated with IUCN Red List categories (i.e., species with low p values are highly threatened; McInerny *et al.*, 2006). Because a constant pre-extinction sighting rate is assumed for this model, I reduced n to n-1 for all calculations (McInerny *et al.*, 2006).

Time-to-Extinction Model

To estimate the time of extinction (T_E) within counties and overall, I applied models that assumed gradual extinction (Solow, 2005).

The optimal linear estimate of T_E on the basis of the k most recent sightings is

$$\hat{T}_E = \sum_{i=1}^{k} w_i t_{n-i+1}. \quad (8)$$

The weight vector $\mathbf{w} = (\mathbf{w}_1, \mathbf{w}_2, \ldots, \mathbf{w}_k)'$ is given by $\mathbf{w} = (\mathbf{e}' \ddot{\mathbf{E}}^{-1} \mathbf{e})^{-1} \ddot{\mathbf{E}}^{-1} \mathbf{e}$,

where $\mathbf{e}$ is a vector of k 1s and Λ is the symmetric k-by-k matrix with typical element:

$$\Lambda_{ij} = \frac{\Gamma(2\hat{\nu} + i)\Gamma(\hat{\nu} + j)}{\Gamma(\hat{\nu} + i)\Gamma(j)} \qquad j \le i, \quad (9)$$

where Γ is the gamma function.

An approximate $1 - \alpha$ confidence interval for T_E is

$$(T_1 + \frac{T_1 - T_k}{s_L - 1}, T_1 + \frac{T_1 - T_k}{s_U - 1}), \quad (10)$$

where $s_L = \left(\frac{-\log(1-(\alpha/2))}{k}\right)^{-\hat{\nu}}$ and $s_U = \left(\frac{-\log(\alpha/2)}{k}\right)^{-\hat{\nu}}$.

I carried out a meta-analysis of p values obtained from the five probabilistic models used to infer threat status. The formula, $-2\sum\ln(p)$, was used to calculate the chi-square (χ^2) value with $2k$ degrees of freedom, where p is the threat probability from each test and k is the number of separate tests (Sokal and Rohlf, 1995).

2.3.4 Rarefaction analysis

When comparing the species distribution of 2 or more species, bias can arise due to sampling effects from different recorders over relatively large periods of time (Gotelli and Colwell, 2001; Solow and Roberts, 2006). Unless all species are censused exhaustively and identically, it is inappropriate to compare distributions. To test whether differences in recording effort (i.e., if one species was preferentially recorded) among species affected the recorded distribution of these species, I performed a rarefaction analysis. Solow and Roberts (2006) developed the following model, which estimates the expected number of locations corrected for sampling effort on the basis of number of sightings of each species.

$$E(L(n)) = k - \sum_{j=1}^{k} \binom{m - m_j}{n} \Big/ \binom{m}{n}, \qquad (11)$$

where k is the total number of locations in which each species was recorded, m_j is the number of sightings made from location j (j = 1, 2, …., k), m is the total number of sightings ($m = \sum_{j=1}^{k} m_j$), and $L(n)$ is the number of locations represented in a random sample of n of these sightings ($n \leq m$), where for non-negative integers a and b, $\binom{a}{b} \equiv 0$ when $a < b$.

I estimated the number of counties in which focal species were expected to occur with rarefaction analysis. The expected number of locations generated from this equation is an exact estimate and there is no need to use simulation (e.g., Gotelli and Colwell, 2001). We first calculated an overall rarefaction that included all species. Because *N. maculata* has a naturally restricted distribution, I calculated the number of expected counties excluding sightings of *N. maculata*. Because there were changes in land use in Ireland before 1980, with fewer changes since 1980, I calculated the expected number of counties for each species for pre-1980 and post-1980 records. *N. maculata* sighting records were generally post-1980 (apart from 3 records); therefore, I excluded this species from our analysis of pre- and post-1980 records.

I performed a chi-square test for homogeneity with Yates' correction for continuity (Sokal & Rohlf 1995) to compare the number of pre- versus post-1980 sightings for each species against the total number of sightings of focal species.

2.4 Results

2.4.1 Field observations

Populations of all focal species were found during surveys made 2004-2006, though not all populations were surveyed in all years. Populations had very low numbers of flowering individuals and were found in few locations (Table 2.1). Grazing was a problem for both *C. longifolia* and *P. albida* where flowering populations were destroyed by trampling and grazing of deer and cattle respectively. It was difficult to estimate the number of vegetative individuals in the field as leaves are very small (e.g. *H. paludosa*), difficult to identify (e.g. *C. longifolia* does not produce basal leaves) or dieback during flowering and fruiting stage. All species showed some evidence of fruit set (Table 2.1).

2.4.2 Threat evaluation models

A total of 178 sightings over a 202 year period were analysed between the focal species (Figure 2.1a). The number of within-county sightings among all species ranged from 1 – 13 (median number of sightings per county = 3) (Figure 2.1b). The *p* values calculated from the five models are shown in Tables 2-5. Low *p* values represent high levels of threat. Overall, the models suggest that *C. longifolia*, *H. paludosa*, *N. maculata*, and *P. albida* are not faced with immediate extinction, except for sighting rate, Solow (1993*a*), and the Weibull models for *N. maculata* (Figure 2.2). Based on sighting data until 2004, the estimated time of extinction, T_E, for *C. longifolia* is 2008 (last sighting is 1999; Confidence Interval (C.I.) = 2000, 2040), for *H. paludosa* T_E is 2003 (last sighting is 1999; C.I. = 1999, 2009), for *N. maculata* T_E is 1997 (last sighting is 1995; C.I. = 1995, 2005), and for *P. albida* T_E is 2007 (last sighting is 2003; C.I. = 2003, 2012). When I included survey sightings made during this study, T_E for *C. longifolia* is 2010 (C.I. = 2006, 2030), for *H. paludosa* T_E is 2008 (C.I. = 2005, 2021), for *N. maculata* T_E is 2011 (C.I. = 2006, 2030), and for *P. albida* T_E is 2007 (C.I. = 2005, 2017). Results from the meta-analysis on overall sightings show *N. maculata* is highly threatened, *C. longifolia* (χ^2 = 8.45; df = 10; $p > 0.05$), *H. paludosa* = (χ^2 = 15.94; df = 10; $p > 0.05$), *N. maculata* (χ^2 = 30.15; df = 10; $p < 0.001$), and *P. albida* (χ^2 = 2.88; df = 10; $p > 0.05$).

C. longifolia has been seen in fourteen of the twenty-six counties in the Republic of Ireland (Figure 2.3a), *H. paludosa* has been seen in thirteen counties (Figure 2.3b), *N. maculata* has been seen in six counties (Figure 3c), and *P. albida* has been seen in sixteen counties (Figure 2.3d). The models predict that it is likely that *C. longifolia* has been extirpated from counties Dublin, Roscommon, Tipperary, and Wicklow (Table 2.2). Counties Clare, Waterford, Carlow, Wexford, and Roscommon have only one or two sightings. Indeed, counties Roscommon and Wexford have had sightings recently (within the past 36 years and 11 years respectively) and should be surveyed immediately. *C. longifolia* still occurs in Co. Roscommon (J Fuller, *personal communication*), while a search was made in 2005 in Co. Wexford where it was not re-found (KJ Duffy, *personal observation*). Counties where *C. longifolia* is most likely to be found are Galway, Kerry, Westmeath and Donegal.

H. paludosa (Figure 2.3b) is likely to have been extirpated from counties Kerry and Kildare (Table 2.3). Counties Cork, Clare, Kilkenny, Louth and Sligo only have one or two records (Table 2.3). However, the Sligo and Kilkenny records were only made five and thirteen years respectively before this study began and highlights that this species deserves specific

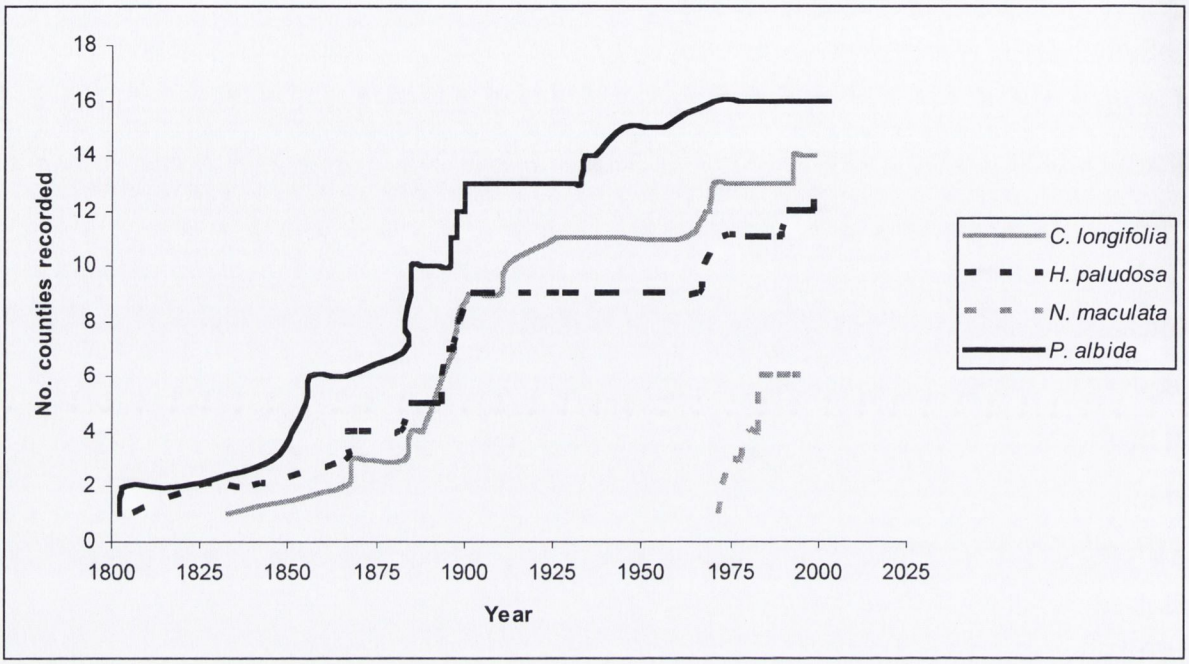
recording attention as it may have been over looked. *H. paludosa* was found in counties Dublin and Mayo during surveys and the statistical models suggest that it should be re-found in counties Carlow, Clare, Donegal, Galway, and Wicklow.

Sighting data is particularly scarce for *N. maculata* (Figure 2.3c) and the sighting rate model suggests that it is unlikely to be re-seen in county Clare. Paradoxically, this was the only county with reliable multiple sighting records (Table 2.4). This result is probably caused by a short observation period relative to the number of sightings made. *N. maculata* has only recently come to the attention of conservation authorities in Ireland as it is believed there is a reduction in its habitats (semi-natural calcareous grassland) in addition to its restricted distribution. Counties Cork, Donegal, Galway, Limerick, and Offaly only have one or two sightings, though models predict that it should be re-found in counties Cork and Donegal.

P. albida has the most complete sighting record of all focal species (Figure 2.3d). The models predict that it has likely been extirpated from counties Kerry, Laois, Mayo, Waterford, and Wicklow (Table 2.5). However, counties Cork, Laois, Limerick, Mayo, Monaghan, and Waterford have only one or two sightings per county. Based on the number of sightings and the time since the last sighting, *P. albida* is likely to be found in counties Cavan, Clare, Donegal, Galway, Leitrim, Limerick, Sligo, and Tipperary.

Figure 2.1. The cumulative number of counties occupied by focal species according to: (a) time in recording history and (b) number of sightings recorded

(a)



(b)

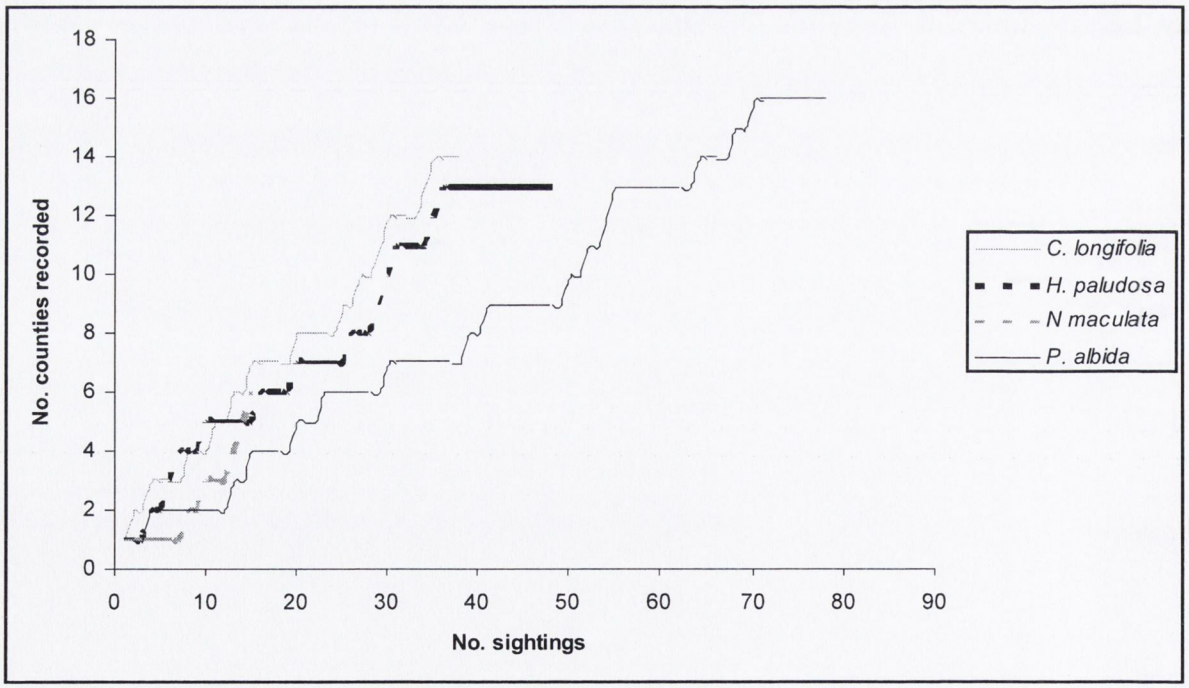


Table 2.1. Population data of focal species in the Republic of Ireland recorded during surveys between 2004-2006. Number of populations damaged refers to grazing (by cattle (c), sheep (s), or deer (d)) which decimated populations during flowering/fruiting.

Species	Year surveyed	Flowering time	No. counties visited	No. populations visited	No. populations recorded	No. populations damaged	Mean no. flowering individuals/populations (± S.E.)	Mean individual height (± S.E.)	Fruit se
C. longifolia	2005, 2006	May-June	4	7	4	2 (d)	28.8 (14.4)	27.6 (1.5)	Yes
H. paludosa	2004, 2005	July-August	2	2	2	0	49.3 (27.1)	5.6 (0.4)	Yes
N. maculata	2005, 2006	April-May	1	7	7	0	10.6 (4.8)	16.9 (0.8)	Yes
P. albida	2005, 2006	May-June	3	12	5	5 (s) and (c)	13.1 (5.9)	17.1 (0.6)	Yes

Table 2.2. Probability values generated by the various models based on sighting records of *C. longifolia* in the Republic of Ireland with values highlighted in bold significant ($p < 0.05$). [a]*n* was reduced to *n*-1 for these calculations [b]Sighting rate [c]C.I. = 95% confidence interval of T_E [d]? = value could not be calculated as $k < -(\log\alpha/2)$. ns = non significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

County	*n*	*T*	t_n	t_{n-1}	Solow 1993a[a]	Solow 1993b	Solow/Roberts	Weibull	SR[a,b]	Last Sighting	T_E (C.I.)[c,d]	χ^2	*p*
Carlow	1	119	0	0	-	-	-	-	-	1885	-	-	-
Clare	2	92	68	0	0.739	0.586	0.739	-	0.695	1980	-	3.01	ns
Cork	4	138	96	68	0.337	0.134	0.400	0.722	0.264	1962	2026 (1972, 4362)	11.34	ns
Donegal	3	171	160	55	0.875	0.919	0.905	0.998	0.871	1993	2299 (2084, *)	0.92	ns
Dublin	2	78	4	0	0.051	**0.020**	0.051	-	**<0.001**	1929	-	33.54	***
Galway	2	103	94	0	0.913	0.839	0.913	-	0.928	1995	-	0.87	ns
Kerry	5	136	131	34	0.861	0.986	0.951	1.000	0.856	1999	2263 (2140, 5274)	0.74	ns
Mayo	5	106	76	42	0.264	0.829	0.531	0.961	0.198	1974	2054 (2000, 2924)	7.62	ns
Roscommon	2	36	7	0	0.194	0.091	0.194	-	**0.011**	1974	-	20.37	**
Tipperary	2	34	8	0	0.235	0.115	0.235	-	**0.031**	1977	-	17.07	*
Waterford	1	110	0	0	-	-	-	-	-	1894	-	-	-
Westmeath	4	113	102	85	0.735	0.595	0.607	0.629	0.720	1993	2015 (1993, 3126)	4.24	ns
Wexford	1	11	0	0	-	-	-	-	-	1993	-	-	-
Wicklow	4	107	34	31	**0.032**	0.088	**0.039**	**0.046**	**0.001**	1931	1935 (1931, 2214)	38.21	***

Table 2.3. Probability values generated by the various models based on sighting records of *H. paludosa* in the Republic of Ireland with values highlighted in bold significant ($p < 0.05$). [a]*n* was reduced to *n*-1 for these calculations [b]Sighting rate [c]C.I. = 95% confidence interval of T_E [d]? = value could not be calculated as $k < -(\log\alpha/2)$. ns = non significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

County	*n*	*T*	t_n	t_{n-1}	Solow 1993a[a]	Solow 1993b	Solow/Roberts	Weibull	SR[a,b]	Last Sighting	T_E (C.I.)[c,d]	χ^2	*p*
Carlow	3	106	92	81	0.753	0.505	0.440	0.641	0.735	1990	2018 (1991, *)	5.08	ns
Cork	1	138	0	0	-	-	-	-	-	1866	-	-	-
Clare	2	138	126	0	0.913	0.840	0.913	-	0.909	1992	-	0.90	ns
Donegal	3	102	88	86	0.744	0.465	0.125	0.351	0.725	1990	2000 (1990, *)	9.02	ns
Dublin	6	110	104	69	0.755	0.947	0.882	0.999	0.744	1998	2098 (2031, 2812)	1.52	ns
Galway	4	109	85	71	0.474	0.679	0.368	0.123	0.422	1980	2022 (1980, 2384)	10.13	ns
Kerry	6	198	90	88	**0.019**	**0.003**	**0.018**	**0.012**	**0.002**	1896	1898 (1896, 1923)	48.85	***
Kildare	3	111	4	3	**0.001**	**<0.001**	**0.009**	0.058	**<0.001**	1897	1899 (1897, *)	56.56	***
Kilkenny	1	13	0	0	-	-	-	-	-	1991	-	-	-
Louth	1	31	0	0	-	-	-	-	-	1973	-	-	-
Mayo	4	36	31	20	0.639	0.653	0.688	0.885	0.601	1999	2017 (2001, 2619)	3.75	ns
Sligo	1	5	0	0	-	-	-	-	-	1999	-	-	-
Wicklow	13	179	170	115	0.538	0.667	0.859	0.936	0.517	1995	2030 (2000, 2155)	3.08	ns

Table 2.4. Probability values generated by the various models based on sighting records of *N. maculata* in the Republic of Ireland with values highlighted in bold significant ($p < 0.05$). [a]*n* was reduced to *n*-1 for these calculations [b]Sighting rate [c]C.I. = 95% confidence interval of T_E [d]? = value could not be calculated as $k < -(\log\alpha/2)$. ns = non significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

County	*n*	*T*	t_n	t_{n-1}	Solow 1993a[a]	Solow 1993b	Solow/Roberts	Weibull	SR[a,b]	Last Sighting	T_E (C.I.)[c,d]	χ^2	*p*
Clare	7	28	19	17	0.098	0.381	0.182	0.137	**0.033**	1995	2002 (1995, 2019)	20.78	*
Cork	2	31	21	0	0.677	0.476	0.677	-	0.614	1993	-	4.02	ns
Donegal	2	21	11	0	0.524	0.355	0.524	-	0.386	1994	-	6.56	ns
Galway	1	21	0	0	-	-	-	-	-	1983	-	-	-
Limerick	1	24	0	0	-	-	-	-	-	1980	-	-	-
Offaly	1	32	0	0	-	-	-	-	-	1972	-	-	-

Table 2.5. Probability values generated by the various models based on sighting records of *P. albida* in the Republic of Ireland with values highlighted in bold significant ($p < 0.05$). [a]*n* was reduced to *n*-1 for these calculations [b]Sighting rate [c]C.I. = 95% confidence interval of T_E [d]? = value could not be calculated as $k < -(\log\alpha/2)$. ns = non significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

County	*n*	*T*	t_n	t_{n-1}	Solow 1993a[a]	Solow 1993b	Solow/Roberts	Weibull	SR[a,b]	Last Sighting	T_E(C.I.)[c,d]	χ^2	*p*
Cavan	3	34	33	25	0.942	0.868	0.889	0.979	0.939	2003	2021 (2004, ?)	0.81	ns
Clare	9	153	142	101	0.551	0.798	0.788	0.982	0.528	1993	2054 (2006, 2301)	3.43	ns
Cork	2	159	55	0	0.346	0.209	0.346	-	0.148	1900	-	11.19	ns
Donegal	5	121	107	96	0.611	0.766	0.440	0.878	0.587	1990	2030 (1993, 2476)	4.87	ns
Dublin	3	200	152	136	0.578	0.916	0.250	0.661	0.528	1956	2941 (2064, ?)	6.15	ns
Galway	7	108	97	56	0.525	0.956	0.788	0.990	0.495	1993	2106 (2059, 2875)	3.28	ns
Kerry	9	149	49	48	**<0.001**	**<0.001**	**0.009**	**<0.001**	**<0.001**	1904	1905 (1904, 1920)	64.68	***
Laois	2	119	13	2	0.109	0.053	0.109	-	**<0.001**	1897	-	13.86	ns
Leitrim	9	70	69	68	0.891	0.682	0.500	0.428	0.884	2003	2004 (2003, 2025)	4.33	ns
Limerick	2	59	46	0	0.779	0.639	0.779	-	0.751	1991	-	2.47	ns
Mayo	2	121	9	0	0.074	**0.034**	0.074	-	**<0.001**	1891	-	30.99	***
Monaghan	1	104	0	0	-	-	-	-	-	1900	-	-	-
Sligo	9	119	116	115	0.815	0.472	0.250	0.161	0.810	2001	2002 (2001, 2018)	8.76	ns
Tipperary	4	106	95	93	0.719	0.347	0.154	0.248	0.703	1993	1997 (1993, 2496)	10.01	ns
Waterford	2	148	40	0	0.270	0.156	0.270	-	0.065	1896	-	14.42	ns
Wicklow	9	202	135	39	**0.039**	**<0.001**	0.590	**0.005**	**0.017**	1937	1938 (1937, 1960)	40.10	***

Figure 2.2. Level of threat inferred from analyses of sightings of four rare orchid species in the Republic of Ireland. Probability values <0.05 (dashed line) imply extinction. Solow (1993*a*; *b*); Solow/Roberts, (Solow and Roberts 2003); Weibull (Solow 2005); SR= Sighting Rate (McInerny *et al.* 2006).

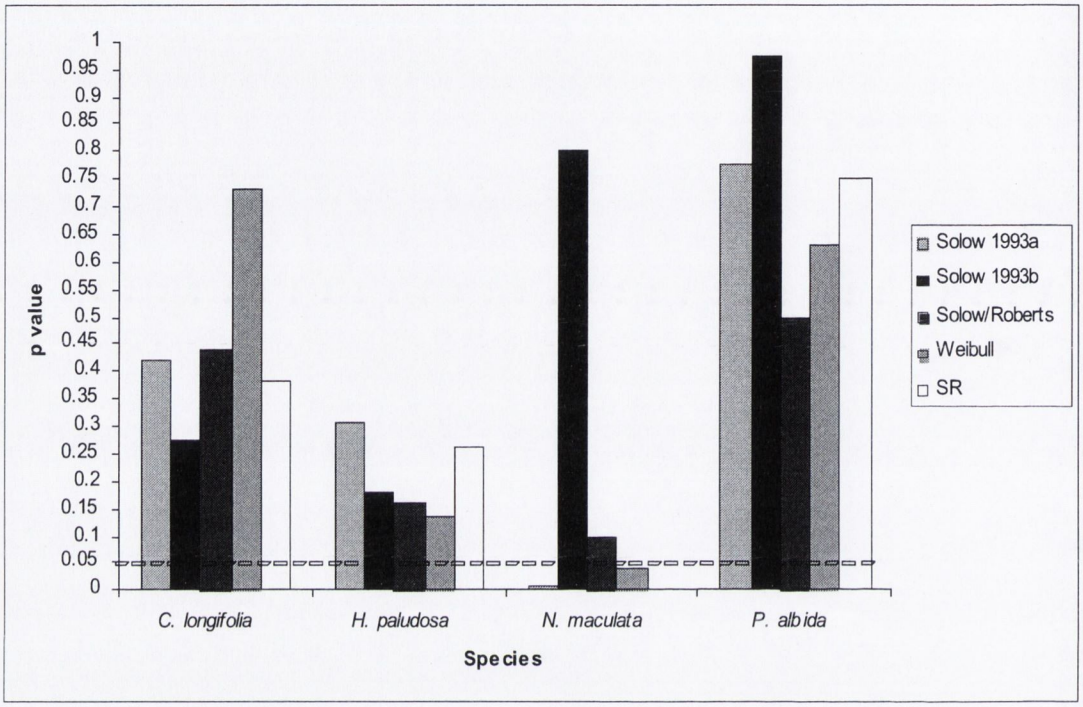
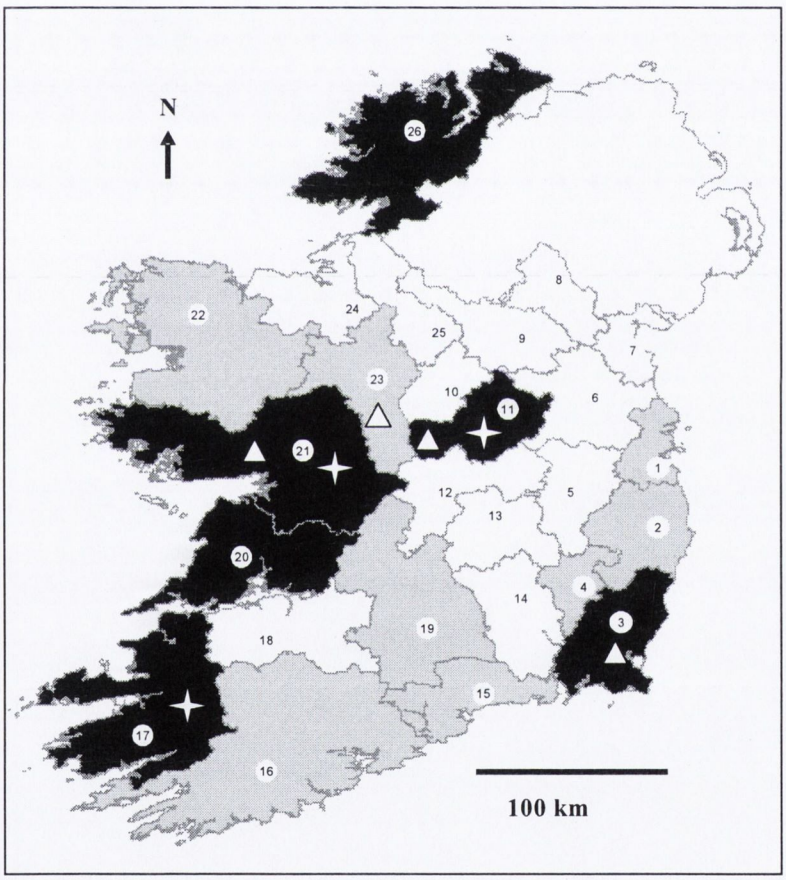
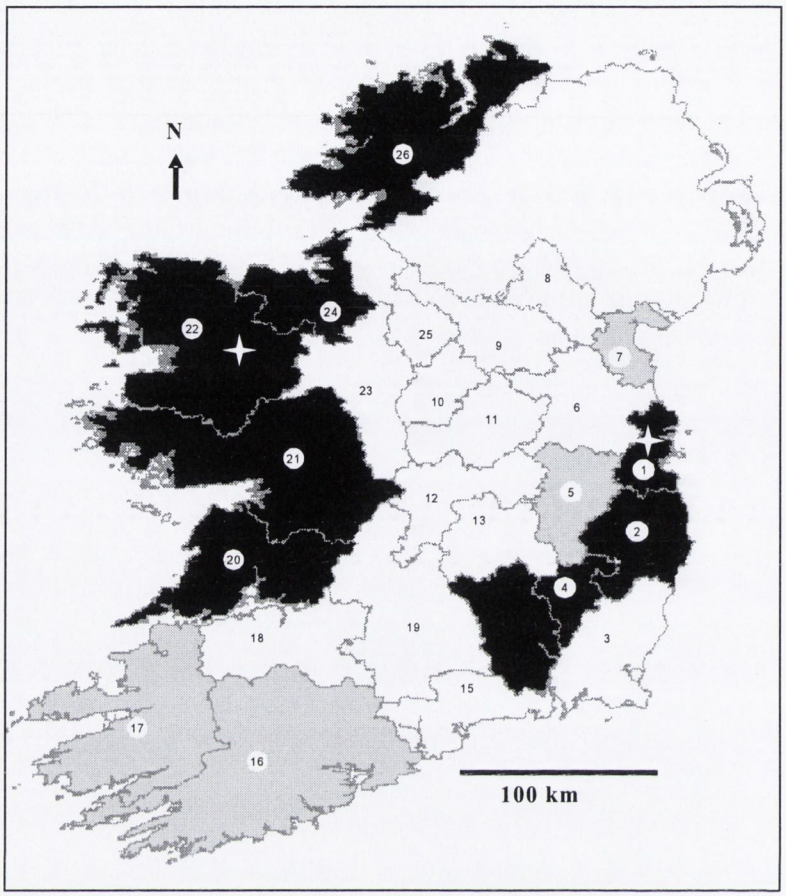


Figure 2.3. Distribution maps of focal orchid species in the Republic of Ireland: (a) *C. longifolia*, (b) *H. paludosa*, (c) *N. maculata*, (d) *P. albida* (black, counties with records after 1980; grey, counties with records before 1980; open star, counties surveyed during 2004-2006 where focal species were found; open triangles, counties where focal species were not found in 2004-2006. Stars do not indicate exact locations of populations within counties, and there maybe more than one population within a given county (1, Dublin; 2, Wicklow, 3; Wexford; 4, Carlow; 5, Kildare; 6, Meath; 7, Louth; 8, Monaghan; 9, Cavan; 10, Longford; 11, Westmeath; 12, Offaly; 13, Laois; 14, Kilkenny; 15, Waterford; 16, Cork; 17, Kerry; 18, Limerick; 19, Tipperary; 20, Clare; 21, Galway; 22, Mayo; 23, Roscommon; 24, Sligo; 25, Leitrim; 26, Donegal).

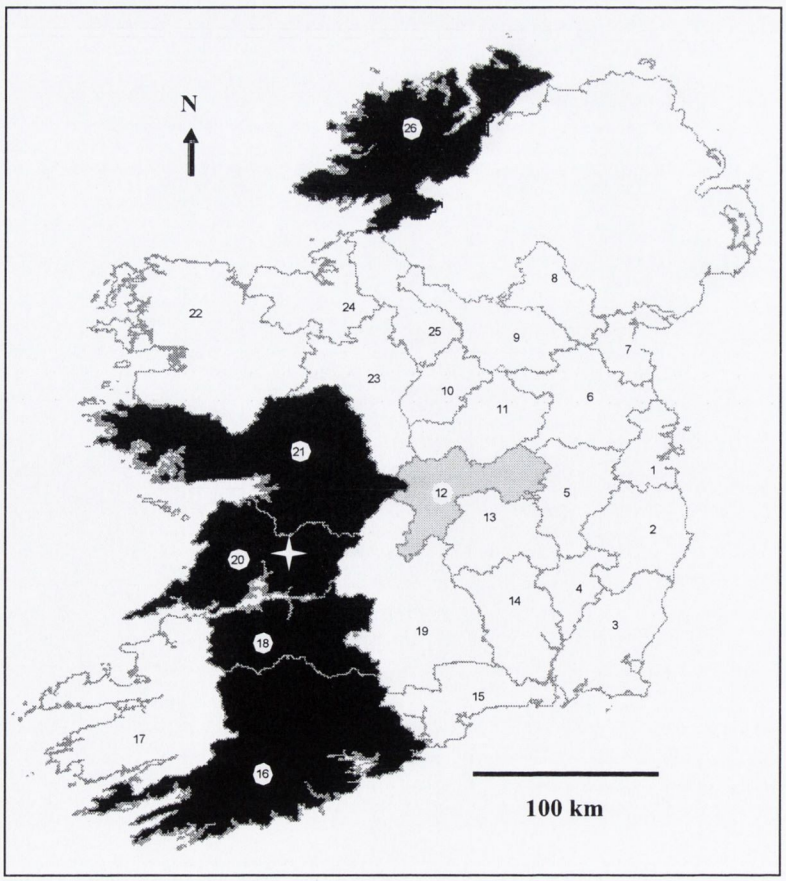
(a) *C. longifolia*



(b) *H. paludosa*



(c) *N. maculata*



(d) *P. albida*

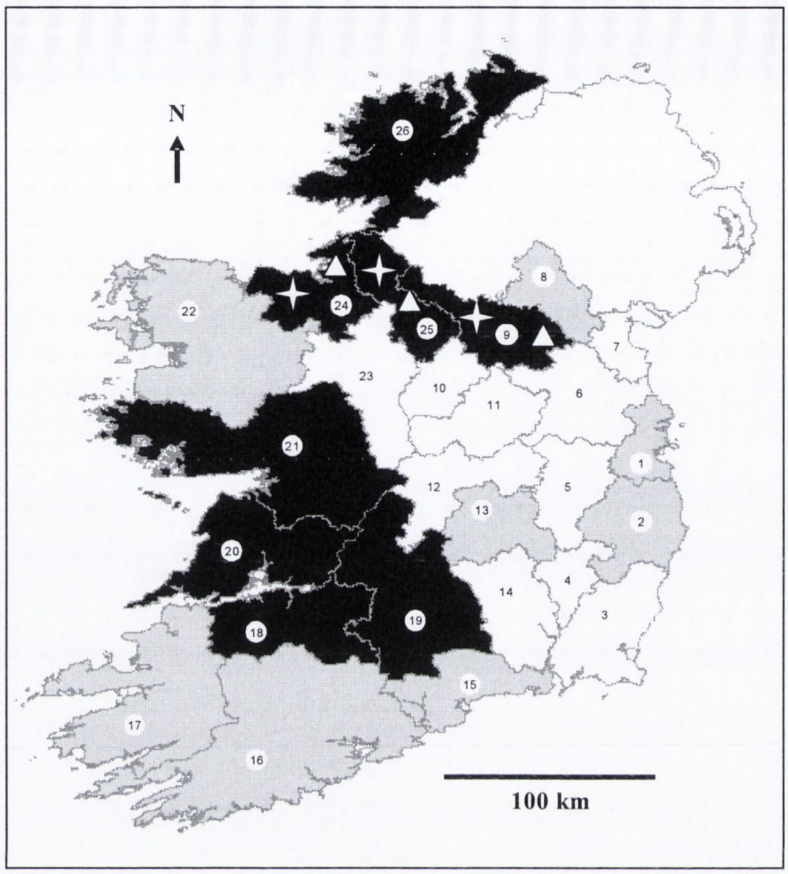


Table 2.6. Number of counties occupied by focal species and after rarefaction; calculated based on (a) total number sightings (values in brackets exclude *N. maculata* sightings), (b) pre-1980 sightings, and (c) post-1980 sightings.

	(a) Total		(b) Pre-1980		(c) Post-1980	
Species	No. counties	Expected (excl. *N. maculata*)	No. counties	Expected	No. counties	Expected
C. longifolia	14	9 (14)	13	13	6	6
H. paludosa	13	8 (12)	11	10	9	6
N. maculata	6	6 (-)	-	-	-	-
P. albida	16	9 (14)	16	13	8	4

2.4.3 Rarefaction analysis

Overall, based on the maximum number of sightings by which species can be compared ($n = 14$ for *N. maculata*), the expected number of counties in which all species should occur is similar; though between 5-7 counties less for *C. longifolia*, *H. paludosa*, and *P. albida* than they actually occur (Table 2.6). This reflects the limited distribution of *N. maculata* in Ireland rather than

recording bias or under recording. When *N. maculata* is excluded from this analysis, based on the number of sighting of *C. longifolia* ($n = 38$), the expected number of counties for *H. paludosa* and *P. albida* to occur is similar (Table 2.6). For pre-1980 records the expected number of counties is similar but only between 1-3 counties lower based on the number of sightings of *C. longifolia* ($n = 32$). There was no difference in the number of locations pre- and post-1980 for any species according to the Chi square test for homogeneity with Yates' correction for continuity (*C. longifolia* $\chi^2 = 2.39$; *H. paludosa* $\chi^2 = 0.05$; *P. albida* $\chi^2 = 1.49$; all $p>0.05$).

2.5 Discussion

2.5.1 Problems inferring extinction

Inference of extinction on the basis of a sighting record is problematic (Diamond, 1987; Solow and Roberts, 2003) due to several factors, including how sightings of a species vary through time (Solow, 2005) and the life history and biology of the species in question (Roberts, 2006). Statistical models used here are a useful tool in such circumstances when testing for extinction (Roberts and Kitchener, 2006). Previously a species was considered extinct when it had not been observed for 50 years (Reed, 1996). This assumption is, however, inappropriate for most taxa (Pimm, 2003; Roberts, 2006). Cryptic species occur in all natural communities, and there is always a chance that species will be overlooked in any survey (Reed, 1996). The IUCN Red List criteria of extinct requires that exhaustive surveys are conducted in known and/or expected habitat at appropriate times throughout its historic range (IUCN, 2001). Such surveys are largely impractical and unlikely to occur except for the most charismatic of species. Prematurely claiming extinction of a species in a given region on the basis of time since last sighting can undermine public support for conservation and lose the chance for last minute conservation intervention (Ladle *et al.*, 2004; Roberts and Kitchener, 2006). Caution should therefore be exercised when making claims of species extinction. In the same way care must also be taken when applying any statistical method and interpretation of results. For example, three models erroneously showed that *N. maculata* was extinct in Ireland (the sighting-rate, Solow 1993*a*, and Weibull models). Also, the estimation of extinction date assumes no further sightings will be made; hence estimates have relatively large confidence interval for T_E. In addition, the probability of future resighting in a county may not be accurate. *N. maculata* was inferred to be highly threatened with extinction in Clare even though this county contained the most populations of this species. Overall, apart from the sighting-rate, Solow 1993*a*, and the Weibull models for *N. maculata*, the models inferred that focal species in this study are not threatened with immediate extinction. In addition, many counties where species have not been seen for some time can still be expected to harbour populations, given suitable habitat. The value of using

different methods is that they detect different trends in the sighting process. This may be due to the power of some of the models, which has been addressed elsewhere (e.g., Solow, 1993*b*; McCarthy, 1998; Solow and Roberts, 2003).

One assumption of the sighting methods used here is that the sighting effort is constant or at least does not fall to zero. McCarthy (1998) proposed using an index of collection effort for each year, e_i, where the probability of an observation falling in year i is equal to e_i/E; E is the sum of all collection effort indices. However, this method will under estimate collection effort at the beginning and end of the sighting records. At the beginning of the sighting process not all species may have been discovered (McInerny *et al.*, 2006). If the species is unknown to science then it is less likely to be collected. Likewise at the end of the sighting record if species are becoming rare or extinct then the collection index, e_i, will fall even if the sighting effort remains the same. This is an area that requires further exploration. A further assumption is the underlying sighting process, whether it is a stationary Poisson process in the case of Solow (1993a) or say a Weibull distribution in the case of Roberts and Solow (2003) and its modification in (Solow, 2005). By using a number of different models it may be possible to elucidate the trajectory of the species being studied (Burgman *et al.*, 2000).

Ireland is a developed country with a limited flora (undoubtedly 815 native plant species, Curtis and McGough, 1988) and yet there is uncertainty regarding the threats facing many species. The records for these orchids are sparse and most likely to be incomplete. For example, in a given year a population may be searched for and it is either: (i) sighted, (ii) present but not sighted or (iii) extinct (McInerny *et al.*, 2006). In a binary record there is no difference between the latter two states; but also when a species has been sighted but not reported. Although I used records from the national database, there may be recorders who have seen these species and have not reported them. Also, the timing of a given survey must coincide with the flowering time of the focal species, which we ensured during our searches in the field. The county analysis applied here is used as opposed to the approach adopted by Preston *et al.* (2002) which involved placing a dot (decline in space) in each 10 x 10km square for a given time period (e.g. 1987-1999) irregardless of previous sighting rates.

2.5.2 Importance of methods for conservation

Orchids are dependent on pollinators for outcrossing and given recent concerns over major pollinator declines (e.g. Kearns *et al.*, 1998; Aguilar *et al.*, 2006) rare species which are dependent on pollinators may be particularly vulnerable to a lack of outcrossing (Spira, 2001).

Indeed, Biesmeijer *et al.* (2006) show there has been a parallel decline of insect-pollinated plants and pollinators in the modified landscapes of Britain and the Netherlands. However, as the flowering populations of focal species were generally small and their sites unmanaged it is difficult to assess the degree to which focal species rely on pollinators.

Declines in these species may be linked with habitat destruction. For example, *C. longifolia* has declined markedly in the 19th and 20th centuries due to cessation of woodland management (Preston *et al.*, 2002). Other factors may contribute to its rarity include grazing by deer during flowering and the increase of invasive species such as *Rhododendron ponticum* (Cross, 1982). *H. paludosa* is an inconspicuous species that occurs in flushes on remote bogs in Ireland. Therefore it probably suffers from under-recording across its range due to its remote habitat. Drainage of bogs, particularly in the lowlands, has caused a dramatic decline of this species. Intensive searches have shown it to be more frequent than previously thought (Preston *et al.*, 2002). *N. maculata* is restricted to the west of the country and flowers in low numbers in all populations observed. It may be under recorded due to its small stature. *N. maculata* is common in central Europe, though it is rare in Ireland with a restricted distribution and small populations where it may be constrained by its habitat (van der Cingel 1995; KJ Duffy *personal observation;* Chapter 5). Many lowland sites for *P. albida* were lost before 1930 due to habitat destruction, agricultural improvement and overgrazing (Preston *et al.*, 2002). *P. albida* needs a short grass sward, a thin litter layer, and some disturbance to create open patches for recruitment from seed (Reinhammar *et al.*, 2002), which did not occur in many sites visited during my surveys.

2.5.3 Conclusions

In conclusion, the use of multiple threat estimates and rarefaction analysis allows detection of overall threat of these species and regions in need of urgent surveying and/or management. Such methods detect different characteristics of the sighting and recording process and therefore should be used in tandem when making a threat evaluation of a species. However, caution must be used when interpreting the output of these models. My use of these models on both a national and regional scale, coupled with field observations, suggest that focal species in Ireland have undergone a decline, which may be linked with habitat destruction. Due to the small flowering populations observed for all species, the need for adequate monitoring programs, management, and data on their biology is urgently required in order to halt their further decline in Ireland.

"It is so good to stand still for a while with the flowers, to see what happens to them and how dependent they are on wind and weather, but many of them also on all kinds of animals. And what a nice contradiction between the silent flowers, that hardly move and the restless insects with their often lightning movement" – **J.P. Thijsse, De bloemen en haar vrienden.**

3. The effects of plant density and nectar reward on bee visitation to the endangered orchid *Spiranthes romanzoffiana*

In Press: Karl J. Duffy and Jane C. Stout. The effects of plant density and nectar reward on bee visitation to the endangered orchid *Spiranthes romanzoffiana*. *Acta Oecologica*

3.1 Abstract

Density can affect attraction of pollinators, with rare plants receiving fewer pollinating visits compared with more common co-flowering species. However, if a locally rare species is very attractive in terms of the rewards it offers pollinators, it may be preferentially visited. *Spiranthes romanzoffiana* is a nectar rewarding, geographically rare, endangered orchid species, which forms small populations in Ireland, co-flowering with more common, florally rewarding species. I examined visitation rates to *S. romanzoffiana* and two nectar rewarding co-flowering species (*Mentha aquatica* and *Prunella vulgaris*) in the west of Ireland. These three plant species were visited by three bee species (*Bombus pascuorum*, *B. hortorum* and *Apis mellifera*). *B. pascuorum* was the most common visitor, while *A. mellifera* was least common. My results suggest that individual *S. romanzoffiana* inflorescences compete intraspecifically for visitation from pollinators at high densities. The relationship between visitation to *S. romanzoffiana* and total floral density appeared to be positive, suggesting interspecific facilitation of pollinator visitation at high densities. Nectar standing crop varied through the season, among species and between open and bagged flowers. Nectar standing crop was not correlated with visitation in *S. romanzoffiana*. Despite relatively high visitation, *S. romanzoffiana* produced no mature fruit during this flowering season. The lack of fruit maturation in this species may be a major factor causing its rarity in Europe.

3.2 Introduction

The density at which plants occur within a population can affect reproductive success. Low density and fragmented populations of outcrossing, seed-producing plants may suffer from limited seed recruitment due to a number of factors including (i) a low number of potential mates (Alexandersson and Ågren, 1996), (ii) inbreeding depression caused by mating with closely related individuals (Fritz and Nilsson, 1994) and (iii) reduced attractiveness to pollinators (Sih and Baltus, 1987; Eriksson and Ehrlén, 1992; van Treuren *et al.*, 1993) and pollination limitation (Burd, 1994; Larson and Barrett, 2000; Spira, 2001). Reduced fecundity of plants in small populations will put them at greater risk from environmental change and other stochastic processes (Ellstrand and Elam, 1993; Kearns *et al.*, 1998). Plant density can be considered at two

scales: either the number of individuals in a local area (i.e. per population) (Kunin, 1993) or the number of populations in a geographic area (Hartley and Kunin, 2003). Rare plants may be either locally rare (a small number of individuals per population) or geographically rare (a low number of populations in a region), or both. In this chapter, I examine how geographical rarity affects pollination success within a locally abundant population.

Many rare plants, whether locally or geographically rare, occur in mixed arrays, often sharing pollinators with more common co-flowering species. Consequently, we must also consider the relative density and impact of co-flowering species on the pollination of rare plants. Individuals of a plant species that occur at low density may compete interspecifically with co-flowering species for pollinator attention (Waser, 1978; Rathcke, 1983; Bell *et al.*, 2005). This is because many pollinators tend to prefer abundant plant species and large floral displays in order to maximise foraging efficiency (Heinrich, 1979; Goulson, 1994; Stout *et al.*, 1998; Mitchell *et al.*, 2004). In addition, plants that occur at a low frequency and in a mixed arrays are more likely to suffer from improper pollen transfer (IPT) (the transfer of pollen to a stigma of a different species) and pollen wastage (Rathcke, 1983; Goulson, 1994; Stout *et al.*, 1998). Ultimately this can result in pollination limitation (Wilcock and Neiland, 2002). Alternatively, if a species is geographically restricted, but locally abundant, individuals may also face intra-specific competition for pollinator attention (Campbell and Motten, 1985; Kunin, 1993; Brown and Mitchell, 2001). In this case, although the population as a whole may receive higher rates of pollinator visitation, individuals may compete with one another for visitation, and mate with closely related individuals, which increases the likelihood of inbreeding (Ellstrand and Elam, 1993; Karron *et al.*, 2004). On the other hand, facilitation may occur whereby co-flowering conspecific or heterospecific plants form an increased floral display and all individuals receive increased visitation (Rathcke, 1983; Geer *et al.*, 1995; Moeller, 2004). This is the "magnet species" effect (Laverty, 1992), and is known mainly in nectarless orchids (e.g. Johnson *et al.*, 2003; Juillet *et al.*, 2007).

In addition to plant density, the quantity and quality of reward provided by a plant can affect the foraging behaviour of pollinators and consequently plant reproductive success. The quantity of nectar reward can affect how many visits a plant receives (attractiveness to pollinators) (Klinkhamer and van der Lugt, 2004) and the duration of visits (Pleasants and Zimmerman, 1979; Pleasants, 1989). However, Rodriguez-Robles *et al.* (1992) found that nectar availability was not a good predictor of effective pollinator visitation. The quality of nectar reward can affect the pollinator species attracted to individuals for example, bumblebees prefer

concentrated nectar, as dilute nectar can be heavy to carry and inefficient to store (Waser, 1983; Wilson *et al.*, 2006). The nectar reward of a rare species compared with more common co-flowerers may further affect its pollination success.

Terrestrial orchid populations often occur in mixed arrays, at variable densities, and with variable nectar rewards (approximately one-third of the Orchidaceae provide no nectar at all) (Nilsson, 1992; Ackerman *et al.*, 1994; Neiland and Wilcock, 1998). Hence orchids are particularly susceptible to competition with co-flowering species and IPT (Ratchke, 1983; Alexandersson and Ågren, 1996; Neiland and Wilcock, 1999). This can be particularly detrimental for orchids as their pollen are amassed as pollinia and so IPT may result in the loss of an individual flower's entire male complement (Dressler, 1981; Johnson *et al.*, 2005).

Spiranthes romanzoffiana is a nectar rewarding orchid species with an uneven amphi-Atlantic distribution (Forrest *et al.*, 2004). Individuals produce lateral buds in the autumn, which over-winter and develop into the following year's vegetative or flowering parts (Forrest *et al.*, 2004). *S. romanzoffiana* may reproduce vegetatively by infrequent production of twin (or, rarely, three to four) lateral buds, which can lead to the formation of multiple inflorescences (Summerhayes, 1968). It occurs widely in North America where it is pollinated by medium- and long-tongued bumblebees and solitary bees (Catling, 1983) but in Europe, it only occurs in Britain and Ireland. The percentage of flowers that set fruit has been reported at relatively high levels (>75%) in North America (Larson and Larson, 1987). Greenhouse hand pollination experiments in North America demonstrated that *S. romanzoffiana* was incapable of agamospermy (seed production without pollen) (Catling, 1982). However, this species is capable of autogamy (within flower self-pollination) with 64% fruit set, with both geitonogamy (within plant self-pollination) and xenogamy (outcrossing) pollination treatments, yielding 100% fruit set (Catling, 1982). In Britain and Ireland, *S. romanzoffiana* has never been reported to set fruit (Forrest *et al.*, 2004). However, Forrest *et al.* (2004) demonstrate that northern Scottish populations (from the Hebridean Islands) are genetically distinct from Irish populations. They suggest that reproduction (hence successful seed set) could be historical or current but infrequent. It is possible that infrequent seed set events may have been overlooked (Forrest *et al.*, 2004) or that the rarity of the species and associated pollinator limitation is responsible for the lack of fruit set.

Little is known of the pollination ecology of orchids in Ireland in general and *S. romanzoffiana* in particular. In Ireland, *S. romanzoffiana* is known from only nine 10 x 10 km

squares (Preston *et al.*, 2002) in the north and west of the country, and often occurs in very small populations. Hence this species can be considered both locally and geographically rare. *S. romanzoffiana* is protected in the Republic of Ireland by the Wildlife Act (1976) and the Wildlife Amendment Act (2000), under the Flora Protection Order (1999). In this study, I investigate the impacts of inflorescence density and nectar production by *S. romanzoffiana* and co-flowering species on pollinator visitation in Ireland. Specifically, I tested the following hypotheses:

1. Pollinator visitation to *S. romanzoffiana* is affected by its density and relative frequency compared with common co-flowering species.
2. Pollinator visitation to *S. romanzoffiana* is affected by nectar rewards, specifically:
   (a) *S. romanzoffiana* has nectar reward that is depleted by insect visitors
   (b) nectar levels vary over the flowering season.
   (c) there is a relationship between nectar standing crop and pollinator visitation rates.
3. Fruit set in *S. romanzoffiana* is related to visitation rates.

3.3 Materials and Methods

3.3.1 Study site

This study was conducted in a population of approximately 200 flowering *S. romanzoffiana* inflorescences (mean no. flowers per inflorescence (± S.D.) = 15.94 ± 5.51; n = 50) in a 1 km x 0.5km area in Co. Mayo, Ireland. This population was selected for study as it is the largest known population (other populations contain < 50, though occasionally up to 100, flowering individuals; KJ Duffy, *personal observation*) and plants formed naturally discrete patches of varying densities. The study site is adjacent to a lakeshore and is regularly flooded during winter months but remains dry during the summer. Other flowering vegetation at the site included *Mentha aquatica, Prunella vulgaris, Anagalis tenella, Leontodon hispidus, Lythrum salicaria,* and *Parnassia palustris*. *P. vulgaris* and *M. aquatica* were selected for further study as potential competitors/facilitators because they are also small herbaceous plants displaying flowers at the same time, in the same patches and at a similar height as *S. romanzoffiana* and flowers are visited by the same insect species foraging for nectar. Other co-flowering species were visited by the same species but to a much lesser extent (KJ Duffy, *personal observation*) and did not form dense patches alongside *S. romanzoffiana*. This study was carried out between July 27th and September 1st 2005.

3.3.2 Pollinator visitation

Six patches (3-4m × 3-4m) within the *S. romanzoffiana* population were selected according to natural density of *S. romanzoffiana* (ranging from 0.33 to 3.25 inflorescences per m^2). Twenty minute observations were carried out in these patches between 08.30-19.30 on 16 days over the flowering season. Observations were only made on days without rain or strong winds (mean temperature taken at ground level during observations (± S.E.) = 19.7 ± 0.18°C; range = 16–21°C; % cloud cover during observations (± S.E.) = 43.6 ± 4.4%; range = 0-100%). Observations of each patch were made randomly throughout the day to minimise the effect of time of day on data collected in a particular patch. All insects entering patches and visiting flowers probing for nectar or collecting pollen, and the number of inflorescences visited by each individual, were recorded. The relationships between the total number of visits to *S. romanzoffiana* (per inflorescence per hour) and density of *S. romanzoffiana* (inflorescences per m^2) and, separately, total flower density (inflorescences per m^2), were analysed using curve-estimation regression analysis. In addition, I controlled for the effect of *S. romanzoffiana* density in observational patches using partial correlation coefficients to examine the relationship between density of co-flowering species and visitation to *S. romanzoffiana*. These analyses were performed using SPSS 14.0.1© (SPSS Inc., 2005).

3.3.3 Pollinia removal and deposition

In order to measure pollinia removal and deposition in *S. romanzoffiana*, I examined 30 flowers from different inflorescences selected at random throughout the population on the 8th of August and 30 flowers on the 20th of August (which corresponds with the beginning and middle of flowering by *S. romanzoffiana*) for the presence or absence of pollinia.

3.3.4 Nectar measurements

On each day pollinator observations were made, 10 randomly selected flowers of *S. romanzoffiana*, *P. vulgaris,* and *M. aquatica* were sampled for nectar standing crop. Nectar was sampled using 1µl glass micropipettes (Drummond "Microcaps", Drummond Scientific Co., U.S.A.). On three days during the flowering season (one day at each of the beginning, middle and end), nectar was taken from flowers that had been bagged for 24 hours. Sugar concentration was measured using a hand-held refractometer modified for small volumes (Ceti-Digit-080, Carl Stuart, Ireland). To test whether standing crop varied over the flowering season among focal species, nectar volumes were compared according to species and period in the season (beginning, middle, and end). Two days within each of the beginning, middle and end of flowering season were selected and analysed using a three-factor mixed model ANOVA (n = 10) with day nested within period of season. To examine differences in nectar standing crop and volume in bagged

flowers among species, I used a three factor ANOVA (n=10), according to species, treatment (bagged vs. unbagged) and day. Nectar volume data were Ln(x+1) transformed prior to analyses where necessary (i.e. when variances were heterogeneous), and were analysed using GMAV 5 (Underwood and Chapman, 1997). Significant terms were analysed using *post hoc* Student-Newman-Keuls (SNK) tests to determine which means differed significantly from each other. To test for the effect of visitation on nectar standing crop I used Pearson correlation on means calculated from the pooled nectar standing crop and visitation rates over the flowering season. These data were analysed using SPSS 12.0.1© (SPSS Inc., 2003).

3.3.5 Fruit production

At the end of the flowering season fruits were sampled randomly from 1,000 individual *S. romanzoffiana* flowers.

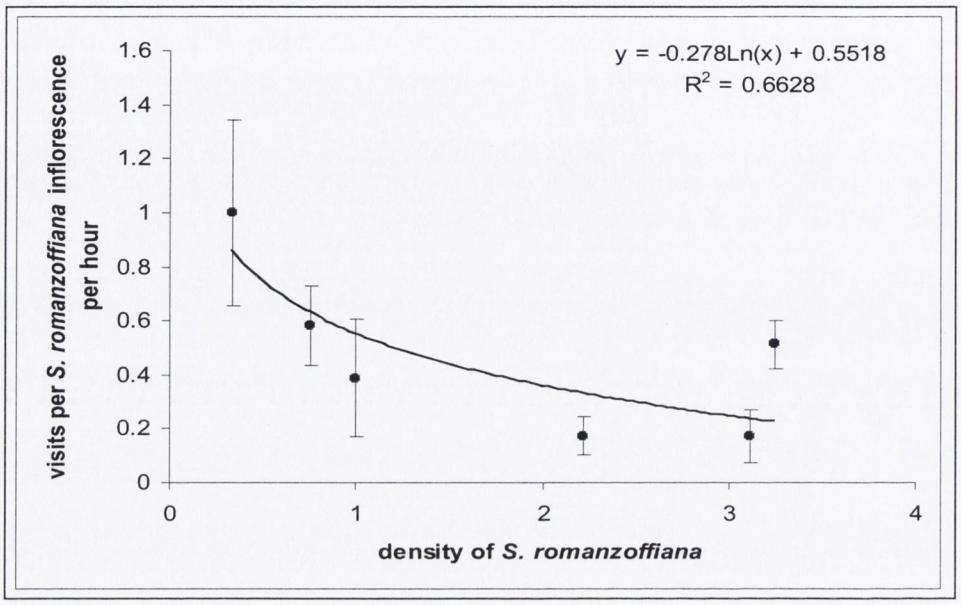
3.4 Results

3.4.1 Pollinator visitation

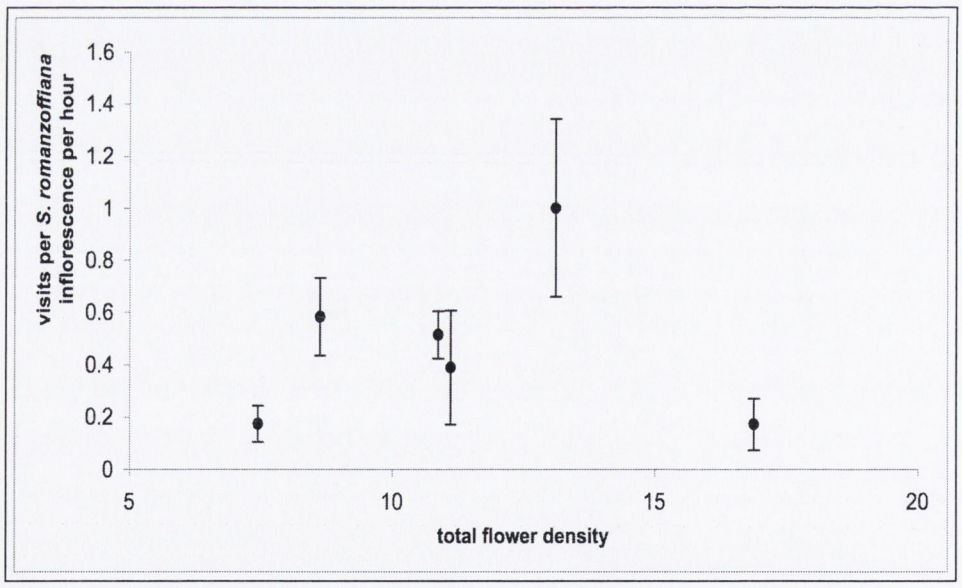
A total of 1,571 visits were observed to the three focal plant species in 21 hours of observations over the flowering season. *Bombus pascuorum* (medium-tongued bumblebee – plate 3.1) (81.47% of all visits ± S.E. 1.84%), *B. hortorum* (long-tongued bumblebee) (17.59% of all visits ± S.E. 1.2%), and *A. mellifera* (short-tongued honeybee) (0.94% of all visits ± S.E. 0.15%) were the only diurnal insects recorded visiting flowers of *S. romanzoffiana*, *P. vulgaris*, and *M. aquatica*. *B. pascuorum* was the most frequent visitor to all focal plant species. When data from all patches are pooled, the three focal species received similar visitation rates per inflorescence per hour (*S. romanzoffiana*: 0.52 visits per hour ± S.E. 0.083; *P. vulgaris*: 0.64 visits per hour ± S.E. 0.102; and *M. aquatica*: 0.73 visits per hour ± S.E. 0.073). Several Lepidopterans were present in the habitat but were not observed to visit the focal plant species. Visits by bumblebees occurred mainly between 10:30 and 17:00 h. I noted that visitors foraged for up to a maximum of 60 seconds per *S. romanzoffiana* inflorescence and visited a maximum of 10 *S. romanzoffiana* inflorescences in sequence. There was a significant negative relationship between density of *S. romanzoffiana* and visitation per *S. romanzoffiana* inflorescence per hour (visitation rate = -0.28 Ln(density) + 0.55, $R^2 = 0.6628$, $F_4 = 7.86$, $p = 0.049$; Figure 3.1a). The relationship between total floral density and visitation to *S. romanzoffiana* appeared to be positive, except in the patch with the highest floral density (Figure 3.1b).

Figure 3.1 Mean number of visits to *S. romanzoffiana* (per inflorescence per hour) (± S.E.) according to (a) density of *S. romanzoffiana* in patches (inflorescences/m^2) and (b) total floral density in patches (inflorescences/m^2).

(a).



(b).



However, none of the curve-estimation regression analyses fitted these data. In addition, when *S. romanzoffiana* density was controlled for, there was no relationship between density of co-flowering species and visitation to *S. romanzoffiana* ($r = 0.165$; $p = 0.068$).

3.4.2 Pollinia removal and deposition

I found pollinia had been removed from 9/30 flowers on the 8th August and 14/30 on the 20th August. Pollinia had been deposited on 1/30 stigmas on the 8th August and 7/30 on the 20th

August. Flowers with their own pollinia intact (i.e. those where the pollinia had not been removed) were not observed to have received pollinia deposition on their stigmas. Pollinia (though not always entire pollinia) were observed on 19 individuals of *B. pascuorum* in flight and 4 individuals of *B. hortorum*. Pollinia were visible on the proboscis and/or thorax of these species from within a 1m distance. No pollinia were seen on *A. mellifera* despite individuals being observed within similar distances. The mean corolla length of *S. romanzoffiana* (± S.E.) in this population is 9.9 mm ± 1.2 mm (n = 50), which may be too long for the tongue of *A. mellifera* (mean tongue length 6.5-6.7mm; Alpatov, 1929).

3.4.3 Nectar levels

There were significant differences in standing crop among the three focal plant species, with most nectar found in *S. romanzoffiana*, and least in *M. aquatica* (Table 3.1, Figure 3.2). There was no significant difference in nectar standing crop through the flowering season in any focal species (Table 3.1). When we examined differences in nectar standing crop and volume in bagged flowers among species, we found a significant interaction between plant species, treatment (bagged vs. unbagged) and day (Table 3.2). SNK post hoc tests revealed that all three species had more nectar in bagged compared with open flowers on day 2 (Figures. 3.2a, b and c). However, *P. vulgaris* had more nectar in open than bagged flowers on day 1. There were other variations among sampling days, but with no consistent pattern. *S. romanzoffiana* had more nectar in bagged flowers on all days compared with *P. vulgaris* ($p < 0.01$) and more than open flowers of *M. aquatica* on one sampling day. Mean sugar concentration (± S.E.) in *S. romanzoffiana* was 24% ± 0.4% (n = 43), 20% ± 0.3% (n = 16) in *M. aquatica*, and 23% ± 0.6% (n = 18) in *P. vulgaris*. No significant correlation was found between mean standing crop and mean number of visits over the flower season of *S. romanzoffiana* ($r = -0.25$; $p = 0.35$) nor in *M. aquatica* ($r = -0.05$; $p = 0.854$), however there was a significant positive correlation between mean standing crop and mean number of visits to *P. vulgaris* ($r = 0.609$; $p = 0.012$).

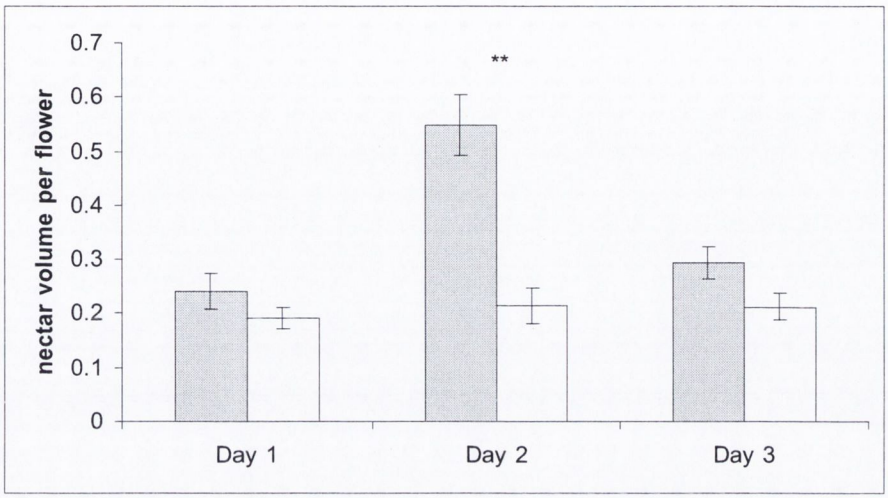
4.4 Fruit production

Fruit maturation was not observed in any *S. romanzoffiana* plants in the population. Capsules were observed to go flaccid and wither within one week after flowers withered.

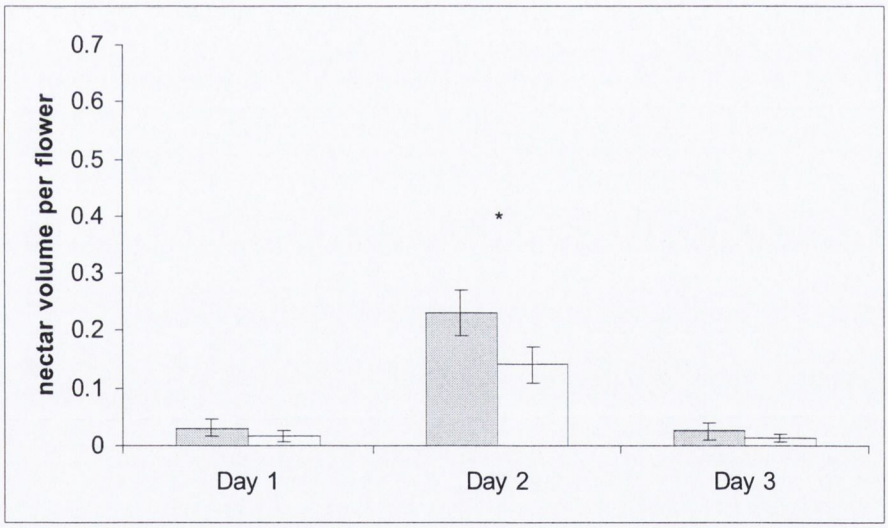
Plate 3.1 *Bombus pascuorum* foraging on an inflorescence of *S. romanzoffiana*

Figure 3.2: Mean nectar volume per flower taken at three days during the flowering season (± S.E.) (a) *S. romanzoffiana* (b) *M. aquatica* (c) *P. vulgaris*. Shaded bars represent bagged flowers; open bars represent standing crop. Significant differences according to SNK tests: * ($p < 0.05$) ** ($p < 0.01$).

(a)



(b)



(c)

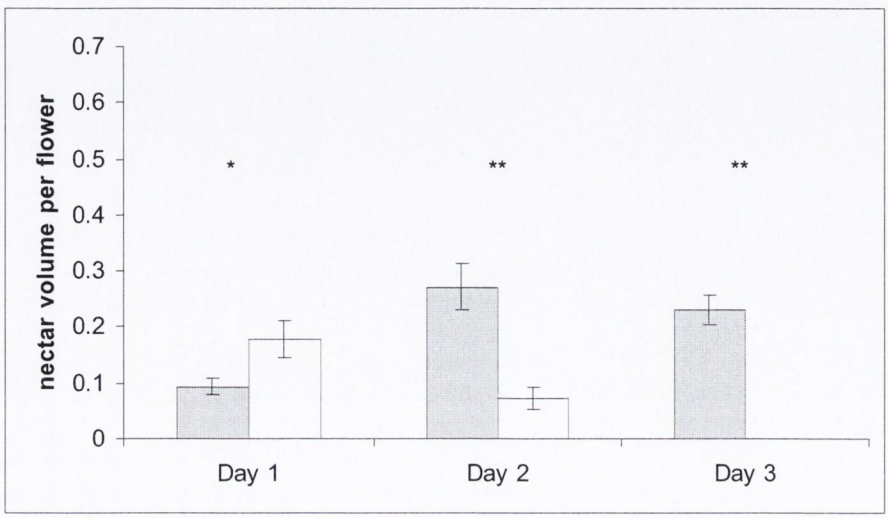


Table 3.1: Three factor mixed model ANOVA of nectar standing crop (microlitres/flower) according to focal plant species (*S. romanzoffiana*, *P. vulgaris* and *M. aquatica*), time in season (beginning, middle and end) and day (nested within time during season). Variances were significantly heterogeneous and so data were transformed using Ln(x+1) to reduce heterogeneity (Cochran's C = 0.1518, $p > 0.05$).

Source of variation	*df*	SS	MS	*F*	*p*
Species	2	1.53	0.765	57.09	0.0001
Time in season	2	0.054	0.027	5.38	0.1017
Day(Time in season)	3	0.015	0.005	0.76	0.5158
Species x Time in season	4	0.103	0.029	1.93	0.2249
Species x Day(Time in season)	6	0.08	0.013	2.02	0.0653
Error	162	1.072	0.006		

Table 3.2: Three factor ANOVA comparing nectar volume (microlitres/flower) in three plant species (*S. romanzoffiana*, *P. vulgaris* and *M. aquatica*), two treatments (bagged or unbagged), on three days. Variances were significantly heterogeneous and so data were transformed using Ln(x+1) to reduce heterogeneity (Cochran's C = 0.1518, $p > 0.05$).

Source of variation	*df*	SS	MS	*F*	*p*
Plant species	2	1.0252	0.5126	17.63	0.0104
Treatment	1	0.1983	0.1983	0.00	<0.0001
Day	2	0.4869	0.2434	8.37	0.0372
Treatment x Day	2	0.2162	0.1081	8.06	0.0395
Treatment x Plant species	2	0.0536	0.0268	2.00	0.2504
Day x Plant species	4	0.1163	0.0291	5.31	0.0005
Treatment x Plant species x Day	4	0.0536	0.0134	2.45	0.0483
Error	162	0.8866	0.0055		

3.5 Discussion

3.5.1 Pollinator visitation

Despite being nationally rare, *S. romanzoffiana* was relatively abundant in the study site and was frequently visited by medium-tongued (*B. pascuorum*) and long-tongued (*B. hortorum*)

bumblebees, and occasionally by short-tongued honeybees (*A. mellifera*). This is the first time that insect visitors to this orchid species have been recorded in Ireland. Only bumblebees were observed to pick up pollinia, and presumably these species act as the primary pollinators of *S. romanzoffiana*. However, although we cannot rule out the possibility that nocturnal insects also visit flowers, most records show that members of *Spiranthes* are adapted to bee pollination and even though some Lepidoptera may visit flowers, none have been recorded carrying pollinia (Catling, 1983; van der Cingel, 1995).

Orchids generally flower at low densities (Tremblay *et al.*, 2005) and inflorescence density has been shown to be an important factor that can influence pollinator activity on orchids (Meléndez-Ackerman and Ackerman, 2001; Internicola *et al.*, 2006; Juillet *et al.*, 2007). Indeed, Juillet *et al.* (2007) show that the nectarless *Traunsteinera globosa* has increased reproductive success in the presence of high densities of the rewarding *Trifolium pratense*. In this study, we found that at low density, *S. romanzoffiana* attracted more visits per inflorescence per hour than at high density. This inverse relationship suggests that in higher density patches, individual *S. romanzoffiana* inflorescences compete intraspecifically for pollinator attention. However, when we considered total floral density within patches (i.e. the total density of all three focal plant species), there appeared to be a trend for an increase in visitation to *S. romanzoffiana* with increasing total density, except at very high densities. This suggests that heterospecific inflorescences may facilitate visitation to *S. romanzoffiana*, up to a point, above which *S. romanzoffiana* competes with heterospecifics for pollinator attention (Rathcke, 1983). Indeed, previous studies have found that the presence of co-flowering species can increase pollinator visitation to rare species (Kwak *et al.*, 1991; Geer *et al.*, 1995; Gibson *et al.*, 2006). Recently, Ghazoul (2006) experimentally demonstrated interspecific facilitation for pollination with *Raphanus raphanistrum*: this species received higher pollinator visits and had increased seed set in the presence of co-flowerers than when it occurred alone. In addition, Ghazoul (2006) found that the balance between facilitation and competition was dependent on relative density of co-flowerers. As well as experiencing competition for pollinator visitation, plants that occur in mixed arrays at high densities may suffer from increased IPT (Heinrich, 1979; Stout *et al.*, 1998). I noted that focal plant species shared individual visitors and that switching between focal species occurred regularly at high densities (KJ Duffy; *personal observation*). However, levels of IPT were not quantified because it was not possible to sample stigmas of *S. romanzoffiana* due to its conservation status.

Despite relatively high visitation rates to *S. romanzoffiana*, only a small number of visitors may effectively pollinate the plants. Effective pollinators need to pick up pollinia from the anther and deposit it on a receptive stigma (Tepedino *et al.*, 1999). We believe effective pollination occurs in *S. romanzoffiana* in this population because pollinia were found on the probosci/thorax of visiting *B. pascuorum* and *B. hortorum* and on the stigmas of randomly checked flowers. Pollinia removal was greater than pollinia deposition suggesting that flower visitors are not entirely efficient at transferring pollinia. Previous studies have shown low pollen transfer is common in orchids; with pollinia removal in temperate, nectar rewarding species at 51.9% (n = 19 studies) and fruit set at 41.8% (n = 58 studies) (Tremblay et al., 2005). No pollinia were observed on stigmas of flowers that had their own pollinia intact: this is due to a protandrous mechanism to encourage outcrossing in *S. romanzoffiana*, which involves the position of the stigma changing when pollinia are removed from a flower (Catling, 1983; Sipes and Tepedino, 1995).

3.5.2 Nectar levels

Randomly selected *S. romanzoffiana* flowers contained more nectar (standing crop) than co-flowerers. Since all three species received a similar rate of insect visitation, this suggests that either *S. romanzoffiana* has a higher nectar production rate, or that insects do not completely drain *S. romanzoffiana* flowers of nectar, but do drain co-flowerers. Examination of bagged *S. romanzoffiana* flowers suggests the former, particularly in relation to *P. vulgaris* (there was more nectar in bagged flowers of *S. romanzoffiana* than bagged *P. vulgaris*). When I examined nectar depletion by comparing bagged and open flowers, I found evidence for nectar depletion (i.e. a difference between bagged and unbagged flowers) in all three species, supporting observations that insects utilised all three species in this site. Since few orchids offer a pollen reward for visitors (because pollen is amassed in pollinia) (Tremblay *et al.*, 2005 and references therein), *P. vulgaris* and *M. aquatica* (along with the other co-flowering species in the population) may be pollen sources for the bee visitors in addition to providing a nectar reward. Nectar sugar concentrations of focal plant species were similar throughout the flowering season (between 20 – 25%), which is attractive for bumblebees and is consistent with other nectar producing orchid species (e.g. 18-23% for *Satyrium hallackii*; Johnson, 1997).

Nectar standing crop and visitation rates were not correlated in *S. romanzoffiana* or *M. aquatica* but they were positively related in *P. vulgaris*. This is unusual because increased visitation should result in reduced nectar standing crops. Perhaps an increase in visitation causes an increase in nectar production in *P. vulgaris*, or an increase in production may be causing the

increase in visitation. Whether this directly affects visitation to *S. romanzoffiana* was not tested in this experiment and merits further investigation.

3.5.3 Fruit set

As orchids contain many dust-like seeds, fruit set is the conventional method to measure reproductive success (Neiland and Wilcock, 1998). Neiland and Wilcock (1998) show that fruit set for nectariferous orchids in Europe is approximately 63%. Despite hand pollination (KD Duffy; *personal observation*) and relatively high visitation over the flowering season, examination of more than 1000 flowers revealed that *S. romanzoffiana* fruit failed to swell in this population. This supports previous observations of no fruit set in this species in Europe, but my findings do not support the suggestion that *S. romanzoffiana*'s failure to produce fruit is due to pollinator limitation. Recent research has demonstrated that seeds, containing viable embryos, are present in the unripened capsules (O'Conner, 2006) and this warrants further investigation. Antlfinger and Wendel (1997) measured the role of floral photosynthesis on reproductive effort in *Spiranthes cernua* over it's flowering season, with the highest rates of photosynthesis occurring when the plants were in bud (9.2 μmol CO_2 m^{-2} s^{-1}) and the lowest for the infructescence (0.2 μmol CO_2 m^{-2} s^{-1}). Capsules of *S. romanzoffiana* were observed to wither within a week after flowering. A rapid reduction of photosynthesis at the end of flowering may affect the formation of mature fruits, though this is unlikely. Willems *et al.* (2001) show that experimentally shaded plants of *S. spiralis* developed more seeds than control plants. The reserves for fruit formation come from the current tuber, with seed maturation occurring when the infructescence is dying. The mechanism for the failure of fruit maturation in European *S. romanzoffiana* needs to be understood urgently for future conservation.

3.5.4 Conclusions

In conclusion, *S. romanzoffiana*, as well as being nationally rare, often occurs in small and low density populations. However, flowers are probably not pollination limited, as they are visited frequently by bumblebees and offer substantial nectar reward to these floral visitors. The failure of fruits to mature in this species may have some other cause. In addition, pollinator sharing among the three flowering species examined here may contribute to overall reproductive success.

"Fate is apparently not kind to the vast majority of pollen grains removed from flowers" – Stanton *et al.*, 1992.

4. Effects of population size, density, and co-flowering species on pollination in a common (*Spiranthes spiralis*) and an endangered (*S. romanzoffiana*) orchid

Submitted as: Karl J Duffy and Jane C. Stout. Effects of population size, density, and co-flowering species on pollination in a common (*Spiranthes spiralis*) and an endangered (*S. romanzoffiana*) orchid. *Journal of Ecology*.

4.1 Abstract

A comparative investigation was made of the effects of population size, patch density, and co-flowering species on the pollination of two congeneric, nectar-rewarding orchid species; *Spiranthes romanzoffiana*, which is endangered, and *S. spiralis*, which is more common. Both species are self-compatible but require pollinators for seed set. Two relatively large and two relatively small populations of each species were studied. Visitation to orchid flowers, pollinia removal and deposition, and fruit and seed set were recorded in replicate quadrats. To estimate potential competitive/facilitative interactions for pollinator attention, I quantified the local density of co-flowering species in replicate quadrats and examined their visitation rates in each population. Larger population size increased bee visitation rates to *S. romanzoffiana* but not *S. spiralis*. However, pollinia removal and deposition, and fruit set did not vary according to population size in either species. Within populations of *S. romanzoffiana*, increased density of co-flowering species enhanced pollinia removal. In addition, higher *S. romanzoffiana* density increased bee visitation rates and pollinia deposition in this species. With *S. spiralis*, higher visitation rates enhanced pollinia removal and higher density reduced pollen loss, which was reflected in increased fruit set. *S. romanzoffiana* flowered earlier in the season and its pollinia removal was facilitated by the presence of co-flowering species, while pollinia deposition was enhanced by bee visitation. Fruit and seed set were abundant in *S. spiralis*, which occurred in larger populations; this species may be more attractive to visitors and avoid competition from co-flowerers due to later flowering. This study highlights the importance of examining the effects of scale and floral abundance to interpret how various components of pollination affects reproductive success in both rare and common plants.

4.2 Introduction

Plants, being sessile, often rely on external vectors such as animal pollinators to achieve successful mating (Darwin, 1876; Barrett and Harder, 1996). A shortage of pollinators and/or

pollinator attention can cause pollination limitation (Moody-Weis and Heywood, 2001; Klein *et al.*, 2003; Knight *et al.*, 2005). Since pollinator activity can vary spatially, reproductive success is often highly variable within and among populations of a species (Dudash and Fenster, 1997; Goodwillie, 2001). Population size (the number of flowering plants in a population) plays an important role in the reproductive success of individuals (Rathcke and Jules, 1993; Kunin, 1997a). Large plant populations tend to be more attractive to pollinators than small ones as they contain greater numbers of flowering individuals (Knight, 2003). This collective large floral display is highly attractive to and maximises the time spent by pollinators in a particular area (Kunin, 1997b), which can lead to the increased reproductive success of individuals and the population as a whole.

In addition to population size, the density of plants within a population (number of flowering individuals per unit area) can affect reproductive success, with plants in lower density populations more pollen limited (Sih and Baltus, 1987; Knight, 2003). This is because lower density patches can increase travelling time for pollinators and decrease pollinator efficiency. Positive relationships between population density and the reproduction of individuals (Allee effects; Allee *et al.*, 1949) are thought to be widespread among flowering plants (Kunin, 1997a). Increases in density are likely to increase the probability that pollen will be transferred between conspecific individuals. Anthropogenic disturbances, such as fragmentation, can decrease both the size and density of a natural population (Fahrig, 2003). Therefore, knowledge of how population size and floral density affects plant-pollinator interactions is of great importance to conservation biologists (Kearns *et al.*, 1998; Wilcock and Neiland, 2002).

Co-flowering species are known to affect the behaviour of pollinators towards rare species, and species that occur at low densities (Kwak, 1991). Pollinators tend to prefer species that occur in dense patches (Heinrich, 1979; Goulson, 1994; Stout *et al.*, 1998; Mitchell *et al.*, 2004), and so a plant that occurs at low density within a patch with more dense or common co-flowering species may suffer from interspecific competition (Waser, 1978; Pleasants, 1980; Rathcke, 1983; Bell *et al.*, 2005). They also can be prone to pollination limitation resulting from improper pollen transfer (IPT) (the transfer of pollen between heterospecifics) and/or pollen wastage (Rathcke, 1983; Goulson, 1994; Stout *et al.*, 1998; Wilcock and Neiland, 2002). In addition, a geographically restricted species may also face intra-specific competition for pollinator attention if it forms dense patches within populations (Campbell and Motten, 1985; Kunin, 1993; Brown and Mitchell, 2001). Overall, the population may receive higher rates of

pollinator visitation, but individuals may compete with one another, and mate with closely related individuals, which increases the chance of inbreeding (Ellstrand and Elam, 1993; Karron *et al.*, 2004). Alternatively, facilitation can occur whereby co-flowering conspecific or heterospecific plants form an increased floral display and all individuals receive increased visitation (Rathcke, 1983; Geer *et al.*, 1995; Moeller, 2004). Such facilitation has been recently demonstrated in natural populations of a nectarless terrestrial orchid (Juillet *et al.*, 2007)

In addition to density, the number of populations in the geographical region a species occupies may affect its reproductive success (Kunin and Hartley, 2003). Hence, the classification of a species as 'rare' or 'common' can be taxon dependent and relative depending on the scale applied (Kunin and Gaston, 1993). Certain traits such as breeding system, reproductive investment, dispersal ability, and resource usage may differ between closely related common and rare species (Gaston and Kunin, 1997). For instance, Karron (1987) found that a rare species of *Astragalus* received lower pollinator attention than more widespread congeners. In addition, Rymer *et al.* (2005) found that rare species of *Persoonia* (*P. mollis* subsp. *maxima* and *P. glaucescans*) suffer from reduced reproductive success due to lower pollinator effectiveness when compared to more common congeners (*P. mollis* subsp. *nectans* and *P. lanceolata*). To date there have been few studies that compare similar aspects of the pollination biology of related common and endangered species (Bevill and Louda, 1999). Such studies can highlight the effect rarity has on the reproductive biology of an endangered plant species.

Plant species such as orchids provide a useful model for the study of pollination limitation as they require pollinators for outcrossing and are sensitive to environmental change (Nilsson, 1992; Silvertown *et al.*, 1994; Kull and Hutchings, 2006). In addition, orchids can be severely pollen limited within a flowering season (Johnson and Bond, 1997). Plants that are dependent on pollinators for outcrossing may be particularly vulnerable to pollen limitation when floral density decreases. Individuals occurring at low densities may suffer from insufficient pollen quantity if they attract fewer pollinators and/or receive fewer conspecific pollen grains per pollinator visit (Sih and Baltus, 1987; Feinsinger *et al.*, 1991). There may also be a negative relationship between female reproductive success and floral density, such as when there is increased competition among plants for both resources and pollinators (Fritz and Nilsson, 1994; Larson and Barrett, 1999; Baker *et al.*, 2000).

Terrestrial orchid populations often occur with other flowering species, at variable densities (Neiland and Wilcock, 1998; Juillet *et al.*, 2007; Duffy and Stout, 2008). Hence orchids are susceptible to competition with co-flowering species and IPT (Ratchke, 1983; Neiland and Wilcock, 1999), although the level of competition may depend on their density and whether they provide a reward (Internicola *et al.*, 2006). Competition can be particularly detrimental for orchids as their pollen are amassed as pollinia and so IPT may result in the loss of the entire male complement of an individual flower (Dressler, 1981), although orchids that have sectile pollinia can, in theory, transfer their pollen among many flowers (Johnson *et al.*, 2005). Consequently, the reproductive success of an orchid species may vary among populations within a flowering season (Fritz and Nilsson, 1994; Sipes and Tepedino, 1995).

Here I investigate two members of the orchid genus *Spiranthes*. Both share similar traits such as (i) self-compatibility (ii) bee pollination and (iii) a nectar reward (Catling, 1983; Willems and Lahtinen, 1997). *Spiranthes romanzoffiana* is considered endangered in the Republic of Ireland and is protected in the Republic of Ireland by the Wildlife Act (1976) and the Wildlife Amendment Act (2000), under the Flora Protection Order (1999). *Spiranthes spiralis*, on the other hand, is relatively common in Ireland, and is not legally protected. *Spiranthes* is a genus with a predominately North American distribution with only three species represented in Western Europe. In Ireland both flower relatively late in the season, but peak flowering for *S. spiralis* is approximately three weeks after *S. romanzoffiana*. Here we investigate the pollination ecology of these two orchid species in order to ascertain which factors influence their reproductive success.

Specifically, I test the following hypotheses:

Pollination and fruit set in both *S. romanzoffiana* and *S. spiralis* are affected by:

1. the size of the orchid population (number of flowering individuals)
2. the density of orchid flowers within a patch (number of flowers in a given area)
3. the density of co- flowerers present
4. the insect visitation rate to co-flowering inflorescences

4.3 Materials and methods

4.3.1 Study species and populations

Spiranthes romanzoffiana (Cham.) is perennial orchid that is widespread and frequent in North America, yet confined to the fringes of Ireland and Britain within Europe (Lupton, 2008). The

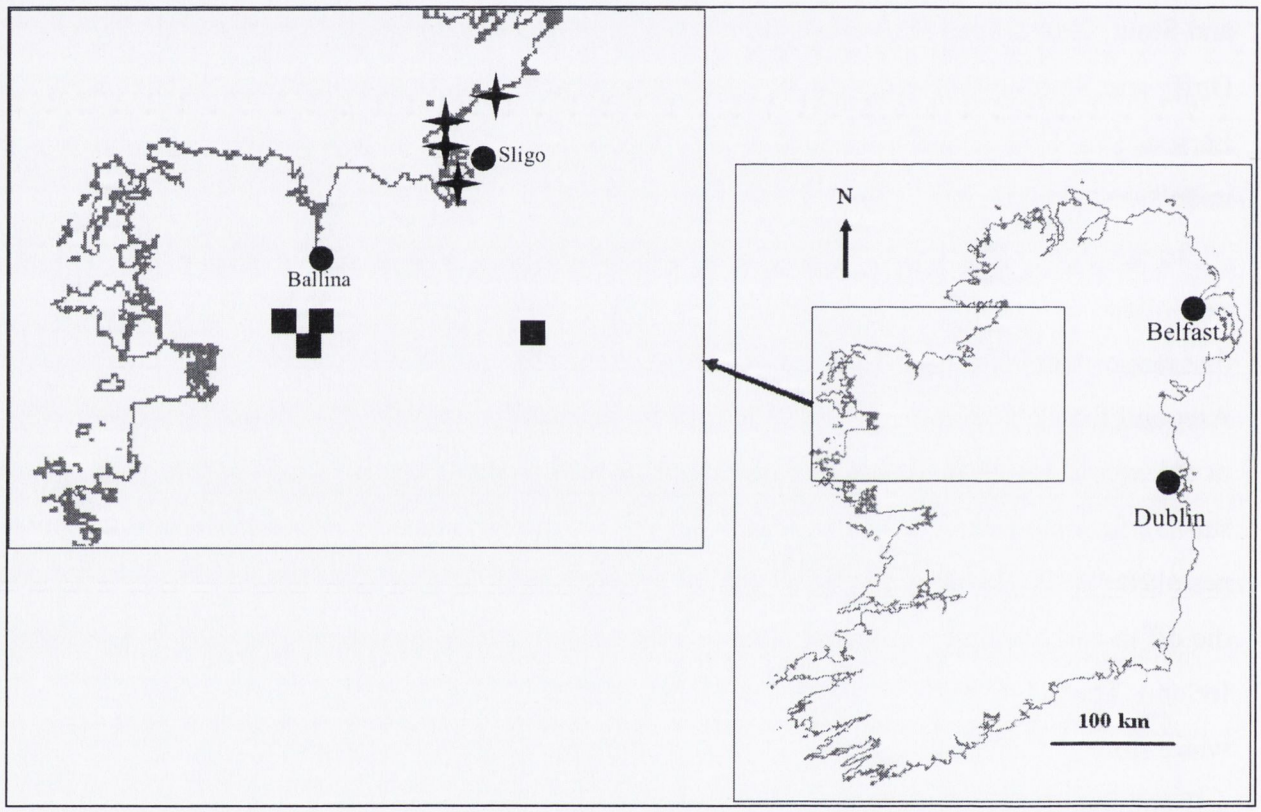
typical habitat is damp peaty meadows, pastures and heaths, especially areas susceptible to inundation of water such as lakeshores (Summerhayes, 1968; Lupton, 2008). Plants produce lateral buds in the autumn, which over-winter and develop into the following year's individuals. There is an occasional production of twin (rarely three or four) lateral buds, which can lead to the formation of multiple inflorescences (Summerhayes, 1968). *S. romanzoffiana* begins flowering in mid- to late-July through to the end of August in Ireland. The height of the inflorescence varies between 5 and 35cm and can bear up to 30 tubular white flowers in a three-ranked arrangement. Flowers are self-compatible, produce nectar and are mainly visited by bumblebees in both North America and Ireland (Catling, 1983; Larson and Larson, 1987; Duffy and Stout, 2008). Fruit set is variable; it is 0% in European populations (Forrest *et al.*, 2004; Duffy and Stout, 2008) while can be up to 75% in North American populations (Larson and Larson, 1987). The reasons for the lack of fruit set in European populations is not yet fully understood (Forrest *et al.*, 2004; Duffy and Stout, 2008; Lupton, 2008).

Spiranthes spiralis (L.) Chevall. is a small, long-lived perennial orchid with a distribution that ranges from North Africa through the Mediterranean north to Denmark and west to western Asia and Russia (Tutin *et al.*, 1980). It typically grows in relatively dry, nutrient poor meadows or calcareous grassland. It occupies open grazed areas with a constant land-use. Individual plants survive for may years and can consist of several rosettes and one or several belowground tubers per plant (Wells, 1967). Leaves are small (between 1 and 5cm long and 0.5 and 1.5cm wide) and die off in early summer. *S. spiralis* begins flowering in late August through to mid-September in Ireland. The height of the inflorescence varies between 5 and 25cm and can bear up to 25 small, white flowers that are arranged as a spiral on the upper half of the flowering stalk. Flowers are self-compatible, produce nectar and are mainly visited by bumblebees. Fruit set can be variable; between 0% and 78% (mean: 35%) (Willems and Lahtinen, 1997). Seeds are wind-dispersed, but most fall in the vicinity of the mother plants (Machon *et al.*, 2003).

This study was conducted in the west of Ireland in 2006. I selected four un-managed populations of varying size (based on the number of flowering orchids) of each of *S. romanzoffiana* and *S. spiralis* (see Figure 4.1). Populations of *S. romanzoffiana* were monitored from 25 July to 26 August and populations of *S. spiralis* were monitored from 2 September to 16 September, representing the entire flowering periods of each species in these sites. Populations of *S. romanzoffiana* contained 14, 21, 71, and 102 flowering individuals. Populations of *S. spiralis* contained 71, 81, approx. 250, and approx. 500 flowering individuals. Other common

flowering vegetation at sites containing *S. romanzoffiana* populations included *Leontodon hispidus*, *Lythrum salicaria*, *Mentha aquatica*, *Potentilla erecta*, and *Prunella vulgaris*. Other common flowering vegetation at sites containing *S. spiralis* populations included *Campanula rotundifolia*, *Leontodon autumnalis*, *Leucanthemum vulgare*, *Mentha aquatica*, *Senecio jacobea*, and *Trifolium repens*.

Figure 4.1. Location of study populations of *Spiranthes romanzoffiana* and *S. spiralis* in the Republic of Ireland; squares indicate *S. romanzoffiana* populations, stars indicate *S. spiralis* populations.

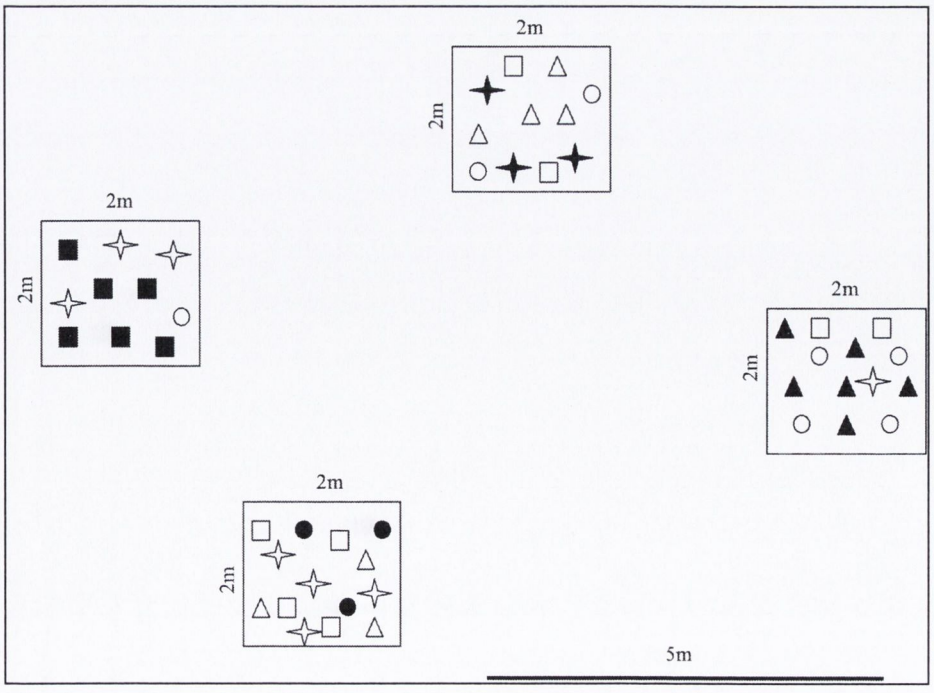


4.3.2 Visitation observations

Replicate 2m × 2m patches within each population were selected to represent the natural range in orchid density, determined by the number of flowering inflorescences. Fifteen-minute observations were made in each patch in dry weather throughout the flowering season. In addition, I selected neighbouring 2m × 2m patches of common co-flowering species (one patch per species) and recorded the number of open inflorescences of co-flowering species and insect visitation rates (Figure 4.2). Each patch was observed once during the flowering season (total number of patches observed was 40 for *S. romanzoffiana* populations, 15 for *S. spiralis*

populations). All insects entering patches, visiting flowers and probing for nectar and pollen, and the number of flowering units (individual flowers for *Spiranthes* spp.; individual flowers, inflorescences or capitula for co-flowering species, depending on the species) visited by each individual were recorded. I identified bee visitors to species level (except *Bombus lucorum/terrestris* complex, whose workers are indistinguishable in the field, Edwards and Jenner, 2005), but grouped other visitors as Lepidopterans, Syrphids or other Dipterans. As observations of direct insect visitation to orchids can be infrequent (Tremblay *et al.*, 2005) I also measured pollinia removal and deposition (presence/absence of massulae on the stigma) in *S. romanzoffiana* and *S. spiralis* using a 10 × hand lens or a 12 × head lens to examine all flowers from inflorescences within each patch at the end of each observation period. I calculated visitation rate per flowering unit per hour as the number of visits to flowering units in 15 mins/total number of flowering units in the patch × 4. One population of *S. spiralis* (71 individuals) was destroyed by cattle grazing during the course of this study, so this population was excluded from subsequent analyses.

Figure 4.2. Experimental design based on a hypothetical arrangement of three common co-flowering plant species along with the orchid species within study populations. This highlights the natural variability in species composition and density within study populations. Each shape represents a different flowering species. The shaded shape in each quadrat represents the species whose insect visitation was recorded for 15 minutes, with insect visitation to the other individuals not recorded. Each quadrat was observed once during the flowering season and its location marked. Therefore, each 2 × 2m quadrat represents an independent observation unit of pollinator visitation to each focal species within each population.



4.3.2 Breeding systems

Previous work has demonstrated *S. romanzoffiana* fruit do not mature despite hand pollinations and visitation from bees (K.J. Duffy, unpublished data). However, a related study on the breeding system of *S. romanzoffiana* showed that seed is present in unripened fruits (O'Conner, 2006) and so I did not repeat this experiment during this study. However, for comparison, I bagged 20 *S. spiralis* inflorescences in bud using a fine (1mm) mesh to exclude flower visitors. I performed hand-pollination experiments (using small wooden sticks to transfer pollen) with the following treatments: (i) facilitated autogamy (pollinia transferred within flower), (ii) geitonogamy (pollinia transferred between flowers on the same inflorescence), (iii) xenogamy (pollinia transferred from individuals at least 5 m away), and (iv) autonomous self-pollination (bagged inflorescences with unmanipulated flowers). I used a separate inflorescence for each treatment and treated five flowers per inflorescence (other flowers were removed by hand).

4.3.4 Fruit and seed production

At the end of the flowering season I recorded the fruit set in *S. spiralis* and *S. romanzoffiana* populations. As I used natural populations, which were openly grazed by cattle, I could not sample fruit from patches used for pollination observations due to grazing in many parts of study populations. However, with *S. spiralis* I tested for the effect of local density on fruit production. I arbitrarily defined relatively 'dense' patches (containing on a mean od more than 12.5 flowers/m^2) and relatively 'sparse' patches (containing a mean of less than 5 flowers/m^2) and sampled from within these patches to see if there are differences in fruit set according to density. Forty fruits were randomly collected from study populations of both *S. romanzoffiana* and *S. spiralis*, except the two small *S. romanzoffiana* populations (for conservation purposes as these populations were extremely small). Fruits were stored individually in separate plastic tubes at 4°C until counted. Seeds from each fruit were liberated by cutting with a scalpel and were spread evenly in a Petri dish. Any remaining seeds stuck in the capsule were washed out with 70% ethanol. Seeds were counted immediately as the alcohol can dehydrate embryos of the seeds if left for long periods. As orchid capsules contain thousands of seeds, to estimate the number of seeds per fruit, I sub-sampled the number of seeds from 10 mm × 10 mm squares using a 90 mm diameter circle of graph paper (to fit the underside of a Petri dish). I counted all the seeds within 10 randomly chosen squares using a × 10 dissecting microscope and multiplied the sum by the total number of squares occupied by seeds.

4.3.5 Statistical analysis

Visitation rate data were highly skewed with significantly heterogeneous variances and so were compared among populations for each species using non-parametric Kruskal-Wallis tests. Mean proportions of pollinia removal, and separately, pollinia deposition were compared among populations for each species using one-way ANOVA.

I used logistic regression to examine the relationship between: (i) the number of orchid flowers in a patch, (ii) the number of orchid inflorescences in a patch, (two metrics of local density) (iii) the visitation rate to orchid flowers, (iv) the mean number of co-flowering units and (v) the mean visitation rate to co-flowering units on the proportion of pollinia removed and the proportion of pollinia deposited for *S. romanzoffiana* and *S. spiralis*. In addition, I calculated the proportion of pollen loss from each patch by: 1 - D/P, where D is the number of flowers with pollinia deposited and P is the number of flowers with pollinia removed. I examined, separately, the relationship between the proportion of pollen loss and the five explanatory variables for both species. Binomial or quasi-binomial errors (when residual deviation was greater than residual degrees of freedom) were used in all regression analyses (Crawley, 2005).

Proportion of fruit set in *S. spiralis* was compared among populations using one-way ANOVA. The proportion fruit set within populations of *S. spiralis* was analysed using logistic regression with binomial errors, with patch size as a categorical explanatory variable. Seeds per fruit were compared among treatments (facilitated autogamy, geitonogamy, xenogamy, spontaneous autogamy and open pollination) using a one-way ANOVA. All data analysis was carried out using R 2.6.1 (R Core Development Team, 2007).

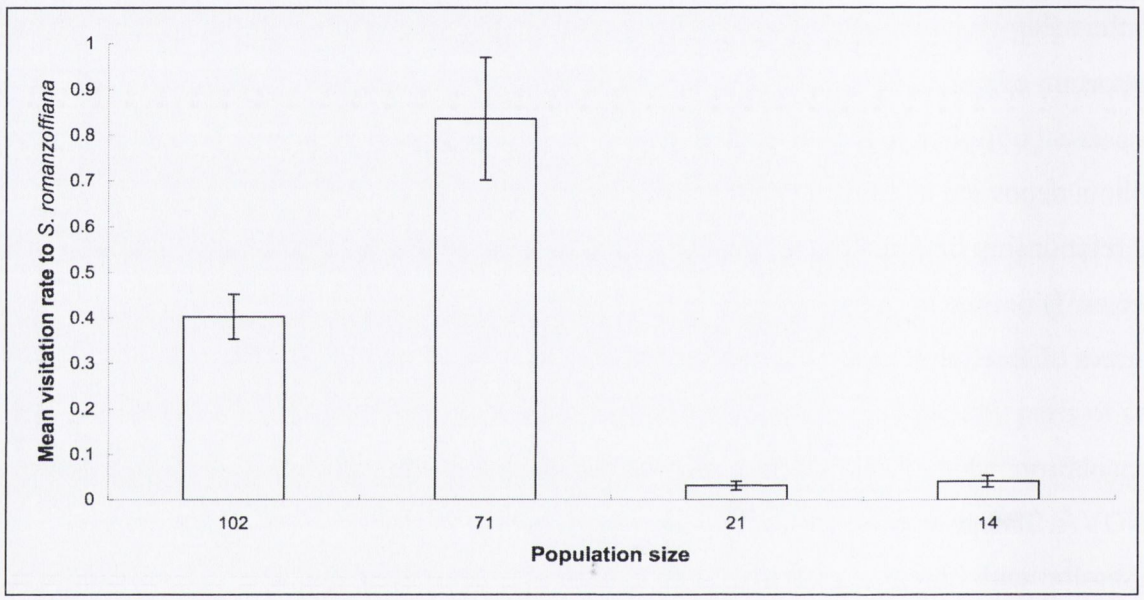
4.4 Results

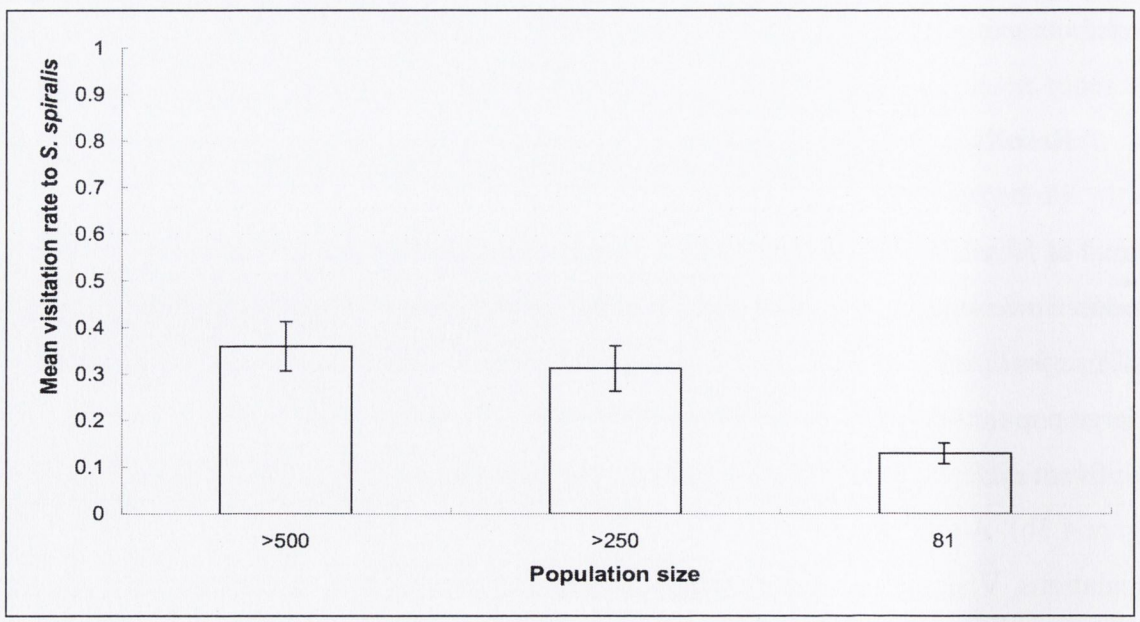
4.4.1 Visitation rates

A total of 52 visits were observed to *S. romanzoffiana* flowers and 48 visits were observed to *S. spiralis* flowers during a total of 13.75 hours of daytime observations. Only bees (*Bombus* spp. and *Apis mellifera*) were observed visiting both species. Visitation rates were significantly higher in large populations of *S. romanzoffiana* ($H_3 = 8.63$, $p = 0.035$; Figure 4.3a). There were no significant differences in visitation rates among populations of *S. spiralis* ($H_2 = 0.23$, $p = > 0.05$; Figure 4.3b). A total of 1,607 visits to co-flowering species were recorded in *S. romanzoffiana* populations. Visitation to co-flowering species in *S. romanzoffiana* sites comprised a range of insect taxa: *Bombus pascuorum* (36.4%), *B. terrestris/lucorum* (27.4%), *Apis mellifera* (16.3%),

Syrphids (8.2%), *B. hortorum* (5.2%), Lepidopterans (0.6%), and Dipterans (5.9%). A total of 373 visits to co-flowering species were recorded in *S. spiralis* populations. Visitation to co-flowering species in *S. spiralis* sites also comprised a range of taxa: *B. pascuorum* (33.8%), *B. terrestris/lucorum* (32.2%), *A. mellifera* (22.5%), Syrphids (8.1%), Dipterans (2.7%), and Lepidopterans (0.8%).

Figure 4.3. Mean (±SE) visitation rates (visits per flower per hour) to (a) *S. romanzoffiana* and (b) *S. spiralis* populations. Population size is given as the number of flowering inflorescences per population at time of sampling.





Pollinia removal and deposition occurred in all populations of both species. There were no significant differences in proportion of pollinia removal or deposition among populations in either species (*S. romanzoffiana*: $F_{3,36} = 1.56$; $p > 0.05$; *S. spiralis*: $F_{2,12} = 0.71$; $p > 0.05$). Data were therefore pooled for all populations for regression analyses.

Logistic regression analyses revealed that for *S. romanzoffiana*, there was a significant positive relationship between the number of co-flowering units and the proportion of pollinia removed (Table 4.1a; Figure 4.4a). There were also significant positive relationships between the proportion of flowers with pollinia deposited and both the visitation rate to flowers and the number of inflorescences in a patch (Table 4.1b; Figure 4.4b; Figure 4.4c). However, there was no relationship between the explanatory variables and the proportion of pollen loss (Table 4.1c). With *S. spiralis*, there was a significant relationship between the proportion of flowers with pollinia removed and the visitation rate (Table 4.2a; Figure 4.4d), but no relationship between pollen deposition and any of the tested variables (Table 4.2b). However, there was a significant negative relationship between the proportion of pollen loss and local floral density as measured by both number of flowers and number of inflorescences per patch (Table 4.2c). Since the number of flowers and number of inflorescences in a patch were correlated ($r = 0.838$; $p < 0.001$), only the effect of the number of inflorescences on pollen loss is shown (Figure 4.5).

4.4.2 Fruit set

Although capsules failed to mature, randomly selected withered fruits of *S. romanzoffiana* from the two large populations contained seed. The estimated number of seed in these fruits of *S. romanzoffiana* ranged from 0 – 473 (mean = 63.42; S.E. = 3.67; N = 80). There were no differences in seed production between the two populations (Wilcoxon rank sum test: $W = 1760.5$; $p > 0.05$) There were no differences according to population size in the proportion of fruit set in *S. spiralis* ($F_{2,117} = 2.59$; $p > 0.05$) or in the estimated number of seeds per fruit ($F_{2,117} = 0.05$; $p > 0.05$). However, there was a significant difference in the proportion of fruit set between large and small patches within populations, with small patches having proportionally less fruit set ($\beta = -0.268$; $df = 91$; $p = 0.014$) (Figure 4.6). There was no correlation between the proportion of fruit set in *S. spiralis* and the number of seeds per fruit ($r = -0.28$; $p > 0.05$).

Table 4.1. Logistic regression models for factors influencing (a) proportion of flowers with pollinia removed, (b) proportion of flowers with pollinia deposited, and (c) pollen loss in *S. romanzoffiana*. Models assume quasi-binomial errors. *df* = 38. †: Values log transformed.

(a)

Predictor	β	S.E. β	e^{β} (odds ratio)	p
No. flowers per patch	0.004	0.023	1.00	0.861
No. inflorescences per patch	0.040	0.124	1.04	0.746
Visitation rate	0.222	0.174	1.25	0.210
Mean no. co-flowering units†	0.450	0.181	1.19	0.017
Mean visitation rate to co-flowering units	-0.021	0.026	0.98	0.430

(b)

Predictor	β	S.E. β	e^{β} (odds ratio)	p
No. flowers per patch	0.058	0.033	1.06	0.089
No. inflorescences per patch	0.325	0.148	1.38	0.034
Visitation rate	0.433	0.173	1.54	0.017
Mean no. co-flowering units	-0.012	0.027	0.99	0.664
Mean visitation rate to co-flowering units	0.016	0.034	1.02	0.640

(c)

Predictor	β	S.E. β	e^{β} (odds ratio)	p
No. flowers per patch	-0.059	0.034	1.03	0.078
No. inflorescences per patch	-0.244	0.134	1.28	0.069
Visitation rate	-0.261	0.169	1.18	0.122
Mean no. co-flowering units	0.040	0.030	1.03	0.183
Mean visitation rate to co-flowering units	-0.027	0.031	1.03	0.532

Table 4.2. Logistic regression models for factors influencing (a) proportion of flowers with pollinia removed, (b) proportion of flowers with pollinia deposited, and (c) pollen loss in *S. spiralis*. Models assume quasi-binomial errors. *df* = 13.

(a)

Predictor	β	S.E. β	e^{β} (odds ratio)	p
No. flowers per patch	-0.002	0.009	0.99	0.797
No. inflorescences per patch	0.044	0.053	1.04	0.410
Visitation rate	1.879	0.757	6.55	0.027
Mean no. co-flowering units	-0.063	0.102	0.94	0.548
Mean visitation rate to co-flowering units	0.144	0.225	1.15	0.532

(b)

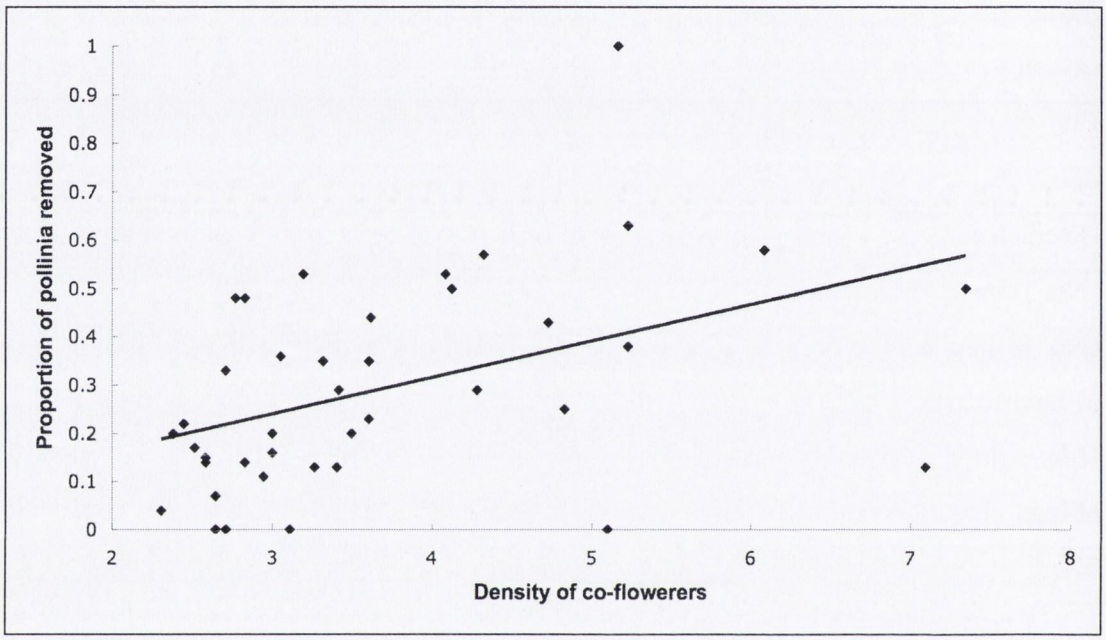
Predictor	β	S.E. β	e^{β} (odds ratio)	p
No. flowers per patch	0.023	0.015	1.02	0.158
No. inflorescences per patch	0.181	0.089	1.19	0.065
Visitation rate	1.102	1.311	3.01	0.416
Mean no. co-flowering units	-0.349	0.209	0.71	0.119
Mean visitation rate to co-flowering units	0.120	0.373	1.13	0.752

(c)

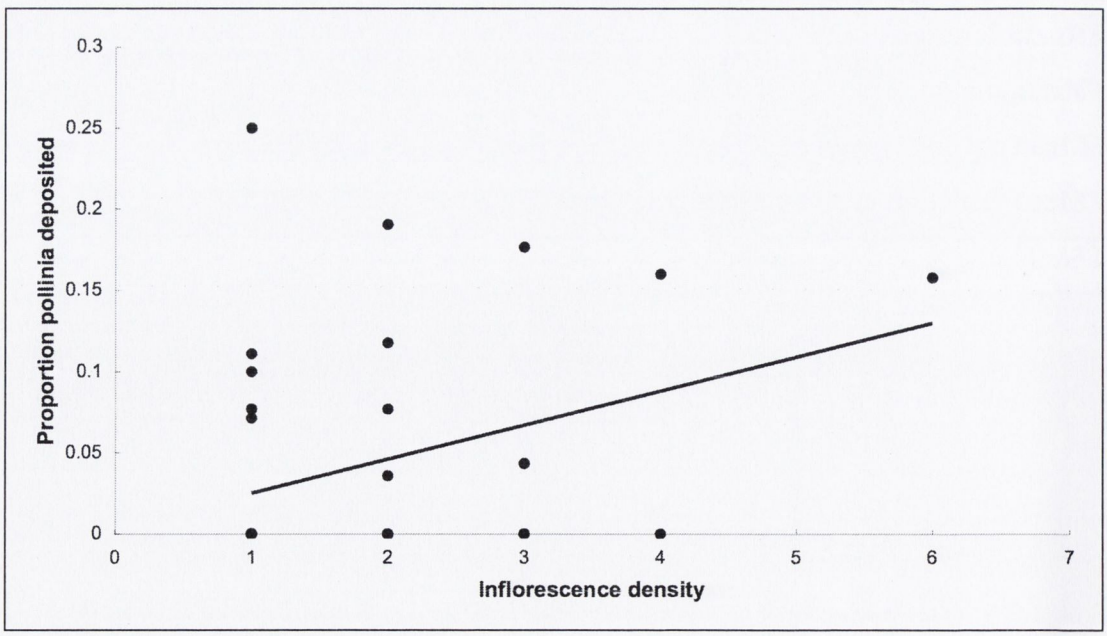
Predictor	β	S.E. β	e^{β} (odds ratio)	p
No. flowers per patch	-0.025	0.010	1.03	0.034
No. inflorescences per patch	-0.123	0.054	1.13	0.041
Visitation rate	-0.522	0.498	1.65	0.266
Mean no. co-flowering units	0.259	0.135	1.30	0.076
Mean visitation rate to co-flowering units	-0.02	0.242	1.02	0.909

Fig. 4. The positive relationship between; (a) the proportion of *S. romanzoffiana* flowers with pollinia removed and the density of co-flowering units (log transformed), (b) the proportion of flowers with pollinia deposited and the number of *S. romanzoffiana* inflorescences in a patch, (c) the proportion of flowers with pollinia deposited and the visitation rate to *S. romanzoffiana* flowers (we tested this relationship including and excluding the presence of an influential outlier and the relationship was still positive, so we retained this data point), and (d) the proportion of flowers with pollinia removed and the visitation rate to *S. spiralis* flowers. See Tables 1 and 2 for statistical output.

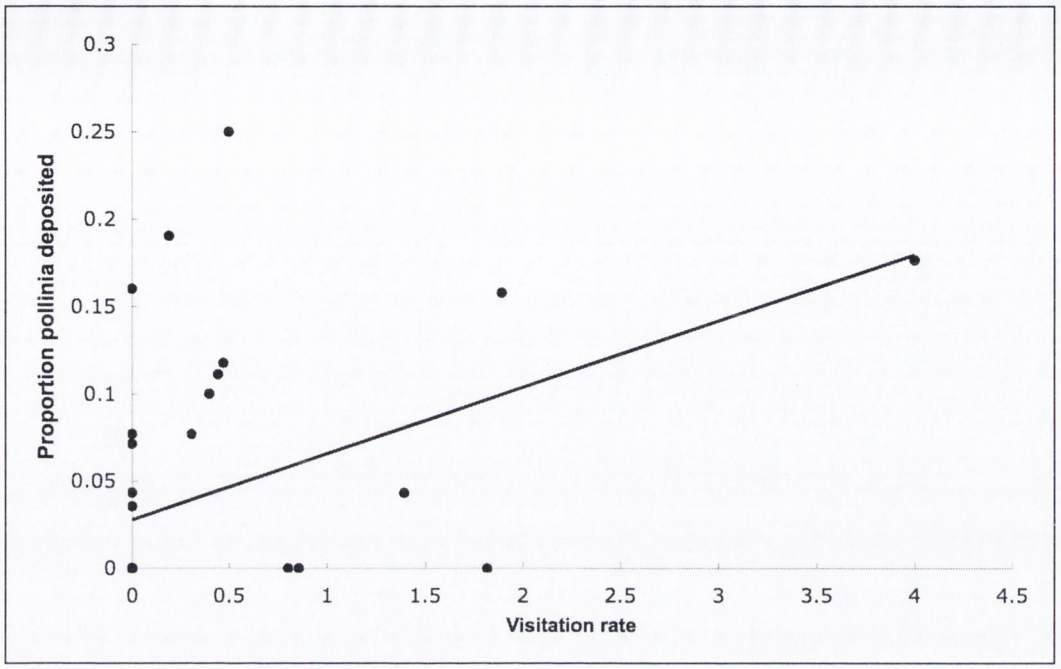
(a)



(b)



(c)


Proportion pollinia deposited
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Visitation rate

(d)

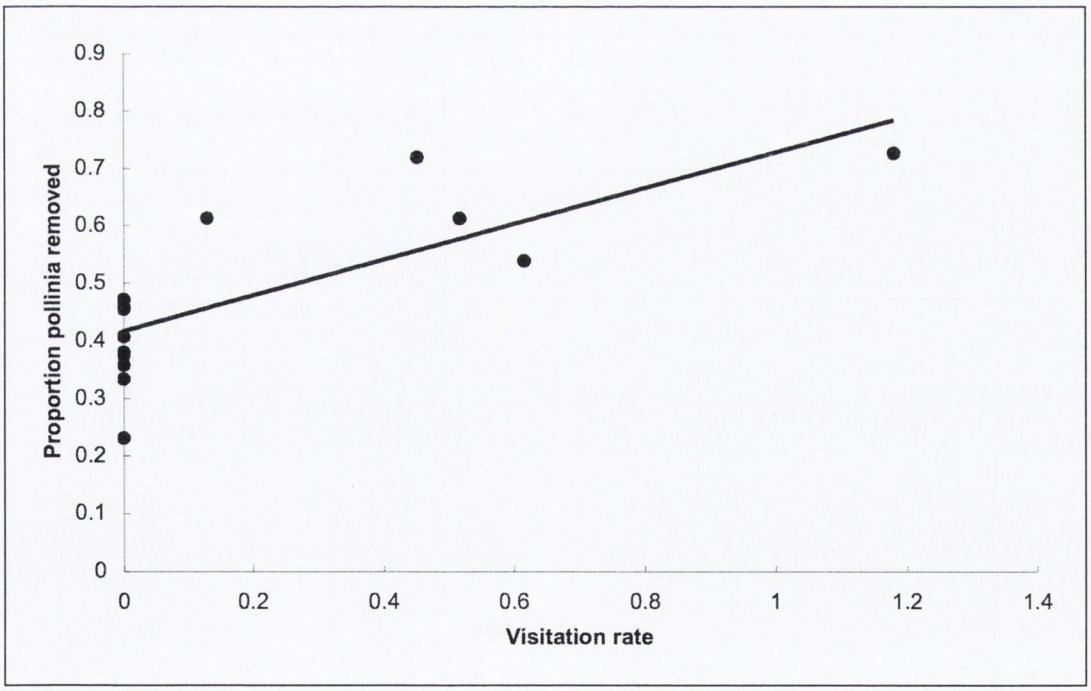

Proportion pollinia removed
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Visitation rate

Fig. 5. The relationship between the proportion of pollinia loss and the number of *S. spiralis* inflorescences per 2 × 2 m patch.

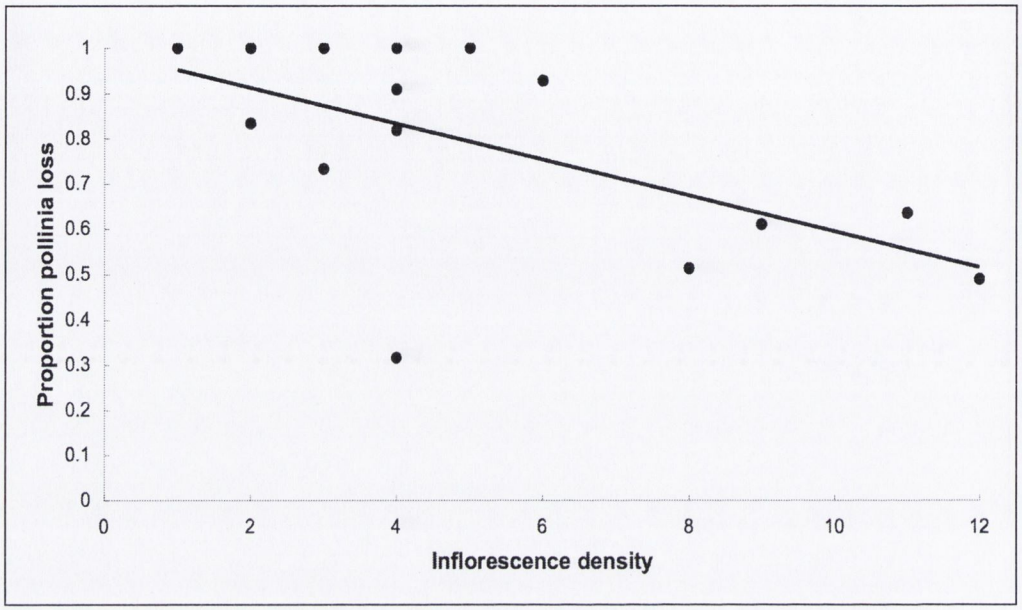
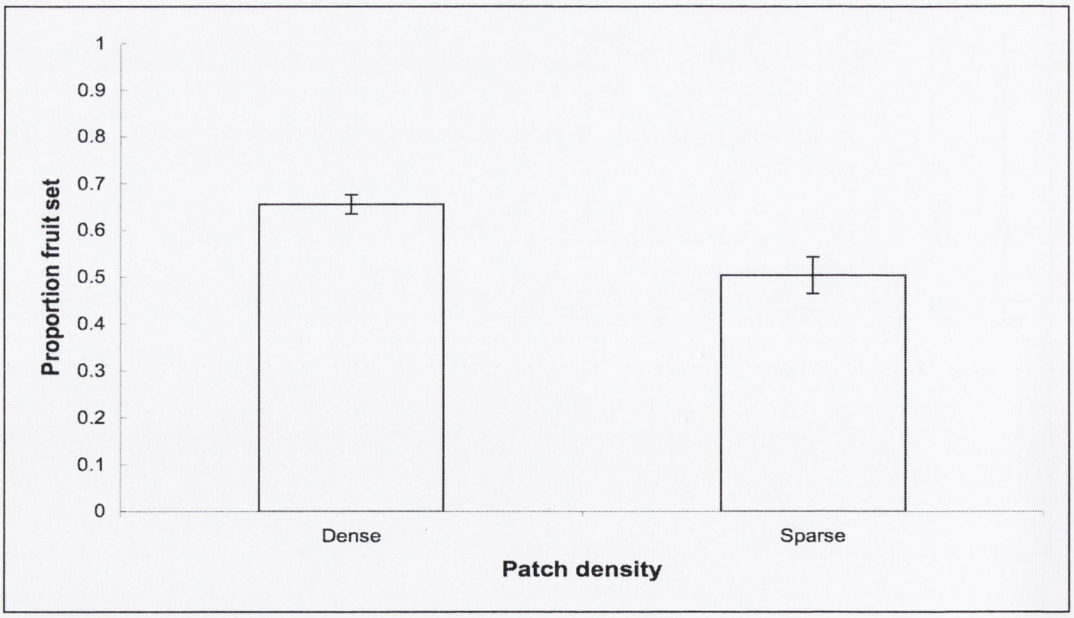


Fig. 6. Proportion of fruit set in *S. spiralis* according to patch density within populations. Dense patches were defined as those with more than 12.5 flower/m^2; sparse patches were defined as those with less than 5 flowers/m^2.



Estimated number of seeds per capsule did not differ among breeding system treatments ($F_{2,72} = 1.33$; $p > 0.05$).

4.5 Discussion

To increase our understanding of the effects of rarity, Bevill and Louda (1999) argue for the direct comparison of attributes of related rare and common species. This study examined the differences of the effects of relative population size, density and co-flowering species on pollination of an endangered and a common orchid. This is one of the few studies to compare the pollination of a common and rare species, and the first study to my knowledge, which attempts to do so with orchids. In a similar study, Karron (1987) found that generalist bees visited four species of both common and rare *Astragalus*, with one rare species, *A. linifolius*, receiving less pollinator attention than the more widespread *A. lonchocarpus*. Such a comparison of related rare species versus common species can inform conservationists of the effects, if any, rarity has on pollination biology. Ideally, experiments would involve both the common and the rare species flowering in similar populations, at the same time, have the same reliance on pollinators, and similar floral rewards, colour and morphology. In this study, both *S. romanzoffiana* and *S. spiralis* have the same breeding system (self-compatible but dependent on insects to set seed), similar floral colour and morphology, though *S. romanzoffiana* tends to contain more nectar than *S. spiralis*, probably because flowers are bigger (K.J. Duffy, unpublished results).

Population size may be important in these orchids for pollinator attraction, but it does not seem to affect overall reproductive success. I only found differences in visitation rate according to population size in *S. romanzoffiana*, with fewer visitors attracted to flowers in small populations. However, I only had one small *S. spiralis* population in my analysis and this was the same size as the large *S. romanzoffiana* populations and there were few observed visits per flower to both species, making it difficult to draw conclusions on whether this is a general pattern. Other variations among populations of both species may have affected pollinator visitation, such as the local pollinator density and microclimate, which may have affected pollinator-flying days (e.g. Steffan-Dewenter, 2003). Sipes and Tepedino (2006) found that pollinia removal rates varied among populations of *Spiranthes diluvialis* and this was due to the variation in the abundance of bees at their study populations.

Other studies have shown that within-population density rather than population size is more important for predicting pollinator visitation, with higher visitation rates in dense patches (e.g. Kunin, 1997b; Knight, 2003). A previous study on *S. romanzoffiana* showed increased competition for pollinator visitation with increasing conspecific inflorescence density (Duffy and Stout, 2008; Chapter 3). In this study, I found that high-density patches of *S. romanzoffiana* received increased visitation and higher pollen deposition. Similarly, high-density patches of *S. spiralis* had reduced pollen loss and increased fruit set. Unfortunately, I could not test for the interaction between population size and patch density due to constraints to my experimental design, but it is possible that impacts of patch scale density may vary with population size (e.g. Kunin, 1997b).

In the rare *S. romanzoffiana*, an increase in co-flowerer density increased pollinia removal, but not deposition. Pollinia deposition was increased with increased conspecific density. This suggests that the presence of co-flowerers makes *S. romanzoffiana* flowers more attractive to pollinators, but that visitors are moving from *S. romanzoffiana* to other co-flowering species, hence causing pollen wastage and/or are losing pollinia en route to the next flower (via grooming or breakage of the sectile *Spiranthes* pollinia). Indeed, *Bombus pascuorum* and *B. terrestris/lucorum* were observed visiting other flowering species immediately after visiting *S. romanzoffiana*. When co-flowering plants are more abundant, foragers may find them more profitable, causing foragers to focus on them (as majors) whilst occasionally including *S. romanzoffiana* in their foraging regime (as minors) (Heinrich, 1976). As *S. romanzoffiana* density increases in the patch, pollinators may remain more faithful to it and so deposition increases. It is possible that bees focus on *S. romanzoffiana* for nectar and collect pollen from co-flowerers; nectar in bagged flowers was higher than in open flowers in both *S. romanzoffiana* and *S. spiralis* (K.J. Duffy, unpublished data), suggesting depletion by insect foragers.

With *S. spiralis*, I detected no intra- or inter-specific effects on either pollinia removal or deposition. This may be because *S. spiralis* flowers later in the season (it typically begins flowering late August/early September) and therefore occurs with fewer co-flowering species and individuals, and at a time when there are fewer bees competing for resources. In the Netherlands, Willems and Lahtinen (1997) found that clumped plants had higher fruit set than sparsely distributed plants and that the removal of co-flowering species increased fruit production in *S. spiralis*, suggesting interspecific competition for pollinators. *S. spiralis* was rarely surrounded by dense vegetation in my study populations. In addition, our experimental

design did not allow us to examine directly within-patch effects of heterospecific flowers on the fruit production of *S. spiralis*.

Fruit maturation failed to occur in *S. romanzoffiana* despite evidence of pollination, which makes it impossible to compare overall reproductive success with patch and population size. However, low numbers of seeds were found in the immature fruits, which make it possible that *S. romanzoffiana* may reproduce sexually. Whether the seeds are viable to establish new individuals in suitable habitats requires further long-term investigation. It may be that fruit failure may be the result of genetic inbreeding or soil nutrient status. Indeed, Forrest *et al.* (2004) found low genetic diversity in Irish populations of *S. romanzoffiana*, which suggest autogamous reproduction or an extreme genetic bottleneck.

As *S. romanzoffiana* is endangered in Ireland and is known from few populations, current populations require monitoring and protection from disturbances such as grazing. Small populations may be prone to pollination limitation since as the number of flowers per unit area declines, so do both pollen import and export. As orchids are not known to provide pollen rewards, other co-flowering species may maintain pollinator species around *S. romanzoffiana* populations. Further investigation into the reasons for the lack of fruit maturation is required. *S. spiralis* does not appear to be in decline in Ireland however, it is rare in other European countries (e.g. the Netherlands; Jacquemyn *et al.*, 2007). However, as cattle grazing during flowering decimated one population, future management should allow for it to flower and set seed.

In conclusion, intra- and inter-specific interactions can vary with spatial scale and affect different components of pollination (visitation, pollen export, import, loss, fruit set) of orchids in different ways. I found there were differences in the pollination of the two species. This is probably due to *S. romanzoffiana* flowering earlier than *S. spiralis*, with co-flowering species facilitating pollinia removal. Both local density of *S. romanzoffiana* inflorescences and insect visitation has a positive effect on pollinia deposition. Insect visitation has a positive effect on pollinia removal in *S. spiralis*, and local density acts against pollen loss and increases fruit set. Flowering population sizes of *S. spiralis* are larger than *S. romanzoffiana*, which likely influences their comparatively greater reproductive success. *S. romanzoffiana* fails to set fruit, however minute quantities of seed is present in unripened capsules. *S. spiralis* sets abundant fruit and seed and overall pollination success is not limited by population size. Studies, such as mine, highlight the importance of taking into account different measures of abundance in quantifying

the effect rarity has on different components of pollination biology. Although a plant species may be rare, it may not necessarily suffer an overall reduction in reproductive success, either within or among populations, and negative effects of rarity can be ameliorated or enhanced by the presence of co-flowering species. Increased knowledge in this area may be important for our understanding of how insect-pollinated plant species respond to increasing habitat reduction and modification.

"*Self-fertilisation is a rare event with orchids.... It is an astonishing fact that self-fertilisation should not have been a habitual occurrence. It apparently demonstrates to us that there must be something injurious in the process. Nature thus tells us, in the most emphatic manner, that she abhors perpetual self-fertilisation.*" – **Charles Darwin. On the various contrivances by which British and foreign orchids are fertilised by insects, 1862**

"*It is clear that a knowledge of the causes which set the limits to the distribution of species is of fundamental importance to the student of plant geography and it is obvious that the termini of the ranges are the only localities favorable to the study of these conditions*" – **Robert F. Griggs. On the behavior of some species at the edges of their ranges. Bulletin of the Torrey Botanical Club, 1914.**

5. The ecology and genetic diversity of the dense-flowered orchid (*Neotinea maculata*) at the centre and edge of its range

Awaiting final acceptance as: Karl J. Duffy, Giovanni Scopece, Salvatore Cozzolino, Michael F. Fay, Rhian J. Smith and Jane C. Stout The ecology and genetic diversity of the dense-flowered orchid (*Neotinea maculata*) at the centre and edge of its range. *Annals of Botany*.

5.1 Abstract

Species may occur over a wide geographical range within which populations can display large variation in reproductive success and genetic diversity. *Neotinea maculata* is a rare orchid of conservation concern at the edge of its range in Ireland, where it occurs in small populations. However, it is relatively common throughout the Mediterranean. A putative reason for its rarity in Ireland is its apparent reliance on self-pollination, which may be due to a depauperate pollinating fauna. This may then affect genetic diversity and overall reproductive strategy of the species. I compared vegetation communities, breeding system, and genetic diversity, using three AFLP primer pairs, among populations in both Ireland and Italy. I quantified vegetation using quadrats taken along transects in study populations and performed hand-pollination experiments to assess reliance of *N. maculata* on pollinators in both Irish and Italian populations. *N. maculata* occupies different vegetation communities in both Italian and Irish populations. Breeding system experiments show *N. maculata* is 100% autogamous and there are no differences in fruit and seed production in selfed, outcrossed or unmanipulated plants. AFLP markers revealed Irish and Italian populations have similar genetic diversity, but that Irish populations are distinct from Italian populations. *N. maculata* does not suffer any negative effects of autogamous reproduction; it self-pollinates and sets seed readily in the absence of pollinators. It has high levels of polymorphism and similar genetic diversity to other orchid species. It can occupy various habitats; however Irish populations are naturally small and rare and should be conserved. This is probably due to climatic factors and the presence or absence of suitable soil mycorrhizae to allow recruitment from seed.

5.2 Introduction

Species that occur over a large geographical area often have populations that occupy a variety of different habitats (Caughley *et al.*, 1988; Hall *et al.*, 1992). However, not all of these habitats provide populations with equal ecological advantages. Differences in both biotic (e.g. competition, genetic structure, presence of mutualists) and abiotic (e.g. light, moisture,

temperature) factors may affect both the abundance of populations and individuals within populations in a given region (Griggs, 1914; Caughley *et al.*, 1988; Lawton, 1993; Carey *et al.*, 1995). Indeed, most species occur at high frequency in only a few sites of their geographical range (Lawton, 1993; Blackburn *et al.*, 1999). One instance in which a species may occur at a lower frequency, in terms of both the number of populations and the number of individuals within populations, is where it occurs at the edge of its geographical range (Hanski, 1982; Lawton, 1993). This may be, at least in part, due to optimal biotic and abiotic conditions for such species occurring at the core of its distribution, while at the edge of the range the species may suffer from a suboptimal environment (Carey *et al.*, 1995; Channell and Lomolino, 2000). Consequently, this has led to the assumption of an "abundant centre" distribution in ecology (e.g. Sagarin and Gaines, 2002)

Populations of plant species that occur at the edge of their range may suffer from reduced reproduction and recruitment (Busch, 2005; Moeller and Geber, 2005). This can be due to a decrease in animal-mediated outcrossing, as pollinators may not be present at the edge of the range (Herlihy and Eckert, 2002) or there may be a lack of specialist pollinators, which occur in the centre of the species range. Populations of self-compatible species may autonomously self-pollinate in order to provide reproductive assurance at the edge of their range (Fausto *et al.*, 2001; Herlihy and Eckert, 2002; Busch, 2005). Theoretically, selfing should be favoured when there is low pollinator attention while outcrossing favoured when there is low vigour of selfed progeny (inbreeding depression) (Barrett and Harder, 1996; Takebayashi and Morrell, 2001). In addition to lower pollinator abundance, other factors such as soil and vegetation communities may not be suitable for individuals to establish and survive owing to competition or a lack of nutrients (Brown, 1984). As a result, populations may be fewer and smaller at the edge of a species range due to habitat constraints (Gaston and Kunin, 1997).

Populations of species that occur at the edge of range have been highlighted for conservation concern, because of their low abundance and unique ecological regimes (Lesica and Allendorf, 1995). Such populations may experience different selection pressures owing to different environmental conditions (e.g. selection for autogamy in times of low pollinator abundance; Kalisz and Vogler, 2003). In addition to low pollinator abundance, expected heterozygosity, number of alleles, and proportion of polymorphic loci are known to increase with increasing population size and can be lower in small populations (Leimu *et al.*, 2006). They may be highly inbred and suffer from low genetic diversity and/or problems with reproductive traits (Ellstrand and Elam, 1993; Vucetich and Waite, 2003) such as impaired stigma receptivity and

low pollen viability. In addition to occurring at the edge of their range, species may be naturally rare in a community or geographical region. Indeed, rare species occur in all natural communities, with most communities containing many species with few individuals (Rabinowitz *et al.*, 1986; Gaston and Kunin, 1997). Human-mediated fragmentation of natural habitats has caused many previously common species to become rare (Rathcke and Jules, 1993; Young *et al.*, 1996). An understanding of the differences between natural and human induced rarity is imperative for appropriate conservation action. Therefore, good knowledge of habitat differences and genetic diversity are necessary for adequate conservation of a species (de Lange and Norton, 2004; Pillon *et al.*, 2007).

Certain plant groups, such as orchids, are adapted for insect-mediated outcrossing (Nilsson, 1992; Tremblay *et al.*, 2005) and are sensitive to habitat requirements (e.g. soil mycorrhizal association for successful establishment from seed; Rasmussen, 2002). In addition, some species of orchid occur across large geographical ranges and many are self-compatible (Darwin 1862; Dressler, 1981; Neiland and Wilcock, 1998). This makes them good model species to test theories related to fitness of populations at the edge of their range, however little work has been done in this area using orchid species. The dense-flowered orchid, *Neotinea maculata*, occurs throughout the Mediterranean in Europe, and reaches its western geographic edge in western Ireland where it has a relatively restricted distribution. In Ireland, *N. maculata* is considered rare, occurring in the west of the country in only thirteen 10 x 10km squares (Preston *et al.*, 2002). It forms small and scattered populations in Ireland with few flowering individuals (approx. from 1 – 20 individuals; KJ Duffy, *personal observation*). However, it is common in the Mediterranean and can form large populations (>100 individuals) (van der Cingel, 1995). Little is known of its reproductive ecology, though it is thought to be self-compatible and reproduce autogamously (>80% of flowers per inflorescence mature fruit, van der Cingel, 1995). *N. maculata* has very small flowers (full anthesis: approx. 2mm corolla diameter), it is scented and may contain a nectar reward, which can help attract potential pollinators (van der Cingel, 1995). Putative pollinators are flies and wasps, though pollinators have never been recorded (van der Cingel, 1995), and pollinating taxa may be present in Italy but not in Ireland. Other members of *Neotinea* are nectarless and depend on pollinators for outcrossing (van der Cingel, 1995; Tali *et al.*, 2004). *Neotinea maculata* has a staggered flowering period throughout Europe, beginning flowering in March-April in the south Mediterranean, while more northerly populations begin flowering in April-May, which allows for comparison of populations within one season.

Here, I investigate factors that may influence the limited distribution of *Neotinea*

maculata in Ireland. I investigate ecology and population genetics of *N. maculata* in populations in both Ireland (edge populations) and Italy (centre populations). Specifically, I hypothesise that at the edge of its range compared with the centre, *N. maculata* populations:

1. are restricted to a smaller number of vegetation communities,
2. experience reduced fruit and seed production, because of a lack of mutualists, and increased reliance on self-pollination,
3. have lower genetic diversity and higher levels of inbreeding.

5.3 Materials and Methods

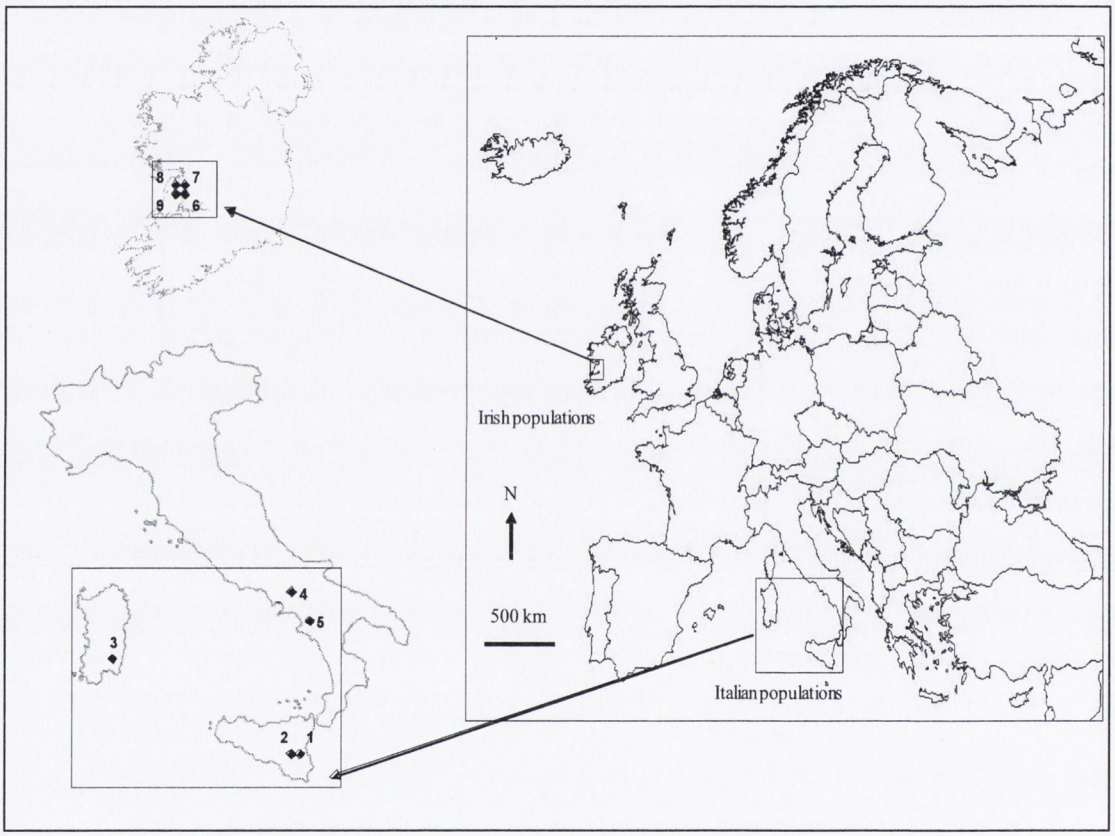
5.3.1 Vegetation analysis

In the spring and early summer of 2006, I quantified the vegetation which co-occurs with flowering *N. maculata* using 1 x 1m quadrats taken at regular intervals (5m intervals in Irish populations; 10m intervals in Italian populations). These were taken along a 100m transect in three Italian populations (two mainland Italy: Roccamonfina and Vesuvio; one in Sardinia: San Gregorio) and along a 50m transect in four Irish populations (Loch Bunny, Loch Gealáin, Mullach Mór, and Oughtmama) (Figure 5.1). The length of the transect reflecting the approximate occurrence of flowering populations of *N. maculata.* Although the Vesuvio population was much larger, all flowering individuals occurred in homogeneous habitat, which was represented in quadrats sampled (KJ Duffy, *personal observation*). All plant species that occurred in each quadrat were recorded and percentage abundance was estimated for each species. Composite soil samples were taken from the 0-5cm layer in at least three patches surrounding *N. maculata* (which is within the range of the entire root length of *N. maculata*; KJ Duffy, *personal observation*) were taken from each population. These were analysed for (i) pH, (ii) loss on ignition (%), (iii) available phosphorous (mg/KG), (iv) total C (%) and (v) total N (%).

Non-metric multidimensional scaling (NMDS) was used to evaluate vegetation composition and abundance data. Vegetation community analyses were performed in PC-Ord 4 (McCune and Mefford, 1999). The autopilot mode of NMDS in PC-Ord 4 was run at the slow and thorough setting, and incorporated a maximum of 400 iterations, a stability criterion of 0.00005 with 20 iterations to evaluate stability, 6 starting axes, 40 runs with real data, and 50 runs with randomised data. Sorensen's distance and a random starting configuration were used (McCune and Mefford, 1999). A multi-response permutation procedure (MRPP) was used to test for vegetation differences between all populations and between Ireland and Italy. This procedure calculates a distance measure in ordination space within each group (Sorensen's distance in this

case), and tests whether the distance between the observed and expected distances is due to chance. The test statistic (*T*) describes the separation between the groups; the more negative *T* is, the stronger the separation between groups. In addition, another statistic (*A*) describes the within-group homogeneity, compared to random expectation (McCune and Grace, 2002). Vegetation diversity within each population was measured using the Shannon diversity index, as calculated in PC-Ord 4 (McCune and Mefford, 1999).

Figure 5.1. Locations of *Neotinea maculata* study populations. Population number codes: 1 = Monti Rossi; 2 = Buccheri; 3 = San Gregorio; 4 = Roccamonfina; 5 = Vesuvio; 6 = Loch Bunny; 7 = Loch Gealáin; 8 = Oughtmama; 9 = Mullach Mór.



5.3.2 Breeding system and pollination

In order to assess the extent to which *N. maculata* relies on pollinators, I performed hand pollination treatments. 20 inflorescences in study populations were bagged prior to anthesis and the following treatments were performed on five flowers from five inflorescences when flowers opened: (i) pollen removal (emasculation) to test for spontaneous seed production (agamospermy); (ii) within-flower pollen transfer (autogamy), (iii) pollen added from an individual >10m away (xenogamy), (iv) control (no manipulation). This was performed in two Italian populations (Roccamonfina and Vesuvio) and two Irish populations (Loch Bunny and

Loch Gealáin). As *N. maculata* has very small flowers (see Plate 5.1), a 12 × head lens with a headlight was used to observe flowers and a fine-tipped wooden stick was used to transfer pollen. Mature capsules were collected and put in Eppendorf® or Falcon tubes with some silica gel and stored at 4°C and were counted within one year of collection. The total number of fruit set was recorded and seed per fruit from treated individuals was estimated. As orchid seeds are minute, I liberated the seeds from the capsule, spread them evenly, and washed the remaining seeds stuck in the capsule with 70% ethanol. Unlike water, ethanol does not have surface tension, which prevents seeds from clumping and allows for a more even spread on the Petri dish. Seeds were counted immediately as I noticed the alcohol dehydrates seeds if left immersed for more than 2 hours. As orchid capsules contain thousands of seeds, to estimate the number of seeds per fruit, we sub-sampled the number of seeds from 10mm × 10mm squares using a nine cm diameter circle of graph paper (to fit the underside of a Petri dish). I counted all the seeds within 10 squares and multiplied by the total number of squares occupied by seeds.

I tested for differences in seed set among (i) pollination treatment (agamospermy, autogamy, xenogamy, and unmanipulated), and (ii) population, using a two-factor ANOVA. For this analysis, I used R 2.6.1 (R Development Core Team, 2007).

In order to test whether *N. maculata* offers a reward to flower visitors I attempted extracting nectar using 0.5µl microcapillary tubes. However, as *N. maculata* has minute flowers it was not possible to extract nectar using microcapillary tubes or other methods (e.g. folded filter paper) (Dafni, 1992). Instead, I tested for the presence/absence of a sugar reward by adding 1µl of distilled water to the base of the labellum with a microcapillary tube and tested the resulting solution for sugar using standard diabetic test kit (Dafni, 1992).

To test for viability, pollen was taken from one flower from each of 10 inflorescences in each of two Italian and two Irish populations and stained with Alexander's stain (Alexander, 1980). Stigmas were taken from different flowers on the same 10 inflorescences per population and tested for receptivity using Peroxtesmo Ko test paper (Dafni and Maues, 1998).

5.3.3 Molecular methods

No more than one-half of a leaf from two- or more-leaved individuals was taken (Table 5.2). All samples were collected and stored in zip-lock plastic bags containing a mean of 14.2g (S.E. = 1.3g; N = 10) of silica gel (Chase and Hills, 1991) for between one and eight months until total DNA was extracted. We sampled leaf material from five Italian populations (Buccheri, Monti

Rossi, San Gregorio, Roccamonfina, and Vesuvio) and three Irish populations (Loch Bunny, Loch Gealáin, and Mullach Mór). Sampling of Irish *N. maculata* was undertaken during the December 2006 when fresh leaves have sprouted, while Italian samples were collected in the field during other experimental work in spring 2006 from individuals in full anthesis.

I employed a DNA fingerprinting method using amplified fragment length polymorphisms (AFLPs) (Vos *et al.*, 1995), which have been successfully used across a wide range of orchid taxa (e.g. Smith *et al.*, 2004; Tali *et al.*, 2006; Pillon *et al.*, 2007; Flanagan *et al.*, 2006). AFLPs were chosen for this study because they require no prior knowledge of the DNA sequence and provide large amounts of data with reproducible results. In addition, AFLPs were recently used on the congeneric *Neotinea ustulata* (Tali *et al.*, 2006). DNA was extracted from approximately 0.05-0.1g of dried material using a modified 2× CTAB (cetryltrimethyl-ammonium bromide) procedure (Doyle and Doyle, 1987), purified on a QIAquick column (Qiagen Ltd., Crawley, West Sussex, U.K.) and quantified using a spectrophotometer. AFLP analysis was performed according to the AFLP Plant Mapping protocol of Applied Biosystems Inc. Sampled DNA was restricted with the endonucleases *Eco*RI and *Mse*I and ligated to appropriate double-stranded adapters according to the manufacturer's protocols. As genome size can have a marked effect on the quality of AFLP traces (Fay *et al.*, 2001) and as the congeneric *Neotinea ustulata* has a large genome (Tali *et al.*, 2006), I modified the Applied Biosystems protocol, which uses three selective base pairs on each primer in the selective amplification, by incorporating an additional base on one primer of each pair. For this second amplification, 12 different primer combinations were tested, of which three pairs with the following selective bases were chosen for the full study: -ctat/-act, -ctaa/-agg, and -ctaa/-acc.

AFLP profiles were manually scored with the presence of each band as '1', and the absence of a band as '0'. Markers with evidence of small, un-scorable peaks were discarded from all samples. Bands ranging from 50bp – 500bp were scored. The following statistical analyses were performed using GenAlEx 6.1 (Peakall and Smouse, 2005). The calculation of genetic distances followed the method of Peakall *et al.* (1995) and outlined in Maguire *et al.* (2002) as:

$$E = n\,[1- (2nxy/2n)]$$

where, n is the total number of polymorphic bands and 2nxy is the number of markers shared by two individuals. Both band-presence and absence are considered informative which gives a Euclidean metric as required for subsequent analysis of molecular variance (AMOVA). Genetic distance matrices for each AFLP primer set on their own, and the total data set (three primer sets combined) were calculated. Genetic structure was tested by AMOVA on the genetic distance

matrix (999 permutations). AMOVA output nomenclature follows that of Excoffier *et al.* (1992) in that variation was summarised both as the proportion of the total variance and as φ-statistics

Plate 5.1. *Neotinea maculata* flowering with *Orchis mascula* in the Burren in the west of Ireland.

Photo: K.J. Duffy

(*F*-statistic analogues). I tested for genetic differentiation (i) within population (ii) between populations and (iii) between Ireland and Italy. Overall genetic diversity and diversity within populations and regions was measured using the Nei diversity indices, as calculated with POPGENE 1.32 (Yeh *et al.*, 2000). Mantel tests were performed (999 permutations) to test whether genetic patterns detected by one AFLP primer set were congruent with the patterns detected by another primer set. Based on the output of individual genetic distance matrix I performed a principle coordinate analysis (PCO) to group individuals. In addition, two UPGMA trees were produced: (i) using PAUP 4.0 (Swofford, 2000) for analysing the relationships between all individuals in the study, (ii) using POPGENE 1.32 (Yeh *et al.*, 2000) for analysing the relationships between populations.

5.4 Results

5.4.1 Vegetation

A total of 70 quadrats were analysed (30 in Italian populations; 40 in Irish populations) with a total of 80 vascular plant species found occurring among all quadrats surveyed. NMDS analysis determined that a 2-D solution had the least stress (stress = 0.14) of vegetation data, with a sum

of 82.5% of the variation explained by this output. This analysis revealed that *N. maculata* occurred within a different species composition in both Italian and Irish populations (Fig. 2). The MRPP analysis revealed significant differences between Irish and Italian vegetation communities (T = -20.78; A = 0.129; p = < 0.001). In addition, there were significant differences within Italian (T = -15.19; A = 0.547; p < 0.001) and Irish populations (T = -18.65; A = 0.304; p < 0.001), indicating that *N. maculata* occupies different vegetation communities in both regions. There was a clear dichotomy in Italian populations with the Roccamonfina population clearly separated from San Gregorio and Vesuvio populations (Fig. 2). Species diversity and soil property values for each population are given in Table 5.1 with a wide range of values given for each. Available P and pH are negatively correlated (spearman rank correlation; r_s = -0.867; p = 0.005); both total C (r_s = 0.812; p = 0.01) and total N (r_s = 0.711; p = 0.032) are positively correlated with organic matter content (LOI). Mean Shannon diversity values (H) ranged from 0.356 - 2.149 per population, highlighting the wide range in species richness of vegetation associated with *N. maculata*.

Breeding system

I found no differences in fruit set according to either the autogamy or xenogamy hand pollination treatment ($F_{1,56}$ = 0.128; p = 0.72) in either Italian or Irish populations, with 100% of flowers producing fruit. However, flowers that were emasculated failed to set fruit and produced no seed. Unmanipulated capsules contained an estimated mean of 1,355 (S.E. ± 52.5) seeds. This indicates that *N. maculata* is fully self-compatible and autonomously autogamous though not agamospermous, as it requires the presence of pollen on its stigma in order to set seed. I observed some flowers in both Irish and Italian populations had self pollinia on the stigma before flower opening (see Plate 5.2).

The diabetic test for the presence of sugars was positive which indicates the presence of a small reward for potential flower visitors at the base of the labellum. All flowers tested in all populations had both viable pollinia and receptive stigmas.

5.4.3 Molecular data

A total of 223 interpretable bands were produced; 90 -ctat/-aca, 78 -ctaa/-acc, and 55 -ctaa/-agg among the 79 *N. maculata* individuals surveyed. Overall, 219 bands were polymorphic (98.2%). Mantel tests revealed significant relationships between primer combinations -ctat/-aca and -ctaa/-acc (r_{xy} = 0.339; p < 0.001), -ctaa/-agg and -ctaa/-acc (r_{xy} = 0.193; p < 0.001) but there were no relationships between primer combinations -ctat/-aca and -ctaa/-agg (r_{xy} = 0.112; p = 0.069).

AMOVA revealed significant genetic differences within populations, between populations and between regions (Table 5.3). However, both the PCO output (Figure 5.3) and the UPGMA tree (Figure 5.4a) on individuals show no clear differentiation of populations or regions, although there is some differentiation of regions. On the other hand, the UPGMA tree based on overall population data (Figure 5.4b) shows a distinction between Irish and Italian populations, which supports the AMOVA results. Similar φ-values were obtained in both Ireland and Italy (φ_{PT} Ireland = 0.103; φ_{PT} Italy = 0.185) indicating that genetic diversity is similar in both regions.

Table 5.1. Soil and vegetation diversity values of populations of *N. maculata*. P = available phosphorus, C = total carbon, N = total nitrogen, LOI = loss on ignition. ANP = Approximate number of plants. For Italian populations, the number of flowering plants only. For Irish populations, it is the approximate number of individuals that produced rosettes.

Population	Location	ANP	pH	P (mg/kg)	C (%)	N (%)	Organic matter (% LOI)	Mean Shannon Diversity Index *H* (± S.D.)
Buccheri	Sicily	?	6.89	32.8	3.71	0.303	27.00	-
Monti Rossi	Sicily	?	6.75	46.4	3.91	0.208	19.00	-
Roccamonfina	Italy	~100	5.76	39.2	2.52	0.195	20.20	2.149 (0.190)
Vesuvio	Italy	~100	5.84	44	30.07	1.204	71.43	0.356 (0.176)
San Gregorio	Sardinia	>1000	6.51	38.4	8.91	0.519	16.38	0.664 (0.340)
Loch Bunny	Ireland	30	7.73	6.4	16.18	1.393	30.35	2.072 (0.194)
Loch Gealáin	Ireland	20	7.10	34.4	26.23	2.056	64.95	1.979 (0.225)
Mullach Mór	Ireland	30	7.55	7.6	17.80	1.470	38.83	1.141 (0.216)
Oughtmama	Ireland		7.84	5.2	11.04	0.679	13.28	1.893 (0.217)

Table 5.2. Details of sample populations, numbers of individuals sampled per population, and AFLP banding patterns within populations. ANP = Approximate number of plants. For Italian populations, the number of flowering plants only. For Irish populations, it is the approximate number of plants with rosettes.

Population	Location	No. individuals analysed	Total no. Bands	No. private bands	Mean genetic diversity (± S.E.)
Buccheri	Sicily	4	121	3	0.137 (0.013)
Monti Rossi	Sicily	5	136	5	0.129 (0.012)
San Gregorio	Sardinia	10	130	0	0.131 (0.012)
Roccamonfina	Italy	10	172	8	0.188 (0.013)
Vesuvio	Italy	10	174	8	0.244 (0.013)
Loch Bunny	Ireland	10	144	1	0.220 (0.015)
Loch Gealáin	Ireland	10	162	2	0.146 (0.013)
Mullach Mór	Ireland	20	157	4	0.212 (0.014)

Figure 5.2. NMDS plot of sample scores of vegetation community data on axes 1 and 2. Open symbols indicate quadrats from Italian populations; closed symbols indicate quadrats from Irish populations. Axis 1 accounts for 39.2% of variation; axis 2 accounts for 43.3% of variation.

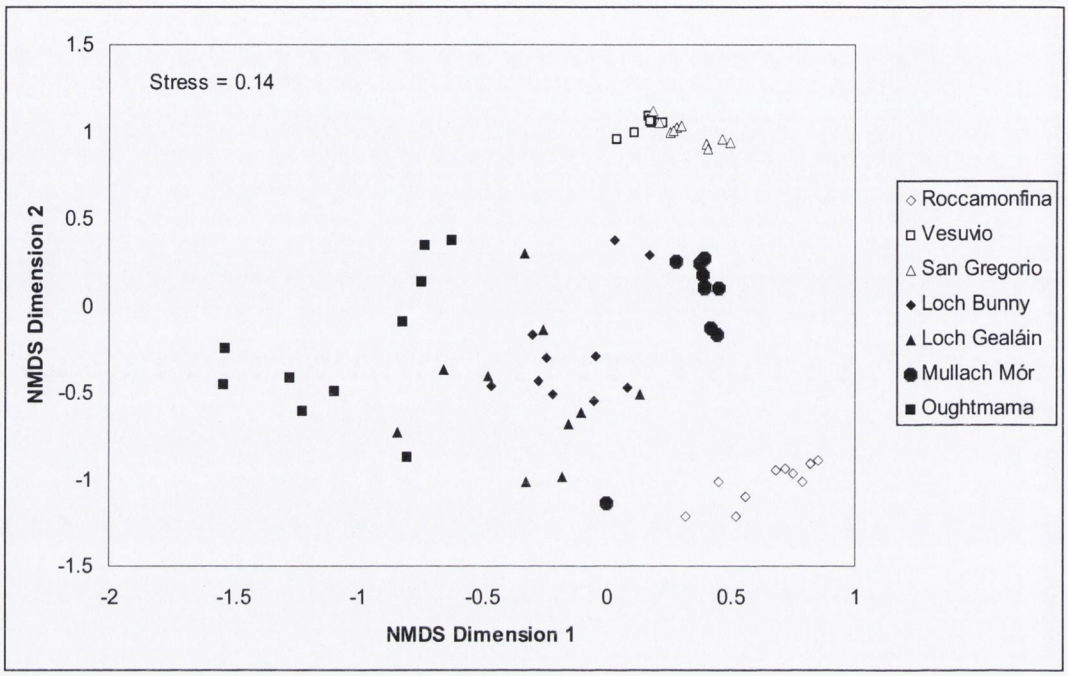


Figure 5.3. Principal co-ordinate (PCO) plot of the first and second axes for the full data matrix based on three AFLP primers. Open symbols indicate Italian populations; closed symbols indicate Irish populations. Axis 1 accounts for 42.72% of variation; axis 2 accounts for 24.61% of variation.

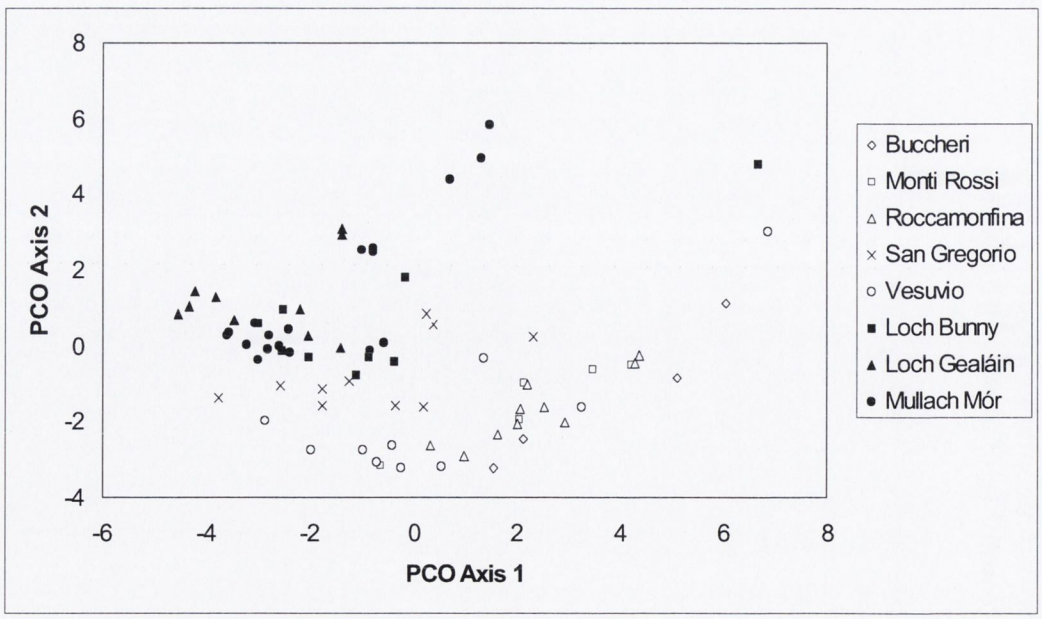


Figure 5.4 (a). Rooted UPGMA tree of the relationship between all individuals of *N. maculata* used in this study based on three AFLP primers. Letters A-E represent individuals from Italian populations; letters F-H represent individuals from Irish populations. Open circles indicate individuals from Italian populations; filled circles indicate individuals from Irish populations.

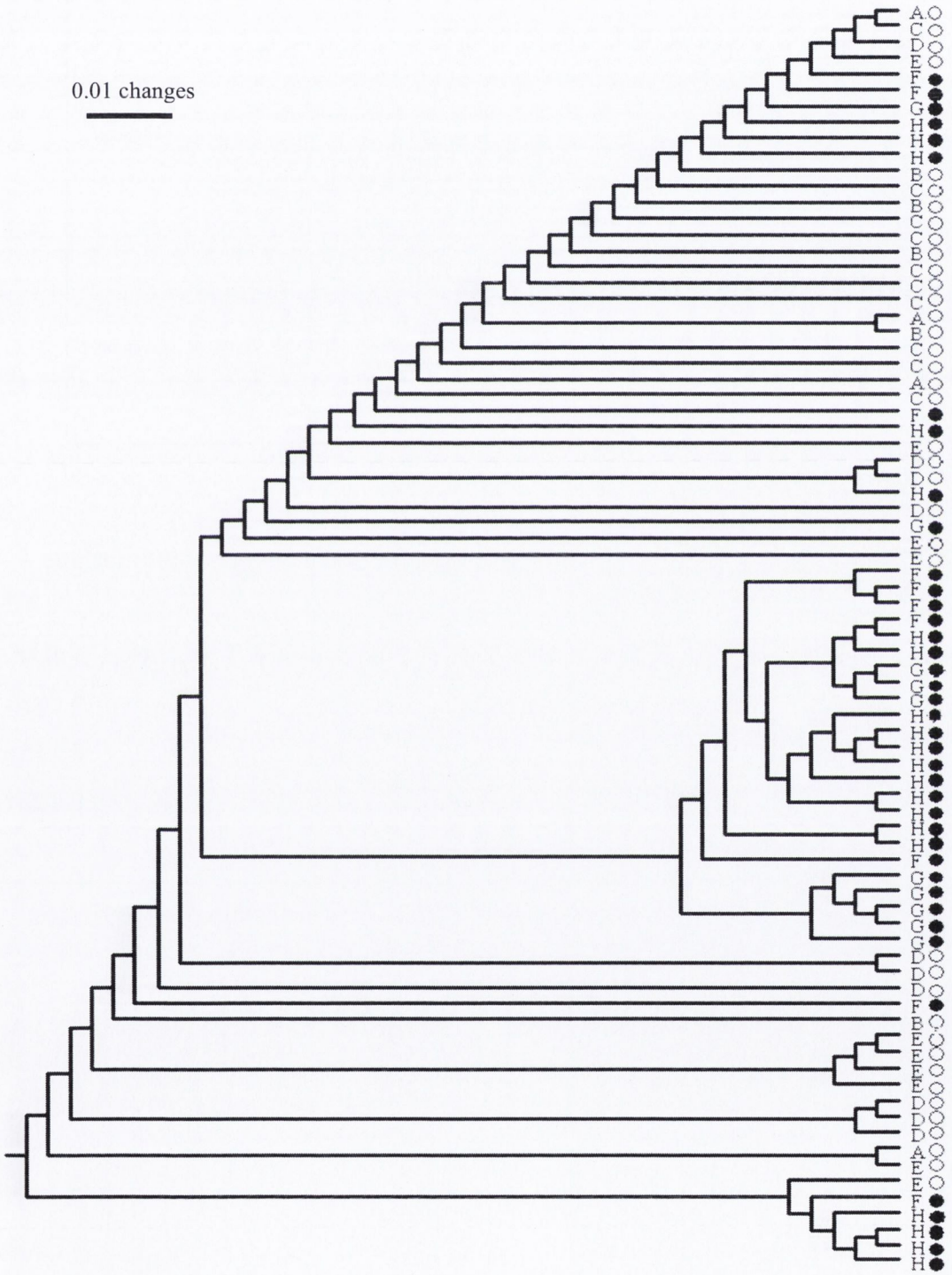


Figure 5.4 (b) Rooted UPGMA tree depicting relationships between the populations studied based on Nei's genetic distance. Branch lengths are above lines.

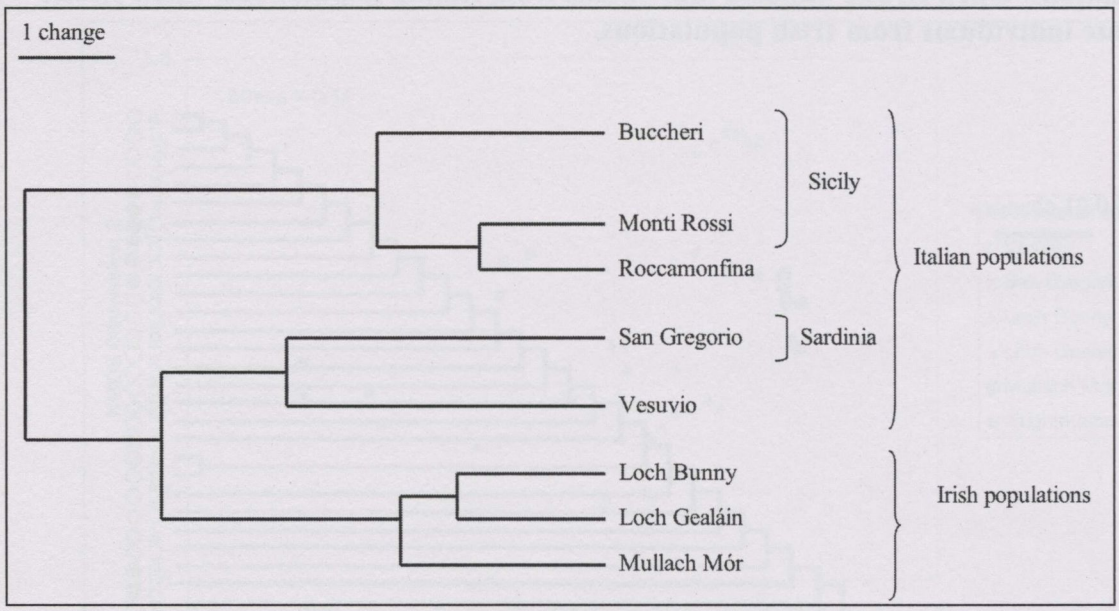
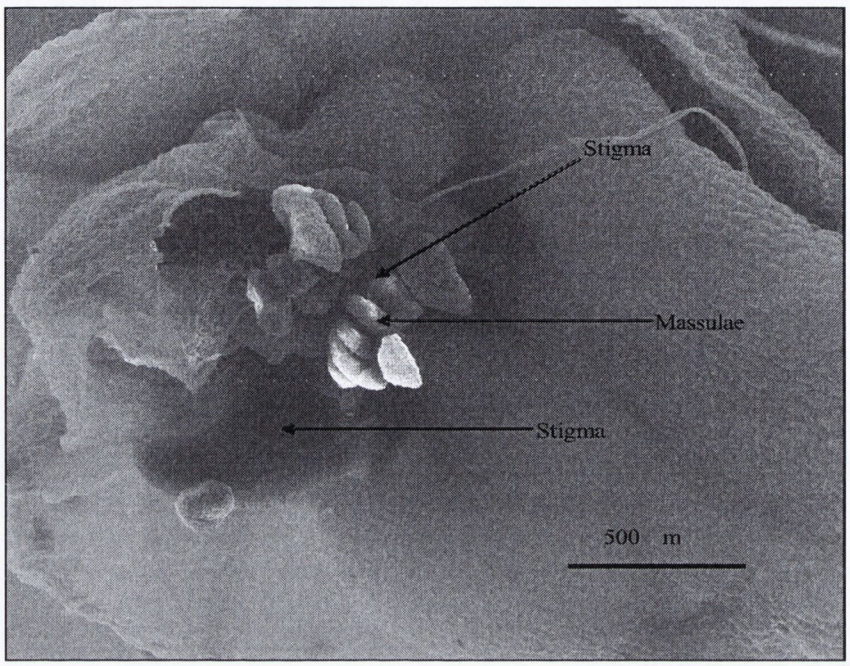


Table 5.3. Results of AMOVA for AFLP data set based on three primer combinations: -ctat/-aca, -ctaa/-acc, and -ctaa/-agg

Source	*df*	SS	MS	Est. Var.	% Variation	Statistic	Value	*p*
Within Populations	71	1556.0	35.3	21.92	72%	φ_{PT}	0.283	0.001
Among Populations	6	353.7	58.9	4.02	13%	φ_{PR}	0.155	0.001
Among Regions	1	252.9	252.9	4.65	15%	φ_{RT}	0.152	0.001
Total	78	2162.6		30.58				

Plate 5.2. S.E.M. image depicting the massulae of *N. maculata* already on the stigma of an un-opened flower from Roccamonfina.



5.5 Discussion

5.5.1 Vegetation

Neotinea maculata occupies a different vegetation community in all populations studied. My analysis showed that *N. maculata* occurs in a wide range of vegetation communities and that it occurs in relatively species rich and species poor habitats. Isolated individuals have been recorded at the margins of woodland in the Burren region in the west of Ireland, which is similar to the common Mediterranean habitat of this species. However, most flowering populations of *N. maculata* in Ireland occur in grassland with limestone outcrops (KJ Duffy; pers. obs.). In Ireland, *N. maculata* is considered to be a member of the Lusitanian species suite where it belongs to a group of species that occur only in the west coast of Ireland and in the Mediterranean (Mitchell and Ryan, 2001). As orchid recruitment from seed is generally dependent on appropriate soil mycorrhizas (Rasmussen, 2002) the distribution of suitable mycorrhizal fungi in habitats may also explain the disjunct European distribution of *N. maculata*. Indeed, *N. maculata* has mycorrhizae taxa such *Tulasnella*, *Leptodontidium* and *Ceratobasidium* associated with its roots from samples examined from both Irish and Mediterranean populations; however the precise role of mycorrhizal symbionts in germination of seeds and seedling development has yet to be established in this species (MI Bidartondo, pers. comm., 2008). In addition, the presence of

suitable local microsite conditions for seedling establishment has been shown to be important for orchids (Jacquemyn *et al.*, 2007; Jersakova and Malinova, 2007) and needs to be established for *N. maculata*. Suitable microsite conditions can include abiotic conditions such as soil characteristics, like those examined in this study, in addition to suitable mycorrhizas. My data suggests that *N. maculata* can grow in a wide range of soil types independent of region. This is similar to *N. ustulata*, which can also occupy different habitats with varying soil chemical properties (Tali *et al.* 2004).

5.5.2 Breeding system and pollination

N. maculata is a fully autogamous species that can set fruit and seed in the absence of pollinators. This pollination system is believed to be an evolutionary 'dead-end' (Stebbins, 1957; Takebayashi and Murrell, 2001). However, it may have advantages such as an escape from a dependency on pollinators (Morgan and Wilson, 1999; Kalisz *et al.*, 2004). In addition, equivalent quantities of seed were produced when cross-pollinated compared with selfed, therefore *N. maculata* likely benefits from occasional cross-pollination, coupled with autonomous self-pollination. Casual daytime field observations of pollinator visitation made between other field work over the course of this study in both Irish and Italian populations of *N. maculata*, only one Syrphid in the Vesuvio population was seen visiting an *N. maculata* inflorescence; the visit lasted approximately 5 seconds and the fly did not visit any other flowers in the vicinity. With such infrequent visitation, autonomous autogamy may evolve if pollinators are unreliable in delivering outcross pollen (Kalisz and Vogler, 2003; Kalisz *et al.*, 2004). I noticed some flowers of *N. maculata* had massulae on their stigma before flower opening, which effectively means these flowers are cleistogamous, a reproductive strategy which may provide reproductive assurance on the population level (Berg and Redbo-Torstensson, 1998; Lu, 2002). However, the timing of pollen drop onto the stigma and whether some individuals purposely have delayed selfing to allow outcrossing, while some individuals automatically self in order to provide reproductive assurance for the population requires further investigation. In addition, the role of the rostellum and whether it breaks down early or late in anthesis in both early and late self-pollinating individuals should be investigated further. In comparison, *N. ustulata* has an average fruit set of 20.9% in Estonian populations with each capsule containing between 2,000-4,000 seeds (Tali *et al.*, 2004).

5.5.3 Population differentiation and genetic diversity

The genetic variability revealed by the AFLP markers was high, as 98.2% of the bands scored were polymorphic. The values of genetic differentiation obtained in this study are relatively

similar compared with other orchid studies (mean G_{ST} value among all species = 0.187; Forrest *et al.*, 2004). Although there is significant variation between populations in Ireland and Italy, the resulting genetic differentiation is lower than the congeneric *Neotinea ustulata* (mean F_{ST} = 0.51; Tali *et al.*, 2006). Given it's strong self-compatibility and autonomous autogamous pollination system *N. maculata* has likely purged its genetic load as I observed high levels of polymorphism among AFLP markers found in this study (Charlesworth and Charlesworth, 1987). The comparatively lower diversity values may be due to a large amount of autonomous selfing in *N. maculata*, which does not occur, for example, in the congeneric *N. ustulata* (Tali *et al.*, 2004). The lack of resolution in the UPGMA analysis based on all individuals' highlights that many individuals share bands. Although there is some evidence of separation of populations, most of the variation (72%) is explained within populations. However, when grouped into discrete populations and regions, both UPGMA and AMOVA revealed significant genetic variation between Ireland and Italy, and between all populations. The self-pollinating mechanism was probably already established in *N. maculata* prior to the glaciation, and individuals persisted in Ireland, owing to a favourable climate. Irish populations may have become isolated from Mediterranean populations over the last 8,000 – 10,000 years. However, it would be interesting to develop specific microsatellite markers that might be useful in revealing fine-scale population structure and detection of alleles, which dominant markers such as AFLP cannot detect (Selkoe and Toonen, 2006; Meudt and Clarke, 2007). Future work should focus on sampling more populations across the range of *N. maculata* to test whether all populations are genetically distinct or whether Irish populations are genetically distinct from all other populations of *N. maculata*.

5.5.4 Conservation issues

The 'abundant centre distribution' hypothesis is a widely held view in biogeographical ecology (Sagarin and Gaines, 2006). However, this hypothesis may not hold true, given that a review of studies addressing this biogeographic question, only 39% of 145 independent empirical tests support the abundant centre hypothesis using lenient criteria (Sagarin and Gaines, 2002). Indeed, Sagarin and Gaines (2006) point out that many studies fail to examine the entire range of focal species, which can lead to potential errors in interpretation. As we have not examined the entire distribution of *N. maculata* we cannot make a generalised statement regarding its fitness over its geographical range. However, we can say that *N. maculata* is not affected by a distribution that is more abundant in the centre of its range, as its population genetic diversity is similar at the edge and core of its distribution, and populations can occupy different vegetation communities in both regions. In addition, this study shows that *N. maculata* does not require pollinators for successful

seed set at both the centre and edge of its distribution and it does not suffer from deleterious effects of self-pollination, in terms of fruit and seed output. However, given (i) the unique vegetation communities in Ireland (e.g. it co-flowers with alpine plants such as *Dryas octopetala* and *Gentiana verna* in some populations), (ii) degree of genetic variation compared with Italian populations, and (iii) the small number of populations and individuals flowering within populations, Irish populations of *N. maculata* merit conservation attention. This should be in the form of annual monitoring in order to gauge natural population flowering fluctuations, and to ensure populations are not extirpated. For instance, in *N. ustulata*, dormancy has been shown to decrease adult survival (Shefferson and Tali, 2007) therefore it would be interesting to investigate the effects of dormancy on the survival of Irish populations of *N. maculata*. Also, research into the role of mycorrhizae on seed germination and seedling establishment in *N. maculata* may inform greatly of their influence on individual establishment and population abundance.

5.5.5 Conclusions

N. maculata is an autogamous self-pollinating orchid species. However, we found it does not suffer from negative effects resulting from self pollination, in populations examined at both the centre and edge of its distribution. It occurs in different vegetation communities each with varying soil properties among populations examined in this study. AFLP analysis revealed populations in the centre and edge of its distribution share similar levels of genetic diversity, however there are significant genetic differences among all populations and Italy and Ireland. In Ireland, it flowers in smaller populations that Italy and is restricted in its distribution. Therefore, Irish populations deserve specific conservation attention, as the effects of habitat destruction and management will have greater effects on population persistence than Italian populations.

"Natural species are the library from which genetic engineers can work.' - **Thomas E. Lovejoy, Time Magazine, 1986**

"*Without habitat, there is no wildlife. It's that simple.*" - **Wildlife Habitat Canada**

6. Population genetic structure and conservation of two endangered orchid species: *Cephalanthera longifolia* and *Pseudorchis albida*

To be submitted as: Karl J. Duffy, Michael F. Fay, Rhian J. Smith, and Jane C. Stout. Population genetic structure and conservation of *Pseudorchis albida* in Ireland.
To be submitted as: Claire Micheneau, Karl J. Duffy, Jane C. Stout and Michael F. Fay. Population genetic structure of *Cephalanthera longifolia* across its European range.

6.1 Abstract

Genetic diversity plays a key role in the conservation of endangered species. Both the number and size of populations of a species affects genetic diversity and are reduced by fragmentation of natural habitats reduces. Here, I examined the effects of small population size on the genetic diversity of two endangered and red-listed orchids in the Republic of Ireland, *Cephalanthera longifolia* and *Pseudorchis albida.* To determine genetic variability within and between populations of *C. longifolia* I used three microsatellite markers, and for *P. albida*, I used AFLP markers and four microsatellite markers. 36 individuals of Irish *C. longifolia* from four populations were compared with individuals from England, Iran, and Syria. All individuals, except for one, were identical at all SSR loci examined. AFLP markers showed that *P. albida* has a high level of polymorphism and significant variation both within and between populations with increasing population genetic isolation through distance. However, the SSR markers used were identical across all loci. Interestingly, when compared with other *Pseudorchis* samples, the SSR markers show all Irish individuals have identical alleles to *P. straminea* rather than *P. albida.* Irish populations of *C. longifolia* and *P. albida* require immediate conservation attention due to their low numbers and low numbers of individuals within populations.

6.2 Introduction

Within and among populations of endangered species, genetic diversity is a key factor that determines whether populations can adapt to stochastic and environmental changes (Ellstrand and Elam, 1993). The level of genetic diversity within endangered species may play a key role in preventing extinction and maintaining evolutionary potential (Charlesworth and Charlesworth, 1987; DeSalle, 2005). Human mediated habitat fragmentation is a leading cause of extinction in natural populations, which involves (i) loss of habitat, (ii) reduced fragment size, and (iii) increased spatial isolation of remnant fragments (Fahrig, 2003). Small populations that exist in fragmented habitats are more prone to environmental and demographic stochasticity (Shaffer, 1981; Lande, 1988). In addition, a rapid decrease in population size caused by fragmentation

may result in loss of genetic variation due to genetic drift and increased selfing (Barrett and Kohn, 1991). Therefore, conservation of populations of endangered species requires evaluation of genetic patterns and structure, in addition to demography (Lande, 1988; Pillon *et al.*, 2007a).

In plant species, genetic differentiation within and among populations can be influenced by a number of factors including; (i) the mating system of the species (Barrett and Kohn, 1991), (ii) demographic patterns (Young *et al.*, 1996; Honnay and Bossuyt, 2005), and (iii) historical factors such as whether the population has gone through a recent bottleneck (Kang *et al.*, 2008). The mating system of a species will influence survival and genetic structure due to their degree of reliance on pollinators (Barrett and Harder, 1996; Aguilar *et al.*, 2006). In addition, demographic patterns such as the longevity of individuals may moderate the loss of alleles through genetic drift (Gitzendanner and Soltis, 2000). These factors impact on genetic diversity and whether populations of an endangered plant species can adapt to increasing fragmentation. In recently reduced and isolated populations, the impact of these factors may be more pronounced on genetic diversity, which affects the short and long-term survival of the species (Reed and Frankham, 2003).

One factor determining the level of genetic diversity within and among populations is the number of populations of a species in a given geographical region (Hanski, 1998; Lammi *et al.*, 1999). If numerous populations occur in a region they can buffer against extinction by re-populating of the extirpated population (Hanski, 1998), however there would be less genetic differentiation among such populations (Lammi *et al.*, 1999). In addition to the number of populations of a species, population size may play a key role in determining the success of individuals (Fritz and Nilsson, 1994). Larger populations may have higher fecundity and greater genetic diversity, due to (i) increased pollinator attraction resulting in increased outcrossing (Fritz and Nilsson, 1994) (ii) higher persistence due to presence of suitable micro-sites for recruitment (Eriksson and Ehrlén, 1992; Jacquemyn *et al.*, 2007) (iii) less inbreeding depression and more heterozygosity (Leimu *et al.*, 2006). However in a recent meta-analysis, Honnay and Jacquemyn (2007) show there are no differences in the susceptibility of either common or rare plant species to the negative genetic effects of habitat fragmentation, therefore relative population size may be of little consequence.

Members of the Orchidaceae are adapted for animal-mediated outcrossing (Nilsson, 1992; van der Cingel, 1995), so may suffer from a lack of outcrossing if there are declines in pollinator species diversity and abundance resulting from habitat fragmentation. Also, there have

been concerns over regional declines of orchid species due to habitat fragmentation (Kull and Hutchings, 2006). *Cephalanthera longifolia* and *Pseudorchis albida* are two endangered orchid species in the Republic of Ireland (Curtis and McGough, 1988) and are protected by the Wildlife Act (1976) and the Wildlife Amendment Act (2000) under the Flora Protection Order (1999). *C. longifolia* is a nectarless, food-deceptive, woodland species, pollinated by solitary bees (Dafni and Ivri, 1981). *P. albida* is a nectariferous, grassland species, pollinated by moths (Baumann and Baumann, 2002). Both have declined because of habitat modification and destruction in Ireland: *C. longifolia* with the destruction of native woodland and abandonment of traditional management practices, *P. albida* with increasing agricultural improvement of grassland (Preston *et al.*, 2002). *C. longifolia* occupies only seven 10 x 10 km squares in Ireland out of a total of 41 occupied in Britain and Ireland while *P. albida* occupies 27 10 x 10 km squares out of a total of 165 occupied in Britain and Ireland (Preston *et al.*, 2002).

To assess genetic variation within both species, I employ both microsatellites (SSRs) and amplified fragment length polymorphisms (AFLPs) (Vos *et al.*, 1995). I used SSRs with *C. longifolia* as plastid microsatellite markers were previously developed for this species (Micheneau, 2002), and previous problems generating AFLP markers (Fay *et al.*, 2005). Both SSRs and AFLPs were used on *P. albida*. AFLPs were used in addition to SSRs with this species because the SSRs employed were not specifically designed for *P. albida*, which may entail problems of transferability among taxa (Peakall *et al.*, 1998). Also, AFLPs require no prior knowledge of the DNA sequence and provide large amounts of data with reproducible results, and have been used on other orchid taxa (e.g. Smith *et al.*, 2004; Tali *et al.*, 2006; Pillon *et al.*, 2007a).

In this chapter, I examine the effect of small population size and low number of populations on the population genetic structure of the endangered *C. longifolia* and *P. albida* in Ireland. Specifically, I test the hypothesis that due to a small number of populations and flowering individuals within populations, individuals and populations of both *C. longifolia* and *P. albida* have low genetic differentiation.

6.3 Materials and Methods

6.3.1 Plant sampling and DNA isolation

No more than one-half of a leaf from two- or more-leaved individuals was taken. All samples were collected during the summers of 2005 and 2006 and stored in zip-lock plastic bags containing silica gel (Chase and Hills, 1991) until total DNA was extracted. I sampled leaf

material from four populations of *C. longifolia* and four populations of *P. albida* in Ireland (see Figure 6.1 for location of populations). These populations were found after intensive searches using historical records. Sampling was undertaken when individuals were in full anthesis and although samples were taken randomly, they represent the range of occurrence within the flowering population. Details of sites and number of individuals sampled are given in Table 6.1. DNA was extracted from approximately 0.01-0.1g of dried material using a modified 2x-CTAB (cetryltrimethyl-ammonium bromide) procedure (Doyle and Doyle, 1987), purified on a QIAquick column (Qiagen Ltd., Crawley, West Sussex, U.K.) and quantified using a spectrophotometer.

6.3.2 Genetic analysis

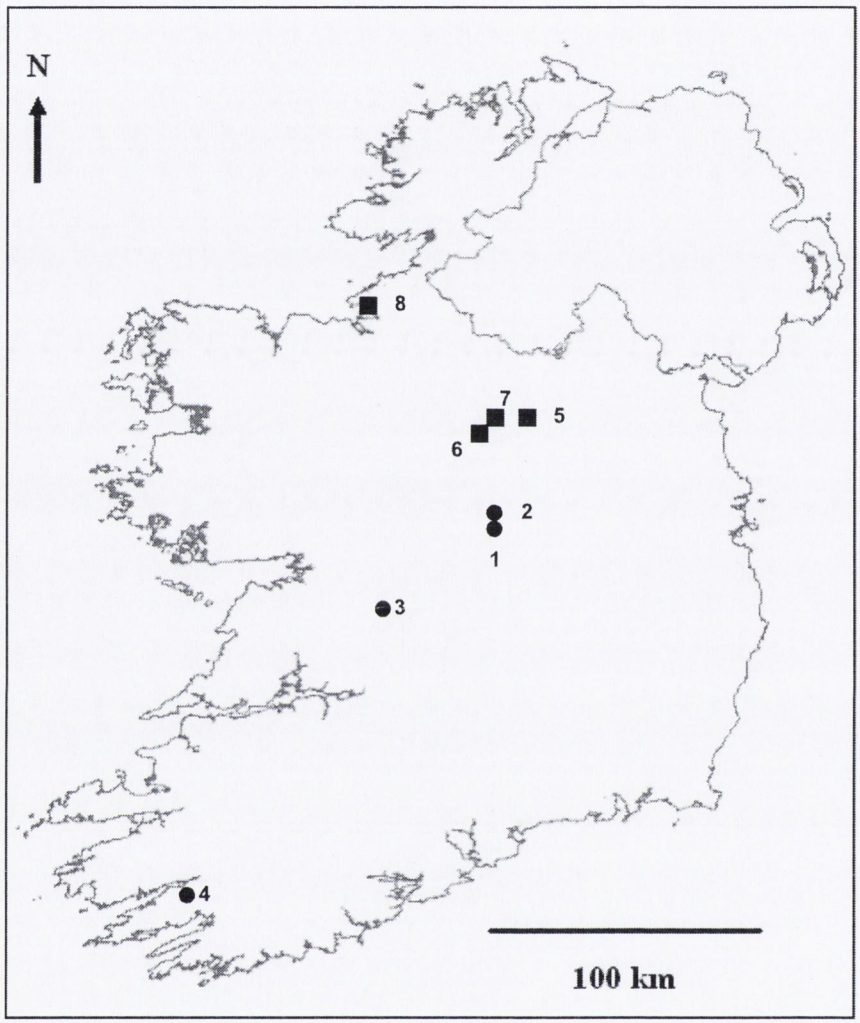
Cephalanthera longifolia - *SSRs*

Primer sequences for the three polymorphic microsatellite loci used are given in Table 6.2. These loci have been successfully used previously on eight species of *Cephalanthera* including *C. longifolia* (Micheneau, 2002). Each reaction contained 17.5 µl 2.5 mM $MgCl_2$ PCR Master Mix (ABgene), 0.5 µl of 0.4% BSA, 0.5 µl each of forward and reverse primer (100 ng/µl), and 1 µl of template DNA. The following PCR program was used: 2 min at 94°C, 28 cycles of 30 sec at 94°C, 1 min at 51°C, and 30s at 72°C, with a final extension of 10 min at 72°C.

Pseudorchis albida - *SSRs*

Primer sequences for the four SSR loci are given in Table 3. Each reaction contained 9.2 µl 2.5 mM MgCl2 PCR Master Mix (ABgene), 0.2 µl of 0.4% BSA, 0.1 µl of each of the eight primers (100ng/µl), and 0.4 µl of template DNA. The following PCR program was used: 4 min at 94°C, 26 cycles of 30 sec at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final elongation of 10 min at 72°C. These primers were previously used on both *P. albida* and *P. straminea* accessions from the DNA bank in RBG Kew (accession reference numbers: 15978 - *P. albida*; 5556 - *P. straminea*).

Figure 6.1. Locations of *Cephalanthera longifolia* and *Pseudorchis albida* populations sampled in this study. Closed circles are *C. longifolia* populations; closed squares are *P. albida* populations. Population number codes: 1 = Coosan Point; 2 = Hare Island; 3 = Rosturra Wood; 4 = Glengarriff; 5 = Shannon Pot; 6 = Corleckagh; 7 = Corratober; 8 = Rosses' Point.



Pseudorchis albida - *AFLPs*

AFLP analysis was performed according to the AFLP Plant Mapping protocol of Applied Biosystems Inc. Sampled DNA was restricted with the endonucleases *Eco*RI and *Mse*I and ligated to appropriate double-stranded adapters according to the manufacturer's protocols. Genome size can have a marked effect on the quality of AFLP traces, with species with large genomes performing better with the addition of an extra base (Fay *et al.*, 2005). Therefore, I initially tested both regular primers (3 + 3 selective bases) and primers that incorporated an additional base (3 + 4 selective bases). In the latter, the pre-selective amplification uses primers, each with one additional base, followed by a selective amplification using primers each with four additional bases, thereby further reducing the number of fragments. Twelve different primer

combinations were tested (six regular primers and six incorporating an additional base), of which two, which incorporated an additional base, were chosen for the full study as they produced reliable bands: -ctac/-aca and -ctaa/-acg.

AFLP profiles were manually scored with the presence of each band as '1', and the absence of a band as '0'. Markers with evidence of small, un-scorable peaks were discarded from all samples. Bands ranging from 50bp – 500bp were scored. All statistical analyses were performed using GenAlEx 6.1 (Peakall and Smouse, 2006). The calculation of genetic distances followed the method of Peakall *et al.* (1995) and outlined in Maguire *et al.* (2002).
Both band-presence and absence are considered informative which gives a Euclidean metric as required for subsequent analysis of molecular variance (AMOVA). Genetic distance matrices for each AFLP primer set on their own, and the total data set (two primer sets combined) were calculated using Nei's distance (Nei, 1972). Genetic structure was tested by AMOVA on the total genetic distance matrix (999 permutations). AMOVA output nomenclature follows that of Excoffier *et al.* (1992) in that variation was summarised both as the proportion of the total variance and as ϕ-statistics (*F*-statistic analogues). I tested for genetic differentiation both within populations and between populations. Genetic diversity within populations was measured using the Nei diversity indices, as calculated with POPGENE 1.32 (Yeh *et al.*, 2000). Mantel tests were performed (999 permutations) to test whether genetic patterns detected by one AFLP primer set were congruent with the patterns detected by the other primer set. In addition, I tested for a genetic-geographic relationship between populations using a Mantel test. This was done by calculating a geographic distance matrix for each population based on lat-long grid references taken in the field during sampling and testing this matrix against the genetic distance matrix. Based on the output of individual genetic distance matrix I performed a principle coordinate analysis (PCO) to group individuals. In addition, UPGMA was used to analyse the relationships between all individuals using PAUP 4.0 (Swofford, 2000).

Table 6.1. Details of sampled populations, number of individuals sampled per population, and AFLP banding patterns within populations (*P. albida* only). Question marks (?) are given because heterozygosity was not calculated due to low diversity at SSR loci.

Species	Population	No. flowering	No. sampled	No. samples DNA analysed	No. bands	No. private bands	Mean gene diversity (± S.D.)	Mean heterozygosity (± S.E.)
C. longifolia	Coosan Point	20	18	10	-	-	-	?
C. longifolia	Hare Island	12	7	7	-	-	-	?
C. longifolia	Rosturra Wood	~80	14	14	-	-	-	?
C. longifolia	Glengarriff	5	5	5	-	-	-	?
P. albida	Shannon Pot	8	8	4	7	2	0.059 (0.129)	0.032 (0.015)
P. albida	Corratober	14	14	8	25	3	0.199 (0.221)	0.199 (0.033)
P. albida	Corleckagh	8	8	4	11	1	0.069 (0.136)	0.069 (0.021)
P. albida	Rosses' Point	39	22	21	28	14	0.198 (0.195)	0.198 (0.029)

Table 6.2a. Microsatellite loci and primer sequences used on individuals of *C. longifolia*.

Locus	Primer	Primer sequence
*psa*1-*acc*D	*psa*1-*acc*D Ceph.mic.F	5' GGA AGT ATA GCA CTA GCT TC 3'
	*psa*1-*acc*D Ceph.mic.R	5' ACT TAT AAC TGA TGT CCC CA3'
*trn*S-*trn*G	*trn*S-*trn*G Ceph.mic.F	5' GTT CAA GGA AAG GAC AAG T 3'
	*trn*S-*trn*G Ceph.mic.R	5' AAC ACG TAT TTT GTT CCT CC 3'
*trn*K-*mat*K	*trn*K-*mat*K Ceph.mic.F	5' ATC TTC CTC CAA AAA ATC CC 3'
	*trn*K-*mat*K Ceph.mic.R	5' GAG CAT TTC AGA ATA CAA ATT C 3'

Table 6.2b. Microsatellite loci and primer sequences used on individuals of *P. albida*.

Locus	Primer	Primer sequence or reference
Dact Ms1	*trn*S	Hamilton (1999)
	Dact Ms1	5' CGT TGG AAC AAA AGA AGT AC 3'
Dact Ms2	*Dact* Ms2	5' GAG TAA TAG TGT TTC TAA GAG 3'
	*trn*G	Hamilton (1999)
*Orch*1	*Orch*1 F	Fay and Cowan (2001)
	*Orch*1 R	Fay and Cowan (2001)
Dact Msf	*Dact* Msf	5' CTA AGA AAT TAA GGG GGC TA 3'
	*trn*L-f	Taberlet *et al.* (1991)

6.4 Results

6.4.1 Cephalanthera longifolia - *SSRs*

36 Irish individuals from four populations were analysed using three microsatellite markers, along with two individuals from each of three English populations, two individuals from Syria, and one Iranian individual all included from collections in RBG Kew (Table 6.2). Among Irish populations, only one individual from the Glengarriff population had a unique cytotype, which is the same as a cytotype found in other English and French populations (data compared with Micheneau, 2002). Individuals from the Rosturra Wood, Hare Island, and Coosan Point populations were identical to each other and individuals from English populations and the Iranian individual (Table 6.3). However, the two Syrian individuals have unique cytotypes compared with other individuals in this study (Table 6.3).

6.4.2 Pseudorchis albida - *SSRs*

41 individuals from four populations were analysed using four microsatellite primers. All loci were identical in all individuals, with allele peaks at 85bp (*Orch*1), 163bp (*Dact* Msf), 232bp (*Dact* Ms1), and 224bp (*Dact* Ms2). When compared against accessions of *P. albida* and *P. straminea* in RBG Kew, we found that all Irish samples matched the alleles of the accession for *P. straminea* rather than *P. albida* (alleles peaks in *P. albida*: *Orch*1 - 92bp, *Dact* Msf - 163bp, *Dact* Ms1 – unknown, and *Dact* Ms2 - 222bp).

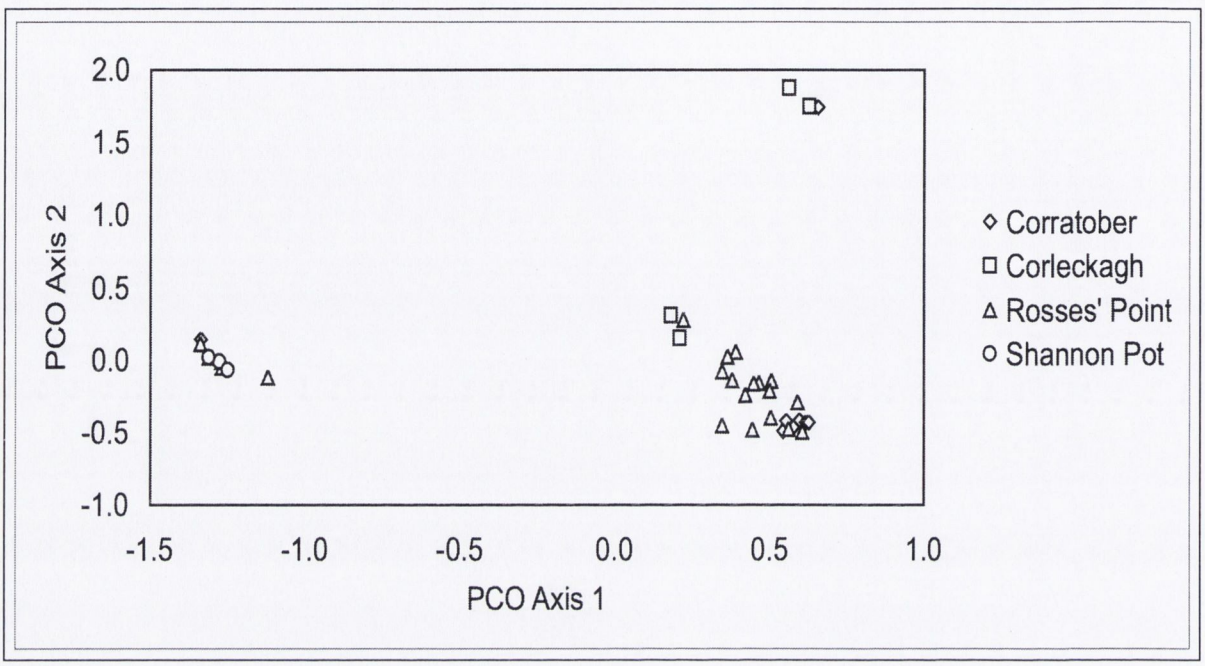
6.4.3 Pseudorchis albida - *AFLP*

44 interpretable bands were produced; 20 -ctac/-aca and 24 -ctaa/-acg among 37 *P. albida* individuals analysed. Overall, 42 bands were polymorphic (95.45%). A Mantel test revealed that datasets derived from individual primer-pair combinations were not correlated (-ctac/-aca vs. -ctaa/-acg: $r_{xy} = 0.078$; $p = 0.158$). Both the PCO analysis (Figure 6.2) and the UPGMA analysis on individuals (Figure 6.3) show no clear differentiation of populations. However, when analysed according to population, AMOVA revealed significant genetic variation within and among populations ($\phi_{PT} = 0.306$; $p < 0.001$), with 69% of variation explained within populations and 31% of variation explained among populations. The Mantel test between genetic and geographic distance revealed a significant relationship ($r_{xy} = 0.234$; $p = 0.003$), indicating increased genetic isolation of populations through distance.

Table 6.3. Alleles, allelic frequency and observed cytotype for each locus based on microsatellite data for individuals of *C. longifolia* from four Irish populations, three English populations, and individuals from Syria and Iran.

Locus	Allele	Code	Coosan	Hare Island	Rosturra	Glengarriff	ENG 1	ENG 2	ENG 3	SYR	IRA
*psa*1-*acc*D	113	A	1	1	1	1	1	1	1	-	1
	118	B	-	-	-	-	-	-	-	0.5	-
	124	C	-	-	-	-	-	-	-	-	-
	128	D	-	-	-	-	-	-	-	0.5	-
*trn*K-*mat*K	169	E	1	1	1	1	1	1	1	0.5	1
	171	F	-	-	-	-	-	-	-	0.5	-
*trn*S-*trn*G	213	G	1	1	1	0.8	1	1	1	-	1
	215	H	-	-	-	0.2	-	-	-	-	-
	216	I	-	-	-	-	-	-	-	1	-
No. of individuals			10	7	14	5	2	2	2	2	1
Cytotype			AEG	AEG	AEG	AEG AEH	AEG	AEG	AEG	BEI DFI	AEG

Figure 6.2. Principle co-ordinate (PCO) plot of the first and second axes for the full data matrix based on two AFLP primer pairs used on *P. albida*. Axis 1 accounts for 49.84% of variation; axis 2 accounts for 26.78% of variation.

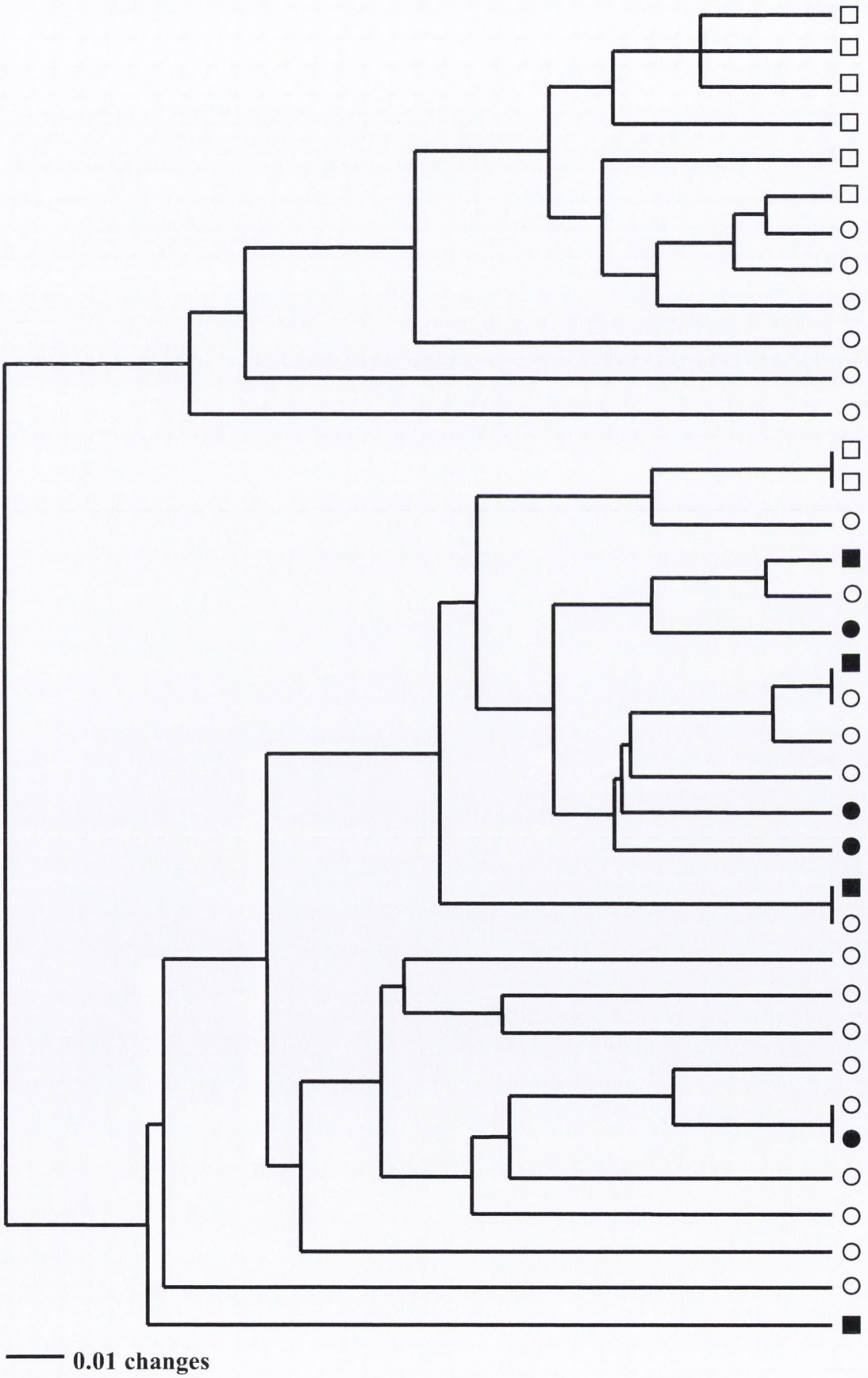


6.5 Discussion

6.5.1 Cephalanthera longifolia

Little genetic differentiation was found among individuals and populations of *C. longifolia* in this study. Irish individuals were found to be mostly identical to English individuals, except one individual that had an allele also found in other English and French samples (Micheneau, 2002). This supports previous work by Micheneau (2002), in that according to these microsatellite markers, the most common cytotype among all populations (in 65 individuals from 17 countries) is the AEG cytotype found this study. Interestingly, the AEH cytotype was found only in five of 92 individuals analysed (Micheneau, 2002), so it represents a rare cytotype. Species that occur in small populations usually have high rates of inbreeding and frequent or severe population bottlenecks and less genetic diversity (Ellstrand and Elam, 1993), which may be the case here, as such species typically have lower polymorphism and heterozygosity (Leimu *et al.*, 2006). However, given that the AEG cytotype in *C. longifolia* is common in other samples across its range, it is likely that this is the major cytotype detected by these primers. The extent of genetic diversity and polymorphism in *C. longifolia* needs to be examined further. Irish populations contain 2 of the 8 total cytotypes found in *C. longifolia* detected by these markers (Micheneau, 2002). Data from these microsatellite markers suggest Irish populations of *C. longifolia* are

Figure 6.3. Rooted UPGMA tree of the relationship between all individuals of *P. albida* used in this study based on the two AFLP primer pairs. Open squares indicate individuals from Corratober; closed squares indicate individuals from Corleckagh; open circles indicate individuals from Rosses' Point; closed circles indicate individuals from Shannon Pot.



probably a subset of European populations, and may have gone through a bottleneck, although further SSR development or successful implementation of ALFP markers would reveal more on overall genetic diversity within Irish populations compared with others.

C. longifolia is pollinated by solitary bees (*Halictus* sp.) in Israel (Dafni and Ivri, 1981), however during other field work I noted only two visits from bumblebees (from the *Bombus terrestris/lucorum* complex, the workers of which are morphologically indistinguishable in the field: Edwards and Jenner, 2005). Genetically, it behaves as an obligate outcrossing species, with a G_{ST} value of 0.104 in Italian populations based on allozyme data (Scacchi *et al.*, 1991). As it is a food deceptive species, it may suffer from low pollination frequency, which is common in deceptive species (Neiland and Wilcock, 1998), hence a lack of outcrossing. However, a lack of nectar reward and subsequent lower pollination success is not necessarily related to extinction (Jacquemyn *et al.*, 2005). Hand pollinations indicate that *C. longifolia* can produce fruit and seed in Ireland, however natural levels of fruit set appear very low, with only 2 fruits observed on separate individuals observed in the Rosturra Wood population, however high levels of browsing from deer, where entire inflorescences were destroyed is common (KJ Duffy, *personal observation*).

6.5.2 Pseudorchis albida

The microsatellites used in this study revealed Irish *P. albida* samples are identical to *P. straminea* at these loci. However, the division of *P. albida* and *P. straminea* needs to be further clarified. These species are divided in Scandinavia on the basis of morphological, isozyme and ecological data, although there is some overlap in characters from individuals in central Europe (Reinhammar, 1995; Reinhammar and Hedrén, 1998; Reinhammar, 1998; Reinhammar *et al.*, 2002). Reinhammar and Hedrén (1998) showed by analysing four populations of *P. albida* and two populations of *P. straminea* in Sweden, *P. albida* had 6.7% polymorphism, while *P. straminea* had 16.7% polymorphism. Five loci were variable between both taxa, with an overlap in four of the five loci. Of 22 alleles found, only two were confined to *P. albida*. Reinhammar (1998) found that there was no separation of these taxa in central Europe on morphological data, but there was a more clear separation in Scandinavia. Indeed, Reinhammar and Hedrén (1998) believe there was immigration into Scandinavia at different times, which led to differentiation of these species. This may not be the case elsewhere in their range and they may comprise one species perhaps as subspecies. One of the key differences between *P. albida* and *P. straminea* is the habitat each species occupies; *P. albida* occupies a wide variety of habitats, while *P.*

straminea occupies arctic-alpine habitats (Reinhammar *et al.*, 2002). SSRs tend to have low transferability across even closely related taxa (Peakall *et al.*, 1998) and the primers used in this study were not developed specifically for *P. albida.* Indeed, they were used in a recent large-scale study of *Dactylorhiza* species (Pillon *et al.*, 2007b) and were used as they were previously successfully tested on individuals of *Pseudorchis* (unpublished data, RBG Kew). Therefore, it would be interesting to develop specific SSR markers for *P. albida*, to see if there is variation according to other microsatellite loci.

AFLP data revealed significant variation among individuals and populations of *P. albida* with most diversity explained within populations, which shows populations have high genetic differentiation. The lack of congruence between the two AFLP primers used is probably due to the relatively few markers produced i.e. there would be a stronger relationship if more bands were produced. The differentiation value ($\phi_{PT} = 0.306$) is higher, but similar to a previous diversity found using allozymes ($G_{ST} = 0.150$ in *P. albida*; $G_{ST} = 0.240$ in *P. straminea*) (Reinhammar and Hedrén, 1998). It is also higher than the average for other terrestrial species (overall mean $G_{ST} = 0.187$) (Forrest *et al.*, 2004), however diversity values are higher for rewarding species than non-rewarding species ($G_{ST} = 0.2 – 0.3$ for rewarding species; $G_{ST} = 0.1 – 0.15$ for non-rewarding species) (Cozzolino and Widmer, 2005). The Mantel test showed increasing genetic isolation with distance. This shows that there is a genetic separation of populations the further away they are from one another. Interestingly, half the bands in the Rosses' Point population are private bands (14 of 28 bands). This population is the furthest population from the others and is ecologically different, insofar as it is coastal and low-lying, while the others tend to be upland and in-land. However, both the UPGMA and PCO analyses failed to group individuals according to population although the Rosses' Point population is generally more separated from the other populations. This is interesting given the results from other tests, but it means that when they are not grouped into discrete populations for analysis, individuals of *P. albida* share bands. Indeed, most variation (69%) was found within populations. Therefore, in restoration efforts, individuals from one population can be transplanted to another site without negative effects of heterosis (outbreeding depression) (Templeton, 1986).

6.5.3 Sampling of endangered orchids

These orchid species occur in very few populations in Ireland, with few flowering individuals in each population. Therefore, even though genetic studies can reveal much about the conservation value and potential of endangered species (Spielman *et al.*, 2004), sampling for reliable genetic data in these species can be problematic. For example, we sampled from four of the seven

currently known populations of *C. longifolia* in Ireland (the presence/absence of some populations is debatable as some may have been recently extirpated), and that most populations tend to flower in low numbers, I would expect to detect if any unique cytotypes were present with our current sampling. Indeed, I found the one unique cytotype in the smallest population, which indicates that, all populations, regardless of size, are of conservation value. With endangered orchids, it is difficult to sample the entire population, due to demographic stochasticity (i.e. individuals can remain dormant, or return to dormancy, for many years), which is characteristic of orchids. Unless populations are monitored over numerous years, it is difficult to get an accurate census of true population size populations of any orchid species and to analyse the role of demography in their rarity (Hutchings, 1987; Lande, 1988). For instance, *C. longifolia* is a long-lived perennial species that can remain dormant for many years (Shefferson *et al.*, 2005). It is known to be prone to adult whole-life dormancy, which may be a response to grazing stress (Shefferson *et al.*, 2005). Therefore, grazing not only destroys the inflorescence, but also may induce dormancy in this species, which in turn makes it difficult to detect and sample this species.

6.5.4 Conservation implications

This study has yielded important findings that have both short and long term conservation implications. Firstly, my SSR data has shown that Irish populations of *C. longifolia* are genetically similar. In addition to occurring in small and few populations, this low genetic variation highlights the need for conservation attention. Secondly, I found *P. albida* has genetic variation within and among populations, and there is increasing genetic difference through distance. Therefore, these populations merit conservation. In addition, the taxonomic status of Irish *P. albida* should be investigated further as to ascertain whether *P. albida* or *P. straminea* occurs in Ireland. Both species, but particularly, *P. albida* requires a short sward and grazed habitats, and is negatively affected by the over abundance of grasses and sedges (Reinhammar *et al.*, 2002). However, in the populations examined, both species suffer from high levels of; (i) deer browsing (*C. longifolia*) and (ii) sheep, cow and horse grazing (*P. albida*) during flowering (KJ Duffy, *personal observation*), which is known to negatively effect the recruitment and survival of orchid populations (Hutchings, 1987a). These results highlight the need for urgent monitoring and conservation of remaining populations of both species. Ultimately this should be in the form of annual monitoring to gauge population fluctuations, examination of the role of mycorrhizae in seed germination and seedling establishment in these species, further genetic characterisation of populations, collection of seed for ex situ propagation, and managing grazing regimes

7. General Discussion

7.1 Summary

The main aims of this thesis (see pages 23-24) were to investigate factors that influence the reproductive biology and conservation of rare orchids in Ireland. Over the course of this thesis, the study species varied according to hypotheses investigated. For instance, in order to infer threat status of rare orchids, I made field surveys and examined sighting records of *Cephalanthera longifolia*, *Hammarbya paludosa*, *Neotinea maculata*, and *Pseudorchis albida* on the basis that these species were found in few, small flowering populations and probably suffered from under-recording. To estimate both regional under-recording and extinction threat, I employed various probabilistic models to test whether the sighting records of each species can infer extinction (Chapter 2). In order to investigate factors that influence pollination limitation, I studied two members of the genus *Spiranthes* (Chapters 3 and 4). Specifically, I examined the role of population size, density, and co-flowering community on the pollination of *Spiranthes romanzoffiana* and *S. spiralis*, as their populations flowered with enough individuals for replicate observations. In addition, I examined the population genetics of *C. longifolia*, *N. maculata*, and *P. albida* (Chapters 5 and 6). With *N. maculata*, I compared population genetic structure using AFLPs in both Italian populations (where it is common) and Irish populations (where it is rare) (Chapter 5). I also examined whether habitat specificity and breeding system affected the rarity of *N. maculata* by comparing Italian and Irish populations. With *C. longifolia* I used SSRs and *P. albida* I used both AFLPs and SSRs to examine genetic variation within and among populations (Chapter 6). This section summarises the results from these investigations and outlines limitations and suggestions for further work.

7.1.1 Inferring extinction of rare orchids

Species extinction is occurring at an increasing rate, however it is difficult to infer precisely when and where a given species goes extinct (Roberts, 2006; Chapter 2). Historical sighting records offer valuable data on the occurrence of a species through time. However, there are inherent problems in the sighting and recording process. For instance, with a binary sighting record we can never be sure if populations were searched for and not found, or not searched for at all (McInerny *et al.*, 2006). This affects the quantification of the sighting (or collection effort for museum and herbarium samples) (e.g. Burgman *et al.*, 1995). An index of recording (or collection) effort is useful for the application of models such as those employed in Chapter 2; indeed the use of the collection effort index has been previously highlighted (McCarthy, 1998). There are other models available for inferring the threat status of a species, for example; the runs

test, the trend analysis, and the partial Solow model, all of which take into account recording effort both within and between years (Burgman *et al.*, 1995; Reed, 1996; McCarthy, 1998). However, given the congruent results generated from among the five models used in Chapter 2, the sparse historical records available, the models I employed gave a robust estimate of threat status based on the records available. I did not include multiple sightings within years when calculating the various threat estimates, although multiple sightings within-years have been included in other studies (e.g. Burgman *et al.*, 1995). This is because one assumption of the models employed is independence of sightings within and among years, and in many cases it is difficult to know if sightings were recorded independently i.e. not as a result of a concerted within-year effort to find focal species. Therefore, the probability values generated by the models are conservative in inferring threat i.e. lower *p* values are produced when multiple within-year sightings are included. In addition, the majority of studies using such methods do not include multiple records within years (e.g. McCarthy *et al.*, 1998; Roberts and Solow, 2003; Roberts and Kitchener, 2006). As discussed in Chapter 2, there is a danger in 'crying wolf' over extinctions as it has the potential to undermine public support for conservation efforts (Ladle *et al.*, 2004). Therefore, while it is important to highlight extinction events, caution is required to ensure that such statements are based on reliable data and analysis.

Analysis of sighting data should be viewed as a preliminary investigation into the causes of species decline. For instance, an individual sighting of a plant species in a given year may constitute just few flowering individuals in one population, which, according to the models used, is quantitatively equivalent to an observation of a thousand individuals in a population. Although such observations yield valuable data on the presence or absence of a species in space and time, it does not give any data on the viability of such populations. As discussed in Chapter 1, rare species in fragmented habitats are prone to demographic, environmental, and genetic stochasticity. Although a regional-scale analysis to infer threat can yield valuable insights to where declines are occurring; demographic, environmental and genetic data are of great importance for the conservation of rare and endangered species. For instance, populations of rare plant species may not be attractive to pollinators (due to low density or otherwise) and they may suffer from reduced seed set (e.g. Waites and Ågren, 2004) and subsequent increased extinction risk (Lennartsson, 2002).

Medium- to long-term annual flowering data of marked individuals (i.e. > 10 years) can greatly improve the sensitivity of models to predict the extinction dynamics of a species (e.g.

Fagan and Holmes, 2006). However, even short-term data can be useful to explore mechanisms of population fluctuations and extinction dynamics in orchid species. For example, Shefferson *et al.* (2005) used three consecutive censuses of marked individuals of *Cephalanthera longifolia* and *Cypripedium calceolus* to infer that dormancy may buffer against stress in these species in Estonia. Most studies that monitored orchid annual flowering population fluxes have data for 10 years (e.g. Hutchings, 1987a; Willems and Dorland, 2000). Such marking of individuals of rare orchids in Irish populations would provide useful data to see how they respond to management strategies.

In an alternative approach to analyse the national decline of orchids, Kull and Hutchings (2006) analysed records of the occurrence of orchids within 10 x 10 km squares in the UK and Estonia and included the habitat characteristics, rather than ecological characteristics (e.g. fruit set: Neiland and Wilcock, 1998; presence of a nectar reward: Jacquemyn *et al.*, 2005) for each species in their analysis. They found that orchid species that occur in calcareous grassland and woodland declined the most, as these habitats have declined greatly. In addition, orchid species confined to dry soils, with a short life span (< 3 year genet half-life), and short in stature (< 30cm tall) declined most rapidly. These findings are in accordance with other studies. For instance, Fischer and Stöcklin (1997), investigated local extinctions in calcareous grasslands between 1950 and 1985, found that species with a short life cycle were more prone to extinction compared to long-lived perennials. Similarly, Honnay and Bossuyt (2005) found that species with a long life span and clonal growth were more persistent to habitat deterioration and fragmentation. Kull and Hutchings' (2006) argued for specific local conservation strategies to prevent national losses of orchid species, because of the specific habitat type some orchid species occur in. The models used in Chapter 2 are useful to highlight such areas in urgent need of recording and conservation action. The use of such models could be expanded to include all orchid species in Ireland, or to other plant species considered rare. This would inform conservation authorities if there is a trend in decline of orchids or whether there are counties where rare species in general are going extinct.

Although three endangered, red-listed orchid species (*Cephalanthera longifolia*, *Hammarbya paludosa*, *Pseudorchis albida*) and the rare *Neotinea maculata* were investigated using probabilistic models, the one other endangered, red-listed orchid, *Spiranthes romanzoffiana*, was not investigated. This is because more is known of its conservation status than the other species. Its population biology, breeding system, and genetics in Ireland have been

recently investigated (Lupton, 2008), it is subject to an All Ireland Species Action Plan (2005), and additional work on its reproductive biology and the factors failure of fruit maturation in this species was recently undertaken (O'Conner, 2006).

7.1.2 The pollination ecology of rare orchids

Recent reviews suggest that increasing fragmentation accelerates the disruption of mutualisms such as plant-pollinator interactions, with rare plants that depend on pollinators for seed set suffering most pollination limitation (Knight *et al.*, 2005; Aguilar *et al.*, 2006; Vamosi *et al.*, 2006). In this thesis, I investigated the pollination biology of the endangered *S. romanzoffiana* at different spatial scales, both within small and large populations, and at varying densities within populations (Chapters 3 and 4). In addition, to test for the negative effects of rarity on pollination, I compared the pollination of *S. romanzoffiana* with the relatively more abundant *S. spiralis* (Chapter 4), as they both share similar ecological traits, such as a reliance on pollinators and nectar rewarding. Such a comparison of the pollination ecology of common-rare congeners can provide useful theoretical and practical data for conservation authorities (see section 1.1.3).

Spiranthes romanzoffiana was visited frequently by bees particularly *Bombus pascuorum*. This is the first record of pollination of *S. romanzoffiana* in Ireland, and rules out a lack of a pollinator as an explanation for its failure to set fruit (Chapter 3). When studied within-population at a local scale, *S. romanzoffiana* inflorescences were shown to compete for pollinator attention as density increased (Chapter 3). This is likely due to individual inflorescences competing for the limiting resource of pollinator attention (sensu Ratchke, 1983). In addition, I examined the effects of nectar reward offered by *S. romanzoffiana* and co-flowering species on pollinator attraction. The quantity and quality of nectar reward was variable over the flowering season, with flowers of *S. romanzoffiana* containing most nectar in the middle of the flowering season. Combined, the investigations into *S. romanzoffiana* pollination highlight the temporal variation among years in pollinator attention to plant individuals. Direct observations of bee visitation to *S. romanzoffiana* varied dramatically between 2005 and 2006. I observed 273 visits to entire inflorescences in 2005 while only 52 visits to individual flowers were recorded in 2006, however the time periods making within-population observations differed among populations between 2005 and 2006, with more observation periods made within-population in 2005 (see Chapters 3 and 4). Due to high visitation rates and rapid movement of bees on flowers in 2005, visitation rate per inflorescence was measured instead of per flower. However, in 2006, there were fewer visits and visitors so visitation per flower was measured instead. This may be due to

the smaller number of flowering individuals and lower densities in 2006, particularly at the larger Loch Cullen population, where the 2005 observations were carried out. Or else it may indicate a decline in the number of bees present at populations. Overall, these results support the prediction that plant-pollinator interactions are variable in space and time (e.g. Jordano *et al.*, 2006).

My investigations into the pollination of both *Spiranthes* species highlight the complexity of competition and facilitation interactions in plant communities. In ecology, the terms 'competition' and 'facilitation' may represent a false dichotomy (e.g. Callaway and Walker, 1997), with either or both effects occurring simultaneously, depending on life stage of the study organism, along with indirect interactions, such as with the physical environment (for example, the availability of nesting sites for pollinators; section 1.2.1). Orchids present a useful group for addressing hypotheses relating to competition and facilitation of pollination. This is because orchids have a dependence on pollinators to transfer pollinia. As their pollen is amassed, there are different components to their reproductive success. For example, I found that the local density of *Spiranthes* flowers plays a facilitative role in determining its own pollination success, either by enhancing the number of flowers visitors, or pollen import and export. This is unsurprising; for example, Willems and Lahtinen (1997) showed there was increased fruit set in dense patches of *S. spiralis*. In addition, they found increased fruit set when co-flowering species were removed. However, they did not measure the varying density within the study population and only examined one population in the Netherlands, albeit there are only two populations remaining in the Netherlands.

In members of *Spiranthes* there should be a large degree of mating between closely related individuals. This is because *Spiranthes* pollinia are sectile (van der Cingel, 1995), with potentially more than one flower pollinated by a single pollinarium attached to an individual pollinator. Mating among close neighbouring plants has been shown in other self-compatible orchids with sectile pollinia. For example, Johnson *et al.* (2005) examined the pollen fates of the South African orchid *Disa cooperi*. They found that more than 95% of pollen was lost during transit by hawkmoth pollinators, and the proportion of pollen lost correlated with the number of pollinaria removed from the plant. In addition, they found that most massulae were dispersed to close neighbouring plants, with rare long-distance dispersal of up to 65m. In a study on two populations of *Platanthera bifolia* one small (100-200 flowering individuals), and one large (>700 flowering individuals) there was no self-pollen on stigmas in either the large or small

population over two years examined, with 23 and 38% geitonogamous pollination in the small population (Maad and Reinhammar, 2004). Therefore, the effects of pollen dispersal in orchids with sectile pollinia can be variable. Indeed, I found no effect of population size on the removal and deposition of pollinia in either *S. romanzoffiana* or S *spiralis* (Chapter 4). However, within populations of *S. spiralis* I found a significant decrease in the proportion of pollen lost as patch density increased. The trend was similar, though non-significant, in *S. romanzoffiana*. This suggests either: (i) pollen is transferred between neighbouring individuals within patches or (ii) dense patches are more successful at importing pollen from other patches. I found self-pollination does not affect fitness, as there was no reduction in fruit or seed set in self-pollinated individuals of *S. spiralis*. As *S. romanzoffiana* produces no fruit and relatively few seeds, it is difficult to quantify the effects of self-pollination on reproductive success. O'Conner (2006) found no differences between selfed and outcrossed individuals. Also, *S. diluvialis* does not suffer negative effects of self-pollination, indeed it produces more seed when geitonogamously pollinated (Sipes and Tepedino, 1995). The rate of pollen carryover in *Spiranthes* has yet to be quantified. For other orchids with sectile pollinia, Johnson and Nilsson (1999) reported a pollen carryover rate of between 67% for *Orchis mascula* and 87% for *Platanthera chlorantha*, and Johnson *et al.* (2004) reported a pollen carryover rate of 78% for *Anacamptis morio*. Sipes and Tepedino (2006) used values from these studies to quantify the rate of pollen carryover in *Spiranthes diluvialis*. They estimated that self-pollination could be as high as 70% if pollinators are in low abundance, due to bees foraging within inflorescence for longer periods in order to maximise their foraging. Flowers of *S. diluvialis* can enter a 'female phase' after two days i.e. the labellum lowers and the stigma is more exposed yet the pollinia are still intact. I noticed this in both *Spiranthes* species, which indicates that the protandrous mechanism to optimise outcrossing proposed by Darwin (1862) may not be efficient for preventing self-pollination in *Spiranthes*. I did not quantify the rate of self-pollination in study populations of either *Spiranthes* species, however such data would be useful to compare to other studies.

The number of co-flowering individuals plays a varied role in *Spiranthes* pollination. In *S. romanzoffiana*, co-flowering species may increase visitation to inflorescences to a point, after which *S. romanzoffiana* inflorescences compete for pollinator attention (Chapter 3). In addition, in *S. romanzoffiana* I found that pollinia removal increases as the density of co-flowering species increase. However, co-flowering species have no effect on pollinia deposition (Chapter 4). Co-flowering species may attract pollinators into a patch and then switch to other species. Such switching among different flowers has been noted previously in *Bombus pascuorum* (e.g. Stout

et al., 1998). Pollinia deposition increases as visitation rate and the density of *S. romanzoffiana* inflorescences increases. With *S. spiralis*, co-flowering species do not affect its pollination, yet although there was a trend for increased pollen loss as the number of co-flowering individuals increased, this was non-significant. One problem encountered in Chapter 4 was the observations of co-flowering species were in separate patches to the orchid patches. This prevented a direct comparison with the pollination of the orchid species within patches. However, as I expected high visitation based on observations in 2005 (Chapter 3), the experimental design ensured that replicate patches were observed independently. These studies highlights the complexity of competition and facilitation for pollination. It was impossible to get reliable pollination data for the other focal species of this thesis, either because (i) they are not visited by pollinators often (due to a lack of pollinators in Ireland or otherwise) or (ii) there are not enough plants in populations to make replicate observations to test for density or other effects on their pollination. However, it would be very interesting to further investigate their pollination ecology to check if there are pollinators present in Ireland, particularly for *Cephalanthera longifolia* and *Pseudorchis albida* as these species have pollinators elsewhere in their range (Dafni and Ivri, 1981; Baumann and Baumann, 2002).

7.1.3 Genetic diversity of rare orchids

Genetic diversity is important in rare species as it reflects their evolutionary potential and, along with demographic and environmental factors, may determine whether a species can adapt to environmental changes (Lande, 1988; Ellstrand and Elam, 1993). Indeed, some previous studies have found that restricted gene flow in orchids may be linked to rarity (e.g. Wallace, 2003; Chung *et al.*, 2004). However, as discussed in section 1.1.3, there can be problems estimating the genetic structure and diversity of species, depending on the marker used and its ability to detect polymorphism. In a review by Forrest *et al.* (2004) 70 out of the 76 studies analysed used isozyme analysis, and no study reported in this paper used AFLP fingerprinting. However, many studies on orchid genetics since then have employed AFLP (e.g. Flanagan *et al.*, 2006; Pillon *et al.*, 2007a; Jacquemyn *et al.*, 2007; Devey *et al.*, 2008), indicating the increased convenience and usefulness of this technique. Only one study used SSRs according to the review of Forrest *et al.* (2004). This may be due to problems identifying reliable polymorphic SSR loci in orchids, due to large genomes (Fay *et al.*, 2005), or perhaps because SSR markers take a longer time to develop compared with dominant markers such as AFLPs. For example, a recent study by Campbell *et al.* (2007) used five SSR loci to identify three separate species of the orchid genus *Gymnadenia* in the UK and Ireland (*G. borealis*, *G. conopsea*, and *G. densiflora*). Also, Forrest

et al. (2004) used five SSR markers in conjunction with AFLP to examine the population genetic structure of *S. romanzoffiana*. These studies contrast with the 13 SSR markers used by Kang *et al.* (2008) to examine the population genetics of the endangered *Berchemiella wilsonii* var. *pubipetiolata* in China.

The three plastid SSR loci used on *Cephalanthera longifolia* in this study revealed little variation in Irish populations, identifying only two cytotypes. Interestingly, one cytotype was found in an individual in the smallest population (Chapter 6). SSRs were also used on *Pseudorchis albida*, but no variation was found at the four loci examined. This is probably due to the fact that these primers were developed on other genera (*Dactylorhiza*) and as such yielded little variability in Irish *P. albida*. Interestingly, the Irish samples of *P. albida* shared their loci with *P. straminea*. However, since there is little to divide these species in central Europe on taxonomic characters (Reinhammar, 1998), they may be the same species or different sub-species within *P. albida* (Chapter 6). More taxonomic work is needed to resolve the status of these *Pseudorchis* species. As discussed in Chapter 1 and Chapter 6, SSRs tend to have low transferability among species. Moreover, a useful marker locus requires identifying a region of the genome with a sufficiently high mutation rate such that multiple alleles can be detected in a population. It also should be located adjacent to a stretch of DNA with a low mutation rate that will bind PCR primers in the majority of individuals of the species. If mutations have occurred in the primer region, some individuals will only have one allele amplify (Selkoe and Toonen, 2006).

AFLP worked well with *Neotinea maculata*, with 223 markers generated (Chapter 5). However, only 44 AFLP markers were generated for *P. albida*. This may be due to the quality of the DNA used, with the *N. maculata* samples having higher quality and quantity of DNA per sample, to which AFLP may be sensitive (Meudt and Clarke, 2007). However, the markers produced for *P. albida* were useful to differentiate populations, with 95.45% polymorphism shared among all individuals. Both *N. maculata* and *P. albida* have significant population differentiation, which indicates reproductive isolation of populations of these species. With, *N. maculata*, this is unsurprising, as it has a strong self-compatibility system, among population gene flow is unlikely. Although orchid seeds are wind dispersed and have the potential for long distance dispersal, some previous studies have shown that most seeds fall close to the maternal plant (e.g. Machon *et al.*, 2003; Jacquemyn *et al.*, 2007). Therefore, it is unlikely *N. maculata* has high gene flow between populations. Surprisingly, given its high level of self-compatibility,

N. maculata was found to have 98.2% polymorphism according to the markers used among all populations. In contrast, the rare, self-compatible *Liparis loeselii* had just 40% polymorphism from two AFLP primer pairs (Pillon *et al.*, 2007a).

7.1.4 Scale effects on the study of rare species

The abundance and range sizes of a species are dynamic both in space and time (Lawton, 1993; Kunin and Gaston, 1997). Therefore, rarity is a scale-dependent concept (Hartley and Kunin, 2003; Chapter 1). Species may be rare in one area or at one time period, but not at another. A major question in biology is the extent to which rare species remain rare in space and time (Gaston, 1997). One aim of this thesis was to examine the differences between common and rare species i.e. to determine whether there are traits or conditions specific to rare Irish orchid species investigated. For this, I compared differences within a species range; *Neotinea maculata* (Chapter 5), and between congeneric species with similar traits; *Spiranthes romanzoffiana* and *S. spiralis* (Chapter 4). As discussed in Chapter 1, designating species rarity can be subjective and in some cases difficult. For instance, just because a species is rare in a given region, it does not necessarily follow that it is more highly threatened. Indeed, Honnay and Jacquemyn (2007) show that the genetic susceptibility to extinction is similar in species that are thought to be common and those that are thought to be rare. Therefore, the study of peripheral populations of a species is important (e.g. Chapter 5). For example, Lammi *et al.* (1999) found that there was significantly less genetic variation in the rare perennial *Lychnis viscaria* at the edge of its range. Also, a demographic analysis of an outlier population of the rare orchid *Orchis militaris* in England showed a gradual increase in population recruitment in response to management strategies (Hutchings *et al.*, 1998). Contrary to Lammi *et al.* (1999), I found that *N. maculata* has similar genetic diversity in peripheral populations, which are smaller, when compared to core populations. I also found that it has significant population differentiation in both areas of its range, which is probably due its heavy reliance on self-pollination. *N. maculata* is probably a naturally rare species in Ireland, although it is relatively common through the Mediterranean region. There have been no records for *N. maculata* outside of the west of Ireland; indeed it only has been recorded in six counties in Ireland (Chapter 2). In addition, it does not seem to suffer fitness costs as a result of its autogamous mode of reproduction, nor does it appear to be extending its range within Ireland. It occupies a variety of habitats in both Ireland and Italy, and has a high level of polymorphism.

When examining the effects of rarity, it is important to consider both population size and

within-population density. As discussed, there were effects of population size on pollinator visitation to *S. romanzoffiana*, however there were no effects of population size on pollinia removal and deposition in either *S. romanzoffiana* or *S spiralis*, or on visitation rate to *S. spiralis*. By making a pairwise comparison between a common and rare congener, I showed that pollinators visit both species, but visitation rates drop significantly below a population size of 71 individuals. I also showed that local density may play a role in the pollination of both species, and their interactions with co-flowerers can be complex. The key difference between the two species is that *S. romanzoffiana* fails to mature fruit. As a result, it is rare as it produces less seed, and therefore has less potential to colonise new sites and form larger populations.

7.2 Implications for the conservation of focal species in Ireland

The findings of this study have implications for the future conservation of rare Irish orchids. I have shown that regional-scale threat estimates are useful for inferring extinction and informing where conservation and re-surveying should occur. However, such analyses depend on the number of observations made in a relatively long time period or else the models can incorrectly infer extinction e.g. *Neotinea maculata* (Chapter 2). Therefore, the output of such analyses should be interpreted with caution. In addition, I have highlighted that all study species have chronically low flowering population sizes, and have few populations within Ireland. Grazing was found to be a problem for all these orchid species during their flowering. Orchids require grazing at their sites to prevent competition from other plant species. However, if they are grazed during their flowering, it will destroy their reproductive output for that year, and may have negatively affect subsequent flowering. I have shown that co-flowering species may play a role in the pollination of an endangered orchid. As orchids are not known to provide pollen to flower visitors, which is essential to insects, other co-flowering species are necessary for attracting and maintaining pollinating insects, such as generalist bumblebees (e.g. Sipes and Tepedino, 1995). Indeed, one of the recommendations of the Millennium Ecosystem Assessment is that landowners should be encouraged to manage land to enhance ecosystem services, such as pollination, and to remove subsidies that are harmful to biodiversity (www.millenniumecosytemassessment.org).

Regular monitoring of populations is essential for all study species and their continued survival in Ireland. The numbers of populations are few and their flowering numbers were low when examined, although flowering population numbers naturally fluctuate greatly in orchid populations (Hutchings, 1987), and therefore in other years there may be increased flowering in

the populations examined in this study. *Cephalanthera longifolia* requires urgent conservation attention. It survives in small populations where there is either human disturbance (e.g. Coosan Point, Loch Ree), or over-grazing and browsing by deer (e.g. Rosturra Wood). *C. longifolia* is a Biodiversity Action Plan species in the UK, and recently one of its populations has increased from 756 flowering plants in 1996 to 3,421 flowering individuals with 944 non-flowering plants in 2007. Conservation authorities recognised that the orchid requires some sunlight to attract solitary bees to pollinate it (Anonymous, 2007). *Hammarbya paludosa* is a species for which we need more data. AFLP was attempted *on H. paludosa*, but with little success. This was probably due to the low quantity of DNA in samples taken, as the leaves of *H. paludosa* are minute. It would be interesting to assess the degree of reliance *H. paludosa* has on asexual reproduction via bulbils. In addition, it would be interesting to find out if the high pollination rates observed by both Darwin (1862) and Reeves and Reeves (1984), occur in Ireland. *Neotinea maculata* appears to be a naturally rare species that has adapted to self-pollination. It does not suffer from high levels of inbreeding depression in the form of reduced fruit and seed set, or genetic diversity. However, the habitats it occupies in Ireland are unique and management should allow for the orchid to flower and set seed without grazing. *Pseudorchis albida* requires urgent conservation attention. Its flowering populations were chronically small and it had the highest incidence of grazing among the species examined. The investigation into its population genetics indicates that it has genetic diversity, and there is a significant level of genetic differentiation among populations, indicating genetic isolation. *Spiranthes romanzoffiana* has a high conservation status in Ireland as it is subject to an All Ireland Species Action Plan (2005). Further research is required to ascertain why fruit maturation does not occur in this species. Whether it is a genetic or environmental factor, or a combination of the two, needs to be investigated, as the lack of fruit maturation is a major cause of its rarity.

7.3 Future research on rare orchids

There is still much work to be done to understand the mechanisms behind why certain species are threatened with rarity more than others. This is the first study, to my knowledge, to quantify the effects of both density and co-flowering species on endangered orchid pollination. As there were limits to the time and resources available, more research is required in order to make long-term inferences of the effect of co-flowering species community on rare orchid pollination. As discussed in Chapter 1, plant communities can vary in their composition of pollinators and plant species and their densities in space and time. The majority of previous studies that investigate the role of co-flowering species on orchid pollination involve rewardless orchids and how co-

flowering "magnet" species facilitate their pollination by providing food for pollinators (e.g. Johnson *et al.*, 2003; Juillet *et al.*, 2007). However, little research has been done to investigate the effects of co-flowering species on the pollination success of nectariferous orchids. The co-flowering species community may drive the evolution of a reward in orchid flowers. As the rate of self-pollination is known to increase with the presence of a reward (e.g. Johnson and Nilsson, 1999), this may affect the breeding system of orchid in order to promote outcrossing. We also need more data on the effects of density on orchid pollination. While recent studies have examined the effects of density in both natural (e.g. Juillet *et al.*, 2007) and experimental populations (e.g. Internicola *et al.*, 2006) of rewardless species, we know little of the effects of density on nectariferous orchids. One study showed that there were no effects of density in *Listera* (*Neottia*) *cordata*, except that highly dispersed plants had lower fruit set (Melendez-Ackerman and Ackerman, 2001). However, this study did not quantify co-flowering species or the quantity of reward offered by the orchid.

Another potential area of further research is to link pollination limitation to mycorrhizal diversity and availability. Recently, Shefferson *et al.* (2008) found that rare orchids in Estonia colonise old mine hills and that the mycorrhizal diversity at these sites was similar when compared with pristine habitats. We need more studies that examine the role of mycorrhizas in orchid distribution. Some studies suggest that orchids can be generalists in their choice of mycorrhizal partner (e.g. Bidartondo *et al.*, 2004), while in the orchid genus *Cypripedium* at least there seems to be some specialised interactions (e.g. Shefferson *et al.*, 2007). It may be that orchids use different mycorrhizal species depending their stage of life cycle. One way of investigating this idea is by seed baiting (Rasmussen and Whigham, 1993) the mycorrhizas of an orchid species in its natural habitat, examining the roots of flowering orchids, and making population counts over several years. Seedling recruitment is a fundamental component in population dynamics models, but it is very difficult to determine in orchids. This is because little is known about the time elapsing between heterotrophic and autotrophic stages and whether orchids (at least some) can form a seed bank. A recent study has shown that seed germination and seedling recruitment rates are highly correlated (Diez, 2007). Jacquemyn *et al.* (2007) examined the within-population genetic structure and recruitment potential within two populations of an orchid species, *Orchis purpurea.* At one population, seed germination was confined to certain microsites, while at the other population both adults and seedlings were not clustered. More data is needed to determine if the restricted germination at the first site was limited by a lack of suitable fungi, or merely by a lack of suitable soil substrate. In addition,

incorporating data, such as life history status, level of fruiting, and levels of grazing into demographic models, along with studies of pollination limitation, will allow us to more fully understand the mechanisms underlying orchid population growth or extinction.

7.4 Concluding remarks

Rare orchid species in Ireland face a variety of threats to their long-term survival, such as, under-recording, a lack of habitat management, low population sizes, low pollinator attention, and inbreeding depression. I found that Irish orchids still occur in small and few populations. In particular, I found that although there have been declines of three of these species (*C. longifolia*, *H. paludosa*, and *P. albida*); there is no obvious regional trend to their decline. In addition, the pollination of the endangered, nectariferous orchid, *S. romanzoffiana*, may be positively affected by population size, but more so by its own density within populations. Also, I found that it displays similar interactions with its more common congener, *S. spiralis*. When examined at the centre and edge of its range, *N. maculata* has a strong reliance on self-pollination, occupies a variety of habitats, and has significant genetic population differentiation, both at the core and edge of its range. *C. longifolia* has low genetic diversity at three SSR loci, while *P. albida* has high levels of polymorphism, it has significant population differentiation, which indicates that there is low gene flow among populations. Although we are beginning to understand the habitat and ecological causes of rare orchid decline, there is still much to be done and to learn in order to prevent the extinction of these species in Ireland and ensure their long-term survival.

References

Ackerman, J.D., Rodriguez-Robles, J. A., and Melendez, E. J. (1994) A meagar nectar offering by an epiphytic orchid is better than nothing. *Biotropica*, **26,** 44-49.

Aguilar R., Ashworth L., Galetto L., and Aizen M.A. (2006) Plant reproductive susceptibility to habitat fragmentation: review and synthesis through a meta-analysis. *Ecology Letters*, **9,** 968-980.

Alexander M.P. (1980) A versatile stain for pollen, fungi, yeast, and bacteria. *Stain Technology*, **55,** 13-18.

Alexander, H.M., Slade, N. A., and Kettle, W. D. (1997) Application of mark-recapture models to estimation of population size of plants. *Ecology*, **78,** 1230-1237.

Alexandersson, R. & Ågren, J. (1996) Population-size, pollinator visitation and fruit production in the deceptive orchid *Calypso bulbosa*. *Oecologia*, **107,** 533-540.

Allee, W.C., Emerson, A.E., Park, O., Park, T., and Schmidt, K.P. (1949) Principles of Animal Ecology. Saunders, Philadelphia, Penn. U.S.A.

Alpatov, V.V. (1929) Biometrical studies on variation and races of the honey bee (*Apis mellifera* L.). *Quarterly Review of Biology*, **4,** 1-58.

Anonymous (2007) Conservation pays off. *Orchid Review*, **115,** 244.

Anstett, M.C., Hossaert McKey, M., and Kjellberg, F. (1997) Figs and fig pollinators: Evolutionary conflicts in a coevolved mutualism. *Trends in Ecology & Evolution,* **12,** 94-99.

Antlfinger, A.E. & Wendel, L.F. (1997) Reproductive effort and floral photosynthesis in *Spiranthes cernua* (Orchidaceae). *American Journal of Botany*, **84,** 769-780.

Araujo, M.B. & Rahbek, C. (2006) How does climate change affect biodiversity? *Science*, **313,** 1396-1397.

Arrhenius, O. (1921) Species and area. *Journal of Ecology*, **9,** 95-99.

Baker, A.M., Barrett, S. C. H., and Thompson, J. D. (2000) Variation of pollen limitation in the early flowering Mediterranean geophyte *Narcissus assoanus* (Amaryllidaceae). *Oecologia*, **124,** 529-535.

Barrett SCH, Harder LD (1996) Ecology and evolution of plant mating. *Trends in Ecology and Evolution*, **11,** 73-79.

Barrett, S.C.H. & Kohn, J. (1991) The genetic and evolutionary consequences of small population size in plants: implications for conservation. *Genetics and the conservation of rare plants* (eds D. Falk & K. E. Holsinger), pp. 3-30. Oxford University Press, Oxford, UK.

Bartareau, T. (1995) Pollination limitation, costs of capsule production and the capsule-to-flower ratio in *Dendrobium monophyllum* F. Muell. (Orchidaceae). *Australian Journal of Ecology,* **20,** 257-265.

Bascompte, J., Possingham, H. and Roughgarden, J. (2002) Patchy populations in stochastic environments: critical number of patches for persistence. *American Naturalist*, **159,** 128-137.

Bateman, A.J. (1948) Intra-sexual selection in *Drosophila. Heredity*, **2,** 349-368.

Bateman, R.M., Hollingsworth, P. M., Preston, J., Yi-Bo, L., Pridgeon, A. M., and Chase, M. W. (2003) Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (Orchidaceae). *Botanical Journal of the Linnean Society*, **142,** 1-40.

Baumann B., & Baumann H. (2002) Zur Bestaubung von *Pseudorchis albida* (L.) A. & D. Loeve (*Leuchorchis albida* (L.) E. Mey.). *Journal Europaischer Orchideen*, **43,** 695-705.

Beissinger, S.R. & Westphal, M.I. (1998) On the use of demographic models of population viability in endangered species management. *Journal of Wildlife Management*, **62,** 821-841.

Bell G. (1985) On the function of flowers. *Proceedings of the Royal Society of London B-Biological Sciences*, **224,** 223-265

Bell, J.M., Karron, J. D., and Mitchell, R. J. (2005) Interspecific competition for pollination lowers seed production and outcrossing in *Mimulus ringens*. *Ecology*, **86,** 762-771.

Berg, H. & Redbo-Torstensson, P. (1998) Cleistogamy as a bet-hedging strategy in *Oxalis acetosella*, a perennial herb. *Journal of Ecology*, **86,** 491-500.

Bevill, R.L. & Louda, S.M. (1999) Comparisons of related rare and common species in the study of plant rarity. *Conservation Biology*, **13,** 493-498.

Bidartondo, M.I., Burghardt, B., Gebauer, G., Bruns, T.D. and Read, D.J. (2004) Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **271,** 1799-1806.

Bierzychudek, P. (1981) Pollinator limitation of plant reproductive effort. *American Naturalist*, **117,** 838-840.

Biesmeijer, J.C., Roberts, S.P.M., Reemer, M., Ohlemuller, R., Edwards, M., Peeters, T., Schaffers, A.P., Potts, S.G., Kleukers, R., Thomas, C.D., Settele, J. and Kunin, W.E. (2006) Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science,* **313,** 351-354.

Blackburn T.M., Gaston K.J., Quinn R.M., and Gregory R.D. (1999) Do local abundances of British birds change with proximity to range edge? *Journal of Biogeography*, **26,** 493-505.

Bobisud, L.E. & Neuhaus, R.J. (1975) Pollinator constancy and survival of rare species. *Oecologia*, **21,** 263-272.

Bond, W.J. (1994) Do mutualisms matter? Assessing the impact of pollinator and disperser disruption on plant extinction. *Philosophical Transactions of the Royal Society of London Series B*, **344,** 83-90.

Bosch, M. & Waser, N.M. (1999) Effects of local density on pollination and reproduction in *Delphinium nuttallianum* and *Aconitum columbianum* (Ranunculaceae). *American Journal of Botany,* **86,** 871-879.

Bremer, K. & Bremer, K. (1994) Asteraceae: Cladistics and Classification. Timber Press, Portland, Oregon, U.S.A.

Bronstein, J.L. (1994) Conditional outcomes in mutualistic interactions. *Trends in Ecology and Evolution*, **9,** 214-217.

Brown, B.J. & Mitchell, R. J. (2001) Competition for pollination: effects of pollen of an invasive plant on seed set on a native congener. *Oecologia*, **129,** 43-49.

Brown, J.H. (1984). On the relationship between abundance and distribution of species. *American Naturalist*, **124,** 255-279.

Brundrett, M.C. (2007) Scientific approaches to Australian temperate terrestrial orchid conservation. *Australian Journal of Botany*, **55,** 293-307.

Buchmann, S.L., & Nabhan, G.P. (1996) The Forgotten Pollinators. Portland Press, Oregon.

Burd, M., 1994. Bateman principle and plant reproduction - the role of pollen limitation in fruit and seed set. *Botanical Review*, **60,** 83-139.

Burgman, M. A., Grimson R. C., and Ferson S. 1995. Inferring threat from scientific collections. *Conservation Biology*, **9,** 923-928.

Burgman, M., B. R. Maslin, D. Andrewartha, M. R. Keatley, C. Boek, and M. McCarthy. 2000. *Inferring threat from scientific collections: power tests and an application to Western Australian Acacia species.* Pages 7-26 in S. Ferson and M. Burgman, editors. Quantitative Methods for Conservation Biology. Springer-Verlag, New York.

Busch, J.W. (2005) The evolution of self-compatibility in geographically peripheral populations of *Leavenworthia alabamica* (Brassicaceae). *American Journal of Botany*, **92,** 1503-1512.

Callaway, R.M. & Walker, L.R. (1997) Competition and facilitation: a synthetic approach to interactions in plant communities. *Ecology,* **78,** 1958-1965.

Calvo, R.N. & Horvitz, C. C. (1990) Pollinator limitation, cost of reproduction, and fitness in plants: a transition-matrix demographic approach. *The American Naturalist,* **136,** 499-516.

Campbell, D.R. & Motten, A. F. (1985) The mechanism of competition for pollination between two forest herbs. *Ecology*, **66,** 554-563.

Campbell, V.V., Rowe, G., Beebee, T.J.C. and Hutchings, M.J. (2007) Genetic differentiation amongst fragrant orchids (*Gymnadenia conopsea* s.l.) in the British Isles. *Botanical Journal of the Linnean Society*, **155,** 349-360.

Carey P.D., Watkinson A.R., and Gerard F.F.O. 1995. The determinants of the distribution and abundance of the winter annual grass *Vulpia ciliata* ssp. *ambigua*. *Journal of Ecology*, **83,** 177-187.

Catling, P.M. (1982) Breeding systems of North Eastern North American *Spiranthes* (Orchidaceae). *Canadian Journal of Botany*, **60,** 3017-3039.

Catling, P.M., (1983) Pollination of North Eastern North American *Spiranthes* (Orchidaceae). *Canadian Journal of Botany*, **61,** 1080-1093.

Caughley, G., Grice, D., Barker, R., and Brown, B. (1988) The edge of the range. *Journal of Animal Ecology*, **57,** 771-785.

Channell, R. & Lomolino, M.V. (2000) Dynamic biogeography and conservation of endangered species. *Nature*, **403,** 84-86.

Charlesworth, D. & Charlesworth, B. (1987) Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics*, **18,** 237-268.

Chase M.W. & Hills, H.G. (1991) Silica gel: an ideal material for field preservation of leaf samples. *Taxon*, **40,** 215-220.

Chase, M.W., Cameron, K.M., Hills, H.G. and Jarrell, D. (1994). *Molecular systematics of the Orchidaceae and other lilioid monocots*. In Pridgeon, A. (Ed.): Proceedings of the 14th World Orchid Conference, pp 61-73. HMSO, London.

Chase, M.W. (2005) Classification of Orchidaceae in the age of DNA data. *Curtis' Botanical Magazine,* **22,** 2-7.

Chung, M.Y., Nason, J. D., and Chung, M. G. (2004) Spatial genetic structure in populations of the terrestrial orchid *Cephalanthera longibracta* (Orchidaceae). *American Journal of Botany*, **91,** 52-57.

Conner, J.K. & Neumeier, R. (1995) Effects of black mustard population size on the taxonomic composition of pollinators. *Oecologia*, **104,** 218-224.

Cormack, R.M. (1964) Estimate of survival from sightings of marked animals. *Biometrika,* 51, 429-438.

Costanza, R., dArge, R., deGroot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., Oneill, R.V., Paruelo, J., Raskin, R.G., Sutton, P. and vandenBelt, M. (1997) The value of the world's ecosystem services and natural capital. *Nature,* **387,** 253-260.

Cozzolino, S. & Widmer, A. (2005) Orchid diversity: an evolutionary consequence of deception? *Trends in Ecology and Evolution*, **20,** 487-494.

Cozzolino, S., Aceto, S., Caputo, P., Widmer, A. and Dafni, A. (2001) Speciation processes in eastern Mediterranean *Orchis* s.l. species: molecular evidence and the role of pollination biology. *Israel Journal of Plant Sciences*, **49,** 91-103.

Crawley, M.J. (2005) Statistics: An Introduction Using R. John Wiley & Sons, Chichester, West Sussex, U.K.

Cross, J. R. (1982) The invasion and impact of *Rhododendron ponticum* in native Irish vegetation. *Journal of Life Sciences: Royal Dublin Society*, **3,** 209-220.

Curtis, T.G.F. & McGough, H.N. (1988) The Irish Red Data Book. 1. Vascular Plants. The Stationary Office, Dublin, Ireland.

Dafni, A. & Ivri, Y. (1981) The flower biology of *Cephalanthera longifolia* (Orchidaceae) - pollen imitation and facultative floral mimicry. *Plant Systematics and Evolution*, **137,** 229-240.

Dafni, A. (1992) Pollination Ecology: A Practical Approach. Oriole Press at Oxford University Press, Oxford, U.K.

Dafni, A., & Maues, M.M. (1998). A rapid and simple procedure to determine stigma receptivity. *Sexual Plant Reproduction*, **11,** 177-180.

Darwin, C. (1862) On the various contrivances by which British and foreign orchids are fertilised by insects. John Murray, London, U.K.

Darwin, C. (1876) On the effects of cross and self fertilisation in the vegetable kingdom. John Murray, London, U.K.

de Lange, P.J. & Norton, D.A. 2004. The ecology and conservation of *Kunzea sinclairii* (Myrtaceae), a naturally rare plant of rhyolitic rock outcrops. *Biological Conservation*, **117,** 49-59.

DeSalle, R. (2005) Genetics at the brink of extinction. *Heredity*, **94,** 386-387.

Devey, D.S., Bateman, R.M., Fay, M.F. and Hawkins, J.A. (2008) Friends or relatives? Phylogenetics and species delimitation in the controversial European orchid genus *Ophrys*. *Annals of Botany,* **101,** 385-402.

Diamond, J.M. (1972) Biogeographic kinetics - estimation of relaxation times for avifaunas of southwest Pacific islands. *Proceedings of the National Academy of Sciences of the United States of America*, **69,** 3199.

Diamond, J.M. (1987) Extant unless proven extinct? Or, extinct unless proven extant. *Conservation Biology*, **1,** 77-79.

Diez, J.M. (2007) Hierarchical patterns of symbiotic orchid germination linked to adult proximity and environmental gradients. *Journal of Ecology,* **95,** 159-170.

Doyle J.J., & Doyle J.L. (1987) A rapid DNA isolation procedure for small quantities of fresh

leaf material. *Phytochemical Bulletin*, **19,** 11-15.

Dreisig, H. (1995) Ideal free distributions of nectar foraging bumblebees. *Oikos*, **72,** 161-172.

Dressler, R.L. (1981) The Orchids: Natural History and Classification. Harvard University Press, London, England.

Dressler, R.L. (1982) Biology of the orchid bees (Euglossini). *Annual Review of Ecology and Systematics*, **13,** 373-394.

Dressler, R.L. (2005) How many orchid species? *Selbyana,* **26,** 155-158.

Dudash, M.R. & Fenster, C.B. (1997) Multiyear study of pollen limitation and cost of reproduction in the iteroparous *Silene virginica. Ecology*, **78,** 484-493.

Duffy, K.J. & Stout, J.C. (2008) The effects of plant density and nectar reward on bee visitation to the endangered orchid *Spiranthes romanzoffiana. Acta Oecologica.* In press.

Edwards, M. & Jenner, M. (2005) Field Guide to the Bumblebees of Great Britain & Ireland. Ocelli Ltd. U.K.

Ehrlich, P.R. (1994) Energy use and biodiversity loss. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, **344,** 99-104.

Ellstrand, N.C. & Elam, D.R. (1993) Population genetic consequences of small population size: implication for plant conservation. *Annual Review of Ecology and Systematics*, **24,** 217-242.

Ellstrand, N.C., Devlin, B. and Marshall, D.L. (1989) Gene flow by pollen into small populations – data from experimental and natural stands of wild radish. *Proceedings of the National Academy of Sciences of the United States of America*, **86,** 9044-9047.

Eriksson, O. & Ehrlén, J. (1992) Seed and microsite limitation of recruitment in plant populations. *Oecologia*, **91,** 360-364.

Excoffier, L., Smouse, P.E., and Quattro, J.M. (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes - application to human mitochondrial DNA restriction data. *Genetics*, **131,** 479-491.

Fagan, W.F. & Holmes, E.E. (2006) Quantifying the extinction vortex. *Ecology Letters*, **9,** 51-60.

Fahrig, L. (2003) Effects of habitat fragmentation on biodiversity. *Annual Review of Ecology Evolution and Systematics*, **34,** 487-515.

Fausto, J.A., Eckhart, V.M., and Geber, M.A. (2001) Reproductive assurance and the evolutionary ecology of self-pollination in *Clarkia xantiana* (Onagraceae). *American Journal of Botany*, **88,** 1794-1800.

Fay, M.F., Cowan, R.S., and Leitch, I.J. (2005) The effects of nuclear DNA content (C-value) on the quality and utility of AFLP fingerprints. *Annals of Botany*, **95,** 237-246.

Fay M.F. & Cowan R.S. (2001) Plastid microsatellites in *Cypripedium calceolus* (Orchidaceae):

genetic fingerprints from herbarium specimens. *Lindleyana*, **16,** 151-156.

Feinsinger, P., Murray, K.G., Kinsman, S. and Busby, W.H. (1986) Floral neighbourhood and pollination success in 4 hummingbird-pollinated cloud forest plant species. *Ecology*, **67,** 449-464.

Feinsinger, P., Tiebout, H. M., and Young, B. E. (1991) Do tropical bird-pollinated plants exhibit density-dependent interactions? Field experiments. *Ecology*, **72,** 1953-1963.

Fischer, M. & Stocklin, J. (1997) Local extinctions of plants in remnants of extensively used calcareous grasslands 1950-1985. *Conservation Biology*, **11,** 727-737.

Fischer, M. & Matthies, D. (1997) Mating structure and inbreeding and outbreeding depression in the rare plant *Gentianella germanica* (Gentianaceae). *American Journal of Botany*, **84,** 1685-1692.

Fischer, J. & D. B. Lindenmayer. (2007). Landscape modification and habitat fragmentation: a synthesis. *Global Ecology and Biogeography*, **16,** 265-280.

Flanagan, N.S., Peakall, R., Clements, M.A., and Otero, J.T. (2006) Conservation of taxonomically difficult species: the case of the Australian orchid, *Microtis angusii*. *Conservation Genetics*, **7,** 847-859.

Forrest, A.D., Hollingsworth, M.L., Hollingsworth, P.M., Sydes, C., and Bateman, R.M. (2004) Population genetic structure in European populations of *Spiranthes romanzoffiana* set in the context of other genetic studies on orchids. *Heredity*, **92,** 218-227.

Fritz, A.L. & Nilsson, L.A. (1994) How pollinator mediated mating varies with population size in plants. *Oecologia*, **100,** 451-462.

Galen, C. & Gregory, T. (1989) Interspecific pollen transfer as a mechanism of competition – consequences of foreign pollen contamination for seed set in the alpine wildflower, *Polemonium viscosum*. *Oecologia*, **81,** 120-123.

Gaston, K.J. & Kunin, W.E. (1997). *Rare-common differences: an overview.* In: The Biology of Rarity: Causes and Consequences of Rare-Common Differences (eds W.E. Kunin & K.J. Gaston), pp. 12-30. Chapman and Hall, London, U.K.

Geer, S.M., Tepedino, V. J., Griswold, T. L., & Bowlin, W. R. (1995) Pollinator sharing by three sympatric milkvetches, including the endangered species *Astragalus montii*. *Great Basin Naturalist*, **55,** 19-28.

Ghazoul, J. (2005) Buzziness as usual? Questioning the global pollination crisis. *Trends in Ecology and Evolution*, **20,** 367-373.

Ghazoul, J. (2006) Floral diversity and the facilitation of pollination. *Journal of Ecology*, **94,** 295-304.

Gibson, R.H., Nelson, I.L., Hopkins, G.W., Hamlett, B.J., and Memmott, J., (2006) Pollinator

webs, plant communities and the conservation of rare plants: arable weeds as a case study. *Journal of Applied Ecology*, **43,** 246-257.

Gitzendanner, M.A, & Soltis, P.S. (2000) Patterns of genetic variation in rare and widespread plant congeners. *American Journal of Botany*, **87,** 783-792.

Gleason, H.A. (1922) On the relation between species and area. *Ecology*, **3,** 158-162.

Goodwillie, C. (2001) Pollen limitation and the evolution of self-compatibility in *Linanthus* (Polemoniaceae). *International Journal of Plant Sciences*, **162,** 1283-1292.

Gotelli, N. J., & R. K. Colwell. (2001) Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. *Ecology Letters*, **4,** 379-391.

Goulson, D. (1994) A model to predict the influence of insect flower constancy on interspecific competition between insect pollinated plants. *Journal of Theoretical Biology*, **168,** 309-314.

Griggs, R.F. (1914) Observations on the behavior of some species at the edges of their ranges. *Bulletin of the Torrey Botanical Club*, **41,** 25-49.

Gulliver, R. & Gulliver, M. (2004) Looking for Irish Ladies on Scottish islands. *Orchid Review*, **112,** 89-110.

Gustafsson, S. & Sjogren-Gulve, P. (2002) Genetic diversity in the rare orchid, *Gymnadenia odoratissima* and a comparison with the more common congener, *G. conopsea*. *Conservation Genetics*, **3,** 225-234.

Hall, C.A.S., Stanford, J.A., and Hauer, F.R. (1992) The distribution and abundance of organisms as a consequence of energy balances along multiple environmental gradients. *Oikos*, **65,** 377-390.

Hamilton, M.B. (1999) Four primer pairs for the amplification of chloroplast intergeneric regions with intraspecific variation. *Molecular Ecology*, **8,** 521-525.

Hamrick, J.L. & Godt, M.J.W. (1996) Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, **351,** 1291-1298.

Hanski I. (1982) Dynamics of regional distribution - the core and satellite species hypothesis. *Oikos*, **38,** 210-221.

Hanski I. (1998) Metapopulation dynamics. *Nature*, **396,** 41-49.

Hartley, S. & Kunin, W.E. (2003) Scale dependency of rarity, extinction risk, and conservation priority. *Conservation Biology*, **17,** 1559-1570.

Heinrich, B. (1979) Bumblebee Economics. Harvard University Press, Cambridge, Mass., U.S.A.

Herlihy, C.R. & Eckert, C.G. (2002). Genetic cost of reproductive assurance in a self-fertilizing

plant. *Nature*, **416,** 320-323.

Herlihy, C.R. & Eckert, C.G. (2005) Evolution of self-fertilization at geographical range margins? A comparison of demographic, floral, and mating system variables in central vs. peripheral populations of *Aquilegia canadensis* (Ranunculaceae). *American Journal of Botany*, **92,** 744-751.

Honnay, O. & Bossuyt, B. (2005) Prolonged clonal growth: escape route or route to extinction? *Oikos*, **108,** 427-432.

Honnay, O. & Jacquemyn, H. (2007) Susceptibility of common and rare plant species to the genetic consequences of habitat fragmentation. *Conservation Biology*, **21,** 823-831.

Huenneke, L.F. (1991) *Ecological implications of genetic variation in plant populations.* Falk, D. A. and K. E. Holsinger (Ed.). Genetics and Conservation of Rare Plants. pp: 31-44. Oxford University Press: Oxford, England, U.K.

Hutchings, M.J. (1987) The population biology of the early spider orchid, *Ophrys sphegodes* Mill. 1. A demographic study from 1975 to 1984. *Journal of Ecology,* **75,** 711-727.

Hutchings, M.J., Mendoza, A. & Havers, W. (1998) Demographic properties of an outlier population of *Orchis militaris* L. (Orchidaceae) in England. *Botanical Journal of the Linnean Society,* **126,** 95-107.

Internicola, A.I., Juillet, N., Smithson, A., & Gigord, L.D.B. (2006) Experimental investigation of the effect of spatial aggregation on reproductive success in a rewardless orchid. Oecologia, 150, 435-441.

Irish Flora Protection Order. 1999. Section 21 of the Wildlife Act, 1976 (Amended 2000); No. 39. S.I. No. 94.

IUCN. 2001. IUCN Red List Categories and Criteria: Version 3.1. IUCN Species Survival Commission. Gland, Switzerland and Cambridge, U.K. .

Jacquemyn, H., Brys, R., Hermy, M., and Willems, J.H. (2005) Does nectar reward affect rarity and extinction probabilities of orchid species? An assessment using historical records from Belgium and the Netharlands. *Biological Conservation*, **121,** 257-263.

Jacquemyn, H., Brys, R., Vandepitte, K., Honnay, O., Roldan-Ruiz, I., and Wiegand, T. (2007a) A spatially explicit analysis of seedling recruitment in the terrestrial orchid *Orchis purpurea. New Phytologist*, **176,** 448-459.

Jacquemyn, H., Brys, R., Hermy, M., and Willems, J.H. (2007b) Long-term dynamics and population viability in one of the last populations of the endangered *Spiranthes spiralis* (Orchidaceae) in the Netherlands. *Biological Conservation*, **134,** 14-21.

Jacquemyn, H., Vandepitte, K., Brys, R., Honnay, O. and Roldan-Ruiz, I. (2007c) Fitness variation and genetic diversity in small, remnant populations of the food deceptive orchid

Orchis purpurea. *Biological Conservation*, **139,** 203-210.

Janecková, P., Wotavova K., Schodelbauerova I., Jersakova J., and Kindlmann P.. (2006) Relative effects of management and environmental conditions and performance and survival of populations of a terrestrial orchid, *Dactylorhiza majalis*. *Biological Conservation*, **129,** 40-49.

Jennersten, O. (1988) Pollination in *Dianthus deltoides* (Caryphyllaceae) – effects of habitat fragmentation on visitation and seed set. *Conservation Biology*, **2,** 359-366.

Jersakova, J., Johnson, S.D. and Kindlmann, P. (2006) Mechanisms and evolution of deceptive pollination in orchids. *Biological Reviews*, **81,** 219-235.

Jersakova, J., & Malinova, T. (2007) Spatial aspects of seed dispersal and seedling recruitment in orchids. *New Phytologist*, **176,** 237-241.

Johnson, S.D. (1997) Pollination ecotypes of *Satyrium hallackii* (Orchidaceae) in South Africa. *Botanical Journal of the Linnean Society*, **123,** 225-235.

Johnson, S.D. & Bond, W.J. (1997) Evidence for widespread pollen limitation of fruiting success in Cape wildflowers. *Oecologia*, **109,** 530-534.

Johnson, S.D. & Nilsson, L.A. (1999) Pollen carryover, geitonogamy, and the evolution of deceptive pollination systems in orchids. *Ecology*, **80,** 2607-2619.

Johnson, S.D., Neal, P.R., and Harder, L.D. (2005) Pollen fates and the limits on male reproductive success in an orchid population. *Biological Journal of the Linnean Society*, **86,** 175-190.

Johnson, S.D., Peter, C.I., Nilsson, L.A., and Ågren, J. (2003). Pollination success in a deceptive orchid is enhanced by co-occurring rewarding magnet plants. *Ecology*, **84,** 2919-2927.

Johnson, S.D., Peter, C. I., and Ågren, J. (2004) The effects of nectar addition on pollen removal and geitonogamy in the non-rewarding orchid *Anacamptis morio*. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **271,** 803-809.

Jolly, G.M. (1965) Explicit estimates from capture-recapture data with both death and immigration-stochastic model. *Biometrika*, **52,** 225-247.

Jordano, P., Bascompte, J. and Olesen, J.M. (2006) *The ecological consequences of complex topology and nested structure in pollination webs*. pp: 173-199. Plant-Pollinator Interactions: From Specialization to Generalization, University of Chicago Press, U.S.A..

Juillet, N., Gonzalez, M.A., Page, P.A., and Gigord, L.D.B. (2007) Pollination of the European food-deceptive *Traunsteinera globosa* (Orchidaceae): the importance of nectar-producing neighbouring plants. *Plant Systematics and Evolution*, **265,** 123-129.

Kalisz, S, Vogler, D.W., and Hanley, K.M. (2004) Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. *Nature*, **430,** 884-887.

Kalisz, S. & Vogler, D.W. (2003) Benefits of autonomous selfing under unpredictable pollinator environments. *Ecology*, **84,** 2928-2942.

Kang, M., Wang, J. and Huang, H. (2008) Demographic bottlenecks and low gene flow in remnant populations of the critically endangered *Berchemiella wilsonii* var. *pubipetiolata* (Rhamnaceae) inferred from microsatellite markers. *Conservation Genetics*, **9,** 191-199.

Karron, J.D. (1987) The pollination ecology of co-occurring geographically restricted and widespread species of *Astragalus* (Fabaceae). *Biological Conservation*, **39,** 179-193.

Karron, J.D., Mitchell, R.D., Holmquist, K.G., Bell, J.M., and Funk, B. (2004) The influence of floral display size on selfing rates in *Mimulus ringens*. *Heredity*, **92,** 242-248.

Karron, J.D., Thumser, N. N., Tucker, R., and Hessenauer, A. J. (1995) The influence of population density on outcrossing rates in *Mimulus ringens*. *Heredity*, **75,** 175-180.

Kearns, C.A. & Inouye, D.S. (1997) Pollinators, flowering plants, and conservation biology - Much remains to be learned about pollinators and plants. *Bioscience*, **47,** 297-307.

Kearns, C.A., Inouye, D. W., and Waser, N. M. (1998) Endangered mutualisms: the conservation of plant-pollinator interactions. *Annual Review of Ecology and Systematics*, **29,** 83-112.

Kéry, M., Matthies D., and Spillmann H. H. (2000) Reduced fecundity and offspring performance in small populations of the declining grassland plants *Primula veris* and *Gentiana lutea*. *Journal of Ecology*, **88,** 17-30.

Kery, M. (2004) Extinction rate estimates for plant populations in revisitation studies: importance of detectability. *Conservation Biology,* **18,** 570-574.

Kery, M. & Gregg, K.B. (2004) Demographic analysis of dormancy and survival in the terrestrial orchid Cypripedium reginae. *Journal of Ecology*, **92,** 686-695.

Klein, A.M., Steffan-Dewenter, I. and Tscharntke, T. (2003a) Fruit set of highland coffee increases with the diversity of pollinating bees. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **270,** 955-961.

Klein, A.M., Steffan-Dewenter, I., and Tscharntke, T. (2003b) Bee pollination and fruit set of *Coffea arabica* and *C. canephora* (Rubiaceae). *American Journal of Botany*, **90,** 153-157.

Klein, A.M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C. and Tscharntke, T. (2007) Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B-Biological Sciences*, **274,** 303-313.

Klinkhamer, P.G.L. & van der Lugt, P-P., 2004. Pollinator service only depends on nectar production rates in sparse populations. *Oecologia*, **140,** 491-494.

Knight, T.M. (2003) Floral density, pollen limitation, and reproductive success in *Trillium grandiflorum*. *Oecologia*, **137,** 557-563.

Knight, T.M., Steets, J. A., Vamosi, J. C., Mazer, S. J., Burd, M., Campbell, D. R., Dudash, M.,

R., Johnston, M. O., Mitchell, R., J., and Ashman, T-L. (2005) Pollen limitation of plant reproduction: pattern and process. *Annual Review of Ecology, Evolution, and Systematics*, **36,** 467-497.

Kocyan, A. & Endress, P.K. (2001) Floral structure and development of *Apostasia* and *Neuwiedia* (Apostasioideae) and their relationships to other Orchidaceae. *International Journal of Plant Sciences*, **162,** 847-867.

Kull, T. & Hutchings, M.J. (2006) A comparative analysis of decline in the distribution ranges of orchid species in Estonia and the United Kingdom. *Biological Conservation*, **129,** 31-39.

Kunin, W.E. & Gaston, K.J. (1993) The biology of rarity - patterns, causes and consequences. *Trends in Ecology and Evolution*, **8,** 298-301.

Kunin, W.E. (1993) Sex and the single mustard - population density and pollinator behavior effects on seed set. *Ecology*, **74,** 2145-2160.

Kunin, W.E. (1997a). *Population biology and rarity: on the complexity of density dependence in insect-plant interactions*. In The Biology of Rarity: Causes and Consequences of Rare-Common Differences (eds W.E. Kunin & K.J. Gaston), pp. 150-173. Chapman and Hall, London, U.K.

Kunin, W.E. (1997b) Population size and density effects in pollination: pollinator foraging and plant reproductive success in experimental arrays of *Brassica kaber*. *Journal of Ecology*, **85,** 225-234.

Kwak, M.M., van den Brand, C., Kremer, P., and Boerrigter, E. (1991) Visitation, flight distances and seed set in populations of the rare species *Phyteuma nigrum* (Campanulaceae). *Acta Horticulturae*, **238,** 303-307.

Kwak, M.M., Velterop, O. and van Andel, J. (1998) Pollen and gene flow in fragmented habitats. *Applied Vegetation Science,* **1,** 37-54.

Ladle, R. J., Jepson, P., Araujo, M. B., and Whittaker, T. J. (2004) Dangers of crying wolf over risk of extinctions. *Nature*, **428,** 799-799.

Lammi, A., Siikamaki, P., and Mustajarvi, K. (1999) Genetic diversity, population size, and fitness in central and peripheral populations of a rare plant *Lychnis viscaria*. *Conservation Biology*, **13,** 1069-1078.

Lande, R., (1988) Genetics and demography in biological conservation. *Science*, **241,** 1455-1460.

Larson, B.M.H., & Barrett, S. C. H. (2000) A comparative analysis of pollen limitation in flowering plants. *Biological Journal of the Linnean Society*, **69,** 503-520.

Larson, R.J. & Larson, K.S., (1987) Observations on the pollination biology of *Spiranthes romanzoffiana*. *Lindleyana* **2,** 176-179.

Laurance, W.F., Lovejoy, T.E., Vasconcelos, H.L., Bruna, E.M., Didham, R.K., Stouffer, P.C., Gascon, C., Bierregaard, R.O., Laurance, S.G. & Sampaio, E. (2002) Ecosystem decay of Amazonian forest fragments: A 22-year investigation. *Conservation Biology*, **16,** 605-618.

Laverty, T.M. (1992) Plant interactions for pollinator visits: a test of the magnet species effect. *Oecologia*, **89,** 502-508.

Lawton, J.H. (1993) Range, population abundance and conservation. *Trends in Ecology and Evolution*, **8,** 409-413.

Lebreton, J.D., Burnham, K.P., Clobert, J. and Anderson, D.R. (1992) Modeling survival and testing biological hypotheses using marked animals – a unified approach with case studies. *Ecological Monographs*, **62,** 67-118.

Leimu, R. & Syrjanen, K. (2002) Effects of population size, seed predation and plant size on male and female reproductive success in *Vincetoxicum hirundinaria* (Asclepiadaceae). *Oikos*, **98,** 229-238.

Leimu, R., Mutikainen, P., Koricheva, J., and Fischer, M. (2006) How general are positive relationships between plant population size, fitness and genetic variation? *Journal of Ecology*, **94,** 942-952.

Lennartsson, T. (2002) Extinction thresholds and disrupted plant-pollinator interactions in fragmented plant populations. *Ecology*, **83,** 3060-3072.

Lesica, P. & Allendorf, F.W. (1995) When are peripheral populations valuable for conservation? *Conservation Biology*, **9,** 753-760.

Levin, D.A. & Anderson, W.W. (1970) Competition for pollinators between simultaneously flowering species. *American Naturalist*, **104,** 455.

Lu, Y.Q. (2002). Why is cleistogamy a selected reproductive strategy in *Impatiens capensis* (Balsaminaceae)? *Biological Journal of the Linnean Society*, **75,** 543-553.

Lupton, D.A. (2008) The Biogeography and Conservation of *Spiranthes romanzoffiana* L. (Cham.). Unpublished PhD Thesis, University of Dublin, Trinity College, Dublin.

Maad, J. & Reinhammar, L.G. (2004) Incidence of geitonogamy differs between two populations in the hawkmoth-pollinated *Platanthera bifolia* (Orchidaceae). *Canadian Journal of Botany-Revue Canadienne De Botanique*, **82,** 1586-1593.

MacArthur, R.H. & Wilson, E.O. (1963) An equilibrium theory of insular zoogeography. *Evolution*, **17,** 373-387.

MacArthur, R.H. & Wilson, E.O. (1967) The Theory of Island Biogeography. Princeton University Press, Princeton, N.J., U.S.A.

Machon, N., Bardin, P., Mazer, S. J., Moret, J. Godelle, B., and Austerlitz, F. (2003) Relationship between genetic structure and seed and pollen dispersal in the endangered

orchid *Spiranthes spiralis*. *New Phytologist*, **157,** 677-687.

Maguire, T.L., Peakall, R., and Saenger, P. (2002) Comparative analysis of genetic diversity in the mangrove species *Avicennia marina* (Forsk.) Vierh. (Avicenniaceae) detected by AFLPs and SSRs. *Theoretical and Applied Genetics*, **104,** 388-398.

Mangal, M., & Tier, C. (1994) Four facts every conservation biologist should know about persistence. *Ecology*, **75,** 607-614.

May, R.M. (1995) Population genetics – the Cheetah controversy. *Nature*, **374,** 309-310.

McCarthy, M.A. (1998) Identifying declining and threatened species with museum data. *Biological Conservation*, **83,** 9-17.

McCune, B. & Mefford, M.J. (1999) PC-ORD. Multivariate Analysis of Ecological Data version 4. Gleneden Beach, Oregon, U.S.A.

McCune, B. & Grace, J.B. (2002) Analysis of Ecological Communities. MjM Software Design, Gleneden Beach, Oregon, U.S.A.

McInerny, G.J., Roberts D.L., Davy A.J., and Cribb P. J. (2006) Significance of sighting rate in inferring extinction and threat. *Conservation Biology*, **20,** 562-567.

Melendez-Ackerman, E.J., Ackerman, J.D. and Rodriguez-Robles, J.A. (2000) Reproduction in an orchid can be resource-limited over its lifetime. *Biotropica*, **32,** 282-290.

Melendez-Ackerman, E.J. & Ackerman, J.D. (2001) Density-dependent variation in reproductive success in a terrestrial orchid. *Plant Systematics and Evolution*, **227,** 27-36.

Memmott, J. & Waser, N.M. (2002) Integration of alien plants into a native flower-pollinator visitation web. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **269,** 2395-2399.

Memmott, J., Waser, N.M. & Price, M.V. (2004) Tolerance of pollination networks to species extinctions. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **271,** 2605-2611.

Meudt, H.M. & Clarke, A.C. (2007) Almost forgotten or latest practice? AFLP applications, analyses and advances. *Trends in Plant Science*, **12,** 106-117.

Micheneau, C. (2002) *Relations phylogénétiques des Neottieae (Orchidaceae) et étude de la variabilité génétique de Cephalanthera longifolia a l'aide de marqueurs moléculaires (séquencage et microsatellites chloroplastiques)*. DEA de Systématique Animale et Végétale. Muséum d'Histoire Naturelle, Paris, France.

Micheneau, C., Fournel, J., and Pailler, T. (2006) Bird pollination in an angraecoid orchid on Reunion Island (Mascarene Archipelago, Indian Ocean). *Annals of Botany*, **97,** 965-974.

Mitchell, F. & Ryan, M. (2001) Reading the Irish Landscape. Town House and Country House, Dublin, Ireland.

Mitchell, R.J., Karron, J. D., Holmquist, K. G., and Bell, J .M. (2004) The influence of *Mimulus ringens* floral display size on pollinator visitation patterns. *Functional Ecology*, **18,** 116-124.

Moeller, D.A. (2004) Facilitative interactions among plants via shared pollinators. *Ecology*, **85,** 3289-3301.

Moeller, D.A. & Geber, M.A. (2005). Ecological context of the evolution of self-pollination in *Clarkia xantiana*: population size, plant communities, and reproductive assurance. *Evolution*, **59,** 786-799.

Montalvo, A.M. & Ackerman, J.D. (1987) Limitation to fruit production in *Ionopsis utricularoides* (Orchidaceae). *Biotropica*, **19,** 24-31.

Moody-Weis, J.M. & Heywood, J.S. (2001) Pollination limitation to reproductive success in the Missouri evening primrose, *Oenothera macrocarpa* (Onagraceae). *American Journal of Botany*, **88,** 1615-1622.

Morgan, M.T. & Wilson, W.G. (2005) Self-fertilization and the escape from pollen limitation in variable pollination environments. *Evolution*, **59,** 1143-1148.

Murcia, C. (1995) Edge effects in fragmented forests – implications for conservation. *Trends in Ecology and Evolution*, **10,** 58-62.

Murcia, C. & Feinsinger, P. (1996) Interspecific pollen loss by hummingbirds visiting flower mixtures: effects of floral architecture. *Ecology*, **77,** 550-560.

Murphy, S.D. & Aarssen, L.W. (1995) Reduced seed set in *Elytrigia repens* caused by allelopathic pollen from *Phleum pratense*. *Canadian Journal of Botany-Revue Canadienne de Botanique*, **73,** 1417-1422.

Nei, M. (1972) Genetic distance between populations. *American Naturalist*, **106,** 283-292.

Neiland, M.R.M. & Wilcock, C.C. (1998) Fruit set, nectar reward, and rarity in the Orchidaceae. *American Journal of Botany*, **85,** 1657-1671.

Neiland, M.R.M., & Wilcock, C. C. (1999) The presence of heterospecific pollen on stigmas of nectariferous and nectarless orchids and its consequence for their reproductive success. *Protoplasma*, **208,** 65-75.

Newmark, W.D. (1987) A land-bridge island perspective on mammalian extinctions in western North American parks. *Nature*, **325,** 430-432.

Nielsen, A. & Bascompte, J. (2007) Ecological networks, nestedness and sampling effort. *Journal of Ecology*, **95,** 1134-1141.

Nilsson, L.A. (1980) Pollination ecology of *Dactylorhiza sambucina* (Orchidaceae). *Botaniska Notiser,* **133,** 367-385.

Nilsson, L.A. (1983) Mimesis of bellflower (*Campanula*) by the red helleborine orchid

Cephalanthera rubra. *Nature*, **305,** 799-800.

Nilsson, L.A. (1992) Orchid pollination biology. *Trends in Ecology and Evolution*, **7,** 255-259.

O'Conner, C. (2006) The Reproductive Biology of the Endangered Orchid *Spiranthes romanzoffiana*. Unpublished M.Sc. thesis, University of Dublin, Trinity College.

Oostermeijer, J.G.B., Vaneijck, M.W. and Dennijs, J.C.M. (1994) Offspring fitness in relation to population size and genetic variation in the rare perennial plant species *Gentiana pneumonanthe*. *Oecologia*, **97,** 289-296.

Oostermeijer, J.G.B., Luijten, S.H., Krenova, Z.V. and Den Nijs, H.C.M. (1998) Relationships between population and habitat characteristics and reproduction of the rare *Gentiana pneumonanthe* L. *Conservation Biology*, **12,** 1042-1053.

Peakall, R., Gilmore, S., Keys, W., Morgante, M., and Rafalski, A. (1998) Cross-species amplification of soybean (*Glycine max*) simple sequence repeats (SSRs) within the genus and other legume genera: implications for the transferability of SSRs in plants. *Molecular Biology and Evolution*, **15,** 1275-1287.

Peakall, R. & Smouse, P.E. (2006) GenAlEx 6: Genetic Analysis in Excel. Population Genetic Software For Teaching and Research. *Molecular Ecology Notes*, **6,** 288-295.

Peakall, R., Smouse, P.E., and Huff, D.R. (1995). Evolutionary implications of allozyme and RAPD variation in diploid populations of dioecious Buffalograss, *Buchloe dactyloides*. *Molecular Ecology*, **4,** 135-147.

Peakall, R. & Beattie, A.J. (1996) Ecological and genetic consequences of pollination by sexual deception in the orchid *Caladenia tentaculata*. *Evolution*, **50,** 2207-2220.

Pellmyr, O., Thompson, J.N., Brown, J.M. and Harrison, R.G. (1996) Evolution of pollination and mutualism in the yucca moth lineage. *American Naturalist*, **148,** 827-847.

Peter, C.I. & Johnson, S.D. (2006) Doing the twist: a test of Darwin's cross-pollination hypothesis for pollination reconfiguration. *Biology Letters*, **2,** 65-68.

Pillon, Y., Qamaruz-Zaman, F., Fay, M.F., Hendoux, F., and Piquot, Y. (2007a) Genetic diversity and ecological differentiation in the endangered fen orchid (*Liparis loeselii*). *Conservation Genetics*, **8,** 177-184.

Pillon, Y., Fay, M.F., Hedrén, M., Bateman, R.M., Devey, D.S., Shipunov, A.B., van der Bank, M., and Chase, M.W. (2007b) Evolution and temporal diversification of western European polyploid species complexes in *Dactylorhiza* (Orchidaceae). *Taxon*, **56,** 1185-1208.

Pimm, S. (2003) Expiry dates. *Nature*, **426,** 235-236.

Pleasants, J.M. (1989) Optimal foraging by nectarivores: a test of the marginal value theorem. *American Naturalist*, **134,** 51-71.

Pleasants, J.M. & Zimmerman, M. (1979) Patchiness in the dispersion of nectar resources:

evidence for hot and cold spots. *Oecologia*, **41,** 283-288.

Pleasants, J.M. (1980) Competition for bumblebee pollinators in Rocky Mountain plant communities. *Ecology*, **61,** 1446-1459.

Preston, C.D., Pearman, D.A., and Dines T.D. (2002) New Atlas of the British and Irish Flora. Oxford University Press, Oxford, U.K.

Preston, F.W. (1948) The commonness, and rarity, of species. *Ecology*, **29,** 254-283.

Preston, F.W. (1962a) Canonical distribution of commonness and rarity 1. *Ecology*, **43,** 185-215.

Preston, F.W. (1962b) Canonical distribution of commonness and rarity.2. *Ecology*, **43,** 410-432.

Proctor, H.C., & Harder, L, D. (1994) Pollen load, capsule weight, and seed production in three orchid species. *Canadian Journal of Botany*, **72,** 249-255.

R Development Core Team (2007). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Rabinowitz, D., Cairns, S., and Dillon, T. (1986) *Seven forms of rarity and their frequency in the flora of the British Isles*. Conservation Biology: The Science and Scarcity of Diversity. (ed M. E. Soule), pp. 182-204. Sinauer Associates Inc., Sunderland, Massachusetts, U.S.A.

Ramirez, S.R., Gravendeel, B., Singer, R.B., Marshall, C.R. and Pierce, N.E. (2007) Dating the origin of the Orchidaceae from a fossil orchid with its pollinator. *Nature*, **448,** 1042-1045.

Ramsey, M. (1995) Ant pollination of the perennial herb *Blandfordia grandiflora* (Liliaceae). *Oikos*, **74,** 265-272.

Rasmussen, H.N. (2002) Recent developments in the study of orchid mycorrhiza. *Plant and Soil*, **244,** 149-163.

Rasmussen, H.N. & Whigham, D.F. (1993) Seed ecology of dust seeds in-situ - a new study technique and its application in terrestrial orchids. *American Journal of Botany*, **80,** 1374-1378.

Rasmussen, H.N. & Whigham, D.F. (1998) The underground phase: a special challenge in studies of terrestrial orchid populations. *Botanical Journal of the Linnean Society*, **126,** 49-64.

Rathcke, B.J. & Jules, E.S. (1993) Habitat fragmentation and plant-pollinator interactions. *Current Science*, **65,** 273-277.

Rathcke, B.J. (1983). *Competition and facilitation among plants for pollination.* In: Pollination Biology (ed L.A. Real), pp. 305-329. Academic Press, Inc., Orlando, Florida, U.S.A.

Reed, D.H. & Frankham, R. (2003) Correlation between fitness and genetic diversity. *Conservation Biology*, **17,** 230-237.

Reed, J.M. (1996) Using statistical probability to increase confidence of inferring species

extinction. *Conservation Biology*, **10,** 1283-1285.

Reeves, L.M. & Reeves, T. (1984) Life history and reproduction of *Malaxis paludosa* in Minnesota. *American Orchid Society Bulletin*, **53,** 1280-1291.

Reeves, T. & Reeves, L.M. (1985) Rediscovery of *Malaxis paludosa* (L.) Sw. (Orchidaceae) in Minnesota. *Rhodora*, **87,** 133-136.

Reinhammar, L.G. (1995) Evidence for two distinct species of *Pseudorchis* (Orchidaceae) in Scandinavia. *Nordic Journal of Botany*, **15,** 469-481.

Reinhammar, L.G. (1998) Systematics of *Pseudorchis albida* s.l. (Orchidaceae) in Europe and North America. *Botanical Journal of the Linnean Society*, **126,** 363-382.

Reinhammar, L.G. & Hedrén, M. (1998) Allozyme differentiation between lowland alpine populations of *Pseudorchis albida* s. lat. (Orchidaceae) in Sweden. *Nordic Journal of Botany*, **18,** 7-14.

Reinhammar, L.G., Olsson, E.G.A., and Sormeland, E. (2002) Conservation biology of an endangered grassland plant species, *Pseudorchis albida*, with some references to closely related alpine *P. straminea* (Orchidaceae). *Botanical Journal of the Linnean Society*, **139,** 47-66.

Richards, C.M., Church, S. and McCauley, D.E. (1999) The influence of population size and isolation on gene flow by pollen in *Silene alba*. *Evolution,* **53,** 63-73.

Roberts, D.L. (2006) Extinct or possibly extinct? *Science*, **312,** 997-998.

Roberts, D.L. & Kitchener A.C.. (2006) Inferring extinction from biological records: Were we too quick to write off Miss Waldron's Red Colobus Monkey (*Piliocolobus badius waldronae*)? *Biological Conservation*, **128,** 285-287.

Roberts, D.L. & Solow A.R. (2003) When did the dodo become extinct? *Nature*, **426,** 245.

Rodrigues, A.S.L., Pilgrim, J. D., Lamoreux, J F., Hoffmann, M., and Brooks, T. M. (2006) The value of the IUCN red list for conservation. *Trends in Ecology and Evolution*, **21,** 71-76.

Rodriguez-Robles, J.A., Melendez, E. J., and Ackerman, J. D. (1992) Effects of display size, flowering phenology, and nectar availability on effective visitation frequency in *Comparettia falcata* (Orchidaceae). *American Journal of Botany*, **79,** 1009-1017.

Rønsted, N., Weiblen, G.D., Cook, J.M., Salamin, N., Machado, C.A. and Savolainen, V. (2005) 60 million years of co-divergence in the fig-wasp symbiosis. *Proceedings of the Royal Society B-Biological Sciences*, **272,** 2593-2599.

Rymer, P.D., Whelan, R.J., Ayre, D.J., Weston, P.H., and Russell, K.G. (2005) Reproductive success and pollinator effectiveness differ in common and rare *Persoonia* species (Proteaceae). *Biological Conservation*, **123,** 521-532.

Sagarin, R.D. & Gaines, S.D. (2002) The 'abundant centre' distribution: to what extent is it a

biogeographical rule? *Ecology Letters*, **5,** 137-147.

Sagarin, R.D. & Gaines, S.D. (2006). Recent studies improve understanding of population dynamics across species ranges. *Oikos*, **115,** 386-388.

Sala, O.E., Chapin, F.S., Armesto, J.J., Berlow, E., Bloomfield, J., Dirzo, R., Huber-Sanwald, E., Huenneke, L.F., Jackson, R.B., Kinzig, A., Leemans, R., Lodge, D.M., Mooney, H.A., Oesterheld, M., Poff, N.L., Sykes, M.T., Walker, B.H., Walker, M. and Wall, D.H. (2000) Global biodiversity scenarios for the year 2100. *Science*, **287,** 1770-1774.

Saunders, D.A., Hobbs R.J., and Margules C.R. (1991) Biological consequences of ecosystem fragmentation - a review. *Conservation Biology*, **5,** 18-32.

Scacchi, R., Deangelis, G., and Corbo, R.M. (1991) Effect of the breeding system on the genetic structure in 3 *Cephalanthera* spp. (Orchidaceae). *Plant Systematics and Evolution*, **176,** 53-61.

Schemske, D.W. (1981) Floral convergence and pollinator sharing in 2 bee-pollinated tropical herbs. *Ecology,* **62,** 946-954.

Schemske, D.W., Husband, B.C., Ruckelhaus, M.H., Goodwillie, C., Parker, I.M., and Bishop, J.G. (1994) Evaluating approaches to the conservation of rare and endangered plants. *Ecology*, **75,** 584-606.

Schiestl, F.P., Ayasse, M., Paulus, H. F., Lofstedt, C., Hansson, B. S., Ibarra, F., and Francke, W. (1999) Orchid pollination by sexual swindle. *Nature*, **399,** 421-422.

Schiestl, F.P. (2005) On the success of a swindle: pollination by deception in orchids. *Naturwissenschaften*, **92,** 255-264.

Schroter, D., Cramer, W., Leemans, R., Prentice, I.C., Araujo, M.B., Arnell, N.W., Bondeau, A., Bugmann, H., Carter, T.R., Gracia, C.A., de la Vega-Leinert, A.C., Erhard, M., Ewert, F., Glendining, M., House, J.I., Kankaanpaa, S., Klein, R.J.T., Lavorel, S., Lindner, M., Metzger, M.J., Meyer, J., Mitchell, T.D., Reginster, I., Rounsevell, M., Sabate, S., Sitch, S., Smith, B., Smith, J., Smith, P., Sykes, M.T., Thonicke, K., Thuiller, W., Tuck, G., Zaehle, S. and Zierl, B. (2005) Ecosystem service supply and vulnerability to global change in Europe. *Science*, **310,** 1333-1337.

Seber, G.A.F. (1965) A note on multiple-recapture census. *Biometrika,* 52, 249-259.

Selkoe, K.A. & Toonen, R.J. (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters*, **9,** 615-629.

Shaffer, M.L. (1981) Minimum population sizes for species conservation. *Bioscience*, **31,** 131-134.

Shefferson, R.P., Sandercock, B. K., Proper, J., and Beissinger, S. R. (2001) Estimating

dormancy and survival of a rare herbaceous perennial using mark-recapture models. *Ecology*, **82,** 145-156.

Shefferson, R.P., Proper, J., Beissinger, S. R., and Simms E.L. (2003) Life history trade-offs in a rare orchid: the costs of flowering, dormancy and sprouting. *Ecology*, **84,** 1199-1206.

Shefferson, R.P., Kull, T., and Tali, K. (2005) Adult whole-plant dormancy induced by stress in long-lived orchids. *Ecology*, **86,** 3099-3104.

Shefferson, R.P. & Tali, K. (2007) Dormancy is associated with decreased adult survival in the burnt orchid, *Neotinea ustulata*. *Journal of Ecology*, **95,** 217-225.

Shefferson, R.P., Kull, T. and Tali, K. (2008) Mycorrhizal interactions of orchids colonizing Estonian mine tailings hills. *American Journal of Botany*, **95,** 156-164.

Sih, A., & Baltus, M. S. (1987) Patch size, pollinator behavior, and pollinator limitation in Catnip. *Ecology*, **68,** 1679-1690.

Silvertown, J., Wells, D.A., Gillman, M., Dodd, M.E., Robertson, H., and Lakhani, K.H. (1994) Short term effects and long term after-effects of fertilizer application on the flowering population of green-winged orchid *Orchis morio*. *Biological Conservation*, **69,** 191-197.

Simberloff, D. (1988) The contribution of population and community biology to conservation science. *Annual Review of Ecology and Systematics*, **19,** 473-511.

Sipes, S.D. & Tepedino, V. J. (1995) Reproductive biology of the rare orchid, *Spiranthes diluvialis* - breeding system, pollination, and implications for conservation. *Conservation Biology*, **9,** 929-938.

Sipes, S.D. & Tepedino, V.J. (2006) "Perfection" subverted? A contrivance for outcrossing in a rare orchid is influenced by pollinator abundance. *Journal of the Torrey Botanical Society*, **133,** 412-420.

Smith, S.D., Cowan, R.S., Gregg, K.B., Chase, M.W., Maxted, N., and Fay, M.F. (2004) Genetic discontinuities among populations of *Cleistes* (Orchidaceae, Vanilloideae) in North America. *Botanical Journal of the Linnean Society*, **145,** 87-95.

Smithson, A. (2002) The consequences of rewardlessness in orchids: reward-supplementation experiments with *Anacamptis morio* (Orchidaceae). *American Journal of Botany*, **89,** 1579-1587.

Snow, A.A., T.P. Spira, R. Simpson and R.A. Klips (1996). *The ecology of geitonogamous pollination*. In: Floral Biology. p. 191-216. D. G. Lloyd and S. C. H. Barrett, Eds. New York, Chapman and Hall.

Sokal, R.R. & Rohlf F.J. (1995) Biometry: The Principles and Practice of Statistics in Biological Research. W. H. Freeman, New York, U.S.A.

Solow, A.R. (1993a). Inferring extinction from sighting data. *Ecology*, **74,** 962-964.

Solow, A.R. (1993b). Inferring extinction in a declining population. *Journal of Mathematical Biology*, **32,** 79-82.

Solow, A.R. & Roberts, D.L. (2003) A nonparametric test for extinction based on a sighting record. *Ecology*, **84,** 1329-1332.

Solow, A.R. (2005) Inferring extinction from a sighting record. *Mathematical Biosciences*, **195,** 47-55.

Solow, A.R. & Roberts D.L. (2006). Museum collections, species distributions, and rarefaction. *Diversity and Distributions*, **12,** 423-424.

Soule, M.E. (1985) What is conservation biology? *Bioscience*, **35,** 727-734.

Soule, M.E. (1986) *Patterns of diversity and rarity: their implications for conservation.* In: Conservation Biology: The Science and Scarcity of Diversity. (ed. M. E. Soule). Sinauer Associates Inc., Sunderland, Massachusetts, U.S.A.

Sowig, P. (1989) Effects of flowering plants patch size on species composition of pollinator communities, foraging strategies, and resource partitioning in bumblebees (Hymenoptera, Apidae). *Oecologia*, **78,** 550-558.

Spielman, D., Brook, B.W. and Frankham, R. (2004) Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Sciences USA*, **101,** 15261-15264.

Spira, T.P. (2001) Plant-pollinator interactions: a threatened mutualism with implications for the ecology and management of rare plants. *Natural Areas Journal*, **21,** 78-88.

Stacy, E.A., Hamrick, J.L., Nason, J.D., Hubbell, S.P., Foster, R.B. and Condit, R. (1996) Pollen dispersal in low-density populations of three neotropical tree species. *American Naturalist*, **148,** 275-298.

Stanton, M.L., Ashman, T.L., Galloway, L.F., and Young, H.J. (1992). *Estimating mate fitness of plants in natural populations.* In: Wyatt, R Ecology and Evolution of Plant Reproduction. London: Chapman and Hall, 62–90.

Stebbins, G.L. (1957) Self fertilization and population variability in the higher plants. *American Naturalist*, **91,** 337-354.

Steffan-Dewenter, I., & Tscharntke, T (1999) Effects of habitat isolation on pollinator communities and seed set. *Oecologia*, **121,** 432-440.

Steffan-Dewenter, I. (2003) Seed set of male-sterile and male-fertile oilseed rape (*Brassica napus*) in relation to pollinator density. *Apidologie*, **34,** 227-235.

Steiner, K.E. (1989) The pollination of *Disperis* (Orchidaceae) by oil-collecting bees in southern Africa. *Lindleyana*, **4,** 164-183.

Stephenson, A.G. (1981) Flower and fruit abortion – proximate causes and ultimate functions.

Annual Review of Ecology and Systematics, **12,** 253-279.

Stout, J.C., Allen, J. A. and Goulson, D. (1998) The influence of relative plant density and floral morphological complexity on the behaviour of bumblebees. *Oecologia*, **117,** 543-550.

Summerhayes, V.C. (1968) Wild Orchids of Britain. 2nd Ed. Collins, London, U.K.

Sunnucks, P. (2000) Efficient genetic markers for population biology. *Trends in Ecology and Evolution*, **15,** 199-203.

Swofford DL (2000) PAUP: Phylogenetic Analysis Using Parsimony (*and other methods). Sinauer Associates, Sunderland, MA, U.S.A.

Tali, K., Fay, M.F., and Bateman, R.M. (2006) Little genetic differentiation across Europe between early-flowering and late-flowering populations of the rapidly declining orchid *Neotinea ustulata. Biological Journal of the Linnean Society*, **87,** 13-25.

Tali, K., Foley, M.J., and Kull, T. (2004). *Orchis ustulata* L. *Journal of Ecology*, **92,** 174-184.

Templeton, A.R. (1986) *Coadaptation and outbreeding depression.* In: Conservation Biology: The Science and Scarcity of Diversity. (ed. Soulé ME), pp. 105-116. Sinauer Associates Inc., Sunderland, Massachusetts, U.S.A.

Tepedino,V.J., Sipes, S.D., and Griswold, T.L. (1999) The reproductive biology and effective pollinators of the endangered beardtongue *Penstemon penlandii* (Scrophulariaceae). *Plant Systematics and Evolution*, **219,** 39-54.

Tilman, D., May, R.M., Lehman, C.L. and Nowak, M.A. (1994) Habitat destruction and the extinction debt. *Nature,* **371,** 65-66.

Thomson, J.D. (1978) Effects of stand composition on insect visitation in 2-species mixtures of *Hieracium. American Midland Naturalist*, **100,** 431-440.

Thomson, J.D., Price, M.V., Waser, N.M. and Stratton, D.A. (1986) Comparative studies of pollen and fluorescent dye transport by bumblebees visiting *Erythronium grandiflorum*, *Oecologia*, **69,** 561-566.

Tremblay, R.L. (1994) Frequency and consequences of multi-parental pollinations in a population of *Cypripedium calceolus* var. *pubescens* (orchidaceae). *Lindleyana*, **9,** 161-167.

Tremblay, R.L., Ackerman, J.D., Zimmerman, J.K. and Calvo, R.N. (2005) Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. *Biological Journal of the Linnean Society*, **84,** 1-54.

Tutin, T.G., Heywood, V.H., Burges, N.A., Moore, D.M., Valentine, D.H., Walters, S.M. & Webb, D.A. (1980) *Flora Europaea* V. Cambridge University Press. Cambridge.

Underwood, A.J. & Chapman, M.G. (1997) General Model Analysis of Variance, 5 factor (GMAV 5). Institute of Marine Ecology, University of Sydney, Australia.

Vamosi, J.C., Knight, T.M., Steets, J.A., Mazer, S.J., Burd, M. & Ashman, T.L. (2006) Pollination decays in biodiversity hotspots. *Proceedings of the National Academy of Sciences USA*, **103,** 956-961.

van der Cingel, N.A. (1995) An Atlas of Orchid Pollination: European Orchids. A. A. Balkema, Rotterdam, The Netherlands.

van der Pijl, L. & Dodson C.H. (1966) Orchids Flowers: Their Pollination and Evolution. University of Miami Press, Coral Gables, Florida, U.S.A.

van Treuren, R., Bijlsma, R., Ouborg, N. J., and van Delden, W. (1993) The effects of population size and plant density on outcrossing rates in locally endangered *Salvia pratensis*. *Evolution*, **47,** 1094-1104.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandelee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. (1995) AFLP - a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23,** 4407-4414.

Vucetich, J.A. & Waite, T.A. (2003) Spatial patterns of demography and genetic processes across the species' range: null hypotheses for landscape conservation genetics. *Conservation Genetics*, **4,** 639-645.

Waites, A.R. & Ågren, J. (2004) Pollinator visitation, stigmatic pollen loads and among-population variation in seed set in *Lythrum salicaria*. *Journal of Ecology,* **92,** 512-526.

Walden-Walsh, B. (2005) Convention on international trade in endangered species of wild fauna and flora: A cites timeline. *Selbyana*, **26,** 92-102.

Wallace, L.E. (2003) The cost of inbreeding in *Platanthera leucophaea* (Orchidaceae). *American Journal of Botany*, **90,** 235-242.

Wallis De Vries, M.F., Poschlod P., and Willems J. H. (2002) Challenges for the conservation of calcareous grasslands in northwestern Europe: integrating the requirements of flora and fauna. *Biological Conservation*, **104,** 265-273.

Waser, N.M. (1978) Interspecific pollen transfer and competition between co-occurring plant species. *Oecologia*, **36,** 223-236.

Waser, N.M. & Real, L.A. (1979) Effective mutualism between sequentially flowering plant species. *Nature,* **281,** 670-672.

Waser, N.M. (1983). *The adaptive nature of floral traits*, In: Pollination Biology. (ed.) L. Real, pp. 242-285. Academic Press, London, U.K.

Waser, N.M., Chittka, L., Price, M. V., Williams, N. M., and Ollerton, J. (1996) Generalization in pollination systems, and why it matters. *Ecology,* **77,** 1043-1060.

Webb, D.A. (1980) The biological vice-counties of Ireland. *Proceedings of the Royal Irish Academy*, **80B,** 179-196.

Webb, D.A. (1983) The Flora of Ireland in its European Context. *Journal of Life Sciences, Royal Dublin Society,* **The Boyle Medal Discourse,** 143-160.

Webb, D.A., Parnell J., and Doogue D. (1996) An Irish Flora. Dundalgan Press (W. Tempest), Dundalk, Ireland.

Wells, T.C.E. (1967) Changes in a population of *Spiranthes spiralis* (L.) Chevall at Knocking Hoe national nature reserve, Bedfordshire, 1962-65. *Journal of Ecology*, **55,** 83.

White, G.C. and Burnham, K.P. (1999) Program MARK: survival estimation from populations of marked animals. *Bird Study,* **46,** 120-139.

Wilcock, C., & Neiland, R. (2002) Pollination failure in plants: why it happens and when it matters. *Trends in Plant Science*, **7,** 270-277.

Wilcove, D.S., McLellan, C.H. & Dobson, A.P. (1986) *Habitat fragmentation in the temperate zone*. Conservation Biology: The Science and Scarcity of Diversity. (ed M. E. Soule), pp. 237-256. Sinauer Associates Inc., Sunderland, Massachusetts, U.S.A.

Willems, J.H. & Dorland, E. (2000) Flowering frequency and plant performance and their relation to age in the perrenial orchid *Spiranthes spiralis* (L.) Chevall. *Plant Biology*, **2,** 344-349.

Willems, J.H. & Lahtinen, M.L. (1997) Impact of pollination and resource limitation on seed production in a border population of *Spiranthes spiralis* (Orchidaceae). *Acta Botanica Neerlandica*, **46,** 365-375.

Willems, J.H., Balounová, Z., and Kindlmann, P., 2001. The effect of experimental shading on seed production and plant survival in the threatened species *Spiranthes spiralis* (Orchidaceae). *Lindleyana*, **16,** 31-37.

Willson, M.F. & Rathcke, B.J. (1974) Adaptive design of the floral display in *Asclepias syriaca* L. *American Midland Naturalist*, **92,** 47-57.

Wilson, P., Castellanos, M.C., Wolfe, A.D., and Thomson, J.D. (2006) *Shifts between bee and bird pollination in Penstemons*, In: Plant-Pollinator Interactions: From Specialization to Generalization. (Eds) N.M. Waser, J. Ollerton, pp. 47-68. The University of Chicago Press, Chicago, U.S.A.

Yeh, F.C., Yang, R., and Boyle, T. (2000) POPGENE, Microsoft windows-based freeware for population genetic analysis, Version 1.32. Edmonton.

Young, A., Boyle, T., and Brown, T. (1996) The population genetic consequences of habitat fragmentation for plants. *Trends in Ecology and Evolution*, **11,** 413-418.