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Population genetic structure in European populations of *Spiranthes romanzoffiana* set in the context of other genetic studies on orchids

AD Forrest[1], ML Hollingsworth[1], PM Hollingsworth[1], C Sydes[2] and RM Bateman[3]
[1]*Royal Botanic Garden Edinburgh, 20a Inverleith Row, Edinburgh EH3 5LR, UK;* [2]*Scottish Natural Heritage, 2 Anderson Place, Edinburgh EH6 5NP, UK;* [3]*Department of Botany, Natural History Museum, Cromwell Road, London SW7 5BD, UK*

Spiranthes romanzoffiana Cham. is restricted in Europe to the British Isles, where it is recognised as a conservation priority species due to frequent extirpation of populations along with no evidence of seed set; vegetative reproduction has been invoked as the sole means of perpetuation and dispersal. To investigate the reproductive ecology of this species, 17 populations have been sampled for chloroplast microsatellites and amplified fragment length polymorphisms (AFLPs). These markers revealed a previously unsuspected genetic–geographic split in the species, which correlates with differences in patterns of within-population variation. Northern populations were fixed for one chloroplast haplotype but showed high levels of AFLP genotypic diversity consistent with sexual reproduction (proportion of genotypes distinguishable, $P_D = 0.98$). More southerly populations showed fixed differences from the northern populations in their chloroplast haplotype and for 10 AFLP markers. They harboured only 12 unique multilocus genotypes among 113 individuals from six populations ($P_D = 0.11$). These genotypes differed mostly by single bands, and none by more than 4/138 loci, with identical multilocus genotypes occurring in widely separated populations. This uniformity in southern populations is consistent with agamospermous or autogamous reproduction, and/or an extreme population bottleneck. Finally, the observed patterns of population differentiation in *S. romanzoffiana* are compared with other studies of orchids, revealing a wide range of values that belie recent contrasting published generalisations that claim that orchids show either higher, or lower, levels of population differentiation than other plant families.
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Introduction

Molecular markers are now routinely used to contribute towards programmes concerned with the conservation of endangered species. Conservation genetics research has proved valuable in assessing the extent and consequences of genetic erosion (Young *et al*, 1996; Saccheri *et al*, 1998), in clarifying taxonomic issues to provide guidelines on which entities to preferentially conserve (Soltis and Gitzendanner, 1998), and in gaining insights into modes of reproduction and patterns of contemporary and historical dispersal (Sork *et al*, 1999).

This latter topic, essentially using molecular markers to investigate reproductive ecology, is relevant to many species of conservation concern, as halting and reversing the decline of a given species ultimately requires successful reproduction and dispersal. The mode of reproduction and the spatial scales of effective dispersal impact on both management strategies of individual sites and the maintenance of networks of populations at the landscape level. In plant species that can reproduce both sexually (via pollen and seed) and asexually (via vegetative spread), it can be exceedingly difficult to gauge the relative importance of each mode from field observations alone. Likewise, physical tracking of pollen and seed dispersal is also problematical beyond extremely local scales. Polymorphic genetic markers are well suited to tackling these issues, and hence are increasingly being applied in a conservation context.

Spiranthes romanzoffiana (Irish Lady's-tresses) is a terrestrial, herbaceous, perennial orchid, widespread and relatively frequent in North America, yet confined to the western fringes of the British Isles within Europe (Preston *et al*, 2002; Figure 1). Its localised European distribution has led to increasing conservation interest; it is now listed in the UK Biodiversity Action Plans as a conservation priority species (UK Biodiversity Group, 1999), and recognised by the IUCN Orchid Specialist group as having 'critically low populations' in Europe (IUCN/Orchid Specialist Group, 1996).

One of the major factors behind this conservation concern relates to the mode of reproduction of European populations of *S. romanzoffiana*. Pollination of this species in North America occurs via unspecialised, medium-sized long-tongued bees, which are attracted to nectar produced from two bosses near the base of the labellum. Similar potential pollinator species occur within the plant's habitat in Europe, and flower visits have been observed (J Robarts, 2002, personal communication). Despite this, no seed set has been recorded in the European range, leading to suggestions

Correspondence: PM Hollingsworth, Royal Botanic Garden Edinburgh, 20a Inverleith Row, Edinburgh EH3 5LR, UK.
E-mail: P.Hollingsworth@rbge.org.uk
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that the plant's persistence there is attributable to vegetative reproduction (Horsman, 1994). Plants produce lateral buds in the autumn, which over-winter and develop into the following year's aerial parts. The only documented mechanism for vegetative reproduction is the occasional production of twin (rarely three or four) lateral buds, which can lead to the formation of multiple aerial parts; these may eventually divide to form different ramets (Summerhayes, 1968). However, while this mechanism may lead to clonal spread over very small areas, it is difficult to imagine this mode of reproduction being effective over larger scales (eg >1 m).

A second area of conservation concern relates to the populations showing a high level of demographic instability; for instance, no population recorded in Scotland prior to 1981 is now known to be extant (UK Biodiversity Group, 1999). New populations have been recorded, but this frequent extirpation of populations leads to difficulties in assessing the distribution and conservation status of this species.

There is thus something of a paradox about European populations of *S. romanzoffiana*. On the one hand, populations appear to have a high turnover, with known populations disappearing and new populations being discovered. On the other hand, there is no satisfactory explanation for how the plant reproduces and disperses. Seed set has not been observed, and although vegetative reproduction has been invoked, the species' apparently limited capacity for clonal spread makes this an unlikely mode of the formation of new populations. We have used chloroplast microsatellites (cpSSRs; Provan *et al*, 2001) and amplified fragment length polymorphisms (AFLPs; Vos *et al*, 1995) to establish whether reproduction is primarily sexual or asexual, and to examine population differentiation in order to infer the spatial scales over which effective gene flow is occurring.

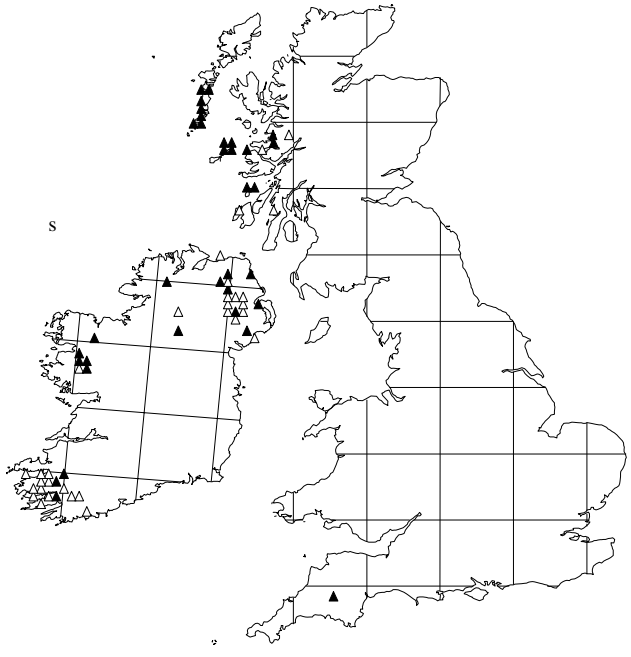

Figure 1 Distribution of *S. romanzoffiana* in Europe. Closed triangles: 1987–1999 records. Open triangles: pre-1987 records (data from Preston *et al*, 2002). The map grid represents 100 km squares.

Materials and methods

Plant material and isolation of genomic DNA

To assess genetic variation in European populations of *S. romanzoffiana*, individuals were sampled from 17 populations: eight from Scotland and nine from Ireland (Table 1; see also Figure 2a). Sampling was designed to assess variation at the within-population, among-population and regional levels. In 10 populations, between 10 and 25 individuals were sampled and examined for both AFLP and cpSSR variation. While this sampling primarily consisted of individuals separated by at least

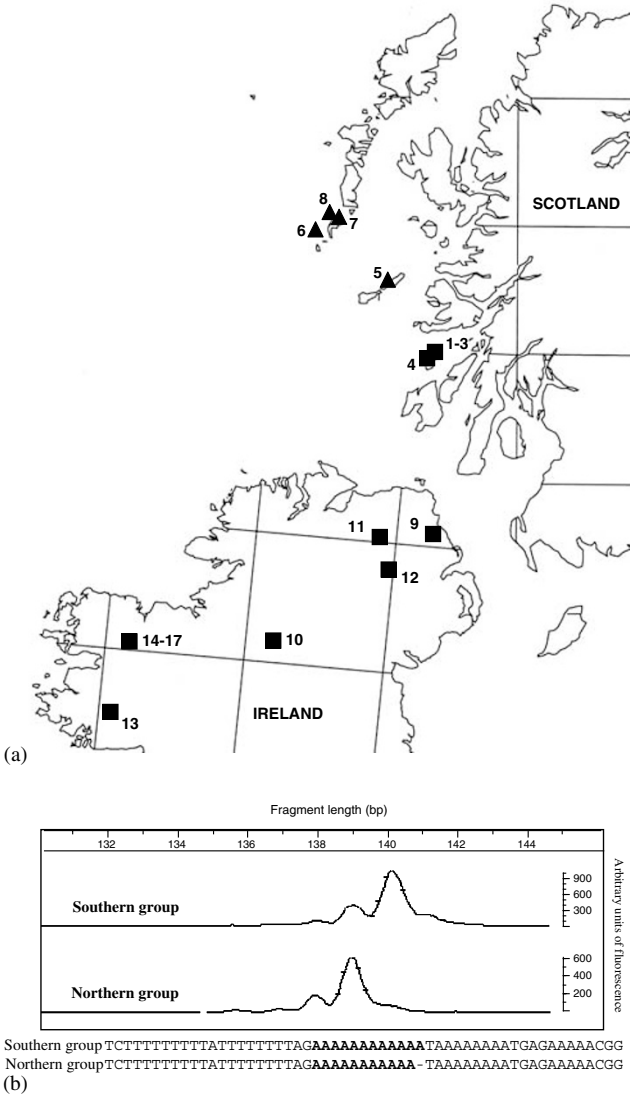

Figure 2 (**a**) Distribution map showing the location of the sampled populations. Numbers correspond to the population names in Table 1, and symbols correspond to different chloroplast haplotypes (squares A_{20}, southern group; triangles A_{19}, northern group). The map grid represents 100 km squares. (**b**) Sample electropherogram and partial sequences showing cpSSR variability. The peak traces correspond to the difference in poly-A repeat number.

Table 1 Accession details of 17 European populations of *S. romanzoffiana*

No.	*Population*	*Name*	*VC*	P_S	*AFLP*	*cpSSR*
	Scotland					
1	SC-CO1	Colonsay, Cnoc Seunta	102	130	25	25
2	SC-CO2	Colonsay, Cnoc nan Caorach	102	85	25	25
3	SC-CO3	Colonsay, Kiloran Dunes C	102	NA	10	10
4	SC-CO4	Colonsay, West Loch Fada	102	30	14	15
5	SC-CL1	Coll, Arileod Field	103	1000	25	25
6	SC-VA1	Vatersay, Causeway	110	65	23	24
7	SC-BA1	Barra, Bruernish A	110	345	23	25
8	SC-BA2	Barra, Bruernish C	110	195	21	25
	Ireland					
9	IR-AN1	Antrim, Gortnagory	H39	205	25	12
10	IR-FE1	Fermanagh, Corraslough Point	H33	5	—	4
11	IR-LO1	Londonderry, Lough Beg	H40	10	—	5
12	IR-TY1	Tyrone, Brookend	H36	45	—	5
13	IR-GA1	Galway, Lough Corrib	H17	10	—	7
14	IR-MA1	Mayo, Lough Cullin NE shore	H27	100	—	5
15	IR-MA2	Mayo, Lough Conn SW shore	H27	50	14	12
16	IR-MA3	Mayo, Lough Conn S shore	H27	30	—	5
17	IR-MA4	Mayo, Lough Conn E shore	H27	80	—	5
	Total				205	234

Exact localities have been omitted at the request of the relevant conservation agencies. VC = Vice County; P_S = approximate population size; AFLP = number of individuals used in AFLP analysis; cpSSR = number of individuals used in chloroplast microsatellite analysis.; NA = Population size estimate not available.

Table 2 cpDNA regions sequenced to search for polymorphic microsatellite loci in *S. romanzoffiana*

Chloroplast region	*Annealing temp. (°C)*	*Extension time (min)*	*Primer source*	*GenBank accession no.*
*atp*B-*rbc*L	53	3	Chiang *et al* (1998)	AY363058, AY363059
*trn*L-intron-*trn*L-*trn*F IGS	55	3	Taberlet *et al* (1991)	AY363055
*psb*C-*trn*S	57	3	Demesure *et al* (1995)	AY363056, AY363057
*trn*S-*trn*fM	62	3	Demesure *et al* (1995)	AY363054
*trn*H-*trn*K	62	3	Demesure *et al* (1995)	AY363060, AY363061

5 m, in eight of the populations five individuals were sampled within a 1 m^2 quadrat to look for evidence of local clonal growth. From a further seven populations, between four and seven individuals were sampled for cpSSR analyses only (Table 1). For all molecular analyses, a section of leaf material approximately 30 mm long was harvested from single leaves and stored in silica gel.

DNA isolation was performed with DNeasy® Plant Mini Kits (Qiagen Ltd, UK). DNA quality and concentration was assessed by running samples alongside Hyperladder concentration marker (Bioline, UK) on a 1% agarose gel in 1 × tris borate/EDTA buffer, and visualised by staining with ethidium bromide.

Chloroplast microsatellite analysis

Five cpDNA regions (Table 2) were screened for microsatellite loci among 11 accessions representing a range of populations. Amplification of chloroplast regions was performed via polymerase chain reaction (PCR) on an MJ Research PTC-200 DNA Engine thermal cycler in 50 µl reactions containing 5 µl 10 × NH_4 reaction buffer (Bioline, UK), 5 µl 2 mM deoxynucleotide triphosphate (dNTP), 2.5 µl 50 mM $MgCl_2$, 1.5 µl of each primer (10 mM), 33.5 µl dH_2O, 1.25 U *Biotaq* polymerase (Bioline, UK) and 1.0 µl DNA template. The thermocycle profile was: initial denaturation for 4 min at 94°C, followed by 30 cycles of 45 s at 92°C, 45 s at 53–62°C (Table 2) and 3 min at 72°C, with a final extension for 10 min at 72°C. PCR products were run on 1% agarose gels to check for amplification success and quality.

Amplified fragments were purified using QIAquick™ PCR purification kits (Qiagen Ltd, UK) following the manufacturer's protocol, and sequenced using the dideoxy chain-termination method. Cycle sequencing was performed in 20 µl reactions containing 4 µl Thermo Sequenase II (Amersham Pharmacia, UK), 0.5 µl primer (10 mM), 13.5 µl dH_2O and 2 µl of purified PCR product, under the following PCR conditions: 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Sequencing PCR products were purified following the manufacturer's instructions, then run on an ABI Prism™ 377 DNA Sequencer, before editing and manual alignment using Sequence Navigator™ 1.0.1 (Applied Biosystems Inc., USA).

Initial screening revealed eight mononucleotide repeat loci >8 bp in length, located in four of the five cpDNA regions amplified (*atp*B-*rbc*L $(T)_{10}$, $(T)_{14}$, $(T)_{\underline{15}}$, $(A)_{\underline{20}}$; *psb*C-*trn*S $(T)_9$; *trn*H-*trn*K $(A)_{13}$; *trn*L-*trn*F $(T)_{\underline{17}}$, $(A)_{\underline{19 \text{ or } 20}}$). Underlined numbers indicate repeat motifs with single base-pair interruptions. Only the poly-A repeat located in the *trn*L intron showed any polymorphism among the 11 accessions studied. Forward and reverse primers were

designed to amplify this microsatellite region (forward primer: 5′-GGTAACTTCCAAATTCAGA-3′; reverse primer: 5′-ACAGCTTCCGTTGAGTCTC-3′) using Primer 3 (www.basic.northwestern.edu/biotools/Primer3.html). Amplification was carried out in 10 µl reactions containing 0.5 µl of each of the two primers (10 mM), 1 µl $10 \times NH_4$ reaction buffer, 0.5 µl 50 mM $MgCl_2$, 1 µl 2 mM dNTPs, 6 µl dH_2O, 0.2 U *Biotaq* polymerase and 0.5 µl DNA template. PCR conditions were as follows: 7 min at 95°C, followed by 30 cycles of 15 s at 95°C, 15 s at 50°C and 15 s at 72°C, with a final extension for 15 min at 72°C. Amplification products were diluted 130-fold in dH_2O and analysed on 5% Long Ranger® acrylamide gels (BMA, USA) on an ABI Prism™ 377 DNA sequencer. Fragment size was determined by comparison with Rox 500 GeneScan size standard using Genotyper 2.0® software (Applied Biosystems Inc., USA).

Amplified fragment length polymorphism

Accessions were analysed following the plant mapping protocol of the PE Applied Biosystems AFLP™ kits. In all, 13 primer combinations were assessed for clarity, reproducibility and polymorphism among 12 accessions representing a range of populations, with three primer combinations selected for further analysis (A = *Eco*RI-ACT/*Mse*I-CTA, B = *Eco*RI-ACT/*Mse*I-CAG, C = *Eco*RI-ACA/*Mse*I-CAG). Selective amplification products were run on 2% agarose gels to check for amplification success, and then electrophoresed on 5% Long Ranger® acrylamide gels; multilocus profiles were visualised using GeneScan Analysis® 3.1.2 software (Applied Biosystems Inc., USA). Markers were scored as present/absent using Genotyper® 2.0, and a binary data matrix constructed encompassing markers in the range 50–400 bp. Markers scoring below 50 arbitrary units of fluorescence were disregarded. Reproducibility of amplification was assessed by replicate extractions and amplifications of a subset of the samples. Only repeatable and unambiguous markers were included in the analysis.

Data analysis

Standard descriptive statistics were obtained to assess patterns of within-population variation: *P*, the proportion of loci that are polymorphic; P_D, the proportion of distinguishable genotypes; A_{PD}, the average number of pairwise differences of markers among individuals. To establish the apportionment of variation into within- and between-population components, an analysis of molecular variation (AMOVA) was undertaken using Arlequin version 2.0 (Schneider *et al*, 2000), and the significance of F_{ST} analogues (ϕ_{ST}) was assessed by permutation tests.

Results

cpSSRs

Polymorphism at the *trn*L microsatellite locus revealed two haplotypes differing by a single base pair (A_{19} or A_{20}), resulting in fragments of 139 or 140 bp, respectively (Figure 2b). No intrapopulation variation was detected, but there was clear geographically structured interpopulation variation (Figure 2a). The A_{19} repeat occurred in all accessions from Coll, Vatersay and Barra (hereafter referred to as the northern group), and the A_{20} repeat occurred in all accessions from Colonsay and Ireland (hereafter referred to as the southern group).

AFLP analysis

A total of 138 unambiguous AFLP markers were generated from the 205 individuals that gave clear amplification profiles. Of these 138 markers, 86 were polymorphic ($P = 0.62$) either within or among populations (Table 3).

Estimation of differentiation among all populations was high ($\phi_{ST} = 0.892$, $P < 0.01$). A hierarchical AMOVA analysis was conducted based on the geographical regions corresponding to the two chloroplast haplotypes (northern and southern groups). Between-group variation accounted for 85.8% of the total variation, with 3.5% of variation among populations within groups and 10.8% among individuals within populations. In total, 10 markers were differentially fixed between the northern

Table 3 Within-population AFLP diversity in *S. romanzoffiana*

Sample unit	N	P	N_{MLG}	P_D	A_{PD}	A_{PD} *range*
Populations						
SC-CO1	25	0.01	2	0.08	0.43	0–1
SC-CO2	25	0.01	2	0.08	0.22	0–1
SC-CO3	10	0.00	1	0.10	0.00	0
SC-CO4	14	0.01	2	0.07	0.09	0–1
SC-CL1	25	0.38	25	1.00	10.74	1–25
SC-VA1	23	0.33	22	0.96	7.81	0–17
SC-BA1	23	0.24	23	1.00	6.72	1–15
SC-BA2	21	0.15	21	1.00	5.61	1–11
IR-AN1	25	0.03	5	0.20	0.56	0–3
IR-MA2	14	0.04	4	0.29	0.90	0–4
Regions						
Northern	92	0.53	90	0.98		
Southern	113	0.06	12	0.11		
Entire data set	205	0.62	102	0.50		

N = number of individuals sampled per population; P = proportion of loci that are polymorphic; N_{MLG} = number of multilocus genotypes detected; P_D = proportion of distinguishable genotypes; A_{PD} = average pairwise difference of markers among individuals within populations.

Table 4 Average pairwise distances (A_{PD}) within and among populations of *S. romanzoffiana* based on 138 AFLP loci

	SC-CO1	*SC-CO2*	*SC-CO3*	*SC-CO4*	*SC-CL1*	*SC-VA1*	*SC-BA1*	*SC-BA2*	*IR-AN1*	*IR-MA2*
SC-CO1	**0.43**	0.39	0.29	1.75	*38.73*	*34.75*	*36.92*	*38.59*	1.35	1.36
SC-CO2		**0.22**	0.11	1.58	*38.54*	*34.39*	*36.72*	*38.41*	1.16	1.27
SC-CO3			**0.00**	1.57	*38.74*	*34.81*	*36.88*	*38.59*	1.20	1.41
SC-CO4				**0.09**	*36.83*	*33.20*	*35.70*	*36.91*	1.60	1.61
SC-CL1					**10.74**	10.35	10.87	10.55	*34.32*	*31.73*
SC-VA1						**7.81**	9.12	9.15	*30.43*	*29.45*
SC-BA1							**6.72**	7.88	*32.71*	*31.07*
SC-BA2								**5.60**	*34.34*	*31.99*
IR-AN1									**0.56**	0.81
IR-MA2										**0.90**

Diagonal elements: A_{PD} within populations. Above diagonal: A_{PD} among populations. Average pairwise distances among populations from different regions (northern *vs* southern) are italicised.

and southern groups. Average pairwise distances among multilocus genotypes were also high when comparing populations from the two groups (Table 4).

The northern populations (Coll, Barra and Vatersay) showed lower levels of differentiation than that among all populations ($\phi_{ST} = 0.193$), but differentiation was still significant ($P < 0.01$). Little differentiation was detected among the southern populations (Colonsay and Ireland), although one population on Colonsay showed a fixed difference for a single marker in all individuals from the other southern populations. Obtaining meaningful estimates of population differentiation within the southern group was hampered by the paucity of polymorphism within and among these populations (see below).

There is a striking difference in the levels of intrapopulation diversity in northern *vs* southern populations (Table 3). The vast majority (90/92) of individuals from the northern populations harboured unique multilocus genotypes ($P_D = 0.98$), the only exception being a pair of accessions from Vatersay, and shared multilocus genotypes among single accessions from Vatersay and Barra (SC-BA1). All multilocus genotypes were unique to single populations except this latter pair. All 20 samples collected within 1 m^2 quadrats (five plants from each of four northern populations) had different multilocus genotypes, consistent with sexual reproduction rather than local clonal spread. In contrast, only 12 unique multilocus genotypes among 113 individuals ($P_D = 0.11$) were detected from the southern populations, most being present only at low frequencies. The majority of these multilocus genotypes differed from each other by only a single marker ($A_{PD} = 0.37$, range 0–4). Artefactual differences attributable to subtle variations in AFLP reaction conditions cannot be excluded as the source of these differences.

Discussion

Regional geographic structure

Both AFLPs and cpSSRs provide strong evidence for a marked genetic–geographic divide in European populations of *S. romanzoffiana*. Differential fixation of chloroplast haplotypes and 10 AFLP markers suggests a lack of contemporary gene flow among the northern (Coll, Barra, Vatersay) and more southerly (Colonsay, Ireland) populations. This split was previously unsuspected, and does not correlate with any documented morphological or ecological differences between these population groups.

Within-region population genetic structure (northern populations)

The northern populations (Barra, Coll, Vatersay) show a moderate degree of population differentiation ($\phi_{ST} = 0.193$) but high levels of intrapopulation genotypic diversity. Although seed set has not been observed in European populations of *S. romanzoffiana*, the genotypic diversity in these northern populations suggests that seed set and sexual recruitment has taken place. The lack of individuals with shared multilocus genotypes (even within 1 m^2 quadrats) indicates that vegetative reproduction does not play a major role in the perpetuation and dispersal of individuals.

It could be argued that the high levels of genotypic diversity, coupled with an absence of observed seed set, could be attributable to trans-atlantic seed dispersal (seed set is frequently observed in North American populations). We feel that this is exceedingly unlikely given (a) the considerable number of distinct genotypes and plants involved, and (b) the marked regional differentiation, which would require differential migration of seeds with the different chloroplast haplotypes into the northern and southern regions.

Instead, the most parsimonious explanation of our data is that sexual reproduction has taken place *in situ*. This reproduction could be historical, or current-but-infrequent. It is difficult to distinguish between these two scenarios, but it is worth noting that an absence of *observed* seed set does not necessarily equate to a genuine absence of seed set. Sexual reproduction could take place via occasional overlooked fertilised flowers; a typical individual terrestrial orchid flower can produce 1000–10 000 seeds (Arditti, 1992). Another potential theory is that a low level of seed set occurs within individual capsules, such that the capsules do not swell and appear fertile, but nevertheless contain a few viable seeds (J Robarts, 2002; M Light, 2003, personal communication). Detailed observations of flowering spikes and capsules, along with pollen viability, seed viability and crossing experiments, are needed to establish the precise conditions under which reproduction occurs.

Until such studies are completed, grazing controls should be established in these northern populations of *S. romanzoffiana* during the flowering and fruiting period. Sheep can have devastating effects on *S. romanzoffiana* flower spikes, and entire populations can be stripped of all flowering spikes in a matter of hours or days when flocks are moved into the orchid-containing pastures. As these populations were believed to reproduce entirely vegetatively (Horsman, 1994), the reproductive consequences of this grazing have been considered to be small. However, as the genetic data from this study suggest that reproduction is predominantly sexual, and yet field observations suggest that seed set is rare, the flowering spikes should be protected from herbivory to maximise the chances of successful seed production and periodic sexual recruitment.

Within-region population genetic structure (southern populations)

In contrast to the data from the northern populations, the more southerly populations are typified by extremely low levels of genotypic diversity, with identical multilocus genotypes being detected in geographically disparate populations. Such genetic uniformity within and among populations could be attributed to efficient clonal growth and dispersal. In clonal plants, if propagules from a single genotype are able to disperse, large areas can be covered by ramets of a single genet (eg Hollingsworth and Bailey, 2000). However, given the lack of evidence for clonal spread in the morphologically similar northern populations, this seems an unsatisfactory explanation. This is particularly evident when one considers the substantial geographical distances involved.

Agamospermy has been reported in some *Spiranthes* species from North America (Catling, 1982; Schmidt and Antlfinger, 1992). Although this has never been observed in *S. romanzoffiana*, several species that usually reproduce via sexual out-crossing have been observed to develop agamospermous races in colonising populations near the edge of their ranges (Catling and Catling, 1991). This offers a potential explanation for the patterns of genotypic similarity among populations in Ireland and Colonsay.

An alternative explanation is that reproduction in the southern populations is sexual, but that the genotypes involved are homozygous at the vast majority of the study loci. An extreme genetic bottleneck could lead to genetic uniformity among sexually reproducing individuals. Alternatively, self-pollination can lead to a rapid reduction in heterozygosity. Autogamy has been observed in other *Spiranthes* species (eg Catling, 1983a, 1990; Sipes and Tepedino, 1995; Sun, 1997). However, Catling's (1983b) observations on North American populations of *S. romanzoffiana* suggest that it is unlikely in this species. This is due to protandry, and also the column structure which prevents the pollinia from coming into contact with the stigmatic surface.

Data from variable codominant markers (eg nuclear microsatellites) are needed to distinguish between these alternative explanations. Hypervariable codominant markers could establish whether the observed genetic uniformity is associated with homozygosity (selfing), or random mating from a very narrow genetic base (bottleneck), or whether there is fixed heterozygosity (indicative of agamospermous reproduction).

Population differentiation

The overall estimate of population differentiation for European populations of *S. romanzoffiana* is high ($\phi_{ST} = 0.892$). However, this figure includes two groups of populations fixed for different markers (apparently not exchanging genes), and potentially showing different reproductive strategies. In terms of evaluating the genetic connectivity of populations, it is perhaps more meaningful to examine patterns of differentiation among the sexual northern populations. Here, there is a smaller, but still significant, level of population differentiation ($\phi_{ST} = 0.193$). It is also worth noting that if one considers pairwise differences among northern populations, an estimate of ϕ_{ST} (0.217) is obtained between the two populations on Barra (separated by only 0.5 km), which is a similar order of magnitude to the estimate for differentiation among the northern populations *per se* (involving interisland distances of 70 km between Coll and Barra). This differentiation, evident even over small scales, may be related to the infrequent production of seeds, which will inevitably reduce opportunities for interpopulation gene flow.

This significant deviation from panmixia among proximal populations of *S. romanzoffiana* could be considered high when compared to average levels of differentiation among orchid populations. Hamrick and Godt (1996) summarised allozyme data from 16 orchid population genetic studies and obtained a mean estimate of G_{ST} for orchids of 0.087. They noted that orchids 'had an exceptionally low mean G_{ST}… perhaps due to the species-specific pollinators characteristic of orchids, and to their tiny wind-borne seeds. Both of these traits could produce high rates of gene flow among populations' (Hamrick and Godt, 1996, p 1297). However, it is worth stressing that allozyme work by Sun and Wong (2001, p 2186) led to exactly the opposite conclusion, that 'gene flow appears to be much more restricted in wild orchids than in other plants'.

These opposing views triggered our own review of levels of differentiation among orchid populations (Table 5). To the best of our knowledge, this is the most comprehensive summary of orchid G_{ST} (or F_{ST}) estimates to date. Our estimates of population differentiation range from $G_{ST} = 0.012$ to 0.924, with mean values of $G_{ST} = 0.187$ (all studies), $G_{ST} = 0.219$ (excluding studies examining less than five populations) and $G_{ST} = 0.161$ (further excluding studies examining dominant marker data sets). These mean estimates are somewhat higher than the 'exceptionally low mean G_{ST}' of 0.087, and within the range recorded for several other plant families by Hamrick and Godt (1996). In this respect it is worth noting that while orchid species share biological attributes such as small, mostly dust-like seeds, they are also a large, heterogeneous and diverse group (Arditti, 1992; Rudall and Bateman, 2002). The Orchidaceae contains ca 19 000 species encompassing an exceptionally wide range of reproductive strategies, variation in generation times, and includes both narrow endemic and widespread species,

Table 5 Genetic diversity among populations of orchid species based on literature survey

Species	N_P	N_S	*Assay*	N_L	G_{ST} (F_{ST})[a,b]	*Ref.*
Caladenia tentaculata G.F.K. Schldl.	9	490	ISO	22	0.034	13
Calypso bulbosa L.	21	779	ISO	3	0.072	25
Catasetum viridiflavum Hook.	16	1442	ISO	17	0.100	41
Catasetum viridiflavum Hook.	16	1442	ISO	17	0.060	41
Cephalanthera longifolia (L.) Fritsch	3	51	ISO	9	0.104	6
Cephalanthera rubra (L.) L.C.M. Richard	7	90	ISO	9	0.247	6
Cymbidium goeringii Reichb. f.	24	1078	ISO	14	0.108	26
Cypripedium acaule Ait.	4	134	ISO	14	0.164	9
Cypripedium calceolus L.	15	425	ISO	12	0.194	8
Cypripedium calceolus L.	3	230	ISO	11	0.016	37
Cypripedium candidum Muhl. ex Willd.	5	107	ISO	14	0.069	9
Cypripedium fasciculatum Kell.	3	69	ISO	12	0.040	20
Cypripedium kentuckiense C. Reed	8	220	ISO	12	0.182	19
Cypripedium reginae Walter	3	97	ISO	14	0.349	9
Dactylorhiza romana (Seb.) Soó	8	306	ISO	19	0.070	32
Dactylorhiza sambucina (L.) Soó	9	410	ISO	19	0.160	32
Diuris sulphurea R. Br.	3	195	ISO	15	0.349	14
Epipactis atrorubens (Hoffm. ex Bernh.) Besser	7	148	ISO	9	0.257	*
Epipactis gigantea Dougl. ex Hook.	4	67	ISO	9	0.214	*
Epipactis gigantea Dougl. ex Hook.	12	c.360	ISO	17	0.493	23
Epipactis helleborine (L.) Crantz	13	401	ISO	9	0.087	27
Epipactis helleborine (L.) Crantz	13	273	ISO	13	0.240	16
Epipactis helleborine (L.) Crantz	47	1170	ISO	9	0.206	34
Epipactis helleborine (L.) Crantz	4	111	ISO	8	0.033	1
Epipactis palustris (L.) Crantz	11	309	ISO	9	0.653	*
Epipactis purpurata G.E. Sm.	12	148	ISO	9	0.150	*
Eulophia sinensis Miq.	7	38	RAPD	97	0.653	35
Goodyera procera (Ker-Gawl.) Hook.	14	343	RAPD	101	0.386	24
Goodyera procera (Ker-Gawl.) Hook.	15	507	ISO	15	0.523	24
Gymnadenia conopsea R. Br.	16	c.300	ISO	11	0.471	2
Gymnadenia conopsea R. Br.	10	174	SSR	3	0.060	28
Lepanthes eltoroensis Stimson	10	96	ISO	6	0.219	36
Lepanthes rubripetala Stimson	11	200	ISO	7	0.266	36
Lepanthes rupestris Stimson	7	140	ISO	7	0.169	36
Leporella fimbriata (Lindl.) A. S. George	4	140	ISO	4	0.044	11
Microtis parviflora R. Br.	5	149	ISO	17	0.296	5
Nigritella rhellicani (Teppner & E. Klein) E. Klein[e]	23	308	ISO	10	0.153	29
Orchis laxiflora Lam.[c]	12	c.600	ISO	25	0.116	12
Orchis laxiflora Lam.[c]	2	47	ISO	9	0.080	3
Orchis longicornu Poir.[c]	6	162	ISO	27	0.015	4
Orchis mascula (L.) L.	3	62	ISO	9	0.083	3
Orchis morio L.[c]	18	346	ISO	27	0.055	7
Orchis morio L.[c]	5	117	ISO	9	0.064	3
Orchis palustris Jacq.[c]	8	325	ISO	25	0.448	12
Orchis papilionacea L.[c]	29	3000	ISO	28	0.038	10
Orchis papilionacea L.[c]	4	88	ISO	9	0.038	3
Orchis pauciflora Ten.	3	92	ISO	9	0.040	3
Orchis provincialis Balb.	2	60	ISO	9	0.023	3
Orchis purpurea Huds.	5	153	ISO	9	0.042	3
Orchis tridentata Scop.[d]	4	143	ISO	9	0.039	3
Pleurothallis adamantinensis Brade	2	35	ISO	12	0.049	31
Platanthera leucophaea (Nutt.) Lindl.	7	c.148	ISO	12	0.750	38
Platanthera leucophaea (Nutt.) Lindl.	10	192	RAPD	64	0.260	38
Pleurothallis fabiobarrosii Borba & Semir	2	65	ISO	12	0.081	31
Pleurothallis johannensis Barb. Rodr.	7	230	ISO	12	0.046	31
Pleurothallis ochreata Lindl.	4	70	ISO	12	0.175	31
Pterostylis aff. *picta* M. A. Clem.	9	139	ISO	16	0.054	39
Pleurothallis teres Lindl.	7	160	ISO	12	0.205	31
Pseudorchis albida (L.) A. & D. Löve s.s.	4	90	ISO	18	0.150	17
Pseudorchis straminea (Fern.) Soó	2	42	ISO	18	0.240	17
Pterostylis aff. *alata* Reichb. fil.	2	24	ISO	15	0.135	33
Pterostylis angusta A.S. George	2	19	ISO	15	0.012	33
Pterostylis aspera D.L. Jones & M.A. Clem.	7	85	ISO	15	0.124	33
Pterostylis gibbosa R. Br.	12	255	ISO	16	0.151	30
Pterostylis hamiltonii Nicholls	4	52	ISO	15	0.143	33
Pterostylis rogersii E. Coleman	9	111	ISO	15	0.136	33
Pterostylis scabra Lindl.	11	155	ISO	15	0.081	33
Spiranthes diluvialis Sheviak	12	651	ISO	14	0.044	18
Spiranthes sinensis (Pers.) Ames	6	181	ISO	22	0.174	15
Spiranthes spiralis (L.) Chevall.	7	857	ISO	4	0.026	40
Tolumnia variegata (Swartz) Braem	14	545	ISO	12	0.110	21

Table 5 *(continued)*

Species	N_P	N_S	*Assay*	N_L	G_{ST} (F_{ST})[a,b]	*Ref.*
Vanilla barbellata Reich. f.	6	87	ISO	7	0.123	22
Vanilla claviculata (W. Wright) Swartz	5	89	ISO	7	0.158	22
Zeuxine gracilis Bl.	6	75	ISO	18	0.333	35
Zeuxine gracilis Bl.	6	74	RAPD	77	0.539	35
Zeuxine strateumatica (Ln.) Schltr.	10	50	RAPD	71	0.924	35
Mean $N=76$ (range = 0.012–0.924)					0.187	
Mean (excl. less than five populations, $N=53$)					0.219	
Mean (excl. dominant data sets, $N=71$)					0.161	
Mean (excl. both the above parameters, $N=48$)					0.184	
Hamrick and Godt (1996) $N=16$					0.087	

N_P = number of populations sampled; N_S = number of individuals sampled; Assay: ISO = isozyme analysis, SSR = microsatellite analysis, RAPD = randomly amplified polymorphic DNA analysis; N_L = number of loci analysed; G_{ST} (or F_{ST}) = proportion of genetic variation partitioned among populations. Source references: 1, Scacchi *et al* (1987); 2, Scacchi and De Angelis (1989); 3, Scacchi *et al* (1990); 4, Corrias *et al* (1991); 5, Peakall and Beattie (1991); 6, Scacchi *et al* (1991); **7,** Rossi *et al* (1992); 8, Case (1993); 9, Case (1994); 10, Arduino *et al* (1995); 11, Peakall and James (1995); 12, Arduino *et al* (1996); 13, Peakall and Beattie (1996); 14, Sharma and Jones (1996); 15, Sun (1996); 16, Hollingsworth and Dickson (1997); 17, Reinhammar and Hedrén (1997); 18, Arft and Ranker (1998); 19, Case *et al* (1998); 20, Aagaard *et al* (1999); 21, Ackerman and Ward (1999); 22, Nielsen and Siegismund (1999); 23, Thornhill (1999); 24, Wong and Sun (1999); 25, Alexandersson and Ågren (2000); 26, Chung and Chung (2000); 27, Ehlers and Pedersen (2000); 28, Gustafsson (2000); 29, Hedrén *et al* (2000); 30, Sharma *et al* (2000); 31, Borba *et al* (2001); 32, Bullini *et al* (2001); 33, Sharma *et al* (2001); 34, Squirrell *et al* (2001); 35, Sun and Wong (2001); 36, Tremblay and Ackerman (2001); 37, Brzosko *et al* (2002); 38, Wallace (2002); 39, Sharma *et al* (2002); 40, Machon *et al* (2003); 41, Murren (2003); * J Squirrell and P Hollingsworth, unpublished.

[a]Species in which no genetic variation was detected are not included in this review.

[b]Different investigators have used different methods of assessing population differentiation (eg G_{ST}, F_{ST}, θ). We have not attempted to standardise here as in some cases the raw data are unavailable, and there is also a strong correlation between measures. Culley *et al* (2002) showed that when calculating G_{ST} in different ways (the average of ratios *vs* the ratio of averages), the difference is trivial in the vast majority of cases. Likewise, Weicker *et al* (2001) found high congruence between F_{ST} and θ in their review of empirical data sets. The variance caused by the spatial scale of sampling schemes among studies is likely to be a far more significant source of error in making comparisons among species.

[c–e]Recent phylogenetic classifications place these species into [c]*Anacamptis*, [d]*Neotinea* and [e]*Gymnadenia* (Bateman *et al*, 1997).

as well as species with relatively continuously distributed ranges and species with only isolated populations. As such, they should be expected to show, and do show, considerable variation in the levels of population differentiation.

Note added in proof

Recent intensive surveys have failed to find any evidence of seed set in *Spiranthes romanzoffiana* populations in the British Isles (R Gulliver, unpublished report to SNH). However, Frank Horsman has brought to our attention the presence of an unpublished drawing from 1969 showing two seeds from an Irish plant, and the drawing of a single unripe seed in RW Butcher's 1961 'New Illustrated British Flora' (London: Leonard Hill).

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