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Genetic structure in the nonrewarding, bumblebee-pollinated orchid *Calypso bulbosa*

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Among- and within-population variation at neutral loci is governed by an interaction between stochastic processes and gene flow. A previous study of pollen dispersal in which the transfer of individually marked pollinia was monitored, indicated not only that populations of the nonrewarding, bumblebee-pollinated orchid *Calypso bulbosa* are connected by substantial levels of gene flow, but also that seed production may be the result of self-fertilization to a considerable extent. We examined the genetic structure of 21 *C. bulbosa* populations in northern Sweden by *F*-statistics analyses of variation at three polymorphic allozyme loci. Four populations each consisted of two or three distinct patches, which also allowed us to evaluate among-patch differentiation. The inbreeding coefficient over all loci within populations was high ($F_{IS} = 0.283$). *F*-statistics indicated that the level of genetic differentiation among patches within populations varied among populations. F_{ST} among patches within populations ranged between −0.021 and 0.119 and was significantly different from zero in two of the populations. There was low to moderate genetic differentiation among populations ($F_{ST} = 0.072$). A Mantel test indicated a positive correlation between geographical and genetic distances among populations, but this correlation was dependent on the difference in allele frequencies between the southernmost population sampled and all other populations. Self-fertilization and substructuring within sampling units (within patches and populations) may have contributed to the high inbreeding coefficients observed in many *C. bulbosa* populations. Long-distance seed and pollen dispersal may account for the low to moderate genetic differentiation among populations.

Keywords: deceptive pollination, *F*-statistics, Orchidaceae, population differentiation.

Introduction

The mating system and the dispersal characteristics of pollen and seeds will influence the genetic structure of natural plant populations (e.g. Hamrick & Godt, 1989; Williams & Gurie, 1994; Giles *et al*., 1998). In highly selfing species with limited seed and pollen dispersal, inbreeding coefficients are expected to be high, and most of the genetic variation at neutral loci is expected to be observed among populations. In contrast, outcrossing species with wide seed and pollen dispersal are expected to be characterized by low inbreeding coefficients and by limited population differentiation at neutral loci.

The geographical structure of genetic variation at neutral loci should depend on patterns of gene flow. If gene dispersal is limited and founders of new populations are more likely to originate from neighbouring than from distant populations (the stepping-stone model), this may contribute to a correlation between geographical and genetic distance (Kimura & Weiss, 1964; Slatkin, 1985). If, on the other hand, founders are drawn at random from populations over a large geographical area (the island model), then this should reduce any correlation between geographical and genetic distance (Wright, 1951; Slatkin, 1985).

In this study, we used allozyme variation and *F*-statistics (Wright, 1951) to document the genetic structure in the bumblebee-pollinated, nonrewarding orchid *Calypso bulbosa*. A previous study of pollen dispersal in two Swedish *C. bulbosa* populations where pollinia were marked individually showed: (i) that the rate of self-pollination was considerable (18–37% of pollinated flowers); (ii) that the pollen dispersal distances within populations were distributed leptokurtically (median dispersal distances to the seventh-closest flowering plant); and, finally (iii) that a substantial

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fraction of the pollen deposited originated from plants outside the study patches (5–14% of pollinated flowers; J. Ågren, R. Alexandersson & A.R. Waites, unpubl. mss). From these observations, we can formulate several predictions concerning the structure of genetic variation at neutral loci in *C. bulbosa*. First, the high rate of self-pollination should result in relatively high fixation indices within populations (F_{IS}). Secondly, the relatively long dispersal distances of pollen within populations and the considerable pollen flow among populations should be associated with low levels of genetic differentiation among populations and among patches within populations (F_{ST}). To examine these predictions, we quantified the fixation indices and the level of among-population differentiation for 21 populations of *C. bulbosa* in northern Sweden. To assess within-population differentiation, we quantified among-patch differentiation in four populations consisting of two or three distinct patches separated by 50–90 m. Finally, we used the Mantel test to quantify the correlation between geographical and genetic distances among *C. bulbosa* populations.

Materials and methods

Study species

Calypso bulbosa L. (Orchidaceae) is a diploid ($2n = 24$; Lid, 1979), self-compatible, nonautogamous, perennial herb (Ackerman, 1981). *Calypso bulbosa* has a circumboreal distribution and the species is divided into four varieties (Hultén & Fries, 1986). The variety *Calypso bulbosa bulbosa* is found in the northern parts of Sweden, Finland and Russia (Hultén & Fries, 1986). In Sweden, it grows in relatively nutrient-rich, mesic to moist spruce (*Picea abies* (L.) Karst.) and spruce–pine (*P. abies – Pinus sylvestris* L.) forests.

The plant forms a single leaf from an underground corm in late summer. In spring, reproductive plants develop a 4–15 cm tall inflorescence with a single flower with pink petals and sepals. In northern Sweden, *C. bulbosa* flowers for 3–4 weeks beginning in late May or early June. The flowers are nonrewarding (i.e. do not offer a reward to flower visitors) and are pollinated by bumblebee queens (Alexandersson & Ågren, 1996; J. Ågren, R. Alexandersson & A.R. Waites, unpubl. mss). The fruit matures in late July and may contain thousands of small seeds [mean length of seeds ($\pm$SD): 0.74 $\pm$ 0.17 mm, $N = 10$]. The old leaf senesces during fruit development. In August, the plant forms one, or sometimes two new corms. In five populations in northern Sweden, an average of 12% of the adult plants had more than one corm (range 9–21%; R. Alexandersson & J. Ågren, unpubl. data).

Allozyme electrophoresis

The study was carried out in 21 populations in the counties of Västerbotten and Norrbotten in northern Sweden. The latitude of the study populations ranged from 63°53′ N to 65°44′ N and their altitude from 75 m to 430 m a.s.l. (Fig. 1, Table 1). A population was defined as a group of plants separated from the nearest conspecific by at least 100 m. Population size was quantified as the mean number of flowering plants 1992–94 and ranged between 19 and 667 (Table 1). Four study populations were subdivided into distinct patches, separated by 50–90 m, each including about 30–70 flowering plants. Population 1 was divided into three patches and populations 2, 17 and 20 into two patches each (Fig. 1, Table 1).

In each population (or patch; populations 1, 2, 17 and 20), we collected the tip of a leaf from 30 to 90 randomly chosen adult plants. In order to avoid sampling the same genet more than once, sampled plants were always separated by at least 1 m.

The sampled leaf tips were transported to the laboratory in Eppendorf tubes that were filled with water and stored on ice. Within 28 h of sampling, the samples were homogenized with a Tris-HCl extraction buffer (Peakall & Beattie, 1991) and a small amount of sand. Enzyme extracts were absorbed onto wicks and frozen at −70°C. Within 3 months, electrophoresis was performed on horizontal starch gels. The electrophoresis was run at 250 V and 50 mA for 6 h. We used Ashton-buffers as gel (pH 8.2) and electrode buffers (pH 8.4; described in Soltis *et al.*, 1983). Stain receipes were modified from Wendel & Weeden (1989), and interpretable bands were obtained for three enzyme systems: aspartate aminotransferase (*Aat*; EC 2.6.1.1), triosephosphate isomerase (*Tpi*; EC 5.3.1.1) and phosphoglucomutase (*Pgm*; EC 5.4.2.2).

Statistical analysis

We used χ^2-tests in order to determine whether there was variation in allele frequencies among populations. In population 7, three alleles were detected at the *Pgm* locus; in this case, the two rare alleles were pooled before testing.

We used *F*-statistics (Wright, 1951) to characterize the genetic structure within and among populations. The *F*-statistics were calculated using the Weir & Cockerham (1984) estimators and the program FSTAT v1.2 (Goudet, 1995). Standard errors were estimated by jack-knifing, and confidence intervals by boot-strapping. The hierarchical levels in the data were denoted in the conventional way: F_{IS}, F_{ST} and F_{IT}, where I = individual, S = population and T = total. In order to quantify genetic

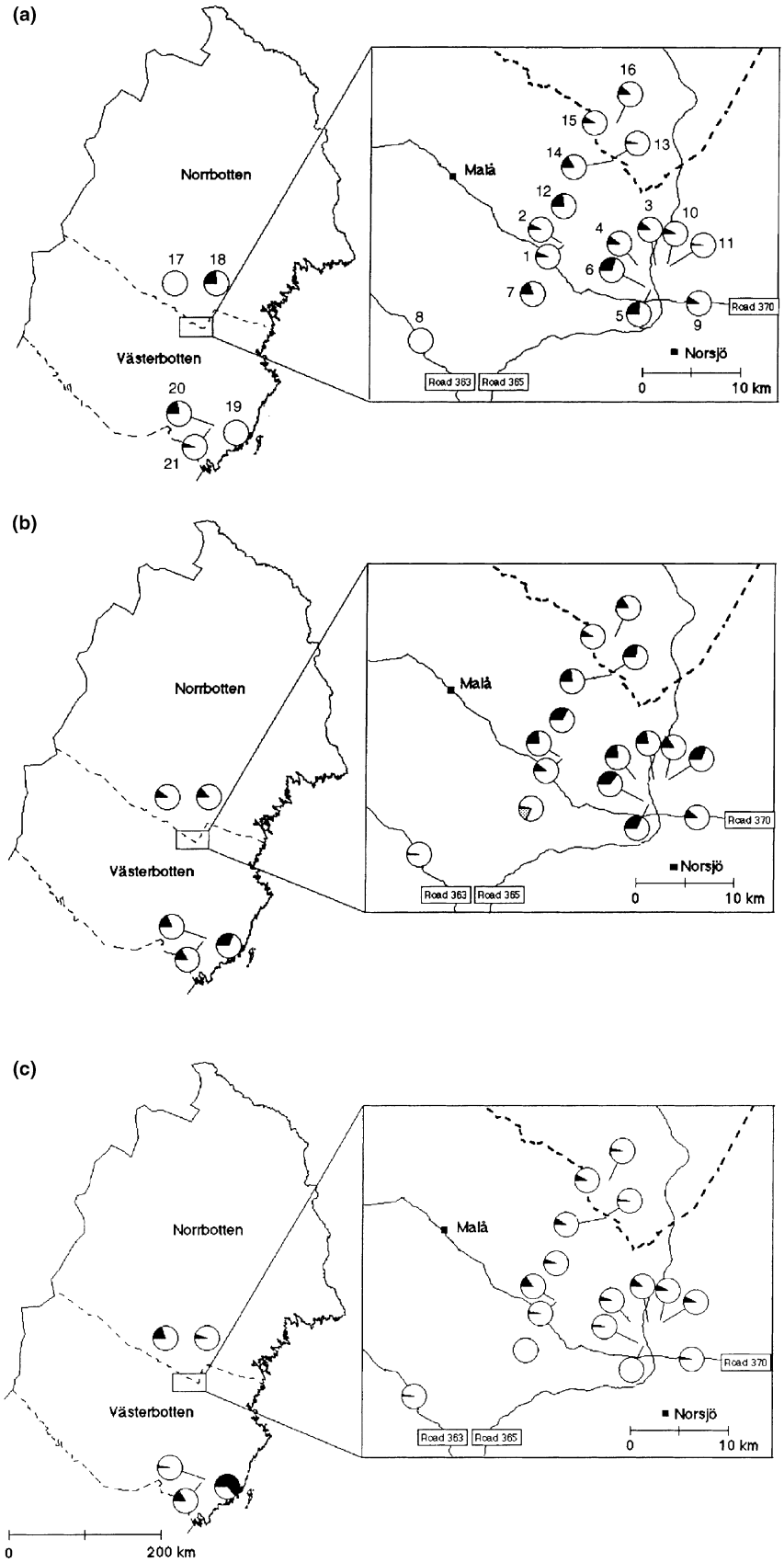

Fig. 1 Allele frequencies at three allozyme loci, in 21 *Calypso bulbosa* populations sampled in the provinces of Västerbotten and Norrbotten in northern Sweden: (a) *Aat*, (b) *Pgm* and (c) *Tpi*. Population numbers (see Table 1) are given in (a).

Table 1 Size (mean number of flowering plants 1992–94), altitude, inbreeding coefficient within population (F_{IS}), sample size (N) and heterozygosity (observed and expected) of 21 *Calypso bulbosa* populations in northern Sweden (see Fig. 1 for location of populations)

					Heterozygosity observed (expected)		
Population	Size	Altitude (m)	F_{IS}	N	*Tpi*	*Pgm*	*Aat*
1 Kron-Olle 1	156	280	0.226	90	0.26 (0.24)	0.21 (0.36)	0.10 (0.14)
2 Kron-Olle 2	137	280	0.439	60	0.05 (0.05)	0.03 (0.18)	0.12 (0.12)
3 Finnliden farm	48	280	0.032	30	0.23 (0.21)	0.25 (0.34)	0.23 (0.21)
4 Ol-ersa	159	235	0.431	30	0.10 (0.10)	0.14 (0.37)	0.13 (0.18)
5 Borup 1	150	280	0.606	30	0.00 (0.00)	0.15 (0.43)	0.17 (0.39)
6 Borup 2	61	305	0.144	30	0.03 (0.03)	0.28 (0.46)	0.50 (0.42)
7 V. Högkulla	263	350	0.516	30	0.00 (0.00)	0.07 (0.07)	0.27 (0.33)
8 Kittelforsheden	86	250	0.000	30	0.03 (0.03)	0.03 (0.03)	0.00 (0.00)
9 Kryddgrovan	19	225	0.229	30	0.07 (0.07)	0.17 (0.21)	0.10 (0.16)
10 Finnliden 1	284	280	0.146	30	0.13 (0.13)	0.19 (0.29)	0.13 (0.13)
11 Finnliden 2	44	280	0.379	30	0.13 (0.13)	0.20 (0.43)	0.03 (0.03)
12 Näsberg	146	290	0.402	30	0.10 (0.10)	0.04 (0.44)	0.39 (0.36)
13 Järvselet 1	20	285	0.011	30	0.03 (0.03)	0.39 (0.40)	0.07 (0.07)
14 Järvselet 2	36	290	0.162	30	0.17 (0.16)	0.32 (0.36)	0.17 (0.26)
15 Sandfors	48	300	0.239	30	0.07 (0.13)	0.11 (0.17)	0.17 (0.16)
16 Lidmyrliden	158	335	0.002	30	0.07 (0.07)	0.23 (0.26)	0.23 (0.21)
17 Döttern	96	430	0.101	59	0.32 (0.32)	0.12 (0.18)	0.00 (0.00)
18 Vistån	667	285	0.405	30	0.03 (0.10)	0.13 (0.24)	0.26 (0.37)
19 Ersmarksberget	49	75	0.144	30	0.47 (0.47)	0.30 (0.43)	0.00 (0.00)
20 Orrböle	72	175	0.147	60	0.05 (0.05)	0.19 (0.30)	0.37 (0.35)
21 Fällfors	107	155	0.089	30	0.23 (0.26)	0.24 (0.27)	0.10 (0.10)

differentiation among patches within populations 1, 2, 17 and 20, we calculated F-statistics separately for each population. A 'P' was added to the conventional subscripts ($F_{IS(P)}$, $F_{ST(P)}$ and $F_{IT(P)}$) in this analysis to avoid confusion with the previous analysis. To test for neutrality among loci, we examined whether the confidence intervals of F_{IS} over populations overlapped or lay between the confidence intervals of F_{IS} over loci (Goudet *et al*., 1995).

A Mantel test (Manly, 1985) was performed to determine whether there was a correlation between geographical and genetic distances among populations.

Results

The over-population confidence intervals of F_{IS} for each locus overlapped or lay between the over-loci confidence intervals (data not shown) and therefore all three loci were assumed neutral and included in the analysis.

Two alleles were detected at the *Tpi* and *Aat* loci, and three alleles at the *Pgm* locus. One of the *Pgm*-alleles was found in only one population (Population 7; Fig. 1). Allele frequencies differed significantly among populations (d.f. = 20; *Tpi*: $\chi^2 = 173$; *Pgm*: $\chi^2 = 66$; *Aat*: $\chi^2 = 128$; $P < 0.0001$). Five populations were monomorphic for one allozyme locus (two populations lacked variation at the *Tpi* locus, and three populations lacked variation at the *Aat* locus; Fig. 1). No population was monomorphic for more than one locus (Fig. 1). In most populations, the expected heterozygosity was highest for *Pgm* (Table 1), indicating that this was the most informative locus.

F_{IS} was positive at all three loci, indicating a deficiency of heterozygotes within populations, and the overall estimate was significantly greater than zero ($F_{IS} = 0.283$; Table 2). The among-locus variation in estimates of F_{IS} was large (range 0.008–0.429; Table 2). Estimates of F_{IS} for individual populations tended to be positively correlated with population size ($r = 0.39$, $P = 0.08$, $N = 21$; Table 1).

The magnitude of genetic differentiation among patches within populations varied ($F_{ST(P)}$; Table 3). The estimates of patch differentiation within populations based on three loci ranged from −0.021 to 0.119, and were statistically different from zero in populations 1 and 2 (Table 3). $F_{IS(P)}$ was higher than $F_{ST(P)}$ in all four populations (Table 3).

Population divergence measured by F_{ST} was low to moderate (Table 2). Population 19 had a strong influence on F_{ST}, because of its strongly deviating allele frequencies at the *Tpi* locus (Fig. 1). If population 19 is excluded from the calculation of F-statistics, the

Table 2 F_{IS}, F_{ST} and F_{IT} (±SE) estimated for all 21 *Calypso bulbosa* populations sampled, and for a data set from which population 19, which had strongly deviating allele frequencies at the Tpi-locus (see Fig. 1), was excluded. Standard errors were estimated by jackknifing and confidence intervals (CI) by bootstrapping

	F-statistics based on 21 populations			*F*-statistics based on 20 populations		
Source	F_{IS}	F_{ST}	F_{IT}	F_{IS}	F_{ST}	F_{IT}
Tpi	0.008 ± 0.033	0.201 ± 0.141	0.208 ± 0.145	0.007 ± 0.039	0.045 ± 0.016	0.052 ± 0.037
Pgm	0.429 ± 0.053	0.034 ± 0.013	0.449 ± 0.055	0.436 ± 0.056	0.034 ± 0.013	0.456 ± 0.057
Aat	0.110 ± 0.066	0.068 ± 0.018	0.170 ± 0.062	0.109 ± 0.066	0.063 ± 0.017	0.166 ± 0.062
Total	0.283 ± 0.159	0.072 ± 0.042	0.329 ± 0.115	0.289 ± 0.161	0.044 ± 0.011	0.319 ± 0.146
95% CI	0.012–0.429	0.035–0.180	0.170–0.449	0.012–0.435	0.035–0.064	0.054–0.455

Table 3 Separate *F*-statistics for four populations of *Calypso bulbosa* subdivided into distinct patches: Kron-Olle 1 (three patches), Kron-Olle 2, Döttern and Orrböle (two patches each). Standard errors of total estimates were calculated by jack-knifing. Estimates significantly different from zero ($P < 0.05$) are indicated in boldface

Population	Level	*Tpi*	*Pgm*	*Aat*	Total (±SE)
Kron-Olle 1	$F_{IS(P)}$	−0.052	**0.422**	**0.271**	**0.238** (0.168)
	$F_{ST(P)}$	−0.013	**0.083**	−0.010	**0.035** (0.036)
	$F_{IT(P)}$	−0.066	**0.482**	**0.263**	**0.270** (0.192)
Kron-Olle 2	$F_{IS(P)}$	−0.036	**0.798**	−0.046	**0.455** (0.392)
	$F_{ST(P)}$	**0.034**	**0.175**	−0.017	**0.119** (0.089)
	$F_{IT(P)}$	0.000	**0.833**	−0.064	**0.557** (0.431)
Döttern	$F_{IS(P)}$	−0.009	**0.327**	m	0.041 (0.197)
	$F_{ST(P)}$	−0.017	0.050	m	−0.004 (0.039)
	$F_{IT(P)}$	−0.026	**0.361**	m	0.046 (0.226)
Orrböle	$F_{IS(P)}$	−0.012	**0.373**	−0.035	0.134 (0.203)
	$F_{ST(P)}$	−0.011	−0.024	−0.019	−0.021 (0.003)
	$F_{IT(P)}$	−0.023	**0.358**	−0.055	0.116 (0.205)

m, monomorphic locus.

single-locus *Tpi*-estimate of F_{ST} is reduced from 0.201 to 0.045 and F_{ST} over all loci is reduced from 0.072 to 0.044 (Table 2). The estimates of other *F*-statistics were affected only marginally by the exclusion of population 19 (Table 2).

There was a positive correlation between genetic distance and geographical distance among populations (Mantel test: $r = 0.40$, $P < 0.05$, $N = 210$; Fig. 2), but this correlation was totally dependent on the difference in allele frequencies between population 19 (which was the southernmost population sampled) and the other populations. If population 19 is excluded, there is no significant correlation between genetic and geographical distances among populations ($r = 0.05$, $P > 0.05$, $N = 190$; Fig. 2).

Discussion

This study has documented a high fixation index within populations (F_{IS}) and a low to moderate level of genetic differentiation among populations of the nonrewarding orchid *Calypso bulbosa*. In two of the four populations investigated, there was significant genetic differentiation among patches within populations.

Both inbreeding and genetic substructuring within sampling units may contribute to the high F_{IS} values. In order to produce the observed fixation indices, selfing rates in the order of 34% ± 5% (mean ± SE, $N = 21$) would be required [based on the formula $s = 2F/(1 + F)$, where s is the selfing rate, and F is the within-population fixation index; Jain, 1979]. Interestingly, these values correspond well with data on pollen transfer in populations 1 and 2. In a three-year study of these populations, where individual pollinia were marked with microtags, 18–37% of the pollinated flowers were pollinated with self-pollen (J. Ågren, R. Alexandersson & A.R. Waites, unpubl. mss). This suggests that the high fixation indices can be explained to a large extent by self-fertilization, provided that inbreeding depression is low. In *C. bulbosa*, there is no significant difference in fruit set after self-and cross-pollination (R. Alexandersson, unpubl. data). However, there is no information on whether the probability of establishment differs between offspring produced after selfing and outcrossing,

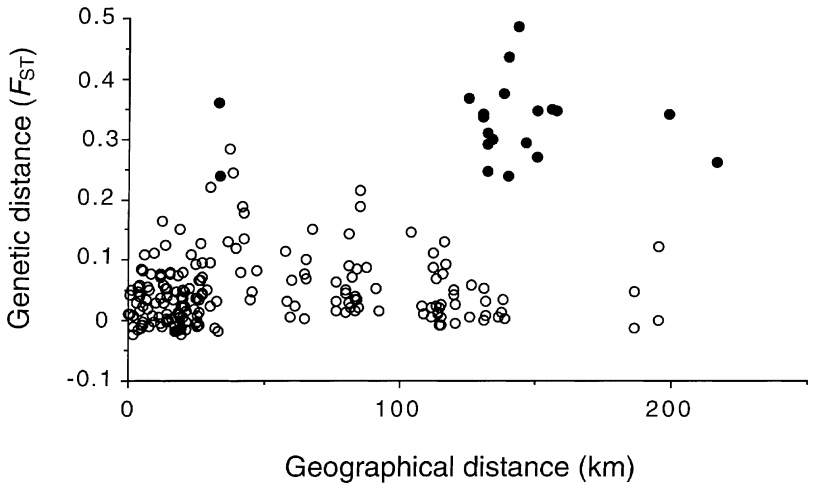

Fig. 2 Relationship between geographical and genetic distances (pairwise F_{ST}) among 21 *Calypso bulbosa* populations in northern Sweden. Filled circles represent pairwise distances from population 19 (see Fig. 1, Table 1).

respectively. Pollinators mediate any self-pollination in *C. bulbosa*, as this species does not have a mechanism for autogamy (Ackerman, 1981; R. Alexandersson, pers. obs.). Moreover, cage experiments indicate that a fair proportion of bumblebee visits may result in the deposition of self-pollen (J. Ågren, R. Alexandersson & A.R. Waites, unpubl. mss).

In this study, plants were sampled throughout the investigated populations. Because large populations should be more likely to be differentiated into subpopulations, the tendency for F_{IS} to be correlated positively with population size ($r = 0.39$, $P = 0.08$) suggests that limited pollen and seed dispersal within sampling units may contribute to the high estimates of F_{IS}, at least in large populations.

Limited pollen dispersal seems to be a less likely reason for the deficiency of heterozygotes within patches, however. The neighbourhood area based on pollen dispersal (N_A) can be estimated using the formula $N_A = 0.25\pi k\sigma^2$, where σ^2 is the variance in absolute pollen dispersal and k is a correction for kurtosis (Crawford, 1984; Fenster, 1991). Using data on within-patch pollen dispersal collected in populations 1 and 2 (J. Ågren, R. Alexandersson & A.R. Waites, unpubl. mss), the neighbourhood area was estimated as 130 m^2 ($\sigma^2 = 184$ m, leptokurtosis $\gamma_2 = 10.4$, $k = 0.9$). This corresponds roughly to the overall sizes of the patches in populations 1 and 2, and suggests that limited dispersal of pollen leaving the paternal plant may contribute to high estimates of F_{IS} in large populations, but is unlikely to contribute to substructuring within patches.

Limited seed dispersal may contribute to the deficiency of heterozygotes within populations of *C. bulbosa*. Orchid seeds are generally assumed to be dispersed widely because of their small size (Murren & Ellison, 1998). Although the seeds of *C. bulbosa* are similar in size to those of many other terrestrial orchids (cf. Rasmussen, 1995, p. 8), seed dispersal in *C. bulbosa* could be more restricted than in many other orchids, because the fruit stalk is short (10–15 cm) and because wind speeds should be reduced significantly close to the ground in the typical forest habitats of *C. bulbosa*. Field observations indicate that *C. bulbosa* seeds are commonly deposited close to the maternal plant (R. Alexandersson, pers. obs.).

The documented substructuring within sampling units is not likely to be caused by vegetative propagation and sampling within clones. Although individual *C. bulbosa* can produce two corms under favourable conditions, different shoots belonging to the same genet will be closely clumped, and care was taken in the present study not to sample more than one shoot from clumps of shoots that could have arisen through vegetative propagation.

There are few published estimates of fixation indices in orchid populations. Peakall & Beattie (1996) documented high F_{IS} (0.275) in populations of the wasp-pollinated, sexually deceptive, orchid *Caladenia tentactulata*. A spatial autocorrelation analysis indicated that their study populations consisted of breeding units with a diameter of 2–7 m. Because the mean pollen dispersal distance was about 15 m, the authors suggested that the high inbreeding coefficients in *C. tentactulata* were caused by limited seed dispersal. In contrast, Peakall & James (1989) estimated F_{IS} as −0.015 (not significantly different from zero) in the clonal, rewarding, ant-pollinated orchid *Leporella fimbriata*. In this species, the low F_{IS} was surprising, as 70% of documented pollinator movements were within clones. The authors suggested that efficient seed dispersal and inbreeding depression could explain the low fixation index.

The level of population differentiation observed in *C. bulbosa* ($F_{ST} = 0.072$, $N = 21$) is lower than the

average level documented for plant species with wind-dispersed seeds (mean ± SE, $G_{ST} = 0.14 \pm 0.02$, $N = 121$) and for animal-pollinated plants with mixed-mating systems ($G_{ST} = 0.22 \pm 0.02$, $N = 60$; Hamrick & Godt, 1989). The low to moderate level of population differentiation in *C. bulbosa* is consistent with data collected for several other orchids (e.g. Scacchi *et al.*, 1990; Rossi *et al.*, 1992; Arft & Ranker, 1998), and may reflect efficient gene dispersal among orchid populations. In *C. bulbosa*, a substantial inflow of pollen from outside the local patch has been documented (J. Ågren, R. Alexandersson & A.R. Waites, unpubl. mss), whereas in other orchids an efficient seed dispersal has been implicated (e.g. Corrias *et al.*, 1991; Rossi *et al.*, 1992).

In animal-pollinated plants, patterns of reward production affect pollinator behaviour, and may therefore be expected to influence pollen dispersal distances and population structure. Reward-producing animal-pollinated plants are often characterized by a high proportion of near-neighbour matings (e.g. Levin & Kerster, 1974; Fenster, 1991), which may restrict pollen dispersal distances and increase genetic differentiation among patches/populations (Turner *et al.*, 1982). In nonrewarding plants, nearest-neighbour matings should be less frequent and pollen dispersal distances longer than in rewarding species (Peakall & Beattie, 1996; J. Ågren, R. Alexandersson and A. R. Waites, unpubl. mss). As a result, the level of genetic differentiation among populations, and among patches within populations, should be lower in nonrewarding than in reward-producing plants. In order to test this hypothesis, one should ideally compare phylogenetically independent pairs of closely related deceptive and rewarding species. At present, there are very few estimates available of genetic differentiation among orchid populations, and they do not indicate a marked difference between deceptive and rewarding orchid species. Published estimates of population differentiation (G_{ST} and F_{ST}) range between 0.015 and 0.45 (median = 0.072, $N = 18$) for nonrewarding species, and between 0.045 and 0.47 (median = 0.083, $N = 3$) for rewarding orchids (Table 4).

The Mantel test indicated no correlation between geographical and genetic distance among *C. bulbosa* populations, if the southernmost population was excluded. No correlation between geographical and genetic distance among populations is expected if founders of new populations are drawn more or less at random from populations over a large geographical area, or if long-distance pollen dispersal is frequent.

The allele frequencies at the *Tpi* locus in the southernmost population sampled (Population 19) differed markedly from those in the other populations sampled (Fig. 2). As a consequence, estimates of F_{ST} and of the correlation between genetic and

Table 4 Estimates of population differentiation (F_{ST} or G_{ST}) in rewarding and deceptive orchids. To indicate the size of the geographical area covered by a given study, the distance between the two most distant populations sampled is given

Species	F_{ST}/ G_{ST}	N	Distance (km)	Reference
Rewarding				
Gymnadenia conopsea	0.471	16	750	Scacchi & de Angelis (1989)
Leporella fimbriata	0.045	4	30	Peakall & James (1989)
Spiranthes diluvialis	0.083	12	690	Arft & Ranker (1998)
Nonrewarding				
Caladenia tentactulata	0.034	9	210	Peakall & Beattie (1996)
Calypso bulbosa	0.072	21	216	Present study
Cephalanthera longifolia	0.104	3	10	Scacchi *et al.* (1991)
C. rubra	0.247	7	17	Scacchi *et al.* (1991)
Cypripedium acaule	0.164	4	500	Case (1994)
C. calceolus	0.194	15	2300	Case (1994)
C. candidum	0.069	5	215	Case (1994)
C. reginae	0.349	3	185	Case (1994)
Orchis laxiflora	0.116	13	2000	Arduino *et al.* (1996)
O. longicornu	0.015	6	190	Corrias *et al.* (1991)
O. mascula	0.083	3	8	Scacchi *et al.* (1990)
O. morio	0.055	18	960	Rossi *et al.* (1992)
O. palustris	0.448	8	2150	Arduino *et al.* (1996)
O. papilionacea	0.038	4	20	Scacchi *et al.* (1990)
O. pauciflora	0.040	3	12	Scacchi *et al.* (1990)
O. provincialis	0.023	2	14	Scacchi *et al.* (1990)
O. purpurea	0.042	5	12	Scacchi *et al.* (1990)
O. tridentata	0.039	4	11	Scacchi *et al.* (1990)

geographical distances were influenced strongly by the inclusion of this population in the data set. Additional sampling of populations to the south and to the east of population 19 might shed some light on whether the deviating allele frequencies reflect an origin that is different from the other populations included in the present study.

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