

## Spatial genetic structure in populations of the terrestrial orchid *Orchis cyclochila* (Orchidaceae)

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**Abstract.** Orchid seeds are minute, dust-like, wind-borne and, thus, would seem to have the potential for long-distance dispersal. Based on this perception, one may predict near-random spatial genetic structure within orchid populations. In reality we do not know much about seed dispersal in orchids and the few empirical studies of fine-scale genetic structure have revealed significant genetic structure at short distances (< 5m), suggesting that most seeds of orchids fall close to the maternal plant. To obtain more empirical data on dispersal, Ripley's  $L(d)$ -statistics, spatial autocorrelation analyses (coancestry,  $f_{ij}$  analyses) and Wright's  $F$  statistics were used to examine the distribution of individuals and the genetic structure within two populations of the terrestrial orchid *Orchis cyclochila* in southern Korea. High levels of genetic diversity ( $H_e = 0.210$ ) and low between-population variation were found ( $F_{ST} = 0.030$ ). Ripley's  $L(d)$ -statistics indicated significant aggregation of individuals, and patterns varied depending on populations. Spatial autocorrelation analysis revealed significant positive genetic correlations among individuals located < 1 m, with mean  $f_{ij}$  values expected for half sibs. This genetic structure suggests that many seeds fall in the immediate vicinity of the maternal plant. The finding of significant fine-scale genetic structure, however, does not have to preclude the potential for the long distance dispersal of seeds. Both the

existence of fine-scale genetic structure and low  $F_{ST}$  are consistent with a leptokurtic distribution of seed dispersal distances with a very flat tail.

**Key words:** Allozymes, *Orchis cyclochila*, Orchidaceae, seed dispersal, spatial distribution, spatial genetic structure, spatial autocorrelation analysis.

### Introduction

Fine-scale genetic structure is evident within plant populations when the spatial distribution of genetic variation among individuals is nonrandom. The development and maintenance of genetic structure within plant populations is influenced by evolutionary forces such as gene flow, genetic drift, and microenvironmental selection, and by ecological processes including adult density, thinning among cohorts, spatial and temporal patterns of seedling establishment, colonization and disturbance history effect (Slatkin and Arter 1991, Sokal and Jacquez 1991, Hamrick et al. 1993, Hamrick and Nason 1996, Kalisz et al. 2001, Parker et al. 2001, Chung et al. 2003a). Fine-scale genetic structure has been widely studied and has primarily been quantified using spatial

autocorrelation analysis to investigate population genetic processes (Sokal and Oden 1978, Epperson 1989, Heywood 1991).

Seeds of members of the Orchidaceae are minute, dust-like, and wind-dispersed, so it is often assumed that they can disperse over long distances (Ackerman and Ward 1999, Tremblay et al. 2005). This assumption leads to the prediction of near random spatial genetic structure within populations of orchid species. Studies of spatial genetic structure within populations may allow us to indirectly infer the extent and patterns of gene movement, particularly seed dispersal within plant populations (e.g. Hamrick et al. 1993, Kalisz et al. 2001, Chung et al. 2000, Chung et al. 2003b). To date empirical studies of within population spatial genetic structure in orchids are few with only five such studies having been conducted, yet all indicate significant genetic clustering on a scale of less than 5 m (terrestrial orchids: Peakall and Beattie 1996; Chung et al. 1998; Chung et al. 2004a,b; epiphytic orchid: Trapnell et al. 2004). It would appear that most seeds fall and seedlings develop in the immediate vicinity of the maternal plant. If such fine-scale spatial genetic structure is in fact typical of orchids, especially terrestrial species, then it counters to general expectations.

To further aid in understanding whether seed dispersal generates near random or significant fine-scale spatial genetic structure, we investigated the non-clonal terrestrial orchid *Orchis cyclochila* (Fr. et Sav.) Maxim. To quantify this structure, individuals were mapped from two undisturbed populations in southern Korea. These populations were then subject to analyses of spatial clustering, fine-scale genetic structure, and Wright's  $F$ -statistics in order to quantify spatial distributions of individuals and genetic structure both within and between populations.

## Materials and methods

**Study plant.** The terrestrial orchid *Orchis cyclochila* is distributed in deciduous forest of temperate

regions in southeastern Russia (Ussuri), Korea, and Japan (Kitamura et al. 1986). In Korea *O. cyclochila* grows in humus soils under deciduous forest overstory (M. Y. Chung and M. G. Chung, personal observation). *Orchis cyclochila* is 7–17 cm tall with 2–4 flowers on one erect raceme. The whitish purple flowers (ca. 1.0 cm long) which bloom May through October are nectarless with a hinged labellum. The breeding systems and pollinators of *O. cyclochila* are not yet known, but it is generally known that members of *Orchis* s. lat (including *Anacamptis* and *Neotinea*, Pridgeon et al. 1997) are outbreeders (Nilsson 1983, 1984; Fritz 1990). The mature fruits (ca. 1.0 cm long) contain large numbers of small seeds.

**Study sites.** A total of 110 visually identified individuals were mapped and sampled from two populations in South Korea (Fig. 1). The first location (JIR, a 5 × 10-m area,  $N = 51$ , altitude 1050 m above sea level [a.s.l.]) is on a north-facing slope of a hill, located at Mt. Jiri, Province Gyeongsangnam-do. The second site (ODA, a 8 × 16-m area,  $N = 59$ , altitude 980 m a.s.l.) is on a south-facing hillside under oak forests, located at Mt. Odae, Province Kangwon-do. No additional *O. cyclochila* were found in the immediate vicinity of the two populations, thus our study plots encompassed virtually all *O. cyclochila* observed at these sites. The two populations are separated by about 280 km. Leaf samples were kept on ice, transported to the laboratory, and stored at 4°C until protein extraction for allozyme analysis (see below).

**Spatial distribution of individuals.** To assess the spatial distribution of individuals, Ripley's  $L(d)$ -statistics (Ripley 1976, 1977) were used. Ripley's  $L(d)$  is calculated from the number of point pairs within concentric circles of increasing radii ( $d$ ) around each plant. Since the use of circles with a radius greater than half the shortest plot side introduces excessive bias due to edge effects, we selected radial distances 0.25 to 2.5 m (JIR) and to 4.0 m (ODA) with a 0.25 m lag (e.g. Parker et al. 1997, Burke et al. 2000, Cruse-Sanders and Hamrick 2004, Ng et al. 2004). Values of  $L(d) = 0$ ,  $L(d) > 0$ , and  $L(d) < 0$  indicate spatial randomness, spatial clustering, and spatial repulsion (hyperdispersion), respectively, up to distance  $d$ . 95% confidence envelopes about the null hypothesis of spatial randomness were determined by Monte Carlo simulation (199 replicates) with a value of

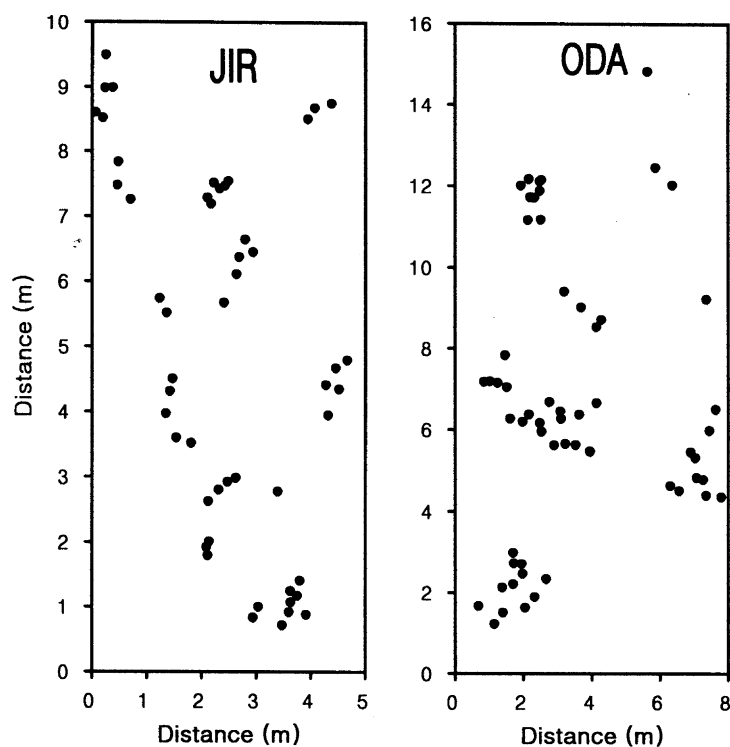


Fig. 1. Distribution of 51 (JIR) and 59 (ODA) *Orchis cyclochila* individuals

$L(d)$  outside of this envelope judged to be a significant departure from the null hypothesis.

All calculations and simulations were performed using a program developed by P. Aldrich (Smithsonian Institution, National Museum of Natural History, USA) and E. Berg (Kenai National Wildlife Refuge, USA).

**Enzyme extraction and electrophoresis.** Leaf samples were cut finely and crushed with a mortar and pestle in a phosphate-polyvinylpyrrolidone extraction buffer (Mitton et al. 1979). Enzyme extracts were absorbed onto  $4 \times 6$ -mm wicks cut from Whatman 3 MM chromatography paper, which were then stored at  $-70$  °C until needed. Allozyme variation was determined with horizontal starch-gel electrophoresis. Gels (11%) were stained for nine enzyme systems, which produced 14 putative loci. A modification (Hauffer 1985) of Soltis et al.'s (1983) system 6 was used to resolve diaphorase (*Dia-1*, *Dia-2*) and leucine aminopeptidase (*Lap-1*, *Lap-2*). A modification (Chung and Kang 1994) of Soltis et al.'s (1983) system 11 was used to resolve formic acid dehydrogenase (*Fdh*), phosphoglucosomerase (*Pgi-1*, *Pgi-2*), phosphoglucosomutase (*Pgm*), and shikimate dehydrogenase

(*Skdh*). A morpholine citrate buffer system (pH 6.1; Clayton and Tretiak 1972) was used to resolve isocitrate dehydrogenase (*Idh-1*, *Idh-2*), malate dehydrogenase (*Mdh-1*, *Mdh-2*), and 6-phosphogluconate dehydrogenase (*6Pgd*). Staining recipes were taken from Soltis et al. (1983), except diaphorase (Cheliak and Pitel 1984). The genetic basis of enzyme banding patterns was inferred from observed segregation patterns in light of typical subunit structure and subcellular compartmentalization (Weeden and Wendel 1989). Putative loci were designated sequentially, with the most anodally migrating isozyme designated "1", the next "2", etc. Likewise, alleles were designated sequentially with the most anodally migrating allele designated "a".

**Spatial autocorrelation analysis of genetic variation.** To obtain a multiallelic, multilocus mean measure of spatial genetic structure per a given distance, the coancestry ( $f_{ij}$ ) between all pairs of individuals within populations from their multilocus genotypes was estimated following the methods of Loiselle et al. (1995) and Kalisz et al. (2001). Mean values of  $f_{ij}$  were obtained for distance intervals of 0.5 and 1 m over all pairs of individuals

located within that interval. When  $f_{ij} = 0$ , there is no significant correlation among individuals at the spatial scale of interest; when  $f_{ij} > 0$ , individuals in a given distance class are more closely related than expected by chance; and when  $f_{ij} < 0$ , individuals within a given distance class are less related than expected by chance. To assess statistical significance at each distance interval, mean  $f_{ij}$  estimates were compared to 95% and 99% confidence intervals estimated via bootstrapping under the null hypothesis of no spatial genetic structure ( $f_{ij} = 0$ ). To generate these intervals, intact multilocus genotypes were drawn at random with replacement, assigned to occupied map locations within the study population, and used to calculate  $f_{ij}$ . This procedure was repeated 399 times with the bootstrap  $f_{ij}$  estimates for each distance class ranked from lowest to highest. For a given distance class, the observed  $f_{ij}$  represents the 400<sup>th</sup> statistic and is significantly different from zero at  $P < 0.05$  (or  $P < 0.01$ ) if it falls in the 2.5% (or 0.5%) tails of this ranked distribution. All calculations and simulations were performed using a program developed by J. D. Nason. Finally, to test whether the slope ( $\beta$ ) of a correlogram is statistically significant,  $f_{ij}$  estimates were permuted with respect to distance (999 times) using the program Permute! (version 3.4 alpha; Casgrain 2001) to construct the distribution of the slope under the null hypothesis  $\beta = 0$ . When testing for negative spatial autocorrelation (one-tailed test), we reject the null hypothesis if there are fewer than 50 random values at least as large as the actual observed  $\beta$  value.

**Analysis of genetic diversity and structure.** To estimate genetic diversity and genetic structure, a locus was considered polymorphic if the frequency of the most common allele did not exceed 0.95. Genetic diversity parameters were estimated using the program POPGENE (Yeh et al. 1999): percent polymorphic loci (%P), mean number of alleles per locus ( $A$ ), observed heterozygosity ( $H_o$ ), and Nei's unbiased gene diversity ( $H_e$ ).

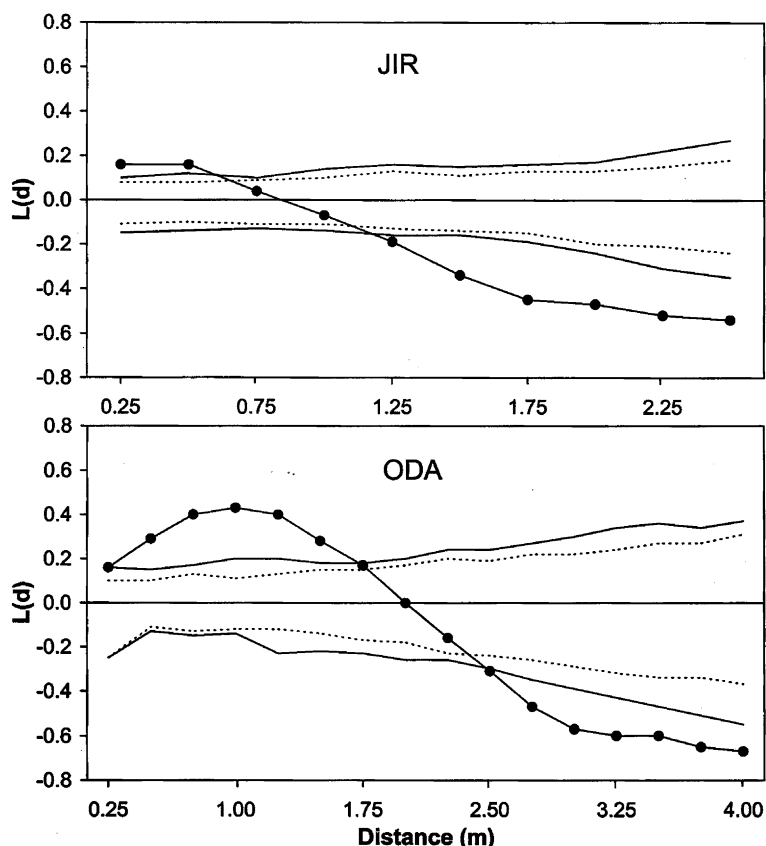
To measure deviations from H-W equilibrium at each polymorphic locus, Wright's (1965)  $F$  statistics ( $F_{IS}$ ,  $F_{IT}$ , and  $F_{ST}$ ) were calculated following Weir and Cockerham (1984). These fixation indices were used to measure deviations from H-W equilibrium attributable to individuals in local populations ( $F_{IS}$ ), variation among local populations ( $F_{ST}$ , an indicator of the degree of differentiation between local populations), and individuals

relative to the total population ( $F_{IT}$ ). Means and standard errors were obtained by jackknifing over polymorphic loci. Bootstrap confidence intervals (95% CI) were constructed around jackknifed means of the  $F$  statistics with 1500 replicates and the observed mean  $F$  statistics were considered significant when confidence intervals did not overlap zero. These calculations were made using the program FSTAT (version 2.9.3.2 by Goudet [2002], see Goudet [1995]). We also calculated  $F_{IS}$  separately for each population with 95% bootstrap confidence intervals (1000 replicates) constructed using the program GDA (Lewis and Zaykin 2001).

## Results

**Spatial distribution of individuals.** Visual inspection of the spatial arrangement of plants within populations suggested a clumped distribution (Fig. 1). In accordance with this, Ripley's  $L(d)$  statistics indicated significant aggregation of individuals at short distance intervals (Fig. 2). As evident from the correlograms both the intensity and spatial scale of this aggregation was greater in ODA than JIR.

**Spatial autocorrelation analysis of genetic variation.** Of the 14 loci examined, seven (*Dia-2*, *Idh-2*, *Mdh-2*, *6Pgd*, *Pgi-2*, *Pgm*, and *Skdh*) and six (*Dia-2*, *Idh-2*, *Mdh-2*, *6Pgd*, *Pgi-2*, and *Skdh*) were polymorphic in both JIR and ODA, respectively, and were used in the analysis of within population genetic structure. Consistent with observations on patterns of growth, inspection of multilocus genotypes failed to reveal evidence of clonal spread. Consequently, spatial autocorrelation analyses describe within population structure among individual genets. Spatial autocorrelation analyses for JIR and ODA indicated significant positive autocorrelation at short distance interval (0.5–1 m at the 99% and 0.5–2 m at the 95% level) with  $f_{ij}$  at 0.5 m of 0.123 in JIR and 0.153 in ODA (Fig. 3). Beyond these distance intervals, no significant  $f_{ij}$  values was detected at the 99% level. At the 95% level, in contrast, significant but weak negative coancestry values were observed at 4 and 8 m in JIR and 5 and 11 m in ODA (Fig. 2). The



**Fig. 2.** Results of Ripley's  $L(d)$ -statistics, observed  $L(d)$  estimates for univariate Ripley analysis of individuals of *Orchis cyclochlila* for the two populations (closed circles with solid lines) and envelopes defined by the 5% (dashed lines) and 1% (solid lines) highest and lowest values generated from 199 Monte Carlo simulations of a randomly distributed population

overall slope of the correlogram for each study site was significantly negative (JIR:  $\beta = -0.926$ ,  $P = 0.001$ ,  $R^2 = 0.846$ ; ODA:  $\beta = -0.749$ ,  $P = 0.001$ ,  $R^2 = 0.561$ ). That is, a significant decrease of the  $f_{ij}$  values was observed with increasing distance. Taken together these results indicate significant spatial genetic structuring in the two populations.

**Analysis of genetic diversity and structure.** Allozyme variation was moderately high, with a mean percentage of polymorphic loci within populations of 46.5%, a mean number of alleles per locus of 1.83, and a mean genetic diversity (expected heterozygosity,  $H_e$ ) of 0.210 (Table 1). At the population level, bootstrap confidence limits about  $F_{IS}$  showed a significant deficit of heterozygosity across polymor-

phic loci in ODA, whereas JIR did not deviate from H-W expectations (Table 1). Over populations, significant deficits of heterozygosity were found within populations and for the population as a whole ( $F_{IS} = 0.113$ , 95% CI = 0.016 to 0.240;  $F_{IT} = 0.140$ , 95% CI = 0.044 to 0.265). Deviations from H-W expectations due to allele frequency differences between populations were also significant, both over the seven polymorphic loci [ $F_{ST} = 0.03$ , 95% CI 0.003 to 0.058] and for four individual loci (results not shown)].

## Discussion

**Fine-scale genetic structure.** Patterns of fine-scale genetic structure can be used to interpret

**Table 1.** Summary of genetic diversity measures and mean fixation ( $F_{IS}$ ) values observed in two populations of *Orchis cyclochila*. Abbreviations:  $N$ , sample size; %P, percentage of polymorphic loci;  $A$ , mean number of alleles per locus;  $H_o$ , observed heterozygosity;  $H_e$ , Hardy-Weinberg expected heterozygosity or genetic diversity; SE, standard error, and 95% CI, 95% confidence intervals

Population	$N$	%P	$A$	$H_o$	$H_e$ (SE)	$F_{IS}$ (95% CI)
JIR	51	50.0	1.86	0.195	0.207 (0.064)	0.071 (-0.117, 0.259)
ODA	59	42.9	1.79	0.183	0.213 (0.070)	0.148 (0.039, 0.266)
Mean	55	46.5	1.83	0.189	0.210	0.113 (0.016, 0.240)*

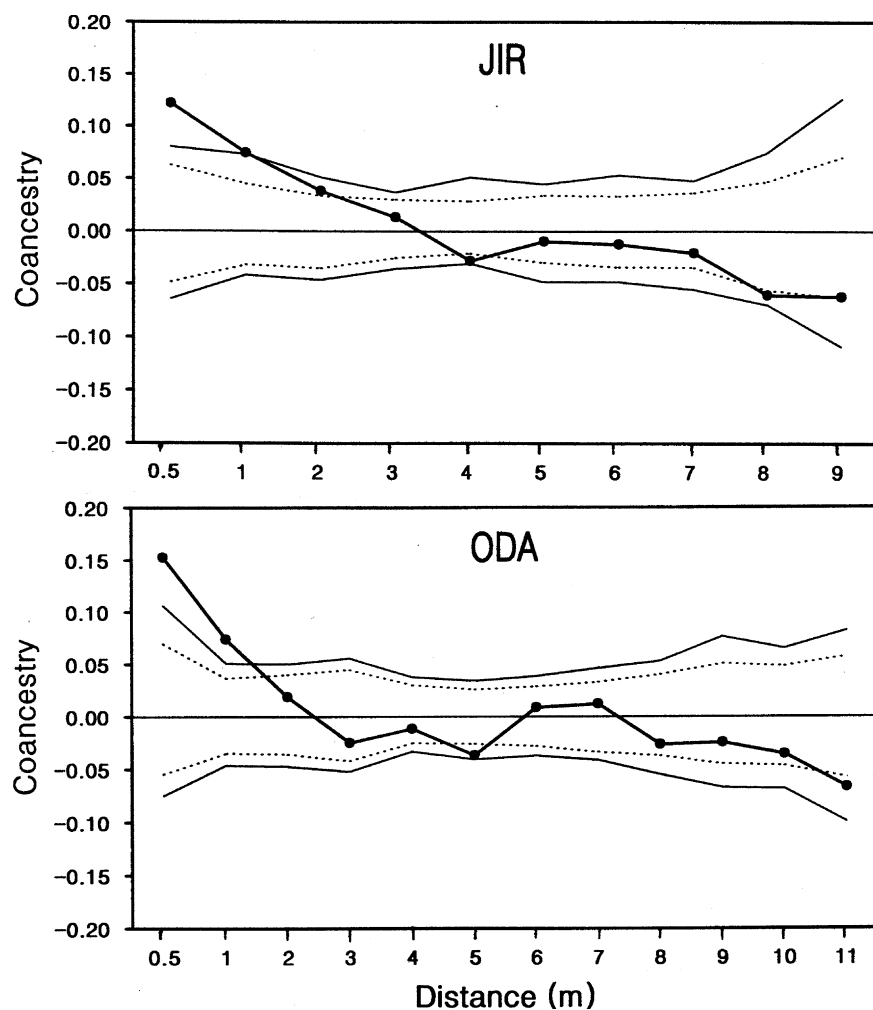
\* Mean is the Weir & Cockerham's (1984) estimate of  $F_{IS}$  over populations.

the extent and pattern of seed dispersal (Epperson 1993, Chung and Epperson 1999, Kalisz et al. 2001, Vekemans and Hardy 2004). Significant fine-scale genetic structuring at the short interplant distances likely reflect spatially localized family structures generated by the limited seed dispersal, particularly in outbreeding species (e.g. Campbell and Dooley 1992, Loiselle et al. 1995, Kalisz et al. 2001, Cruse-Sanders and Hamrick 2004). In *Orchis cyclochila* we found significant fine-scale genetic structure at the shortest distance classes:  $f_{ij} = 0.123$  and  $0.153$  at 0.5 m in JIR and ODA, respectively;  $f_{ij} = 0.075$  and  $0.074$  at 1 m in JIR and ODA, respectively. As confirmed in this study, *Orchis cyclochila* is a non-clonal terrestrial orchid, and thus clonal spread is not an explanation for the significant fine-scale genetic structure of the populations. Rather, the observed pattern of genetic structure can be attributed primarily to localized seed dispersal and recruitment around maternal plants (Peakall and Beattie 1996; Chung et al. 1998; Kalisz et al. 2001; Trapnell et al. 2004; Chung et al. 2004a,b).

Under random mating, the coancestry between individuals is a measure of the inbreeding coefficient of their hypothetical offspring, with expected values of 0.250 for full-sib or parent-off-spring comparisons, 0.125 for half-sib comparisons (Cockerham 1969). Because orchids have pollinia, the seeds within a fruit are usually full-sibs (Proctor and Harder 1994, Pacini and Hesse 2002). Thus, if a plant typically only produces a single fruit then the level of coancestry of the seeds should

be 0.25. If progeny are the products of several reproductive events, the relatedness might be full-sibs within an annual cohort and half-sibs among cohorts. For the 0–0.5 m interplant distance class, the mean coancestry values for JIR and ODA were 0.123 and 0.153, respectively. These values are considerably lower than the expected value of 0.25. One explanation would be that even at the shortest distance classes there is overlap in the seed shadows of plants which would bring down the observed level of relatedness to that approximating half sibs.

Although Ripley's  $L(d)$ -statistics revealed a stronger pattern of spatial aggregation in ODA than JIR, the fine-scale genetic structure of the two *O. cyclochila* populations was generally similar in terms of coancestry values at short inter-plant distances, the distance at which mean coancestry values first intersect the x-axis, and the slope of the correlograms (Fig. 3). The general pattern of genetic structure found for this species is quite similar to that reported for three local populations of the neotropical epiphytic orchid, *Laelia rubescens* in the Pacific lowlands of northwest Costa Rica (Trapnell et al. 2004). Significant positive fine-scale genetic structure at distance of 0.5 m with mean  $f_{ij}$  values of 0.106 to 0.153 was evident in *L. rubescens*. The authors attributed the observed spatial pattern to the establishment of sexually derived progeny within and near maternal clusters. Very similar results were also observed for local populations of three terrestrial orchids *Cymbidium goeringii*, *Cephalanthera longibracteata*, and *Cremastra*



**Fig. 3.** Correlograms of estimated coancestry ( $f_{ij}$ ) at 0.5 m for the first distance and 1 m lags for populations JIR and ODA. Closed circles indicate mean coancestry values for successive distance classes. The solid and dashed lines represent upper and lower 99% and 95% confidence envelopes, respectively, around the null hypothesis of  $f_{ij} = 0$

*appendiculata* in southern Korea (Chung et al. 1998; Chung et al. 2004a,b). Again, significant fine-scale genetic structure was detected at 0.5, 3 and 5 m with mean  $f_{ij}$  values of 0.147, 0.147, and 0.102, respectively (M. Y. Chung et al. unpublished data; Chung et al. 2004a,b). Peakall and Beattie (1996) in Australia reported evidence of the significant fine-scale genetic structure at 1 m distance [mean  $f_{ij} = 0.086$  across five loci; recalculated from Peakall and Beattie (1996)] in a population of the terrestrial orchid *Caladenia tentaculata*. Although studies of fine-scale genetic structure of orchids are

still limited, these five studies together, suggest that a significant component of seed dispersal and establishment occurs in the immediate vicinity of the maternal plant, generating significant fine-scale spatial genetic structuring in populations of both terrestrial and epiphytic orchid species. Consistent with these results, using mathematical models (e.g. a simple ballistic model) for wind-dispersed seeds and wind-tunnel experiments, Murren and Ellison (1998) showed that mean expected seed dispersal distances for the neotropical epiphytic orchid *Brassavola nodosa* L. were less than 6 m

under conditions approximating those found in its natural habitats (mangrove island in Belize, Central America)

Since the seeds of orchids are so small and contain no endosperm, germination and seedling establishment are impossible without symbiotic relationships with endomycorrhizal fungi (Dressler 1981, Warcup 1981). So, even with long-distance dispersal of seeds, they could not successfully colonize vacant areas without the fungi. This may explain the complete absence of *O. cyclochila* away from the immediate vicinity of the study populations. To test this hypothesis, it would be necessary to study the distribution of the associated fungi around the study area.

**Genetic diversity and structure.** Populations of *O. cyclochila* possess moderately high levels of genetic variation ( $H_e = 0.210$ ), relative to mean values for 13 European *Orchis* s. l. spp. ( $H_e = 0.120$  to  $0.160$ : Schlegel et al. 1989, Scacchi et al. 1990, Corrias et al. 1991, Rossi et al. 1992). The two populations of *O. cyclochila* are only weakly differentiated (mean  $F_{ST} = 0.030$ ) though separated by about 280 km. According to a comprehensive review on orchids (Tremblay 2005), *Orchis* s. l. species typically have low estimates of  $F_{ST}$  among populations (mean  $F_{ST} = 0.010$  to  $0.083$  within 20 km to 1,000 km in Europe, Scacchi et al. 1990, Corrias et al. 1991, Rossi et al. 1992, Arduino et al. 1995). On the one hand, these authors attributed these low estimates to numerous and minute seeds, which could be dispersed by wind, coupled with predominantly outcrossing breeding system and/or probable phylogenetic constraints. On the other hand, it is worth noting that the spatial scale of sampling causes differences in  $F_{ST}$  estimates calculated for the same species. For example, Scacchi et al. (1990) estimated  $F_{ST} = 0.080$  for two populations of *O. laxiflora* separated by 6 km in central Italy, whereas Arduino et al. (1996) reported  $F_{ST} = 0.116$  for 13 populations distributed across Italy and southeastern Greece (up to 2,000 km apart).

Within populations, a significant deficiency of heterozygotes relative to H-W expectations

indicates inbreeding and/or population substructure (Hartl and Clark 1997). Although members of *Orchis* appear to be outbreeders as a rule, it is likely that the slight, but significant deficit of heterozygosity found in populations of *O. cyclochila* (mean  $F_{IS} = 0.113$ ) is indicative of proximity-dependent mating among relatives due to limited seed dispersal around maternal individuals. If heterozygote deficiencies are associated with significant intrapopulation spatial genetic structure, they may also be attributable to a spatial Wahlund effect, which will arise when genetically divergent subpopulations are unwittingly included in a population sample. Coancestry analyses do indicate significant fine-scale genetic structure at distances less than 1 m within *O. cyclochila* populations and the correlograms do continue to decline after crossing the x axis ( $\beta = -0.926$  and  $-0.749$  for JIR and ODA). Thus the significant  $F_{IS}$  values for ODA appear to reflect a spatial Wahlund effect (Barbujani 1987) and partial mating with relatives.

In summary, levels of genetic diversity in *O. cyclochila* populations were moderately high. Adult individuals within populations were spatially aggregated and spatial autocorrelation analyses revealed significant local spatial structure in populations, suggesting that restricted seed dispersal around maternal plants has primarily contributed to shaping spatial genetic structuring in *O. cyclochila*. Reckless collection of wild orchids in South Korea is common (M.Y. Chung and M.G. Chung, personal observation). Since *O. cyclochila* populations exhibit significant fine-scale genetic heterogeneity, collection activity that removes local spatial aggregations should eliminate significant portions of the genetic diversity within populations. Thus this study suggests protection of genetic variation across an entire population.

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