

# Patterns of hybridization and population genetic structure in the terrestrial orchids *Liparis kumokiri* and *Liparis makinoana* (Orchidaceae) in sympatric populations

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## Abstract

We investigated the potential for gene flow and genetic assimilation via hybridization between common and rare species of the terrestrial orchid genus *Liparis*, focusing specifically on sympatric and allopatric populations of the common *Liparis kumokiri* and the rare *Liparis makinoana*. We utilized analyses of genetic diversity, morphology, and the spatial distributions of individuals and genotypes to quantify the dynamics of interspecific gene flow at within- and among-population scales. High levels of allozyme genetic diversity ( $H_E$ ) were found in populations of the rare *L. makinoana* (0.317), whereas the common *L. kumokiri* ( $N = 1744$  from 14 populations) revealed a complete lack of variation. This contrast may reflect different breeding systems and associated rates of genetic drift (*L. makinoana* is self-incompatible, whereas *L. kumokiri* is self-compatible). At the two known sympatric sites, individuals were found that recombined parental phenotypes, possessing floral characteristics of *L. kumokiri* and vegetative characteristics of *L. makinoana*. These putative hybrids were the only individuals found segregating alleles diagnostic of both parental species. Analysis of these individuals indicated that hybrid genotypes were skewed towards *L. kumokiri* and later generation recombinants of *L. kumokiri* at both sympatric sites. Furthermore, Ripley's bivariate  $L(r)$  statistics revealed that at one site these hybrids are strongly spatially clustered with *L. kumokiri*. Nonetheless, the relatively low frequency of hybrids, absence of ongoing hybridization (no  $F_1$ s or first generation backcrosses), and strong genetic differentiation between morphologically 'pure' parental populations at sympatric sites ( $F_{ST} = 0.708\text{--}0.816$ ) indicates that hybridization was not an important bridge for gene flow. The results from these two species suggest that natural hybridization has not played an important role in the diversification of *Liparis*, but instead support the view that genetic drift and limited gene flow are primarily responsible for speciation in *Liparis*. Based on genetic data and current status of the species, implications of the research for conservation are considered to provide guidelines for appropriate conservation and management strategies.

**Keywords:** allozymes, conservation, fine-scale genetic structure, genetic diversity, hybrids, *Liparis kumokiri*, *Liparis makinoana*, Orchidaceae, speciation

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## Introduction

In general terms, speciation involves the formation of barriers to gene flow and reproductive isolation between diverging

populations (Mayr 1942, 1963). From a mechanistic perspective, however, the relative importance of different evolutionary processes and their interplay with the ecology of the organism is often complex. Unravelling this complexity is an exciting challenge for evolutionary ecologists, especially so for those working on orchids. Many orchids, particularly terrestrial species, are relatively rare and occur in small,

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spatially isolated populations. On the one hand, such isolation likely promotes diversification by contributing to barriers to gene flow that increase the effectiveness of local drift and selection (reviewed in Tremblay *et al.* 2005). On the other hand, many orchids are of special concern for conservation because such isolation increases the risk of extinction. Although differences in flowering phenology, floral fragrance, oils, colour and form, combine with specialized pollinators to enhance reproductive isolation between orchid taxa (van der Pilj & Dodson 1966; Dressler 1982), natural hybridization is known to occur in a number of orchid genera (e.g. *Cypripedium*, Case 1993; *Dactylorhiza*, Hedrén 1996; *Epipactis*, Harris & Abbott 1997; *Orchis*, Catling & Brownell 1999; *Ophrys*, Nielsen & Siegismund 1999; *Platanthera*, Soliva & Widmer 2003; and *Vanilla*, Pellegrino *et al.* 2005). Hybridization may further exacerbate the decline of rare species through a combination of genetic assimilation and pollen swamping (Ellstrand & Elam 1993; Levin *et al.* 1996). If hybrids do not exhibit reduced fitness relative to parental taxa, they may ultimately displace pure populations of one or both parental taxa. This process may especially favour the genetic assimilation of the rarer taxa due to a proportionally greater frequency of interspecific pollination and recombination with hybrids. Even if hybrids have reduced fitness relative to parental species and do not contribute to introgression, the rarer taxon may yet decline because its growth rate may fall below that necessary for replacement due to pollen swamping from the more common taxon.

Here, we investigate the potential for gene flow and genetic assimilation in hybridizing populations of common and rare members of *Liparis*, a diverse genus of terrestrial orchids (c. 250 species, Mabberley 1989). *Liparis kumokiri* is the most common member of the genus in Korea and Japan whereas *Liparis makinoana*, which is distributed over this area and northeastern China, is rare throughout its range. Despite the broad overlap in geographic distribution, the two species are known to be sympatric at only two locations in South Korea, and at both locations putative hybrids of recombinant morphology occur. These individuals possess floral characteristics of the more common parental species (*L. kumokiri*) and vegetative characteristics of the rarer parental species (*L. makinoana*). On the one hand, the similarity of floral traits suggests asymmetrical hybridization towards *L. kumokiri*, in which case interspecific recombination is less likely to result in the genetic swamping of the rarer *L. makinoana*. On the other hand, the combined floral and vegetative phenotype suggests that the morphological signature of hybridization may be obscured by dominance at loci underlying these traits. As a consequence, patterns of recombination and role of hybrids to function as a bridge for introgressive gene flow will be difficult to resolve from morphological data alone.

To quantify the potential for interspecific gene flow in sympatric populations of *L. kumokiri* and *L. makinoana*, and

gain insights into evolutionary diversification of *Liparis*, we examined the multilocus genotypes of morphologically classified parental species and putative hybrid individuals. A preponderance of F<sub>1</sub> hybrids would indicate restricted hybridization with little potential for gene flow between parental species. High frequencies of backcross or introgressed individuals, in contrast, would indicate a high potential for interspecific gene flow. The similarity of floral characteristics between the putative hybrids and the more common *L. kumokiri* may interact with pollinator foraging behaviour to promote positive assortative mating between these classes of individuals and negative assortative mating between them and the rarer *L. makinoana*. Such asymmetry in hybridization would be indicated by higher frequencies of backcross classes towards *L. kumokiri* than towards *L. makinoana*. Further insight into interspecific gene flow between sympatric populations may be revealed by patterns of spatial aggregation within and between parental species and hybrid individuals. Specifically, if putative hybrids are spatially clustered with one parental species (but not the other), that species is likely to be the maternal parent of F<sub>1</sub> and backcross hybrids that may serve as foci for interspecific gene flow. Finally, to quantify spatial clustering, the scale of dispersal, and thus the potential for hybridization, we used spatial statistics and spatial autocorrelation methods within sympatric putative hybrids and parental species populations. We discuss these results with respect to causes and consequences of natural hybridization between the two species, the role of hybridization for diversification of *Liparis*, and their conservation.

## Materials and methods

### *Plant species*

*Liparis kumokiri* and *Liparis makinoana* grow in pine–oak forests in Japan and Korea, with *L. makinoana* also found in northeastern China (Kitamura *et al.* 1986). *L. kumokiri* is relatively common compared to other *Liparis* species in Korea, whereas *L. makinoana* is extremely rare in southern Korea and Japan (Kitamura *et al.* 1986; Oh *et al.* 2004; M. Y. Chung & M. G. Chung, personal observations). Each spring, new roots develop from the overwintering corm, and each mature or adult plant usually produces two basal leaves. By autumn senescence, the parent corm of each plant completely disappears and is replaced by a new corm. Thus, although vegetative reproduction via corms is possible, with new plants or ramets remaining close to the original plant (Whigham & O'Neill 1991), within populations most individuals are likely distinct genets rather than clonal ramets.

*Liparis makinoana* flowers from the middle of May through late June, whereas *L. kumokiri* flowers in middle of June to early July in South Korea (M. Y. Chung & M. G. Chung, personal observations). Inflorescences bear 3–23 flowers

**Table 1** Collection sites of *Liparis makinoana*, *Liparis kumokiri*, and their putative hybrids. *N* denotes the total sample size for each population

Species	Population	<i>N</i>	Location
<i>L. makinoana</i>	SOA	374	Mount Sobaek, Yeongchun-myeon, Danyang-gun, Chungcheongbuk-do
	SOB	51	Mount Sobaek, Gagok-myeon, Danyang-gun, Chungcheongbuk-do
Putative hybrids	SOA	12	Mount Sobaek, Yeongchun-myeon, Danyang-gun, Chungcheongbuk-do
	SOB	34	Mount Sobaek, Gagok-myeon, Danyang-gun, Chungcheongbuk-do
<i>L. kumokiri</i>	SOA	610	Mount Sobaek, Yeongchun-myeon, Danyang-gun, Chungcheongbuk-do
	SOB	184	Mount Sobaek, Gagok-myeon, Danyang-gun, Chungcheongbuk-do
	LK1	65	Mount Dongrak, Gokseong-eup, Jollanam-do (65)
	LK2	252	Mount Wolak, Woalarsan National Park, Chungcheongbuk-do
	LK3	67	Mount Chiak, Chiaksan National Park, Gangwon-do
	LK4	79	Mount Jiri, Seongsamjae, Jirisan National Park, Jollanam-do
	LK5	113	Mount Palgong, Palgong Provincial Park, Gyeongsangbuk-do
	LK6	59	Mount Juwang, Juwangsan National Park, Gyeongsangbuk-do
	LK7	27	Mount Yongchwi, Habuk-myeon, Yangsan-si, Gyeongsangnam-do
	LK8	60	Mount Yeonhwa, Yeonhwasan Provincial Park, Gyeongsangnam-do
	LK9	48	Mount Waryong, Sacheon-si, Gyeongsangnam-do
	LK10	68	Mount Geumo, Hadong-gun, Gyeongsangnam-do
LK11	49	Mount Kumo, Dolsan Island, Jollanam-do	
LK12	63	Mount Jogye, Songgwang-myeon, Sooncheon-si, Jollanam-do	

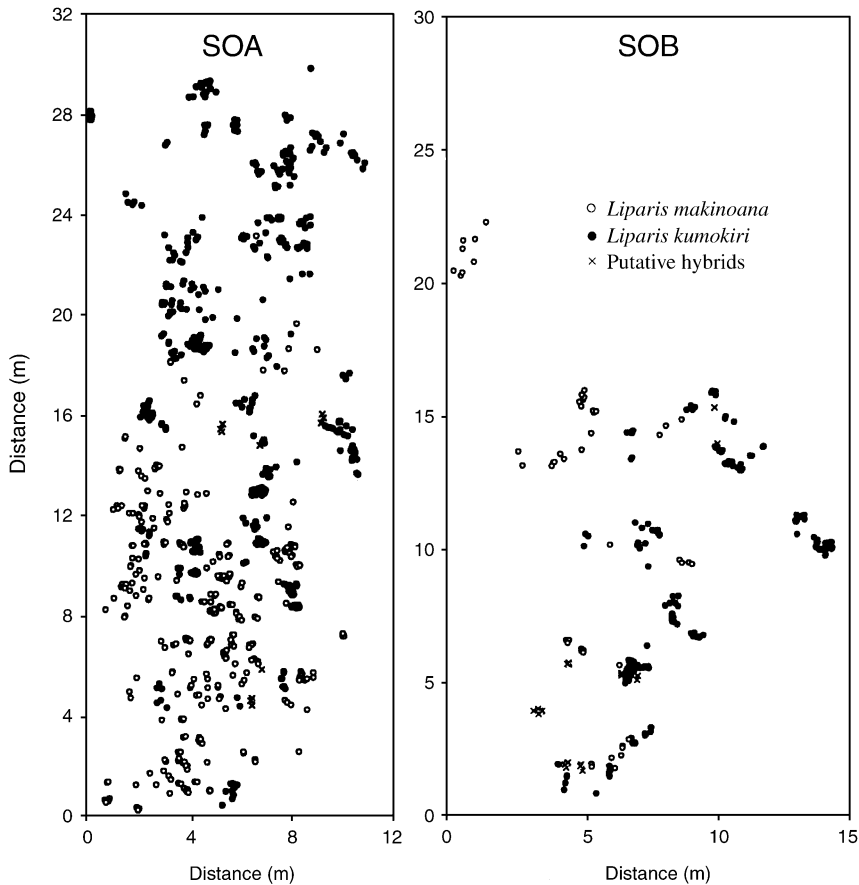
in 10–35 cm tall inflorescences. The two species can be distinguished easily by colour and size of the labellum (greenish yellow and width of *c.* 5 mm for *L. kumokiri* and brownish purple and 8–12 mm for *L. makinoana*), cross-sectional shape of the scape (triangular for *L. kumokiri* and square for *L. makinoana*), and leaf shape and margin (cordate and undulate for *L. kumokiri* and ovate to ovate-lanceolate and entire for *L. makinoana*). The basal part of the dorsal sepal, column, and labellum of flowers of the two species is shiny or glossy, and is thought to function as a ‘nectar mimic’ (Whigham & O’Neill 1991). Although the two species differ in breeding system, with *L. kumokiri* being self-compatible and *L. makinoana* self-incompatible, both exhibit low fruit set in natural conditions (10.2–12.2% for *L. kumokiri* and 0.1–0.2% for *L. makinoana*) and increased fruit set following hand-pollination (Oh *et al.* 2001). The much lower percentage of fruit set observed in *L. makinoana* than *L. kumokiri* may reflect combined effects of pollinator limitation and self-incompatibility. During 4-week field surveys conducted in July 1998, 1999, 2000, 2003, and 2004, we failed to observe any pollinators in the populations of either species examined in this study. Typical of orchids, fruits (2.0–2.5 cm long) contain large numbers of minute seeds.

Scattered through the two study sites where *L. kumokiri* and *L. makinoana* are known to be sympatric (SOA and SOB, described below) were apparent hybrid individuals. These individuals combined morphological features of both species: each hybrid possessed floral and inflorescence characteristics similar to *L. kumokiri* while also expressing vegetative characters (leaf shape and margin) similar to *L. makinoana*. Such individuals, hereafter referred to as ‘putative hybrids’, are not known from any other locations in South Korea.

#### Sample collection

To permit the analysis of allozyme genetic variation within and among populations, we collected 1 cm<sup>2</sup> leaf samples from adult and juvenile individuals from 14 populations of *L. kumokiri* located across the range of the species in South Korea (Table 1). At two of these sites, SOA and SOB, located on hillsides of Mount Sobaek, we also encountered and sampled *L. makinoana* and putative hybrid individuals. These two sympatric sites currently represent the only known occurrences of *L. makinoana* in South Korea; we failed to find this species at other historical locations identified from herbarium records, indicating that this species is extremely rare in South Korea and may be declining (Oh *et al.* 2004). In total, 2215 samples (*N* = 1744, 425, and 46 for *L. kumokiri*, *L. makinoana*, and putative hybrids, respectively) were assayed in the course of this study (Table 1).

To quantify and compare fine-scale patterns of spatial aggregation and genetic structure within populations of the two species, we mapped all *L. kumokiri*, *L. makinoana*, and putative hybrid individuals found in the two sites where they co-occur (small individuals were not collected to protect them). Site SOA (12 × 30 m area, *N* = 610, 374, and 12 for *L. kumokiri*, *L. makinoana*, and putative hybrids, respectively, Fig. 1) was located at 980 m above sea level (a.s.l.) on a north-facing slope supporting the growth of *Pinus densiflora* as well as deciduous trees. Additional *L. kumokiri* were found in the immediate vicinity, but our study plot encompassed virtually all *L. makinoana* and putative hybrids observed at this site. The second site, SOB (15 × 25 m area, *N* = 184, 51, and 34 for *L. kumokiri*, *L. makinoana*, and putative hybrids, respectively, Fig. 1) was located 5 km south of SOA at an



**Fig. 1** Distribution of individual *Liparis makinoana* (○,  $N = 374$  and 51 in SOA and SOB, respectively), *Liparis kumokiri* (●,  $N = 610$  and 184 in SOA and SOB, respectively), and their putative hybrids (×,  $N = 12$  and 34 in SOA and SOB, respectively) in two populations on Mount Sobaek, Chungcheongbuk-do, South Korea.

altitude of 780 m a.s.l. in a relatively flat area under a pine-oak overstorey near a stream. Here our study plot encompassed all observed individuals of the study taxa. All samples were collected by M. Y. Chung and M. G. Chung.

#### Electrophoretic procedures

All sampled leaf material was kept on ice until it could be transported to the laboratory, where it was stored at 4 °C until protein extraction. Leaf samples were finely cut and then crushed with a mortar and pestle in a phosphate-polyvinylpyrrolidone extraction buffer (Mitton *et al.* 1979). Enzyme extracts were absorbed onto 4 × 6-mm wicks cut from Whatman 3MM chromatography paper, which were then stored at -70 °C until needed. Levels and distribution of allozyme variation were determined with horizontal gel electrophoresis. Starch gels (12%) were stained for nine enzyme systems, which resolved 15 putative loci. A modification (Haufler 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 morpholine citrate buffer system (Clayton & Tretiak 1972) was used to resolve isocitrate dehydrogenase (*Idh-1*, *Idh-2*), malate dehydrogenase (*Mdh-1*, *Mdh-2*), and 6-phosphogluconate dehydrogenase (*6Pgd-1*,

*6Pgd-2*). A modification (Chung & Kang 1994) of Soltis *et al.*'s (1983) system 11 was used to resolve formate dehydrogenase (*Fdh*), phosphoglucosomerase (*Pgi-1*, *Pgi-2*), phosphoglucosomutase (*Pgm*), and shikimate dehydrogenase (*Skdh*). Staining recipes were from Soltis *et al.* (1983), except diaphorase (Cheliak & Pitel 1984) and formate dehydrogenase (Wendel & Weeden 1989). The genetic basis of enzyme banding patterns was inferred from observed segregation patterns in light of typical subunit structure and subcellular compartmentalization (Weeden & Wendel 1989; Wendel & Weeden 1989). Putative loci were designated sequentially, with the most anodally migrating isozyme designated as 1, the next most 2, etc. Likewise, alleles were designated sequentially with the most anodally migrating allele designated as superscript a.

#### Data analysis

*Clonal structure and allozyme diversity.* *Liparis* species reproduce sexually and vegetatively. Thus, to determine whether shoots with identical marker genotypes are clones, the available genetic markers must provide the statistical power to discriminate clonal genotypes from identical sexually produced genotypes. The discriminating power of our

allozyme markers was measured for each population as  $1 - P_G$ , where  $P_G$  (the probability that two random, sexually produced genotypes will be identical) was calculated as the product over loci of observed genotypic frequencies of genets assuming linkage equilibrium (Berg & Hamrick 1994). Because our power was high for the two populations of *L. makinoana* (see Results) and identical genotypes were spatially clustered as expected for growth via vegetative spread (Chung *et al.* 2004a), we identified putative clonal ramets by simple inspection of the genotypic data.

The following allele frequency and genetic diversity parameters were estimated using the program POPGENE (Yeh *et al.* 1999): percent polymorphic loci (%*P*; a locus was considered polymorphic if the frequency of the most common allele does not exceed 0.95), mean number of alleles per locus (*A*), observed heterozygosity ( $H_O$ ), and Nei's gene diversity ( $H_E$ ). These estimates were calculated for total samples and for samples excluding clones (hereafter referred to as 'genets').

*Wright's F-statistics.* To measure the average level of inbreeding within and genetic differentiation among populations of each species, Wright's (1965)  $F_{IS}$  and  $F_{ST}$ , respectively, were estimated for total samples and genets over polymorphic loci according to the method of Weir & Cockerham (1984). We also used  $F_{ST}$  to quantify genetic differentiation between parental taxa at sympatric sites. These estimates, and their 95% bootstrap confidence intervals (1000 replicates), were obtained using the program FSTAT (Goudet 2002). For each population,  $F_{IS}$  was also calculated separately with 95% bootstrap confidence intervals (1000 replicates) constructed using the program GDA (Lewis & Zaykin 2001).

*Patterns of hybridization.* We used multilocus genotypes and the hybrid index of Rieseberg *et al.* (1998) to characterize patterns of hybridization for SOA and SOB populations of *Liparis*. For each individual plant this index was calculated as the probability of a randomly selected gene originating in *L. kumokiri* divided by the sum of the probabilities of it originating at random in *L. kumokiri* or *L. makinoana*. An individual's multilocus hybrid index was obtained by averaging indices over alleles and then loci. In our analyses, then, hybrid index values approaching one should characterize *L. kumokiri*-like genotypes while values approaching zero should characterize *L. makinoana*-like genotypes. At each site, differences in hybrid index values between *L. kumokiri*, *L. makinoana*, and putative hybrids were tested using analysis of variance (ANOVA). The utility of this index requires the accurate characterization of allele frequencies in *L. kumokiri* and *L. makinoana* source populations giving rise to the populations of interest. At both SOA and SOB we used local populations of morphologically 'pure' *L. kumokiri* and *L. makinoana* to estimate these frequencies.

In complementary analysis, we used the maximum-likelihood analysis of population admixture of Nason *et al.*

(2002) to obtain, for SOA and SOB, population-level estimates of the relative frequencies and variances of six candidate genealogical classes to which individuals in mixed populations might belong: pure *L. kumokiri* ( $P_1$ ), pure *L. makinoana* ( $P_2$ ), first ( $F_1$ ) and second ( $F_2$ ) generation hybrids between these parental taxa, and first ( $B_1P_1$ ,  $B_1P_2$ ) or second ( $B_2P_1$ ,  $B_2P_2$ ) generation backcrosses to either parental taxa. These six classes represent all possible combinations of first- and second-generation recombination between species. Additional recombinant types are possible with later generation hybridization, but the number of possible hybrid types rises exponentially and their enumeration is problematic. As discussed in Nason & Ellstrand (1993) and Nason *et al.* (2002), however, despite assuming that hybridization is restricted to the production of these six genealogical classes, their estimated frequency spectrum is often indicative of more advanced generation hybridization.

*Spatial distribution of parental taxa and hybrids.* We used Ripley's univariate *L*-function (Ripley 1976) to summarize the spatial patterning of *L. kumokiri*, *L. makinoana*, and putative hybrid individuals at sites SOA and SOB. The *L*-function is calculated from the counts of plants (ramets) and genets in concentric circles of radius *r* about each plant. Since the use of circles with a radius greater than half the shortest plot side introduces excessive bias due to edge effects, radial distances was selected from 1 m to 6 m for SOA and to 7.5 m for SOB (e.g. Parker *et al.* 1997). Values of  $L(r) = 0$ ,  $L(r) > 0$ , and  $L(r) < 0$  indicate spatial randomness, spatial clustering, and spatial repulsion (hyperdispersion), respectively, up to distance *r*. Ninety-five per cent (95%) confidence envelopes about the null hypothesis of spatial randomness were determined by Monte Carlo simulation (199 replicates) with a value of  $L(r)$  outside of this envelope judged to be a significant departure from the null hypothesis.

To determine the spatial clustering of putative hybrids with parental species, and parental species with each other, we used Ripley's bivariate *L*-function. The bivariate *L*-function is calculated in a manner analogous to the univariate function but involving the counts of plants of one type in concentric circles of radius *r* about plants of the other type. Values of  $L_{12}(r) = 0$ ,  $L_{12}(r) > 0$ , and  $L_{12}(r) < 0$  indicate spatial independence, spatial clustering, and spatial repulsion, respectively, of the two plant types up to distance *r*. Again, 95% confidence envelopes about the null were determined by Monte Carlo simulation. 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).

*Fine-scale genetic structure.* Measurements of fine-scale spatial genetic structure provide information concerning the scale of dispersal within populations of a species and hence

the potential for dispersal between sympatric congeneric populations. To characterize fine-scale genetic structure within SOA and SOB populations of both *Liparis* species and their putative hybrids, we used the spatial autocorrelation procedures of Loiselle *et al.* (1995; see also Vekemans & Hardy 2004). The mean kinship coefficient ( $f_{ij}$ ) between pairs of individuals  $i$  and  $j$  within each population was plotted as a function of the distance interval between them. For distance intervals of  $\leq 1$  m and  $> 1$  m we used 0.25-m and 1-m lags, respectively, with right-hand binning. Using randomization procedures, 95% confidence intervals (CIs) about the null hypothesis of no genetic structure ( $f_{ij} = 0$ ) were constructed at each distance interval (Loiselle *et al.* 1995). Given the relatively small number of putative hybrid individuals at SOB, we binned samples into distance intervals  $\leq 1$  m, 1 to  $\leq 3$  m, 3 to  $\leq 4$  m, and then used 4-m lags thereafter. Putative hybrids at SOA were not analysed due to a small sample size ( $N = 12$ ). At both sites the effects of clonal structure were evaluated by conducting the spatial autocorrelation analyses, as described above, for total samples (including clones) vs. genets (with a single representative ramet per clone). In this latter case the  $x, y$  coordinate of each genet was placed at the genet's centre of mass.

To test the overall pattern of spatial genetic autocorrelation for each data set (total samples and genets of *L. makinoana* in SOA and SOB, and putative hybrids in SOB) across all distance classes, the slope ( $\beta$ ) of pairwise kinship coefficients ( $f_{ij}$ ) on the logarithm of distance interval was evaluated using a Mantel test (1000 permutations). The null hypothesis ( $\beta = 0$ ) of no genetic structure was rejected if the actual  $\beta$  was less than or equal to the 50th smallest permuted  $\beta$  (one-tailed test,  $\alpha = 0.05$ ). To test for differences in  $\beta$  (and hence the strength of fine-scale genetic structure) between data sets, standard errors for each  $\beta$  were obtained by jackknifing across loci (as suggested in Vekemans & Hardy 2004). The

approximate 95% CIs for each  $\beta$  were thus obtained as  $\pm 1.96$  times the jackknifed standard errors. Slopes were considered to be significantly different if their 95% CIs did not overlap.

Finally, to test whether mean values of  $f_{ij}$  at less than 1 m between total samples and genets, and among populations are significantly different from each other, we also used the approximation of a 95% CIs ( $\pm 1.96$  times the standard error). All analyses, including estimation of jackknifed standard errors, were conducted using the program SPAGED1 (Hardy & Vekemans 2002).

## Results

### Allozyme diversity and clonal structure

Allozyme variation was high within populations of *Liparis makinoana* and homogeneous across total samples and genets with a mean percentage of polymorphic loci within populations of 73.3 and mean number of alleles per locus of 2.20 (Table 2). Genetic diversity ( $H_E$ ) calculated from both total samples and genets were 0.317 and 0.319, respectively (Table 2). In contrast, all 14 populations of the more common *Liparis kumokiri* (1744 samples) were completely homozygous and allozymically indistinguishable (no polymorphic loci). The putative hybrids, in turn, maintained low levels of genetic variation within SOA ( $H_E = 0.072$ ) and SOB ( $H_E = 0.087$ ) (Table 2).

Consistent with high levels of genetic diversity in populations of *L. makinoana*, our power to discriminate clonal genotypes from sexually produced genotypes identical by chance alone was close to 1 ( $P_G = 0.00005$  and  $0.0001$  at SOA and SOB). Given this power, spatially proximal ramets sharing the same genotype were treated as clones. Hereafter we referred subscripts ( $r$ ) and ( $g$ ) to total samples and

**Table 2** Summary of genetic diversity measures and mean fixation ( $F_{IS}$ ) estimates observed in *Liparis makinoana*, *Liparis kumokiri*, and their putative hybrids

Species	Population	$N$	% $P$	$A$	$H_O$ (SE)	$H_E$ (SE)	$F_{IS}$ (95% CI)
<i>L. makinoana</i>	SOA (total)	374	73.3	2.27	0.267 (0.059)	0.332 (0.066)	0.197 (0.069, 0.337)
	SOA (excluding clones)	350	73.3	2.27	0.267 (0.059)	0.333 (0.066)	0.199 (0.072, 0.336)
	SOB (total)	51	73.3	2.13	0.275 (0.058)	0.301 (0.058)	0.098 (-0.013, 0.212)
	SOB (excluding clones)	48	73.3	2.13	0.278 (0.058)	0.304 (0.058)	0.099 (-0.003, 0.202)
Putative hybrids	SOA (total = genets)	12	20.0	1.20	0.033 (0.032)	0.072 (0.041)	—
	SOB (total)	34	40.0	1.60	0.078 (0.043)	0.087 (0.045)	0.118 (-0.018, 0.377)
	SOB (excluding clones)	21	40.0	1.60	0.083 (0.043)	0.096 (0.047)	0.163 (-0.020, 0.394)
<i>L. kumokiri</i>	All 14 populations	1744	0.0	1.00	0 (0.000)	0 (0.000)	—

$N$ , 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 CI, confidence intervals. Dash indicates analysis not conducted due to a small sample size (putative hybrids at SOA) and monomorphism at all loci examined (all 14 populations of *L. kumokiri*).

samples restricted to genets. At SOA the number of genets containing one, two, three, and four ramets was 330, 17, 2, and 1, respectively [total  $N_{(r)} = 374$  and genets  $N_{(g)} = 350$ ] while at SOB the number of genets containing one, two, three, and four ramets was 46, 1, 1 and 0, respectively [total  $N_{(r)} = 51$  and genets  $N_{(g)} = 48$ ; Table 2]. The mean distance separating clonal ramets was  $7.41 \text{ cm} \pm 0.96 \text{ cm}$  (standard error) at SOA and  $7.75 \text{ cm} \pm 0.89 \text{ cm}$  at SOB. This spatial proximity is consistent with the mode of vegetative spread in *Liparis* in which new corms are formed adjacent to the parental corm (Whigham & O'Neill 1991). Patterns of clonal spread could not be quantified for *L. kumokiri* owing to the complete lack of allozyme polymorphism. Finally, for putative hybrids, all 12 ramets were distinct genets at SOA, whereas 21 genets were screened from 34 ramets at SOB (Table 2).

#### Wright's $F$ -statistics

Wright's  $F$ -statistics calculated for total samples and genets of *L. makinoana* and putative hybrids were similar, so we report results only for genets here.  $F_{IS}$  calculated for the two populations of *L. makinoana* was significantly greater than zero (mean  $F_{IS} = 0.187$ , 95% CI: 0.067–0.326), with individual population fixation indices of 0.199 (significant) for SOA and 0.099 (marginally significant) for SOB (Table 2). Putative hybrid populations at SOA and SOB had relatively high  $F_{IS}$  estimates but were not significantly different from zero given the small number of genets (Table 2).

Wright's  $F_{ST}$  between SOA and SOB populations of *L. makinoana* was moderate (given their spatial proximity, populations are separated by 5 km) and significant ( $F_{ST} = 0.040$ , 95% CI: 0.021–0.060) whereas  $F_{ST}$  between parental taxa was large and highly significant ( $F_{ST} = 0.708$ , 95% CI: 0.610–0.758 at SOA;  $F_{ST} = 0.816$ , 95% CI: 0.698–0.861 at SOB). Underlying the large degree of allele frequency differentiation between taxa was a number of taxon-specific alleles. Of the 33 alleles found at 11 polymorphic loci, 8 alleles were present in both taxa whereas 22 alleles were unique to *L. makinoana* and 3 alleles were unique to *L. kumokiri* (Table 3). Moreover, allelic differences at three loci (*Mdh-2*, *6Pgd-1*, and *Pgi-2*) were diagnostic, and the frequencies of some shared alleles were also highly skewed (e.g. *Dia-2a*, *Fdhb*, and *Idh-1c*). Putative hybrid individuals segregated for both *L. makinoana* and *L. kumokiri* specific alleles, as described below.

#### Patterns of hybridization

At SOA, of the 22 alleles unique to *L. makinoana*, four were also present in the putative hybrids (range of allele frequency in hybrids: 0.083–1.0) (Table 3). With respect to *L. kumokiri*, in contrast, of three unique alleles, two were present in putative hybrids (range: 0.917–1.0). At SOB, of 20 alleles unique to *L. makinoana*, nine were also present in the putative

hybrids (range: 0.015–1.0) whereas all three alleles unique to *L. kumokiri* were also present in putative hybrids (range: 0.177–1.0). We did not observe any allele in the putative hybrids that were not found in the two parental taxa.

Analysis of hybrid index values calculated for parental species and putative hybrids revealed greater genetic input of *L. kumokiri* than *L. makinoana* into the putative hybrid populations. At SOA, the mean hybrid index values for each population were significantly different (ANOVA,  $P < 0.05$ ; *L. kumokiri*  $\bar{x} = 0.90 \pm 0.000$ , *L. makinoana*  $\bar{x} = 0.13 \pm 0.002$ , and putative hybrids  $\bar{x} = 0.80 \pm 0.011$ ), and the distributions of scores for each population were nonoverlapping (Fig. 2). Similarly at SOB, the mean hybrid index values for each population were significantly different (ANOVA,  $P < 0.05$ ; *L. kumokiri*  $\bar{x} = 0.88 \pm 0.000$ , *L. makinoana*  $\bar{x} = 0.14 \pm 0.005$ , and putative hybrids  $\bar{x} = 0.66 \pm 0.012$ ) and the distributions of scores for each population were again nonoverlapping (Fig. 2). At both locations, SOA especially, the putative hybrids had index values closer to those of *L. kumokiri*, indicative of proportionally greater genetic contributions of this parental species to the hybrid populations.

At both SOA and SOB, maximum-likelihood analysis of population admixture indicated that morphologically pure populations of *L. kumokiri* and *L. makinoana* do indeed consist of 100% *L. kumokiri* ( $P_1$ ) and 100% *L. makinoana* ( $P_2$ ) individuals, respectively. For both taxa there was no evidence of first or second generation backcross classes of individuals indicative of introgression between taxa.

At SOA, the putative hybrids were estimated to consist of 50.2% ( $\pm 14.5\%$ )  $F_2$  and 49.8% ( $\pm 14.5\%$ ) second generation backcrosses to *L. kumokiri* ( $B_2P_1$ ), whereas at SOB, the putative hybrids were estimated to consist of 91.3% ( $\pm 6.47\%$ )  $F_2$  and 8.7% ( $\pm 6.47\%$ ) second generation backcrosses to *L. kumokiri* ( $B_2P_1$ ). Given inference of no  $F_1$  or first-generation backcross individuals, we interpreted this pattern of classification to indicate that the inferred  $F_2$  and  $B_2P_1$  hybrids are representative of later generation recombinants skewed towards *L. kumokiri*, particularly at SOB.

#### The spatial distribution of parental taxa and hybrids

Ripley's  $L(r)$  analyses of the spatial distributions of *L. makinoana* and putative hybrids did not differ qualitatively whether we used total samples or genets (identified by allozyme analysis). Ramets and genets could not be distinguished for *L. kumokiri* (this species was monomorphic at all locations), however, so for all taxa, we simply report results for total samples. Within SOA, univariate analyses of Ripley's  $L(r)$  indicate that the parental taxa and putative hybrids were each significantly spatially clustered ( $L(r) > 0$ ) at near neighbour distances (Fig. 3).  $L(r)$  remained significant and positive for all values of  $r$  in *L. makinoana* but declined and became negative in *L. kumokiri* and putative hybrids, with  $L(r) = 0$  at approximately 2.5 m and 3.75 m, respectively. At

**Table 3** Allele frequencies for 11 polymorphic loci of *Liparis makinoana*, putative hybrids, and *Liparis kumokiri* in SOA and SOB. Boldface text indicates alleles unique to *L. makinoana* (22 alleles) or *L. kumokiri* (three alleles). Boxes identify alleles in the putative hybrids that were unique to one or the other parental species

Locus	Alleles	SOA (total)			SOB (total)		
		<i>L. makinoana</i>	Putative hybrids	<i>L. kumokiri</i>	<i>L. makinoana</i>	Putative hybrids	<i>L. kumokiri</i>
<i>Dia-2</i>	a	0.259	1.000	1.000	0.441	1.000	1.000
	b	<b>0.741</b>	0.000	0.000	<b>0.559</b>	0.000	0.000
<i>Fdh</i>	a	<b>0.702</b>	0.333	0.000	<b>0.873</b>	0.794	0.000
	b	0.298	0.667	1.000	0.127	0.206	1.000
<i>Idh-1</i>	a	<b>0.443</b>	0.000	0.000	<b>0.275</b>	0.029	0.000
	b	<b>0.418</b>	0.000	0.000	<b>0.598</b>	0.000	0.000
	c	0.124	1.000	1.000	0.128	0.971	1.000
	d	<b>0.015</b>	0.000	0.000	0.000	0.000	0.000
<i>Idh-2</i>	a	0.594	1.000	1.000	0.775	0.971	1.000
	b	<b>0.406</b>	0.000	0.000	<b>0.226</b>	0.029	0.000
<i>Lap-1</i>	a	0.428	1.000	1.000	0.686	1.000	1.000
	b	<b>0.326</b>	0.000	0.000	<b>0.088</b>	0.000	0.000
	c	<b>0.186</b>	0.000	0.000	<b>0.128</b>	0.000	0.000
	d	<b>0.060</b>	0.000	0.000	<b>0.098</b>	0.000	0.000
<i>Mdh-2</i>	a	<b>0.467</b>	1.000	0.000	<b>0.461</b>	0.000	0.000
	b	0.000	0.000	<b>1.000</b>	0.000	1.000	<b>1.000</b>
	c	<b>0.148</b>	0.000	0.000	<b>0.078</b>	0.000	0.000
	d	<b>0.385</b>	0.000	0.000	<b>0.461</b>	0.000	0.000
<i>6Pgd-1</i>	a	<b>0.599</b>	0.000	0.000	<b>0.677</b>	0.000	0.000
	b	<b>0.401</b>	0.000	0.000	<b>0.324</b>	0.000	0.000
<i>6Pgd-2</i>	c	0.000	1.000	<b>1.000</b>	0.000	1.000	<b>1.000</b>
	a	<b>0.015</b>	0.000	0.000	0.000	0.000	0.000
	b	0.630	1.000	1.000	0.784	1.000	1.000
	c	<b>0.355</b>	0.000	0.000	<b>0.216</b>	0.000	0.000
<i>Pgi-2</i>	a	<b>0.488</b>	0.083	0.000	<b>0.412</b>	0.309	0.000
	b	0.000	0.917	<b>1.000</b>	0.000	0.177	<b>1.000</b>
	c	<b>0.361</b>	0.000	0.000	<b>0.343</b>	0.471	0.000
	d	<b>0.151</b>	0.000	0.000	<b>0.245</b>	0.044	0.000
<i>Pgm</i>	a	0.988	1.000	1.000	0.931	0.971	1.000
	b	<b>0.012</b>	0.000	0.000	<b>0.069</b>	0.029	0.000
<i>Skdh</i>	a	<b>0.096</b>	0.583	0.000	<b>0.039</b>	0.074	0.000
	b	0.889	0.417	1.000	0.843	0.912	1.000
	c	<b>0.015</b>	0.000	0.000	<b>0.118</b>	0.015	0.000

SOB, in contrast, *L. kumokiri* and putative hybrids exhibited significant spatial clustering for all values of  $r$ , whereas *L. makinoana* exhibited significant spatial clustering only at distances of  $r < 4.5$  m (Fig. 3).

At SOA, bivariate analyses indicated a general pattern of significant spatial repulsion between parental taxa and between these taxa and putative hybrids at virtually all distances (Fig. 3). At SOB, in contrast, whereas *L. kumokiri* and *L. makinoana* exhibited a general pattern of repulsion, putative hybrids were significantly spatially clustered with *L. kumokiri* and *L. makinoana* at  $r < 5.5$  m. At shorter distance intervals ( $r < 2.0$  m), however, aggregation of putative hybrids with *L. kumokiri* was much stronger and less idiosyncratic than with *L. makinoana*.

#### *Fine-scale genetic structure in L. makinoana and hybrids*

For *L. makinoana* at both SOA and SOB,  $f_{ij}$  estimates were significant and positive at smaller distance intervals ( $< 2$  m) with the highest  $f_{ij}$  values found at the smallest distance interval (0.25 m). At this distance, within-sites  $f_{ij}$  was higher for total samples (including clones) than for genets, as expected, though not significantly so, and between-sites  $f_{ij}$  was higher at SOB than at SOA for both total samples and genets, although again not significantly so [ $f_{ij}$  at 0.25 m (95% CIs) at SOA, total samples: 0.132 (0.085, 0.180), genets: 0.110 (0.068, 0.151); and at SOB, total samples: 0.211 (0.159, 0.263), genets: 0.172 (0.121, 0.224)]. Because the number of clones at SOA and SOB was small (Table 2), their weak

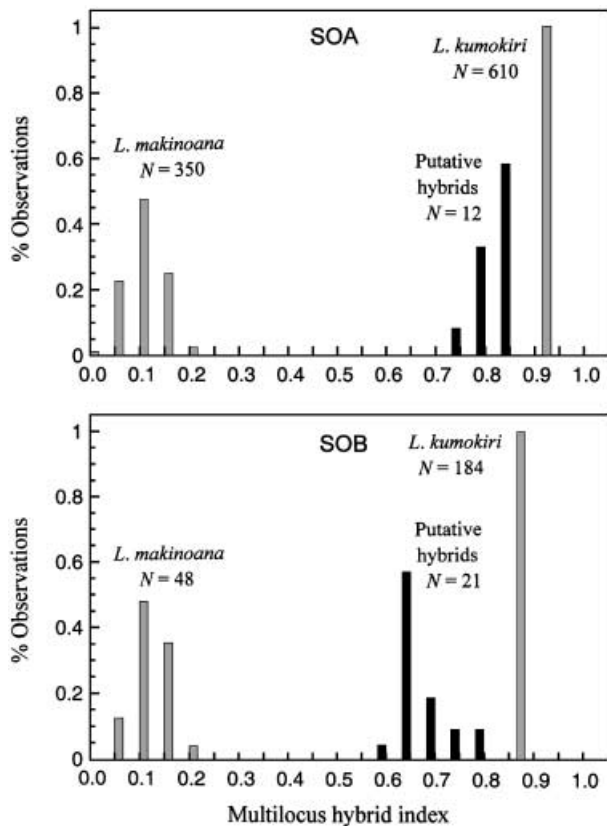


Fig. 2 Hybrid index values calculated for parental species and putative hybrids at SOA and SOB.

effect on fine-scale genetic structure is not unexpected. For putative hybrids at SOB,  $f_{ij}$  estimates at the smallest distance interval (1 m) were not significantly different from zero for either total samples or genets. For comparison, we calculated mean  $f_{ij}$  (and jackknifed CIs) at the 0–1 m distance interval for *L. makinoana* at SOB, finding significantly higher values in this species than in putative hybrids for both total samples and genets [ $f_{ij}$  at 1 m (95% CIs), *L. makinoana*, total samples: 0.111 (0.073, 0.149), genets: 0.092 (0.047, 0.137); putative hybrids, total samples: 0.028 (–0.007, 0.059), genets: –0.036 (–0.034, 0.034)]. Because differences between total samples and genets were not significant, in Fig. 4 we present correlograms for genets only.

The overall slopes ( $\beta$ ) of the four correlograms obtained from two populations of *L. makinoana* were similar and significantly different from the null hypothesis of no spatial genetic structure ( $\beta = 0$ ): at SOA, for total samples  $\beta = -0.024$  (95% CIs = –0.001, 0.001) and for genets  $\beta = -0.022$  (95% CIs = –0.002, 0.002); at SOB, for total samples  $\beta = -0.034$  (95% CIs = –0.009, 0.007) and for genets  $\beta = -0.029$  (95% CIs = –0.010, 0.008). For total samples of putative hybrids at SOB ( $N = 34$ ), the slope was significantly different from zero ( $\beta = -0.032$ , 95% CIs: –0.021, 0.014), whereas for the

reduced sample consisting of genets ( $N = 21$ ) the slope was not significant ( $\beta = -0.002$ , 95% CIs: –0.028, 0.020).

Differences between slopes for total samples vs. genets and SOA vs. SOB of *L. makinoana* were not significant as 95% jackknifed confidence intervals overlapped for all pairwise comparisons. For putative hybrids at SOB, the difference in slopes between total samples and genets, although considerable, was not significant. Difference in slope between *L. makinoana* and putative hybrid total samples and genets also were not significant (even when *L. makinoana* was re-analysed at 1-m distance intervals to correspond with analyses of the putative hybrids).

## Discussion

### Characterization and consequences of hybridization

Where the common *Liparis kumokiri* and the rare *Liparis makinoana* co-occur we have observed putative hybrid plants recombining the floral characteristics of *L. kumokiri* with the vegetative characteristics of *L. makinoana*. In all cases, the identity of these plants as hybrids was confirmed by analysis of their multilocus allozyme genotypes. Moreover, because the two parental species differ in the identity and frequency of alleles at a number of loci, we were further able to resolve the nature and origins of hybrid individuals.

Analyses of hybrid index values indicate that, genotypically, hybrids are skewed towards *L. kumokiri* at both sympatric sites. Maximum-likelihood analyses of population admixture revealed an absence of  $F_1$  and first-generation backcross hybrids, from which the inferred high frequencies of  $F_2$  hybrids and the second generation backcrosses to *L. kumokiri* are likely indicative of later generation recombinants skewed towards this species. As predicted, such asymmetry in hybridization is probably due to the similarity of floral characteristics between the hybrids and the more common *L. kumokiri*, which may promote pollinator-mediated positive assortative mating between these classes of individuals. Ripley's bivariate  $L(r)$  statistics revealed that hybrids are spatially clustered with *L. kumokiri* at SOB (Fig. 3), which further enhances the potential positive assortative mating.

Given these patterns, what can we infer about the mechanisms of hybrid formation, the microevolutionary dynamics of hybrid populations, and introgression and reproductive isolation between *L. kumokiri* and *L. makinoana*? On the one hand, the presence of advanced generation hybrids at the two sites where these species are known to co-occur suggests that barriers to interspecific recombination must be relatively weak and that the potential for introgression in sympatry is high. On the other hand, a closer investigation of the genetic data indicates that the hybrid and parental-species populations at SOA and SOB are currently evolving independently of one another. Not only is there no evidence of recent recombination between

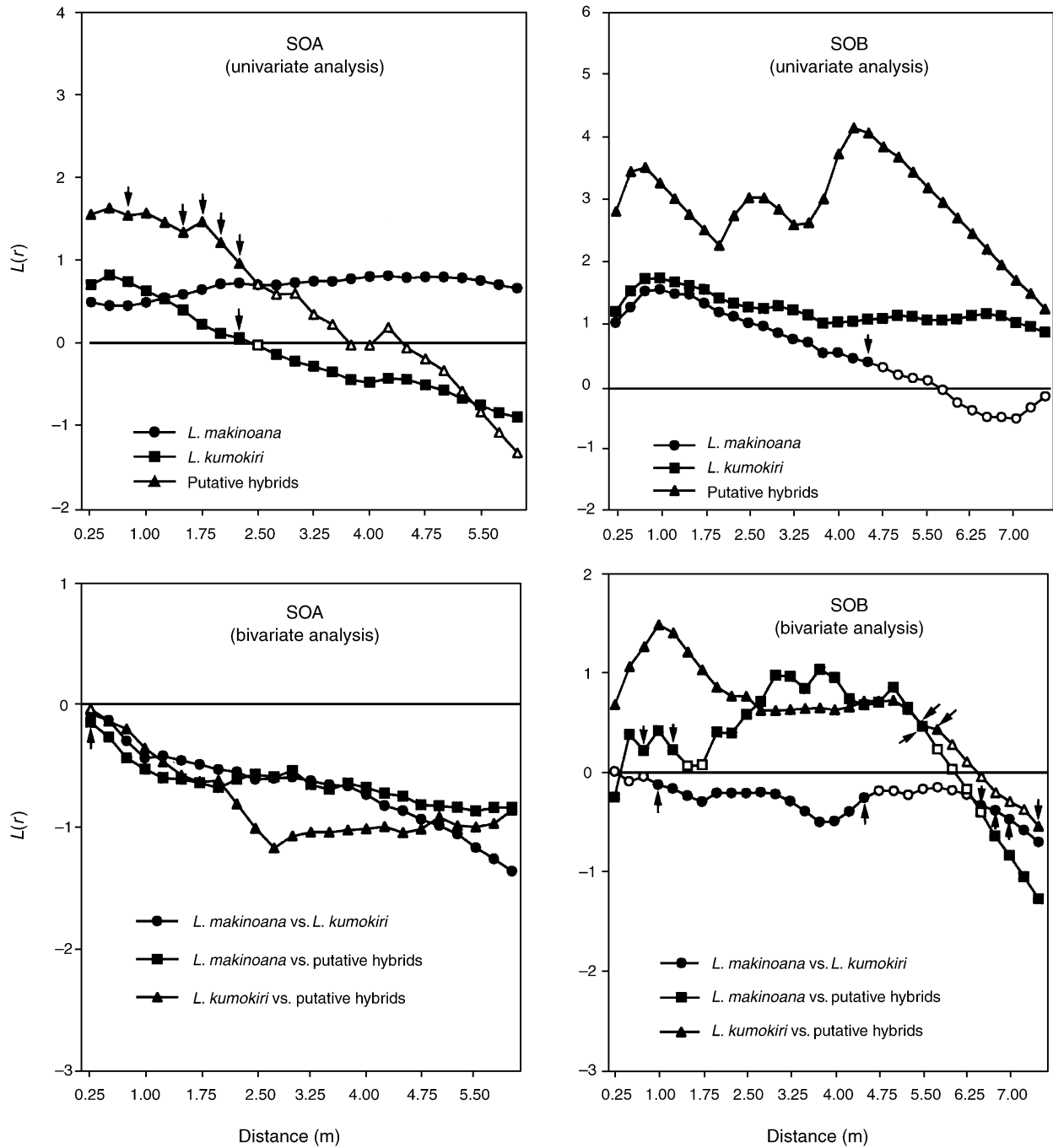
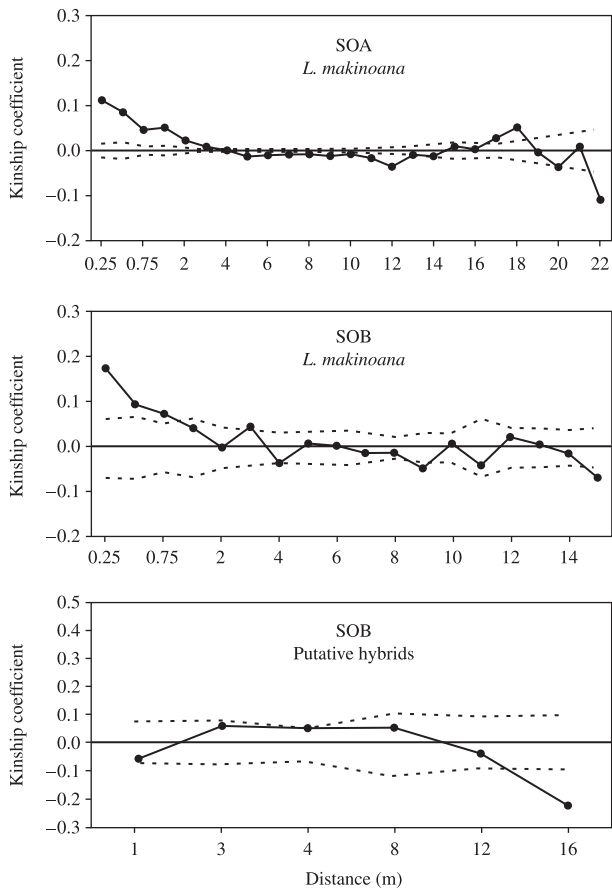


Fig. 3 Ripley's  $L(r)$ -statistics for univariate and bivariate analyses of total individuals of *Liparis makinoana*, *Liparis kumokiri*, and their putative hybrids. The symbols ●, ■, ▲ with arrows (↑) and the symbols ●, ■, ▲ without arrows represent  $L(r)$  values were above and below the 5% and 1% highest and lowest values generated from 199 Monte Carlo simulation of a randomly distributed population. Open symbols (○, □, and △) represent  $L(r)$  values were within the 5% and 1% highest and lowest values.

species, but allele frequencies in both hybrid populations exhibit significant genetic drift from parental-species source populations. For example, alleles that are fixed in *L. kumokiri* were found to be rare or absent at some loci in hybrids at SOA (i.e. *Mdh-2b*) and SOB (i.e. *Fdhb* and *6Pgi-2b*), despite

the general similarity of their hybrid index values. Likewise, allele frequency differences between *L. makinoana* and hybrids were substantial at a number of loci in SOA (e.g. *Dia-2*, *Idh-1*, *6Pgd-1*, and *Pgi-2*) and SOB (e.g. *Dia-2*, *Idh-1*, *Mdh-2*, and *6Pgd-1*).



**Fig. 4** Spatial autocorrelation analyses of co-ancestry ( $f_{ij}$ ) estimated from genets of *Liparis makinoana* at SOA and SOB and genets of putative hybrids at SOB. The dashed lines represent the upper and the lower 95% confidence envelopes, respectively, around the null hypothesis of  $f_{ij} = 0$ .

Further evidence of genetic drift within the hybrid populations and away from the parental taxa comes from the low genetic diversity of the hybrids. Given the large differences in allele frequency between parental taxa at both sites (mean  $F_{ST}$  at SOA: 0.708; at SOB: 0.816), genetic diversity in recently formed hybrids should be high, even if somewhat skewed towards the monomorphic *L. kumokiri*. In contrast, the genetic diversity of hybrids was low at both sites (Table 2). Taken together, these results suggest that the formation of  $F_1$  hybrids is historically rare at both sympatric sites, that the hybrid populations are currently evolving largely independently of the parental taxa, and that the greater genetic similarity of hybrids to *L. kumokiri* is historical and not indicative of ongoing recombination (backcrossing).

What, then, are the ecological and evolutionary consequences of hybridization between *L. kumokiri* and *L. makinoana*? In total, our results indicate that neither recent hybridization ( $F_1$  formation), introgression, nor genetic assimilation of the rarer parental species (Ellstrand & Elam 1993; Levin *et al.* 1996), is ongoing where the two species occur in sympatry.

### Causes of infrequent hybridization between two *Liparis* species

Compared to the parental taxa, *L. kumokiri* and *L. makinoana*, the frequency of hybrids among total samples was low at both sympatric sites (1.2% at SOA and 15.5% at SOB). We can distinguish among several possible reasons for why hybrids are relatively rare. One possibility is that the apparent low frequency of hybrids is an artefact of the low power of our genetic markers to distinguish them from parental taxa. This is not the case, however, as our power to distinguish hybrids from parental taxa on the basis of their multi-locus genotypes is high (Fig. 2). Another possibility is that *L. kumokiri* and *L. makinoana* have only recently come into sympatry so that the low frequency of hybrids reflects the earliest stages of recombination between these taxa. This is unlikely to be the case as the profile of hybrid class frequency estimates obtained from the maximum-likelihood model indicates not recent recombination between taxa (no  $F_1$  or first-generation backcross hybrids) but advanced generation recombination among hybrids ( $F_2$  generation and beyond).

The most likely scenario for the uncommonness of hybrids is that their formation and persistence have been limited by a combination of pre- and/or post-mating barriers to reproduction between parental taxa coupled with lower overall fitness. Ecological factors contributing to reproductive isolation may include differences in flowering phenology, pollinators, and spatial demography. Although flowering phenologies of *L. kumokiri* and *L. makinoana* are not entirely distinct (*L. kumokiri* flowers from mid-June through mid-July, whereas *L. makinoana* flowers from mid-May through late June in the study populations, M.Y. Chung & M.G. Chung, personal observations), differences between taxa should impede hybridization between the two species, even in sympatry. *L. kumokiri* and *L. makinoana* also differ substantially in flower size and colour. During 4-week field surveys conducted in July 1998, 1999, 2000, 2003, and 2004, we (M.Y.C. & M.G.C.) failed to observe any pollinators in the populations examined in this study. However, these differences in flower form, coupled with the fact that *L. kumokiri* is self-compatible whereas *L. makinoana* is self-incompatible, suggest that the two species may attract different sets of pollinators.

Interspecific hybridization may also be impeded by the nonrandom spatial distribution of individuals within sympatric populations. In both SOA and SOB, *L. kumokiri*, *L. makinoana*, and hybrids each exhibited significant spatial clustering, whereas the two parental taxa exhibited a pattern of spatial repulsion (Fig. 3). This spatial demographic pattern could be due, in part, to localized patterns of seed dispersal. Consistent with this prediction, our fine-scale genetic structure analyses of *L. makinoana* indicate a leptokurtic distribution of seed dispersal and recruitment around maternal plants (< 2 m, Fig. 4). Although seeds of orchids

are minute and dust-like with high dispersal potential (Tremblay *et al.* 2005), this pattern of fine-scale within-population genetic structure has been reported in studies of five terrestrial and one epiphytic orchid species (Peakall & Beattie 1996; Chung *et al.* 1998, 2004a, b, 2005; Trapnell *et al.* 2004). If coupled with localized pollen dispersal, such fine-scale genetic structure may decrease chances of hybridization between species. The significant excess of homozygotes relative to Hardy–Weinberg expectations in *L. makinoana* (Table 2), which is self-incompatible, suggests that pollen dispersal is localized, generating biparental inbreeding in spatially structured populations.

Post-pollination barriers to successful hybridization are also likely given the high degree of genetic differentiation between *L. kumokiri* and *L. makinoana*. Such differentiation could underlie prezygotic mechanisms of reproductive isolation, such as interspecific incompatibilities between pollinia and stigma. Post-zygotic barriers are also likely including the inviability and sterility of early generation hybrids, and surviving hybrid genotypes that are less well adapted to local environments than the parental taxa.

#### *Contrasting levels of genetic diversity between two Liparis species: conservation implications*

Populations of the rare, self-incompatible *L. makinoana* possess considerably higher levels of genetic variation (% $P = 73\%$ ,  $A = 2.20$ ,  $H_E = 0.318$ ) than the average genetic diversity of other herbaceous plants (% $P = 38\%$ ,  $A = 1.6$ ,  $H_E = 0.131$ ) as reported by Hamrick & Godt (1989). In contrast, a complete lack of allozyme variation was found in the common, self-compatible congener *L. kumokiri*. Inbreeding and genetic drift can drive the loss of genetic variation within populations, but fixation of the same alleles across populations suggests that current populations originated from the same genetically depauperate ancestral population following a severe population bottleneck.

The finding that two populations of *L. makinoana* maintain high levels of genetic diversity relative to *L. kumokiri* could lead to the conclusion that this species is not at risk at present. However, our long-term monitoring site at SOA indicates a dramatic decline of the number of individuals (M.Y. Chung & M.G. Chung, unpublished). In parallel with this, we also detected evidence of a recent population genetic bottleneck, indicating that levels of genetic variability have been decreasing and, given the current small population sizes, will continue to decline. Furthermore, there are few known surviving populations of this species, indicating its rarity in South Korea. Considering these observations, action should be taken to ensure its long-term genetic variability by implementing appropriate conservation and management strategies. First, the two study populations should be protected for *in situ* conservation. There is a small rest area near the SOA population, and thus people occasionally

visit the site and may trample the small fragile orchids. The SOB population faces similar threats, being a small population located near a trail on a hillside of Mount Sobaek. Our allozyme study found that the two populations are genetically different, though the degree of genetic differentiation is small. In sum, it is recommended that the two populations, as well as other populations that may be discovered, should be protected for the long-term survival of this species in South Korea.

To create a comprehensive conservation strategy, *ex situ* conservation should also be considered. Developing an appropriate sampling strategy serves as an important base for the effective *ex situ* conservation of plant species considered to be facing high risk of extinction in the wild. The sampling strategy for obtaining seed stocks within a population may benefit greatly from estimation of the  $x$ -intercept of the population's spatial genetic correlogram (Diniz-Filho & Telles 2002). The intercept for total samples (about 4 m) of *L. makinoana* was nearly the same as that for samples excluding clones. Thus germplasm collection at 4-m intervals should maximize genetic diversity and minimize genetic redundancy of genotypes within populations.

Since a complete lack of allozyme variation was found in *L. kumokiri*, we cannot suggest guidelines in terms of conservation genetics for this species. However, given the consistent finding of significant fine-scale genetic structure in populations of *L. makinoana* and hybrids at SOA and SOB, thus we suggest a similar strategy for obtaining seed stocks within populations of *L. kumokiri*. Moreover, large populations from spatially distant regions in South Korea should be targeted for genetic evaluation and potential *in situ* and *ex situ* conservation.

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This study is part of an ongoing collaboration between the laboratories of Myong Gi Chung and John D. Nason on the population genetic structure, the mechanisms of orchid diversification, and conservation of terrestrial orchids. Mi Yoon Chung is a research associate in the laboratory of John D. Nason. She uses molecular markers to understand the evolutionary factors that shape patterns of genetic variation in natural populations of orchids in South Korea. This work was initiated when she was in the laboratory of Myong Gi Chung as a PhD student and reflects her deep interest in the Orchidaceae. John D. Nason is interested in the population and conservation genetics of plants and their associated insect herbivores and pollinators. Myong Gi Chung studies on the genetic structure of plant populations with an emphasis on the fine-scale genetic structure in a variety of plants.

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