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Conservation genetics of the endangered terrestrial orchid *Pogonia minor* in South Korea

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Levels of allozyme variation, population genetic structure, and fine-scale genetic structure (FSGS) of the rare, endangered terrestrial orchid *Pogonia minor* were examined for three small and isolated populations (n = 185) in South Korea using 20 putative allozyme loci resolved from 14 enzyme systems. Of the three populations, only one was polymorphic at four loci. Thus, extremely low levels of allozyme variation within populations were found: mean percentage of polymorphic loci was 6.7%, mean number of alleles per locus was 1.07, and mean expected heterozygosity was 0.015. Polymorphic population exhibited a significant deficit of heterozygotes relative to Hardy-Weinberg expectations (F_{IS} = 0.257), suggesting selfing (rate, s = 0.349) through autogamy and biparental inbreeding. Analysis of *O*-ring function revealed significant aggregation of individuals suggests restricted seed dispersal and patchy distribution of microhabitats within populations. Spatial autocorrelation analyses revealed a significant fine-scale genetic structure (up to ≤ 2 m) within a polymorphic population, and a significantly high degree of population differentiation was found among populations (F_{ST} = 0.196). These results suggest that genetic drift, coupled with inbreeding, limited gene dispersal and founder effects would be the main explanatory factors for the extremely low levels of genetic diversity and for shaping the population genetic structure of *P. minor* in South Korea. Considering the current genetic structure of *P. minor*, *in situ* and *ex situ* conservation of the known populations of the species is suggested.

Key words: allozymes, conservation, genetic diversity, Orchidaceae, *Pogonia minor*, spatial distribution, spatial autocorrelation analysis

Introduction

Recent increasing human-mediated disturbance to natural habitats of plant species (e.g., mass collection for commercial value, habitat fragmentation and destruction, and logging) has reduced large populations into small and isolated ones, lowered the levels of genetic diversity, and altered the population genetic structure (Ellstrand & Elam 1993, Rajora *et al*. 2000, Lowe *et al*. 2005, Degen *et al*. 2006, Chung & Nason 2007). In the short term, the decreased genetic variability could lower individual plant fitness and population viability due to the expression

of deleterious alleles (Ellstrand & Elam 1993, Keller & Waller 2002). In the long term, eroded genetic variability can decrease the potential of populations to adapt to directional abiotic and biotic selective pressures as well as reduce their flexibility to response to pathogens and herbivores, if levels of quantitative genetic variation related to environmental adaptability are decreased (Lande & Shannon 1996, Booy *et al*. 2000, Keller & Waller 2002, Frankham *et al*. 2004). Thus, the loss of genetic diversity in the small and isolated populations and increased population genetic divergence between populations are of particular concern to the preservation of species (Falk & Holsinger 1991, Young *et al*. 1996, Hooftman *et al*. 2004).

Populations of many terrestrial orchids in the northern temperate regions are small and isolated, probably due to historical and/or human activities (Cribb & Sandison 1998, Tremblay *et al*. 2005, Brzosko *et al*. 2006). Their current status is susceptible to genetic drift, which leads to random fixation of alleles and reduction in heterozygosity and thus, low levels of genetic diversity in populations of the group (Bornbusch *et al*. 1994, Case 1994, Li *et al*. 2002, Brzosko & Wróblewska 2003, Brzosko *et al*. 2006, Li & Ge 2006, Chung & Chung 2007, but *see* Chung *et al*. 2007a). In a small and isolated plant population in general, if reproduction is pollinator-limited at lower population densities, increased selfing (for self-compatible orchids; Allee effect, *see* Allee *et al*. 1949, Lande 1999) and biparental inbreeding (for outcrossing orchids) would be expected as compared with those in a larger population (Bawa 1990). Under this condition, effective population size will further be decreased and random genetic drift coupled with limited inter-population gene flow will be accelerated over generations (Ellstrand & Elam 1993, Oostermeijer *et al*. 1996, Chung *et al*. 2004a). Thus, information on the genetic and demographic structure of populations of rare and endangered terrestrial orchids is essential for formulating comprehensive plans for their short- and long-term conservation (e.g., Sieg & Ring 1995, Wallace 2002). In spite of critical needs for understanding of population genetics of many rare and endangered terrestrial orchids, empirical conservation genetic studies of this group are still limited (Sun & Wong 2001, Brzosko & Wróblewska 2003, Li & Ge 2006, Ávila-Díaz & Oyama 2007, Chung & Chung 2007).

In this study, we investigated the allozyme diversity in three populations of the non-clonal terrestrial orchid *Pogonia minor*. Based on comparison between herbarium records (six locations have been identified) and recent field surveys (five locations were recorded), the species appears to be rare since the early 20th century (Oh *et al*. 2004, 2005, Lee & Choi 2006). Currently the species is extremely rare in South Korea and its populations are small and spatially isolated (< 100 individuals in 100 m^2 areas; M.Y.C. pers. obs.). Although orchid seeds are dust-like and it is often perceived that they are dispersed over long distances by wind (Arditti & Ghani 2000), such isolation exhibited by *P. minor* likely contributes to interrupted gene flow, which increases the effectiveness of genetic drift, resulting in low levels of genetic diversity within populations and a high degree of inter-population differentiation (Hamrick & Godt 1996, Forrest *et al*. 2004, Tremblay *et al*. 2005). Unless some of the populations of *P. minor* are lost, genetic drift should not cause the overall loss of genetic diversity. It just causes the loss of variation within populations and increases differentiation among populations. With this information, we expect low levels of genetic variation within populations of *P. minor* and high degrees of inter-populational genetic differentiation, due primarily to genetic drift and limited gene dispersal. To test the prediction, we estimated levels of genetic diversity in populations of *P. minor*. Then, to further explain gene flow within and between populations, we analyzed the spatial distribution of individuals and genotypes.

Material and methods

Study plants and sampling

Pogonia minor, a non-clonal terrestrial orchid, is 15–25 cm tall and grows on hillsides in the warmer parts of southern Korea, Japan, and Taiwan (Kitamura *et al*. 1986). *Pogonia minor* is highly self-compatible, and the small, pale

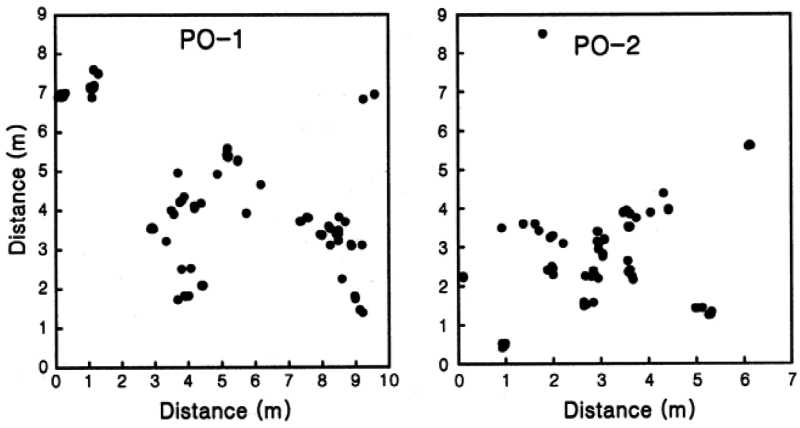

Fig. 1. Spatial distribution of individuals of *Pogonia minor* (PO-1 and PO-2) located in Haenam-gun in South Korea.

rose-purple flower (1.5–3 mm long) is solitary and blooms in June and July, but is scarcely open (M.Y.C. unpubl. data). Although pollinators of the species are unknown, about 60%–70% fruit set was observed in a pollinator-free screened greenhouse and natural habitats, suggesting that autogamy is highly likely. The selfing rates (s = 0.349) estimated in this study (*see* Results) also suggest occurrence of autogamy. The mature capsules (2.5 cm long) contain large numbers of small seeds.

In 2003 and 2004, South Korean research teams carrying out inventory of the vascular flora of South Korea found only three locations of *P. minor* in southern Korea (Oh *et al.* 2004, 2005). Since 1997, we have searched for additional populations of *P. minor* recorded in herbarium specimens as a larger study of population genetics and demography of orchids in Korea, but failed to locate them. In this study, we have studied the three populations from southern Korea recognized since 1997. As the population sizes of *P. minor* are small (< 100 mature individuals), the species can be classified as 'endangered' according to IUCN Red List criteria at regional levels (Gärdenfors *et al.* 2001, IUCN 2001: B1a, 2a, D).

In July 2003, a total of 185 individuals were mapped with x, y coordinates and sampled from three populations of *P. minor* in Haenam-gun: (PO-1, a 8 × 10 m, n = 89: PO-2, a 7 × 9 m, n = 70: PO-3, a 4 × 5 m, n = 26). PO-1 and PO-2 were separated by 258 m, PO-1 and PO-3 by 7.22 km, and PO-2 and PO-3 linearly distance 7.13 km apart. The individuals in PO-1 and PO-2 grow under a small tree and shrubs (*Pinus thunbergii*, *Vaccinium bracteatum*, *Eurya japonica*, and *Rhododendron schlippenbachii*, which occurred at a low frequency in the coastal populations), whereas those in PO-3 were in an open grassland.

All sampled leaf material was kept on ice in a small icebox until it could be transported to the laboratory, where it was stored at 4 °C until protein extraction for allozyme analyses.

Spatial distribution of individuals

To gain insights about seed dispersal within populations of *P. minor*, we assessed the spatial distribution of individuals in PO-1 and PO-2 (Fig. 1) (since area of PO-3 was too small, it was excluded from the analysis). First, we calculated the univariate O-ring statistic $O(r)$ (Wiegand & Moloney 2004) from the mean number of individuals in an annulus of radius (r) around each plant and plotted against the spatial scale r. Since the use of ring widths greater than half the shortest plot side introduces bias due to edge effects, the maximal ring width was set smaller than half the shortest plot side at the starting ring width of 1 m and with a 1-m interval. For testing the significance of $O(r)$ for each r, we used the common null model of complete spatial randomness (CSR). We calculated the first order intensity, λ, as a reference to the point pattern expected under CSR. Ninety-nine percent confidence envelopes around

λ were constructed from the highest and lowest $O(r)$ of 199 spatial randomizations by Monte Carlo simulation for each study population. An observed value of $O(r)$ outside of this envelope was judged a significant departure from CSR, with an observed value above, within, or below the envelope indicating spatial clustering, spatial randomness, or spatial repulsion (hyperdispersion), respectively, at radius r. We conducted all calculations and simulations using the program PROGRAMITA (Wiegand 2003).

Electrophoretic procedures

For extraction, we ground leaf samples with a pestle in a mortar 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. We determined levels and distribution of allozyme variation with horizontal gel electrophoresis using 12.5% starch gels and two types of gel/electrode buffer systems. The first buffer system we used, was a modification (Haufler 1985) of the system 6 of Soltis *et al*. (1983) to resolve alcohol dehydrogenase (*Adh*), diaphorase (*Dia-1*, *Dia-2*), florescent esterase (*Fe*), leucine aminopeptidase (*Lap-1*, *Lap-2*), malic enzyme (*Me*), peroxidase (*Per*), phosphoglucoisomerase (*Pgi-1*, *Pgi-2*), phosphoglucomutase (*Pgm*), and triosephosphate isomerase (*Tpi-1*, *Tpi-2*). The morpholine citrate buffer system (pH 6.1) of Clayton and Tretiak (1972) was used to resolve fructose-1, 6-diphosphatase (*F1,6-1*, *F1,6-2*), isocitrate dehydrogenase (*Idh*), malate dehydrogenase (*Mdh-1*, *Mdh-2*), 6-phosphogluconate dehydrogenase (*6Pgd*), and shikimate dehydrogenase (*Skdh*). Stain recipes were from Soltis *et al*. (1983), except for diaphorase (Cheliak & Pitel 1984). Putative loci were designated sequentially, with the most anodally migrating isozyme designated as 1, the second most anodally migrating isozyme as 2, etc. (Wendel & Weeden 1989). A total of 20 putative loci were resolved from the 14 enzyme systems. Alleles were designated sequentially with the most anodally migrating allele indicated with a superscript 'a' and those that follow with a superscript 'b'.

Data analyses

For allozyme analyses, a locus was considered polymorphic if the frequency of the most common allele did not exceed 0.99 (Young *et al*. 1996). The following allele frequency and genetic diversity parameters were estimated using the program POPGENE (Yeh *et al*. 1999): percentage of polymorphic loci (%*P*), mean number of alleles per locus (*A*), observed heterozygosity (H_o), and Nei's gene diversity (H_e).

To test the overall pattern of genetic structure in each population (isolation by distance model), we calculated the regression slope (b_F) of kinship coefficients (F_{ij}, Loiselle *et al*. 1995, Kalisz *et al*. 2001), the pairwise kinship coefficient between individuals i and j on the natural logarithm of r_{ij} (the distance between i and j), and then evaluated for significance using a Mantel test (999 replicates) under the null hypothesis of no spatial genetic structure ($b_F = 0$). To briefly visualize fine-scale genetic structure and to estimate the mean F_{ij} at the first distance, we calculated mean values of F_{ij} for successive distance intervals (lags, r = 1 m) and then plotted them against distance. For each distance interval, we constructed 95% confidence intervals (CI) for the null hypothesis of no genetic structure ($F_{ij} = 0$) using randomization procedures (Loiselle *et al*. 1995, Vekemans & Hardy 2004). These analyses were conducted using the program SPAGEDI (Hardy & Vekemans 2002). Since b_F depends to some extent on the sampling scheme (Fenster *et al*. 2003), we further calculated a statistic, Sp, which reflects better the rate of decrease of pairwise kinship with distance (Vekemans & Hardy 2004). The Sp statistic was calculated for each locus as $-b_F/(1 - F_{ij(d)})$, where $F_{ij(d)}$ is the mean F_{ij} calculated for enough pairs of individuals at the smallest distance interval (hence $F_{ij(d)} = F_{ij(1\,m)}$), and inverse of the Sp is an estimate of neighbor size, Nb (Vekemans & Hardy 2004).

Using the program FSTAT (Goudet 2000), we estimated Wright's (1965) F_{IS} and F_{ST} over polymorphic loci following the method of Weir and Cockerham (1984) to measure the mean level of inbreeding within and genetic differentiation among populations, respectively. For each population, F_{IS} was also calculated separately with 95% bootstrap confidence intervals (1000

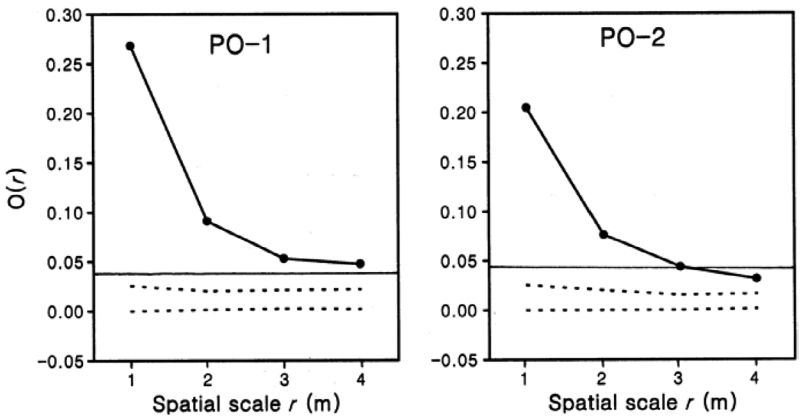

Fig. 2. Spatial aggregation of individuals within populations (PO-1 and PO-2) of *Pogonia minor* as measured using the *O*-ring statistic [*O*(*r*)]. Dots indicate the mean *O*(*r*) for an annulus of radius *r* with 1-m lags. Dashed lines indicate 99% confidence envelopes with 199 replicates about the null hypothesis of random spatial structure. The solid line indicates the first-order intensity of the point pattern within populations: 0.036 in PO-1 and 0.045 in PO-2.

replicates) constructed using the program GDA (Lewis & Zaykin 2001). To determine the relative contribution of biparental inbreeding and selfing, we obtained an equilibrial estimate of the selfing rate, $s = 2(F_{IS} - F_{ij(d = 1\ m)})/(1 + F_{IS} - 2F_{ij(d = 1\ m)})$, which assumes that pollen dispersal is restricted to very short distances (Fenster *et al.* 2003, Vekemans & Hardy 2004).

Results

Analyses of PO-1 and PO-2 using the *O*-ring function revealed a significant positive spatial aggregation of individuals at the scales of $r = 1$ – 4 m (Fig. 2). Although *O*(*r*) value of PO-1 was higher than in PO-2 at $r = 1$ m, the magnitude of patterns of spatial structure with increasing radius (*r*) was similar to each other (slope of the *O*-ring analysis; $b_O = -0.070$ and –0.055 in PO-1 and PO-2, respectively).

Of all the putative loci examined, the four loci *Fe*, *Me*, *Pgi-2*, and *Pgm*, harboring two alleles at each locus were polymorphic only in PO-2, resulting in low levels of genetic variation: the percentage of polymorphic loci within the population (*%P*) was 20%, mean number of alleles per locus (*A*) was 1.2, and mean genetic diversity (H_e) was 0.046 (Table 1). Furthermore, no allozyme variation was found in PO-1 and PO-3 (Table 1). Thus, the means of the three genetic parameters were extremely low: *%P* was 6.67%,

Table 1. Allele frequencies at four polymorphic loci, levels of genetic diversity, and genetic structure (F_{IS} and F_{ST}) in populations of *Pogonia minor* in South Korea. Abbreviations: *n* = sample size; *%P* = percentage of polymorphic loci; *A* = mean number of alleles per locus; H_e = Nei's gene diversity; F_{IS} = fixation index a population and 95% confidence interval (95% CI); F_{ST} = fixation index deviated from Hardy-Weinberg equilibrium attributable to each local population subdivision; – = analysis was not conducted because of monomorphism of all loci examined.

Population	Locus								*%P*	*A*	H_e	F_{IS} (95% CI)	F_{ST}
	Fe		*Me*		*Pgi-2*		*Pgm*						
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*					
PO-1 (*n* = 89)	1.000	0.000	1.000	0.000	0.000	1.000	0.000	1.000	0	1.0	0.000	–	
PO-2 (*n* = 70)	0.764	0.236	0.986	0.014	0.100	0.900	0.221	0.779	20	1.2	0.046	0.257 (0.067, 0.321)	
PO-3 (*n* = 26)	1.000	0.000	1.000	0.000	0.000	1.000	0.000	1.000	0	1.0	0.000	–	
Mean *n* = 61.7									6.67	1.07	0.015		0.196

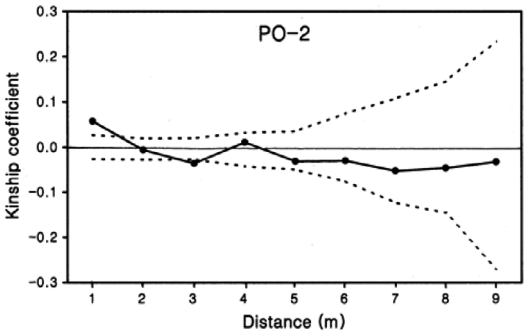

Fig. 3. Correlogram of mean estimated kinship coefficients (mean F_{ij}, filled circles over distance intervals, 1-m lags) within PO-2 of *Pogonia minor*. Dashed lines represent 95% CI constructed for the null hypothesis of $F_{ij} = 0$. The number of comparisons (pairs) represented by each distance class (from 1 m to 9 m) was 486, 643, 591, 323, 233, 76, 29, 26, and 8, respectively.

A was 1.07, and H_e was 0.015 (Table 1).

The spatial autocorrelation analysis revealed significant evidence of fine-scale genetic structuring within PO-2 (Fig. 3): $b_F = –0.045$ (95% CI for $b_F = 0$ is from –0.017 to 0.012) and Sp = 0.047. In addition, significant F_{ij} values were observed at 1-m and 3-m distances: $F_{ij(d = 1\ m)} = 0.058$ (95% CI for $F_{ij} = 0$ is from –0.026 to 0.027) and $F_{ij(d = 3\ m)} = –0.035$ (95% CI for $F_{ij} = 0$ is from –0.027 to 0.021) (Fig. 3). The estimate of neighbor size (Nb) was 21. A significant deficiency of heterozygotes was detected in PO-2 ($F_{IS} = 0.257$), since 95% CI (0.067 to 0.321) did not include zero (Table 1). Estimate of selfing rate, *s*, was 0.349. A significant degree of population differentiation was detected among populations (mean $F_{ST} = 0.196$, $P < 0.001$, 1000 randomizations of genotypes among samples; Table 1), suggesting limited gene flow between isolated populations.

Discussion

Low levels of genetic variation

As we expected, the two populations (PO-1 and PO-3) of *P. minor* harbor extremely low levels of genetic variation (no allozyme variation across 20 loci), and a low level of genetic variation was also revealed in PO-2. Evolutionary processes such as genetic drift, inbreeding, limited gene dispersal, and founder effects would be considered as explanatory factors for the observed genetic diversity in populations of *P. minor*.

Theoretically, the consequences of genetic drift are fixation of alleles and loss of heterozygosity. Although PO-1 and PO-2 are closely located (the linear distance is 258 m), alleles were fixed at four loci (*Fe*[a], *Me*[a], *Pgi-2*[b], and *Pgm*[b]) in PO-1, whereas two alleles at each locus were detected in PO-2 (Table 1). No allozyme variation was detected in PO-1 and PO-3 and low levels were found in PO-2. These results may be indirect evidence of genetic drift operating in the small and isolated populations of *P. minor*. Similar results were found other widely spread, but locally rare, endangered terrestrial orchids (*Listera ovata* in NE Poland, Brzosko & Wróblewska 2003; *Epipactis atrorubens* in NE Poland, Brzosko *et al*. 2006; *Epipactis thunbergii* in South Korea, Chung & Chung 2007) and epiphytic and lithophytic orchids (*Bulbophyllum drymoglossum* and *Sarcanthus scolopendrifolius* in South Korea, Chung *et al*. 2007b; *Amitostigma gracile* in South Korea, Chung & Park 2008).

The breeding system is considered as the main factor influencing genetic variation within plant populations (Hamrick & Godt 1990). Since *P. minor* is self-compatible, non-clonal, one-flowered orchid, autogamy is likely but geitonogamy is unlikely. Estimated selfing rate through probable autogamy was 0.349, which should contribute to the levels of genetic variability within populations. It should be noted that the fixation index ($F_{IS} = 0.257$) are attributable to selfing, biparental inbreeding, and a spatial Wahlund effect. Absence of allozyme diversity ($H_e = 0$) has been reported in highly inbred terrestrial orchids: *Cephalanthera damasonium* (Scacchi *et al*. 1991), *Epipactis phyllanthes* (Ehlers & Pedersen 2000), *Eulophia sinensis* and *Zeuxine gracilis* (Sun & Wong 2001), and *Liparis kumokiri* (Chung *et al*. 2005a).

The analyses of *O*-ring function, fine-scale genetic structure, and Wright's F_{ST} suggest a leptokurtic distribution of seed dispersal with most seeds recruitment around maternal plants, though we expect that a few seeds would dis-

perse a long distance (Trapnell & Hamrick 2004, Alcantara *et al*. 2006). In addition, the patterns suggested by these analyses (i.e., non-random spatial distribution of individuals, greater genetic relatedness among physically close individuals, and significant degree of population differentiation) suggest a pattern of isolation by distance at various levels. The analysis of *O*-ring function revealed significant aggregation of individuals, suggesting restricted seed dispersal within populations. In addition, the comparable slope (b_0) estimates in PO-1 and PO-2 may parallel with similar habitats and similar patchy distribution of microhabitats within populations.

The high estimate of Sp statistic (0.047) and the small neighbor size (Nb = 21), which was about one-third of the total sample (n = 69) found in PO-2 are further indicative of restricted pollen and seed dispersal in populations of *P. minor*. These as well as a significant allozyme divergence between populations provide indirect evidence of limited gene dispersal. One thing noted here is that the significant fine-scale genetic structure detected in *P. minor* is also found in populations of 12 other terrestrial and one epiphytic orchid species (Peakall & Beattie 1996, Chung *et al*. 1998, 2004a, 2004b, 2005a, 2005b, Machon *et al*. 2003, Trapnell & Hamrick 2004, Jacquemyn *et al*. 2006, Wallace 2006, Chung & Chung 2007, Chung & Nason 2007, Chung & Park 2008).

It is expected that a few seeds (founders) could occasionally be dispersed by a strong wind, and thus they are established in new habitats (Trapnell & Hamrick 2004, Alcantara *et al*. 2006), resulting in a loss of genetic diversity during colonization (founder effects). We suspect that PO-3 was recently established via colonization, since the population size and area (26 individuals in 20 m^2) were relatively small in the open habitat. Even though populations of *P. minor* are colonized recently from a few sources from genetically diverse populations (e.g., PO-2) and thus newly established populations consist of a few genetically distinct individuals, it is expected that alleles will be lost via random genetic drift, coupled with restricted gene flow. In sum, a combination of these factors and other unknown ecological processes would contribute to shape the contemporary population genetics of *P. minor* in South Korea.

Implications for conservation

The scarcity of allozyme variation found in *P. minor* warrants conservation concerns. We expect that the standing levels of genetic variation within populations of *P. minor* would be decreased further due to habitat destruction (e.g., building roads). Considering the current status of *P. minor*, we suggest that all known populations should be preserved for *in situ* conservation. When considering reintroduction, as many capsules as possible could be collected from individuals at 2 m intervals from PO-2, because individuals are genetically homogeneous within this distance (Fig. 3) and seeds can be propagated *in vitro* as a restoration strategy (Zettler *et al*. 2007). Based on the spatial information on the spatial distribution of individuals and fine-scale spatial genetic structure, these newly propagated individuals can be reintroduced to restore in the similar habitats located in Haenam-gun and coastal areas in southern Korea to minimize negative impacts (e.g., outbreeding depression and competitive exclusion of less-adapted genotypes; Turkington 1989, Templeton 1991). In addition, gene flow via pollinia transfer from genetically diverse populations (e.g., PO-2) to genetically depleted populations (e.g., PO-1 and PO-3) could artificially be made by hand cross-pollination between populations. In an attempt to develop an *ex situ* conservation, we also suggest collecting seeds from all known populations sampled at 2-m intervals within populations and propagating them in botanical gardens or other appropriate locations. At the same time, we suggest taking an appropriate amount soil from natural *P. minor* habitats in Haenam-gun to ensure an association with mycorrhizal fungi in *ex situ* habitats.

As other terrestrial orchids (*Bletilla striata*, *Platanthera mandarinorum, Pogonia japonica*, and *Spiranthes sinensis*) and four rare, carnivorous plants (*Drosera rotundifolia*, *D. peltata* var. *nipponica*, *Utricularia racemosa* and *U. bifida*) grow in the same and in nearby habitats as *P. minor* in Haenam-gun, it is recommended that

all these areas should be protected by regulations (i.e. designated as 'natural reserves' in South Korea) to maintain the total genetic diversity, to prevent further decrease of effective population size through human activities, and thus to ensure a long-term survival of these rare plants in South Korea.

Finally, to reach a comprehensive management of these terrestrial orchids, ecological studies (e.g., demographic dynamics, pollination biology, germination ecology, seedling establishment, and relationship with mycorrhizal fungi) also should immediately be initiated (Sieg & Ring 1995, Brzosko 2002, Nicolé *et al*. 2005, Ávila-Díaz & Oyama 2007, Yamato & Iwasa 2008).

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