Journal of Heredity 2012:103(5):692–702
doi:10.1093/jhered/ess032
Advance Access publication May 4, 2012

Genetic Diversity in the Common Terrestrial Orchid *Oreorchis patens* and Its Rare Congener *Oreorchis coreana*: Inference of Species Evolutionary History and Implications for Conservation

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Abstract

We hypothesized that the main Korean mountain ranges provided many refugia for boreal plant species, where they likely found relatively stable habitats and maintained large population sizes. Under this scenario, high levels of genetic variation and low degree of differentiation among populations within these species were anticipated. To test this hypothesis, we examined levels of allozyme diversity (17 loci) in 12 populations of the common terrestrial montane orchid *Oreorchis patens* from the main ranges in Korea and 4 populations of its rare congener *O. coreana*, which is restricted to the Korean island of Jeju. As expected, *O. patens* harbored high levels of genetic variation within populations (%$P = 62.8$, $A = 1.96$, $H_o = 0.211$, and $H_e = 0.237$). Allele frequency differences among populations were low ($F_{ST} = 0.075$), and the species also displayed a significant correlation between pairwise genetic differentiation and geographical distance. All these results suggest that extant populations were founded by multiple genetically diverse individuals and that most of this initial diversity would have been maintained in the stable mountainous conditions during Quaternary climatic oscillations. In contrast, we were unable to detect any genetic diversity in *O. coreana*, suggesting that contemporary populations likely originated from a single ancestral source population that had lost all genetic variability. From a long-term conservation genetics perspective, extreme rarity and small population sizes, coupled with its apparent genetic uniformity, place *O. coreana* at a high risk of extinction. Thus, both in situ and ex situ conservation efforts should be of particular importance for this species.

Key words: *allozymes, founder effect, Korea, montane, population history*

As expected from population genetics theory, widely distributed common plant species typically harbor higher genetic diversity than narrowly distributed, rare ones (reviewed in Hamrick and Godt 1989; Gitzendanner and Soltis 2000 and references therein). However, many exceptions have been found, and thus information on current distribution of plant species and present-day population sizes may not always be applicable to predict levels and distribution of genetic diversity in plant species. Many authors (Case 1994; Godt et al. 1995; Lewis and Crawford 1995) have suggested historical factors (e.g., founder effects and genetic bottlenecks caused by the Pleistocene glacial/interglacial cycles) as a plausible

explanation for those cases where levels of genetic diversity are difficult to explain by the usual suite of life-history and ecological traits (Loveless and Hamrick 1984; Hamrick and Godt 1989). In general, higher levels of genetic variation have been found in plant species growing in unglaciated areas (and other favorable areas for plant persistence, e.g., glacial refugia) relative to species in glaciated areas (Godt and Hamrick 2001; Kennedy and Walker 2007 and references therein, but see Wallace and Case 2000) as species occurring in glaciated regions would have lost genetic diversity through founder effects and bottlenecks a result of multiple stepwise colonization events (Cwynar and MacDonald 1987; Hewitt 1996; Broyles 1998; Widmer and Lexer 2001).

In the Korean Peninsula, during the last glaciation, glaciers were not present in the vast majority of the Peninsula and were restricted to the northernmost part of the country in high-elevation mountains of 2200–2800 m above sea level (a.s.l.), near the border with China (Kano 1937; Sasa and Tanaka 1938; Kong and Watts 1993; Shi 2002) (Figure 1). However, the nature of the vegetation present in unglaciated areas during the last glacial maximum (LGM, ca. 20,000 years ago) is under dispute. On the one hand, many paleovegetation reconstructions suggest the near absence of the currently occurring temperate vegetation, which would have experienced an important southward retreat, and that the Korean Peninsula was covered by nonforest vegetation (mainly steppe; Adams and Faure 1997) and/or by a mixture of boreal forests and treeless vegetation (Choi 1998; Harrison et al. 2001; Chung et al. 2006). On the other hand, others argue that temperate deciduous forests were present, perhaps with a scattered distribution (Kong 2000; Chung et al. 2010; Yi and Kim 2010). Indeed, Kong (2000) state that numerous thermophilous paleogenera have survived in Korea till now owing to the presence of refugia for temperate species. Thus, the country would have constituted suitable refugia for both boreal and temperate flora. For the former, the Korean Peninsula would have served as southern glacial refugia, as boreal flora suffered an important southward retreat during glacial periods, reaching the Korean Peninsula and the Japanese Archipelago (e.g., Harrison et al. 2001). For the temperate species, the peninsula would have harbored northern refugia, most likely as small "microrefugia" (sensu Rull 2009) since the bulk of the temperate flora suffered an equally significant glacial southward retreat.

Mountains have provided suitable refugia for plants in many parts of the world because of their ecoclimatic stability along the Quaternary climatic cycles and also because these allowed elevational shifts of plant species to track warm interglacials/cold glacials (Hewitt 2000; Tzedakis et al. 2002). In East Asia, mountains may have even played a more important role for the survival of boreal and temperate flora (López-Pujol et al. 2011; Qiu et al. 2011).

During the cold periods of the Pleistocene, extant boreal plant species are considered to have migrated south into the Korean Peninsula from more north located ancestral populations of these species (Im 1992; Kong and Watts 1993). Today, these species are widely distributed in both

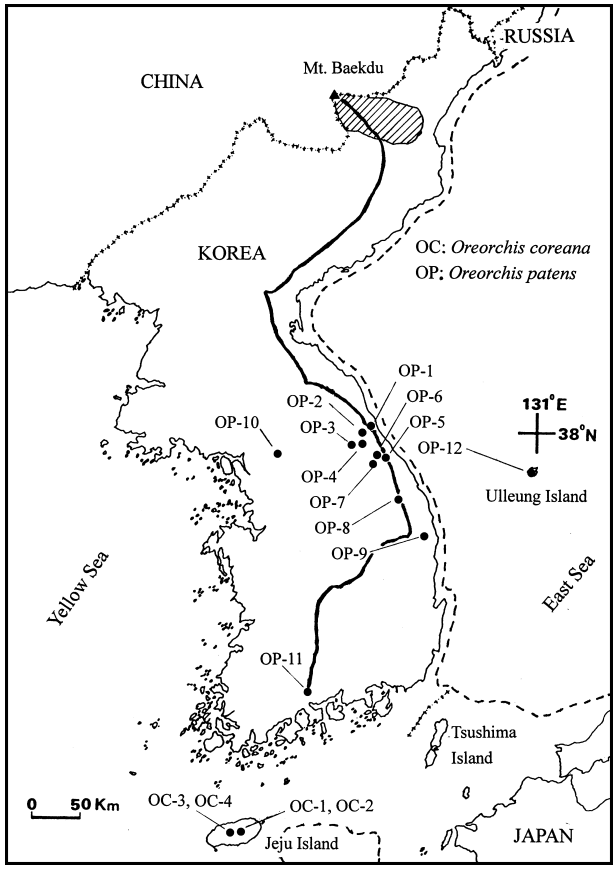

Figure 1. Locations of sampled populations of *Oreorchis coreana* (OC-1 to OC-4) and *O. patens* (OP-1 to OP-12) in the Korean peninsula. Solid line indicates a main mountain range of the so-called "*Baek-du-dae-gan*," which runs north to south along the Korean Peninsula, and slashed area represents the Pleistocene-glaciated high-elevated mountains in the Korean Peninsula: Mountains Baekdu (2744 m), Kwanmo (2451 m), Solryeong (2442 m), Nampodae (2435 m), and Pukpodae (2289 m). Dashed line represents exposed coastal lines during the LGM (Shi 2002); however, the dating and configuration of Late Tertiary/Quaternary land bridges between southwestern Korea and southern Japan (Korea/Tsushima Strait) are still controversial (Ota 1998).

subarctic regions (Amur, Kamchatka, Sakhalin, and Ussuri in Far East Russia) and temperate northeastern Asia (China, Japan, and Korea). Considering this, together with the information available on the Quaternary paleoecology in the Korean Peninsula (see above), we hypothesize 2 scenarios in relation to the levels of genetic variation within populations and degree of genetic divergence among populations of boreal plant species native to the Korean Peninsula. In the first scenario, boreal plant species would have found large refugial areas during the cold periods of the Quaternary, inhabiting "stable" habitats throughout the main Korean mountain ranges (the so-called "*Baek-du-dae-gan*," which runs north to south along the Korean Peninsula; Figure 1). The

topography of these mountain ranges would have allowed these boreal plant species to have persisted there throughout the glacial/interglacial cycles (simply through altitudinal shifts), presumably maintaining large effective population sizes (Kong and Watts 1993). Under this scenario, we expect that levels of genetic variation within populations would be moderate or high and the degree of differentiation among populations would be moderate or low in these species (e.g., Chung et al. 2009). Similar to the first scenario, the second scenario is also of southward migration of boreal flora, but refugial glacial populations in Korea would be small, consistent with the predictions of Adams and Faure (1997) that steppe largely dominated the Peninsula. Thus, small spatially isolated populations would have been maintained in favorable pockets (e.g., microrefugia) along the mountainous regions of the Peninsula. If the extant populations are derived from multiple refugial source populations that had experienced substantial random genetic drift (because of their small size), we may expect low levels of genetic variation within and a high degree of genetic divergence among extant populations. Alternatively, if contemporary Korean populations of boreal species originated from a single ancestral population that had lost most of its genetic variability, one may expect low levels of genetic variation within and among extant populations. To date, these scenarios have never been empirically tested for boreal plant species native to the Korean Peninsula.

Populations of many orchids, but in particular of rare or endemic species, are small and isolated, and have increasingly been a major subject of conservation concerns (Swarts and Dixon 2009a, 2009b; Seaton et al. 2010; Vereecken et al. 2010; Phillips et al. 2011). Studies on conservation genetics of rare orchid species have often been conducted by comparing them with their common congeners (e.g., Sun 1996; Case et al. 1998; Gustafsson and Sjögren-Gulve 2002; Li et al. 2002; Chung et al. 2005; Campbell et al. 2007; Chung et al. 2009). Plant congeners usually share life-history traits, in particular breeding systems and seed dispersal mechanisms; thus, comparisons of congeneric species pairs allow for partial control of "background variation" caused by different life-history traits (Karron 1987, 1991, reviewed in Godt and Hamrick 2001).

In this study, we selected 2 congeneric terrestrial orchids native to Korea, *Oreorchis patens* and *O. coreana*, to infer the recent (the Quaternary) evolutionary history of these 2 montane congeneric species based on their genetic structure. *Oreorchis patens*, a boreal species, has a wider geographic distribution, encompassing Far East Russia (Amur, Kamchatka, Sakhalin, and Ussuri), China, Japan, Korea, and Taiwan (Lee 2007). In Korea, *O. patens* commonly occurs in temperate deciduous forest (main altitudinal range: 600–1460 m a.s.l.) in the main ranges of *Baek-du-dae-gan* (Figure 1), and the number of shoots per population ranges from tens to hundreds. It also occurs on Ulleung Island off the coast of eastern central Korea (OP-12 in Figure 1), but not on Jeju Island (Lee 2011). The current distribution patterns of *O. patens* (i.e., the occurrence of many large populations throughout the Korean Peninsula, particularly in central and northern Korea) suggest that the past history of this species would be closer to the first scenario. Thus, we would expect levels of genetic variation to be high or moderate within populations and the degree of differentiation to be moderate or low among populations of this species. In contrast, its congener *O. coreana* is restricted to hillsides of mixed forests of deciduous and broad-leaved evergreen shrubs and trees (altitude 400–800 m a.s.l.) on Jeju Island off the coast of the southern Korea (Figure 1). The species exhibits a strong preference for wet habitats (damp areas), and only a few populations of low density ($<$50 shoots/100 m^2 per local population) are known. Considering these traits, we predict low levels of genetic diversity for this rare orchid both within and among populations. Genetic data obtained from this study will provide guidelines for the recovery and management of the rare orchid *O. coreana*.

Materials and Methods

Study Plants

From May to July, adults of *O. patens* produce scapes (30–50 cm tall), each bearing 20–35 whitish-yellow flowers (7–15 mm long, 1.5–3 mm in diameter). These flowers, which have faint odor, are pollinated by the large syrphid fly *Melansostoma scalare* (Diptera: Syrphidae) (Sugiura et al. 1997). Flowering scapes (10–60 cm tall) of *O. coreana* are produced during June and July, each bearing 15–30 orange-brown flowers (5.1–7 mm long, 1–2 mm in diameter). The pollinators of *O. coreana* are unknown. This species was described as a new species one century ago, but it was subsequently placed in its own monotypic genus (*Diplolabellum*) because it lacked the caudicle (a stalk attached to pollinium) that is a diagnostic character in *Oreorchis* (Maekawa 1935). More recently, however, molecular phylogenetic analysis using sequence data (ITS, *mat*K, *trn*L-*trn*F, and *trn*T-*trn*L) strongly support treating the species as *O. coreana* (Eum et al. 2008). *Oreorchis patens* and *O. coreana* reproduce both sexually and vegetatively via newly formed pseudobulbs (ca. 1 cm per year). As found in many orchids, fruits (capsules) of both species contain large numbers of tiny seeds.

Population Sampling

For *O. patens*, we collected 849 leaf samples (N_T; Table 1) from 12 populations (OP-1 to OP-12), including 2 isolated populations (OP-11 and OP-12; Figure 1). For *O. coreana*, we sampled a total of 50 leaf samples (N_T) from 4 populations from Jeju Island (OC-1 to OC-4; Figure 1). Populations OC-1 (N_T = 13) and OC-2 (N_T = 16) are separated by approximately 1.3 km, and population OC-3 (N_T= 12) is located approximately 1.5 km north of OC-4 (N_T = 9). Since the number of shoots within *O. coreana* is small, we collected all visually identified shoots; for *O. patens*, the larger size of its populations allowed us to sample one shoot from each patch to avoid collection of the same ramets. To minimize the damage to these orchids, we cut only 2 cm from the tip of leaf per shoot.

Table 1 Summary of clonal diversity measures observed in 12 populations of *Oreorchis patens*

Populations	N_T	N_G	P_{gen} F_{IS}	P_{sex} F_{IS}	R	D
OP-1	144	118	6.64^{-5}	0.0059	0.818	0.996
OP-2	22	21	4.60^{-4}	0.0407	0.952	0.996
OP-3	78	64	2.05^{-4}	0.0081	0.818	0.987
OP-4	20	15	4.00^{-4}	0.0055	0.737	0.958
OP-5	98	87	1.96^{-4}	0.0239	0.887	0.999
OP-6	35	27	7.78^{-5}	0.0005	0.765	0.978
OP-7	60	56	1.78^{-4}	0.0111	0.932	0.998
OP-8	147	104	3.49^{-4}	0.0319	0.705	0.992
OP-9	24	22	2.10^{-3}	0.0405	0.913	0.993
OP-10	109	104	7.36^{-5}	0.0005	0.954	0.999
OP-11	83	56	3.40^{-4}	0.0209	0.671	0.987
OP-12	29	26	4.75^{-4}	0.0051	0.893	0.990
Average	71	58	4.10^{-4}	0.0162	0.837	0.989

Abbreviations: N_T, the number of individuals sampled (including clonal ramets); N_G, the number of individuals excluding clonal ramets; P_{gen} F_{IS}, probability of the identical MLGs occurring by chance due to sexual reproduction by taking into account departures from Hardy–Weinberg (H-W) equilibrium; P_{sex} F_{IS}, probability of the second encounter of identical MLGs via sexual reproduction by taking into account departures from H-W equilibrium; R, genotypic richness; and D, Simpson diversity index of clonal heterogeneity.

Electrophoresis Procedures

Leaf samples were wrapped in damp paper towel, placed in plastic bags, returned to the laboratory, and then stored at 4 °C until protein extraction. For extraction, leaf samples were crushed using chilled mortars and pestles by adding a phosphate-polyvinylpyrrolidone buffer (Mitton et al. 1979) and enzyme extracts were absorbed onto 4 × 6 mm paper wicks (Whatman 3MM chromatography paper). We conducted electrophoresis on 13% starch gels, with 2 buffer systems. We used a modification (Haufler 1985) of the system 6 of Soltis et al. (1983) to resolve alcohol dehydrogenase (*Adh*), cathodal peroxidase (*Per*), diaphorase (*Dia-1*, *Dia-2*), fluorescent esterase (*Fe*), leucine aminopeptidase (*Lap*), malic enzyme (*Me*), phosphoglucomutase (*Pgm*), and triosephosphate isomerase (*Tpi-1*, *Tpi-2*). We also used the morpholine-citrate buffer system (pH 6.1) of Clayton and Tretiak (1972) to resolve fructose-1,6-diphosphatase (*F1,6*), isocitrate dehydrogenase (*Idh-1*, *Idh-2*), malate dehydrogenase (*Mdh-1*, *Mdh-2*, *Mdh-3*), and 6-phosphogluconate dehydrogenase (*6Pgd*). We followed stain recipes from Soltis et al. (1983) except for diaphorase (Cheliak and Pitel 1984). We designated putative loci sequentially, with the most anodally migrating isozyme designated as *1*, the next as *2*, and so on. We also designated different alleles within each locus sequentially by alphabetical order. The observed enzyme banding patterns were consistent with their typical subunit structure and subcellular compartmentalization in diploid plants (Weeden and Wendel 1989).

Data Analysis

Multiple ramets (N_T) representing allozyme-based identical multilocus genotypes (MLGs) could result either from distinct sexual reproduction events or clonal propagation. As it is important to discriminate these cases, we calculated P_{gen} F_{IS}, the probability of the MLG occurring by chance due to sexual reproduction by taking into account observed departures from Hardy–Weinberg (H-W) equilibrium (Parks and Werth 1993; Arnaud-Haond et al. 2007). We calculated P_{gen} F_{IS} for each MLG and obtained an average over distinct MLGs within each population. We also estimated P_{sex} F_{IS}, the probability of a second encounter of an identical MLGs produced via sexual reproduction, again taking into account departures from H-W equilibrium (Parks and Werth 1993; Arnaud-Haond et al. 2007). We obtained an average P_{sex} F_{IS} for each population calculated over individual ramets. We used a $P < 0.05$ cutoff for the discrimination of ramets versus genets. P_{sex} F_{IS} is considered a conservative estimate of clonal identity (Arnaud-Haond et al. 2007). Once clones were identified, we prepared a second data set (N_G) in which each distinct MLG was only represented once and clonal ramets were excluded. Using N_G, we calculated genotypic richness (R) and Simpson diversity index of clonal heterogeneity (D), the probability of encountering distinct MLGs when randomly taking 2 units in a population (Arnaud-Haond et al. 2007). We calculated genotypic richness according to the equation $R = (N_G - 1)/(N_T - 1)$ (Dorken and Eckert 2001). For all these calculations, we used the program GenClone 2.0 (Arnaud-Haond and Belkhir 2007). We also used N_G to calculate genetic diversity and structure parameters (see below).

We considered a locus as polymorphic within a population if the frequency of the most common allele did not exceed 0.99 (Young et al. 1996). We estimated the following allele frequency and genetic diversity parameters using the programs POPGENE (Yeh et al. 1999) and FSTAT (Goudet 1995): mean percentage of polymorphic loci (%*P*), mean number of alleles per locus (*A*), allelic richness (*AR*) using a rarefaction method that compensates uneven population sample sizes (Hurlbert 1971; El Mousadik and Petit 1996), observed heterozygosity (H_o), and Nei's (1978) unbiased gene diversity (H_e).

To gain insight into the role of bottlenecks in relation to genetic diversity within populations, we conducted correlation analyses between log-transformed sample size (N_G) and *AR*, %*P*, *A*, and H_e. Among these parameters, *AR* is known to be highly dependent on effective population size and is, therefore, more useful for identifying historical processes such as bottlenecks (Luikart, Sherwin, et al. 1998; Widmer and Lexer 2001). To test for recent decreases in effective population size (bottlenecks), we evaluated for individual loci, the difference between the H-W H_e and the equilibrium heterozygosity (H_{eq}) expected from the number of alleles assuming mutation-drift equilibrium. These differences were evaluated using a sign test and a Wilcoxon sign-rank test conducted across loci under an infinite allele model using the program BOTTLENECK (Cornuet and Luikart 1996). Since allelic diversity is generally lost more rapidly than H_e (Nei et al. 1975), recently bottlenecked populations are expected to exhibit an excess of H-W equilibrium H_e

relative to H_{eq} (Cornuet and Luikart 1996; Luikart, Allendorf, et al. 1998).

To measure deviations from H-W equilibrium, we calculated Wright's (1965) F_{IS} and F_{ST} over loci following Weir and Cockerham (1984). These fixation indices measure the average deviation from H-W equilibrium of individuals relative to their local populations (F_{IS}, a measure of local inbreeding) and local populations relative to the total population (F_{ST}, also a measure of differentiation among local populations). The significance of multipopulation F_{IS} and F_{ST} estimates was determined by a permutation test (1000 randomizations of alleles among individuals within samples and 1000 randomizations of genotypes among populations, respectively). These calculations were performed using FSTAT (Goudet 1995). Furthermore, we used the program SPAGeDi (Hardy and Vekemans 2002) to calculate population-level F_{IS} (inbreeding) and its significance level by 1000 permutations under the null hypothesis of $F_{IS} = 0$. To test for differences between populations, we approximated 95% CI around F_{IS} as 1.96 times the jackknifed standard error (Loiselle et al. 1995).

To determine the relative importance of gene flow and genetic drift at a regional scale, we analyzed the correlation analysis between pairwise F_{ST} (Weir and Cockerham 1984) and linear geographic distance (kilometer). This approach (Hutchison and Templeton 1999) is based on a stepping-stone model of population genetic structure, that is, a form of isolation-by-distance model with migrants moving only among neighboring discrete populations in 1D and 2D habitats. If a positive linear relationship exists, it suggests that populations are at regional equilibrium between gene flow and drift, that is, isolation by distance. Hutchison and Templeton (1999) also identified 3 other cases of non-equilibrium between gene flow and drift based on degrees of scatter in plotted points: gene flow relatively more important than genetic drift; gene flow relatively less important than genetic drift; and gene flow more important at shorter distances and drift more important at greater distances. We tested a linear regression model using the Mantel test (Mantel 1967) by making 999 replicates. Finally, to determine the degree of genetic divergence among populations of each species and between *O. coreana* and *O. patens*, we calculated Nei's (1978) unbiased genetic identity (I) and distance (D) between pairs of populations and between the 2 species. Using the Nei's D values, we clustered populations into a phenogram using the unweighted pair groups method with arithmetic averages (UPGMA).

Results

Identification of Clones

For *O. patens*, 13 of the 17 isozyme loci were polymorphic while 4 loci, *Adh*, *Dia-2*, *Me*, and *Per*, were monomorphic across the study 12 populations. All the MLGs in the 12 populations had P_{gen} F_{IS} and P_{sex} F_{IS} values < 0.05 (Table 1). Then we conservatively considered that ramets sharing identical MLGs were repeated samples from the same clone. Using this criterion, we identified a total of 702 MLGs (N_G) of 849 total samples (N_T) (Table 1). Estimates of genotypic richness, calculated as $R = (N_G - 1)/(N_T - 1)$, were high, ranging from 0.671 (OP-11) to 0.954 (OP-10) with a mean of 0.837 (Table 1). All Simpson diversity indices were >0.90 and similar across populations with a mean of 0.989 (Table 1). In contrast, we were unable to detect any genetic variation in *O. coreana*. Thus, we failed to separate genets (N_G) from total samples (N_T) in this species.

Levels of Genetic Diversity in *O. patens*

At the population level, the mean percentage of polymorphic loci (%P) and the mean number of allele per locus (A) were 62.8 and 1.96, respectively, and the means of observed (H_o) and expected heterozygosity (H_e) were 0.211 and 0.237, respectively (Table 2). Higher values for these genetic diversity measures were estimated from pooled samples over all populations ($N_G = 702$): %$P = 76.5$; $A = 2.53$; $H_o = 0.214$; and $H_e = 0.258$. Populations OP-9 and OP-10 harbored the lowest and highest allelic richness ($AR = 1.68$ and 1.99, respectively), and populations OP-9 and OP-6 showed the lowest and the highest expected heterozygosity ($H_e = 0.209$ and 0.260, respectively; Table 2). There were significant correlations between population size (N_G) and mean AR (Spearman's rank correlation coefficient, $r_s = 0.709$, $P = 0.010$), %P ($r_s = 0.752$, $P = 0.005$), and A ($r_s = 0.819$, $P = 0.001$). However, we found no significant correlation for H_e ($r_s = 0.515$, $P = 0.087$). Consistent with these findings, we detected significant indications of recent bottlenecks in several populations based on both sign (4 populations) and Wilcoxon sign-rank tests (7 populations) conducted using BOTTLENECK (Table 3).

Population Genetic Structure and Inbreeding in *O. patens*

Population-level F_{IS} estimates calculated over 13 polymorphic loci did not differ significantly among populations (broadly overlapping 95% CIs; Table 2). Except for OP-5 ($F_{IS} = -0.036$), all F_{IS} values were positive, ranging from 0.014 (OP-2) to 0.206 (OP-4) (Table 2). With the exception of 4 populations (OP-2, OP-5, OP-6, and OP-9), F_{IS} values were significant at 0.05 level (Table 2). These results, as well as a significant multipopulation level F_{IS} ($F_{IS} = 0.115$, $P = 0.001$; Table 2), indicate a deficit of heterozygotes within populations that is consistent with local inbreeding. Allele frequency differentiation calculated over polymorphic loci among populations was low but significant ($F_{ST} = 0.075$, $P = 0.001$).

Following the method of Hutchison and Templeton (1999), we found a significant positive linear relationship between pairwise F_{ST} and linear geographical distance ($r = 0.541$, $P = 0.000$), suggesting that populations are at regional equilibrium of gene flow and drift, following an isolation-by-distance pattern (Figure 2). About 70% of the variation in genetic differentiation was due to unknown factors other than geographic distance. In agreement with this, the UPGMA phenogram showed a moderate association between populations in relation to their geographic

Table 2 Summary of genetic diversity measures and mean fixation (F_{IS}) values observed in 4 populations of *Oreorchis coreana* and 12 populations of *O. patens*

Species/populations	Alt (m)	%*P*	*AR*	*A*	H_o (SE)	H_e (SE)	F_{IS} (95 CI)
Oreorchis coreana							
4 populations	715–730	0.0	1.00	1.00	0.000 (0.000)	0.000 (0.000)	—
O. patens							
OP-1	680	70.6	1.93	2.18	0.219 (0.050)	0.246 (0.059)	0.116[a] (0.017, 0.214)
OP-2	1420	58.8	1.84	1.88	0.224 (0.059)	0.227 (0.055)	0.014 (−0.156, 0.184)
OP-3	600	70.6	1.88	2.12	0.214 (0.056)	0.240 (0.063)	0.108[a] (−0.090, 0.306)
OP-4	880	47.1	1.71	1.71	0.188 (0.059)	0.237 (0.066)	0.206[a] (−0.087, 0.499)
OP-5	840	64.7	1.86	2.00	0.258 (0.068)	0.249 (0.065)	−0.036 (−0.217, 0.145)
OP-6	680	64.7	1.92	1.94	0.244 (0.064)	0.260 (0.063)	0.062 (−0.145, 0.269)
OP-7	1020	76.5	1.96	2.18	0.198 (0.050)	0.245 (0.060)	0.193[a] (−0.003, 0.388)
OP-8	1350	64.7	1.85	1.94	0.190 (0.047)	0.228 (0.058)	0.167[a] (−0.020, 0.355)
OP-9	620	58.8	1.68	1.71	0.195 (0.063)	0.209 (0.056)	0.064 (−0.342, 0.469)
OP-10	220	70.6	1.99	2.18	0.220 (0.054)	0.251 (0.061)	0.122[a] (−0.011, 0.255)
OP-11	920	58.8	1.84	1.94	0.191 (0.047)	0.235 (0.057)	0.122[a] (−0.028, 0.271)
OP-12	780	47.1	1.69	1.71	0.188 (0.053)	0.217 (0.062)	0.135[a] (0.001, 0.269)
Average	834	62.8	1.85	1.96	0.211 (0.007)	0.237 (0.004)	0.115[b]
Pooled samples	—	76.5	—	2.53	0.214	0.258	—

Abbreviations: Alt (m), altitude above sea level (a.s.l.) in meters; %*P*, percentage of polymorphic loci; *AR*, mean allelic richness (adjusted for a sample size of 15 plants); *A*, mean number of alleles per locus; H_o, observed heterozygosity; H_e, H-W expected heterozygosity or genetic diversity; and F_{IS}, fixation index within populations. SE and 95% CI denote standard error and 95% confidence intervals, respectively.

[a] Denotes significance ($P < 0.05$) based on permutation (999 replicates) under the null hypothesis of $F_{IS} = 0$.

[b] Significant Weir and Cockerham (1984) estimate of F_{IS} over populations.

locations (Figure 3). As sometimes expected in geographically isolated populations, the 3 isolated populations (OP-11, OP-9, and OP-12) clustered basally relative to the remaining populations (Figure 3). Finally, pairwise Nei's (1978) *I* values for *O. patens* ranged from 0.925 (OP-6 vs. OP-12) to 0.992 (OP-7 and OP-8) with a mean of 0.967 ± 0.015 (SD), and the average *I* value between *O. coreana* and *O. patens* was 0.588 ± 0.027. These estimates fall well within the range of values for most conspecific orchid populations and congeneric orchid species pairs (average $I = 0.955 \pm 0.051$, $N = 84$ and average $I = 0.453 \pm 0.274$, $N = 190$; Chung MY and Chung MG 2012).

Table 3 Results of statistical tests for evidence of recent population bottlenecks in *Oreorchis patens*

Population	Sign test	Wilcoxon sign-rank test
OP-1	0.259	0.075
OP-2	0.128	0.312
OP-3	0.266	0.102
OP-4	0.004	0.002
OP-5	0.178	0.033
OP-6	0.029	0.042
OP-7	0.219	0.188
OP-8	0.163	0.049
OP-9	0.284	0.138
OP-10	0.038	0.031
OP-11	0.119	0.007
OP-12	0.032	0.004

Numbers reported are *P* values of sign and Wilcoxon sign-rank tests conducted using the program BOTTLENECK.

Discussion

Lack of Genetic Diversity in *O. coreana*: Evolutionary Clues

As sometimes occurs for extremely rare species with small population sizes, a complete lack of allozyme variation within and among populations was found in *O. coreana* on Jeju Island. In contrast, high levels of genetic variation within populations and a low degree of genetic differentiation between populations were detected in *O. patens* ($H_e = 0.237$;

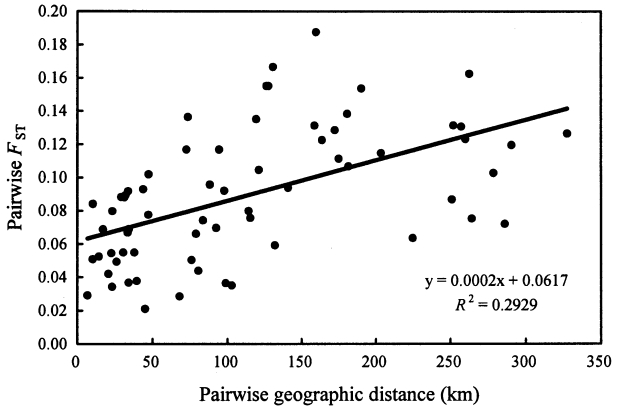

Figure 2. Differentiation among *Oreorchis patens* populations. Multilocus estimates of pairwise differentiation (F_{ST}) are plotted against linear geographical distances (in kilometers) according to Hutchison and Templeton (1999). There was a significant positive linear relationship between pairwise F_{ST} and linear geographical distance ($r = 0.541$, $P = 0.000$).

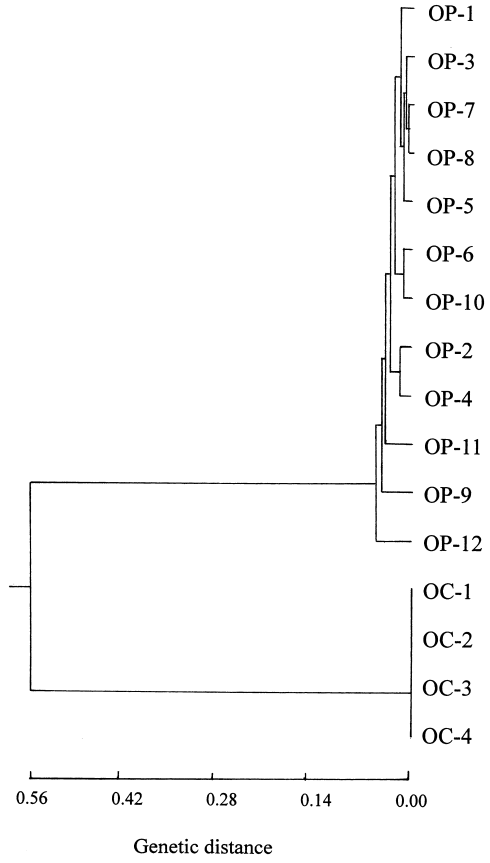

Figure 3. UPGMA phenogram based on Nei's (1978) genetic distances between populations of *Oreorchis coreana* (OC-1 to OC-4) and *O. patens* (OP-1 to OP-12).

Table 4 Weighted average allele frequencies for 13 polymorphic loci of *Oreorchis coreana* and *O. patens*

Locus	Allele	*O. coreana*	*O. patens*	Locus	Allele	*O. coreana*	*O. patens*
Mdh-1	*a*	**1.000**	0.000	*F1,6*	*a*	1.000	0.491
	b	0.000	**0.053**		*b*	0.000	0.282
	c	0.000	**0.947**		*c*	0.000	0.197
					d	0.000	0.026
Mdh-2	*a*	**1.000**	0.000		*e*	0.000	0.005
	b	0.000	**0.007**				
	c	0.000	**0.889**	*Lap*	*a*	0.000	0.605
	d	0.000	**0.104**		*b*	1.000	0.395
Mdh-3	*a*	1.000	0.583	*Pgm*	*a*	0.000	0.189
	b	0.000	0.398		*b*	1.000	0.791
	c	0.000	0.018		*c*	0.000	0.020
6Pgd	*a*	0.000	0.006	*Dia-1*	*a*	**1.000**	0.000
	b	1.000	0.038		*b*	0.000	**0.519**
	c	0.000	0.956		*c*	0.000	**0.479**
					d	0.000	**0.001**
Idh-1	*a*	0.000	**0.010**				
	b	**1.000**	0.000	*Fe*	*a*	1.000	0.001
	c	0.000	**0.193**		*b*	0.000	0.079
	d	0.000	**0.282**		*c*	0.000	0.920
	e	0.000	**0.213**				
	f	0.000	**0.303**	*Tpi-1*	*a*	1.000	0.813
					b	0.000	0.187
Idh-2	*a*	1.000	0.786				
	b	0.000	0.212	*Tpi-2*	*a*	0.000	0.001
	c	0.000	0.002		*b*	1.000	0.999

Boldface text indicates alleles unique to *O. coreana* (4 alleles) or *O. patens* (13 alleles).

F_{ST} = 0.075). These contrasting levels of genetic diversity and population genetic structure suggest that the 2 *Oreorchis* species have had different evolutionary histories. Rare species with small population sizes are highly susceptible to random genetic drift, promoting allelic fixation at neutral loci within extant populations. The original population of *O. coreana* was probably established with low levels of genetic diversity. Subsequently, the small ancestral population lost its genetic diversity via genetic drift. Populations established after all or most of the variation that had been lost from the ancestral population would most likely be fixed for the same alleles.

Among *Oreorchis* species, only *O. coreana* grows on Jeju Island (Lee 2011). The low estimate of Nei's (1978) genetic identity between *O. coreana* and *O. patens* (average I = 0.588) and the high number of species specific alleles each at high frequency (4 alleles unique to *O. coreana* and 13 alleles unique to *O. patens*; Table 4) suggest that the 2 species may have been genetically isolated for a long time. In addition, a strict consensus tree (Eum et al. 2008) based on combined ITS, *mat*K, *trn*L-*trn*F, and *trn*T-*trn*L regions revealed that *O. coreana* (endemic to Jeju Island) is sister to a clade formed by *O. patens* and *O. fargesii* (endemic to mainland China and Taiwan) (bootstrap = 98%), which confirms the clear genetic divergence between *O. coreana* and *O. patens*.

It should be taken into account that transgressions and regressions of the East China Sea (including the Jeju Strait, a small sea between southern Korean coast, and Jeju Island; Figure 1) were frequent since the late Miocene (ca. 10 Ma), but particularly during the Quaternary (Qiu et al. 2011). During these periods, plant species have had many opportunities for migration between southern China and southern Korea (and also southern Japan). In addition, migrations could also occur from East China and even North China through the Yellow Sea (see Figure 1 for exposed coastal areas during the LGM). Thus, we hypothesize that the ancestor of *O. coreana* (probably the same ancestor that also gave rise to *O. patens* and *O. fargesii*) arrived via seed dispersal on Jeju Island not much later than the island started to be formed approximately 2 Ma (Yoon 1997), originating from the continental Korean Peninsula or even from southern China. This hypothesis should be tested by employing more adequate markers for phylogeographic inference (e.g., chloroplast and nuclear DNA sequence) and molecular dating techniques.

Genetic Diversity and Structure in *O. patens*: Insights into Glacial Refugia for Boreal Montane Species

Levels of within-population genetic diversity are high in *O. patens*, with the average proportion of polymorphic loci

(%P) equal to 62.8%, the number of alleles per locus (A) ranging from 1.71 to 2.18, and the expected heterozygosity (H_e) ranging from 0.209 to 0.251 (Table 2), all of which are substantially higher than typical of allozyme-based studies of other terrestrial orchid species (means %P = 46.2%, A = 1.83, H_e = 0.119; Case 2002). Genetic differentiation among populations of *O. patens* is low (F_{ST} = 0.075), a value substantially below the average for orchids (a mean of 0.187 from F_{ST} or from analogous statistics, N = 76; Forrest et al. 2004).

This high diversity and low degree of genetic differentiation indicate that extant populations of *O. patens* were probably founded by multiple genetically diverse individuals and that most of this initial diversity has been maintained in the stable mountainous conditions throughout the Quaternary glacial/interglacial cycles via a combination of relatively large population sizes and recurrent interpopulation gene flow. Indeed, the low value of F_{ST}, coupled with the significant correlation between pairwise genetic differentiation and geographical distance, suggests that historical gene flow among neighboring populations of this orchid has been high along the main mountainous ranges in the Korean Peninsula. The spatial distribution of genetic diversity in *O. patens* is, thus, consistent with the first scenario proposed in the Introduction, that this montane species found many areas for survival (i.e., glacial refugia) along the *Baek-du-dae-gan,* where it maintained relatively large population sizes and where gene flow could occur. Despite the evidence of recent bottlenecks that we detected in several populations (Table 3), combined with interpopulation gene flow, the populations appear to have been large enough to limit the loss of alleles through random genetic drift (Sun 1996). In fact, differences in population size alone between the 2 orchid species can easily explain their contrasting observed levels of genetic variation. The role of glacial refugia in maintaining large population sizes of plant species and, consequently, high levels of genetic diversity is generally acknowledged (e.g., Hewitt 1996, 1999, 2000; Comes and Kadereit 1998; Médail and Diadema 2009; Qiu et al. 2011; but see Petit et al. 2003).

The slight deficit of heterozygotes within populations (mean F_{IS} = 0.115; Table 2) is likely caused by non-random mating among relatives (resulting from limited seed dispersal and localized pollinator movements) and/or geitonogamous selfing (because *O. patens* is self-compatible; Sugiura et al. 1997; Chung MY and Chung MG, unpublished data). Comparison between the 2 estimates F_{IS} and F_{ij}, kinship coefficients between individuals i and j on the natural logarithm of distance interval (Loiselle et al. 1995), could provide some clue about possibility of the occurrence of selfing. If F_{IS} is substantially higher than the near-neighbor F_{ij}, then selfing is at least partially responsible for excess homozygosity within populations. Consistent with this, we found considerably higher F_{IS} (0.122; Table 2) than F_{ij} (0.059) at the smallest distance interval in OP-11 (Chung MY and Chung MG, unpublished data). Finally, sampling may have occurred over a substructured population (i.e., the Wahlund effect), which could also increase observed homozygosities.

Since predominantly outcrossing or mixed-mating orchid species generally harbor higher levels of genetic variation than predominantly selfing or apomictic orchids (Scacchi et al. 1991; Sun 1996; Sun and Wong 2001), it is reasonable to think that the mixed-mating system of *O. patens* would contribute to harbor high levels of genetic variation within populations. Nevertheless, detailed field studies are needed to shed light on the contribution of the reproductive system to the current levels of genetic variability of this orchid.

Besides *O. patens*, other boreal montane species native to Korea have also showed high levels of genetic diversity and low differentiation, suggesting a common pattern. The only allozyme-based study for terrestrial orchids from the main mountainous ranges in the Korean Peninsula is that of *Cypripedium macranthos*, a boreal and widely distributed species in northeastern Asia. Chung et al. (2009) examined 4 populations of *C. macranthos* and, despite the orchid's alarming decline due to overcollection, they found that allozyme diversity was high within populations (%P = 46.7%, A = 1.47, H_e = 0.185) and low among populations (F_{ST} = 0.077). High levels of genetic variation within populations and moderate or low interpopulation differentiation were also found in 2 herbaceous plants occurring in the main mountainous ranges in the Korean Peninsula (%P = 67.7%, A = 2.06, H_e = 0.182, G_{ST} = 0.132 for *Hanabusaya asiatica*; Chung et al. 2001; and %P = 59.4%, A = 2.56, H_e = 0.259, G_{ST} = 0.027 for *Adenophora grandiflora*; Chung and Epperson 1999) and one coniferous species (%P = 45%, A = 1.78, H_e = 0.192, G_{ST} = 0.056 for *Taxus cuspidata*; Chung et al. 1999). All these studies are in agreement with the scenario of multiple refugia and recurrent gene flow along the *Baek-du-dae-gan*. Populations of montane species could have tracked the climatic oscillations by altitudinal migrations (descending in the glacials and ascending in the interglacials), as occurred in other places of East Asia such as China (for a review, see Qiu et al. 2011), and also in the mountain ranges of southern Europe (Hewitt 1999). In the glacial periods, populations would have colonized the lowlands adjacent to mountain ranges, where they had more opportunity for gene flow among them, thus promoting genetic diversity and genetic cohesion. The much longer length of the glacial periods (which accounted for approximately 80% of the Quaternary, whereas the remaining 20% consisted of shorter interglacial periods; Birks and Willis 2008), would have enhanced population's genetic uniformity. Since this study is the first to investigate levels of genetic diversity in boreal montane plant species with regard to paleoecology in Korea, more studies are needed to confirm a common pattern of multiple extensive glacial refugia for the plant species in the mountainous areas of the Peninsula.

Implications for Conservation

Since *O. patens* harbors high levels of genetic variation and low degree of divergence and is relatively common in northeastern Asia, conservation priority should be given to *O. coreana*. Since this species is restricted to a single island (Jeju Island, of less than 2,000 km^2) where it is extremely

rare, it is mandatory to evaluate its current threatened degree according to the IUCN criteria (IUCN 2001). Because the "extent of occurrence" ("the area contained within the shortest continuous imaginary boundary") estimated for *O. coreana* is less than 100 km^2 and the "area of occupancy" ("the area within its extent of occurrence which is occupied by a taxon") is very small (<10 km^2), this orchid can be categorized as "Critically Endangered" following the criteria B1b(iii)c(iv).

From a long-term conservation genetics perspective, the extreme rarity and small population sizes, coupled with its apparent genetic uniformity, place *O. coreana* at a high risk of extinction. Thus, in situ and ex situ conservation efforts should be of particular importance for this species. First of all, we recommend all known populations to be protected in situ by law to prevent further decreases in population size (e.g., Ramsay and Stewart 1998). Concerning the ex situ conservation measures, collections of seeds, tissues for tissue cultures, and a few ramets in each known population would be useful for the success of any conservation action (e.g., further reinforcements/reintroductions). In addition, detailed ecological studies, including demographic dynamics, pollination biology, germination ecology, seedling establishment, and relationships with mycorrhizal fungi, should be immediately initiated to achieve an effective conservation of this rare orchid (Kull 1999; Swarts and Dixon 2009a, 2009b).

Funding

This work was supported by the Basic Science Program through the National Research Foundation of Korea (KRF) funded by the Ministry of Education, Science and Technology (KRF-2007-521-C00280 to M.G.C.); the Korea Environmental Industry and Technology Institute (KEITI) of Eco-star project (project number 052-08-71 for "the development of technology for conservation and management of higher plants in Korea" to M.G.C. through to B.-Y.S.).

Acknowledgments

The author thanks Jin Seok Kim, Chung Pyo Chun, Gyu Young Chung, Byoung-Un Oh, Myung-Ok Moon, Chan-Soo Kim, and Hyoung-Tak Im for helping us in locating populations of *Oreorchis coreana* and *O. patens*; Beom Jin Shim and Myeong Soon Park for laboratory assistance; Ann C. Chung for checking the English on an earlier version of the manuscript; and John D. Nason for providing comments and editing a final version of the manuscript.

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Received September 27, 2011; Revised March 24, 2012; Accepted April 5, 2012

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