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REGULAR PAPER

Genetic diversity of the extremely rare *Habenaria dentata* and the rare *Habenaria linearifolia* (Orchidaceae) in South Korea: implications for population history and conservation

Mi Yoon Chung[1,*], Hoa Thi Quynh Le[1,*], Sungwon Son[2], Huai Zhen Tian[3] & Myong Gi Chung[1,]**

[1]Division of Life Science and the Research Institute of Natural Science, Gyeongsang National University, Jinju 52828, Republic of Korea
[2]Plant Conservation Division, Korea National Arboretum, Pocheon 11186, Republic of Korea
[3]School of Life Sciences, East China Normal University, Shanghai 200241, China
*Both authors contributed equally to this work, and should be considered co-first authors
**Author for correspondence: mgchung@gnu.ac.kr

Background and aims – Since historical events often leave an indelible mark on levels of genetic diversity of plant populations, one may indirectly infer their evolutionary history with the help of current patterns of genetic diversity. The terrestrial orchid *Habenaria dentata*, an element of warm-temperate/subtropical vegetation, reaches its northernmost limits in the Korean Peninsula, and thus it is extremely rare there. As *H. dentata* was absent from the Peninsula during the Last Glacial Maximum (LGM), it is likely to be of post-glacial origin having arrived from either a single refugium or multiple refugia. However, its rare, temperate/boreal congener *H. linearifolia* might have persisted *in situ* in either macrorefugia or microrefugia on the Peninsula during the LGM.
Methods – To test which hypothesis is most appropriate for each species, we investigated levels of allozyme-based (17 loci) genetic diversity and population genetic structure in the two only known populations of *H. dentata* and in 12 populations of *H. linearifolia*.
Key results – No allozyme diversity was found in *H. dentata* ($H_e = 0.000$), whereas *H. linearifolia* exhibited low within-population variation ($H_e = 0.060$) and high among-population differentiation ($F_{ST} = 0.237$). We found little association between populations in relation to their geographic location; several populations presented individuals belonging to different clusters.
Conclusions – Our results suggest that *H. dentata* likely originated from a single ancestral population (perhaps from southern Japan or southern China) through post-glacial dispersal, whereas *H. linearifolia* probably survived the LGM *in situ* in microrefugia situated at low to mid-elevated regions. We further suggest that separate conservation strategies for each species should be employed, given that the two taxa have different ecological and demographic traits and harbour different levels of genetic diversity.

Key words – Conservation, genetic diversity, *Habenaria*, historical events, population history.

INTRODUCTION

The current distribution of plant species at a large scale is mainly determined by range shifts (e.g. contractions/expansions and range displacements) that occurred as consequence of the Quaternary glacial-interglacial oscillations (Hewitt 1996, 2000, Davis & Shaw 2001, Willis & Niklas 2004). Beyond changes in their distribution, the past climatic oscillations greatly influenced current genetic diversity and structure in plant species. Thus, understanding the patterns of current genetic variation usually offers valuable insights into species' recent (e.g. Pleistocene) evolutionary and biogeographical history (e.g. Comes & Kadereit 2003, Hu et al. 2009, Qiu et al. 2011, López-Pujol et al. 2016).

On the Korean Peninsula, the few but increasing pollen records (Choi 1998, Chung et al. 2006, 2010, Yi & Kim 2010) and the vegetation reconstructions (Harrison et al. 2001, Hope et al. 2004, Prentice et al. 2011) suggest that the warm-temperate and subtropical vegetation (typically broad-leaved evergreen/warm mixed forests) would have vanished from the Peninsula during the Last Glacial Maximum (LGM) (reviewed in Chung et al. 2017). This vegetation, currently forming a narrow strip on the southern and southwestern coast, is, thus, of post-glacial origin, likely arriving through

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migrations from more southerly located glacial refugia (that would have been located in southern Japan, southern China, or even some areas of the exposed East China Sea; e.g. Harrison et al. 2001, see the top map in fig. 1). This process could frequently take place in orchids, as they produce tiny seeds which are potentially capable of long-distance dispersal by wind or storms (Dressler 1981, Arditti & Ghani 2000, Trapnell & Hamrick 2004, Yukawa et al. 2012, Takashima et al. 2016).

A distinctive genetic signature may be often evident within and among recently established plant populations that reflects the migration routes from source populations. Two alternative scenarios have been hypothesized for the origin of the current populations of warm-temperate species in mainland Korea (Chung et al. 2012a, 2013a, 2013b). If extant Korean populations of orchid species originated from a single source (a single glacial refugium), then we would expect low levels of genetic diversity due to founder effects. Alternatively, if populations of orchid species were founded by multiple source populations (i.e. from multiple glacial refugia), we should anticipate high levels of genetic variability (Hamrick & Nason 1996). For both scenarios, the genetic differentiation among populations would be low or high depending on rates of recurrent gene flow among Korean populations.

In contrast to the warm-temperate and subtropical vegetation (which is of post-glacial origin), the boreal and temperate plants probably survived the LGM *in situ* in the continental Korean Peninsula (Chung et al. 2017 and references therein). According to Chung et al. (2017), the larger and most significant of the Korean glacial refugia ("macrorefugia" *sensu* Rull 2009) would have been located within or near the main mountain range of the Peninsula (the so-called the "Baekdudaegan", hereafter "BDDG"; see the bottom map in fig. 1). If this scenario is true for boreal or temperate orchids growing on the Korean Peninsula, one might expect high/moderate within-population and low or moderate between-population genetic variation, at least for those species endemic or mainly confined to the BDDG. Under this scenario, we may expect a significantly positive correlation between elevation and levels of genetic diversity (e.g. expected heterozygosity). If boreal or temperate orchids survived in smaller refuge areas ("microrefugia" *sensu* Rull 2009) that persisted in favourable enclaves in the hilly lands and lowlands, we would anticipate low levels of genetic variability within populations and moderate or high differentiation among populations for those orchids.

On the Korean Peninsula, several orchids currently growing in the narrow warm-temperate and subtropical climatic zone along the coast also occur in the more extensive warm-temperate regions of Japan, subtropical China, or rarely tropical Southeastern Asia (Ohwi 1965, Hou 1983, Wu et al. 2009). Thus, current Korean populations of several orchids actually represent the northern margin of their distribution ranges. In this study, to test which hypothesis regarding the origin (single/multiple refugia) for the native Korean warm-temperate or subtropical plant species is more likely, we chose the extremely rare, self-compatible terrestrial orchid *Habenaria dentata* (Sw.) Schltr., of which the only two known populations in Korea constitute the northern edge of its distribution. The understanding of comparative patterns of genetic diversity between congeners, in conjunction with palaeovegetation information, has been proven to be useful in providing insights into species' recent evolutionary and biogeographical history (Chung et al. 2013a, 2013b, 2014a, 2015, 2017) because, among other reasons, congeneric comparisons allow for partial control of the "phylogenetic inertia" (Godt & Hamrick 2001). Having this in mind, we also selected the boreal/temperate terrestrial orchid *H. linearifolia* Maxim. as another study species to test which hypothesis for the extent of refugia (macrorefugia vs. microrefugia) is more appropriate. In addition, genetic data obtained here could provide guidelines for the management and recovery of the extremely rare *H. dentata* and the rare *H. linearifolia* in the Korean Peninsula.

MATERIALS AND METHODS

Plant materials

Habenaria dentata, 35–90 cm tall, is a non-clonal terrestrial orchid that occurs in forests on slopes or along valleys at elevations of 200–2300 m (Chen & Cribb 2009). It is broadly distributed from India, over Nepal, Myanmar, Thailand, Cambodia, Laos, Vietnam, southern China, Taiwan, southern Korea, and to southern Japan. Thus, Korean populations constitute its northern range limit. Inflorescences (racemes) are 5–12 cm long and bear many, white flowers; sepals and petals are ciliate, and petals are falcate-lanceolate, 8–9 × 2–2.5 mm; labellum is broadly obovate, 15–18 × 12–13 mm, 3-lobed with lateral lobes subrhombic or flabellate, 7 × 8 mm wide; spur is pendulous, 3.5–4 cm long. Flowers are present from August to October (Chen & Cribb 2009, Lee et al. 2013a). *Habenaria dentata* is self-compatible (H.H. Yang, Korea National Arboretum, pers. comm.), and nectar-producing white flowers appear to be pollinated by hawkmoths and butterflies (skippers) (K. Suetsugu, Kobe University, pers. comm.). Fruits (capsule, 1.5–2.5 cm long) contain numerous small seeds. The chromosome number is $2n = 64$ (Chen & Cribb 2009).

The two only known populations of *Habenaria dentata* in Korea are located in Hapcheon County, Gyeongsangnam Province (HD-1 and HD-2; fig. 1), and they were newly recorded in 2013 (Lee et al. 2013a); only 30 and 70 juveniles were counted in each population. Only five adults were identified in HD-1, but we failed to find any adults in HD-2. Perhaps owing to its recently discovery in Korea, the species was not listed as an endangered species in both the list of rare plants of Korea (KNA 2012) and the red list of threatened species (MOE 2014), but they are extremely rare in South Korea.

Habenaria linearifolia, 25–80 cm tall, is also a non-clonal terrestrial orchid that occurs in forests, grasslands, bogs or wet grassy places at elevations of 200–1500 m (Ohwi 1965, Chen & Cribb 2009). The species is an element of cool-temperate and boreal vegetation, and it is distributed from central to north eastern China, Russian Far East, northern Honshu and Hokkaido in Japan, and Korea, including Jeju Island (Ohwi 1965, Chen & Cribb 2009). Racemes are 5–16 cm long and comprise 8–20 white or greenish white flowers; petals are 2-lobed with upper one 5–5.5 × 3.5–4 mm; labellum is

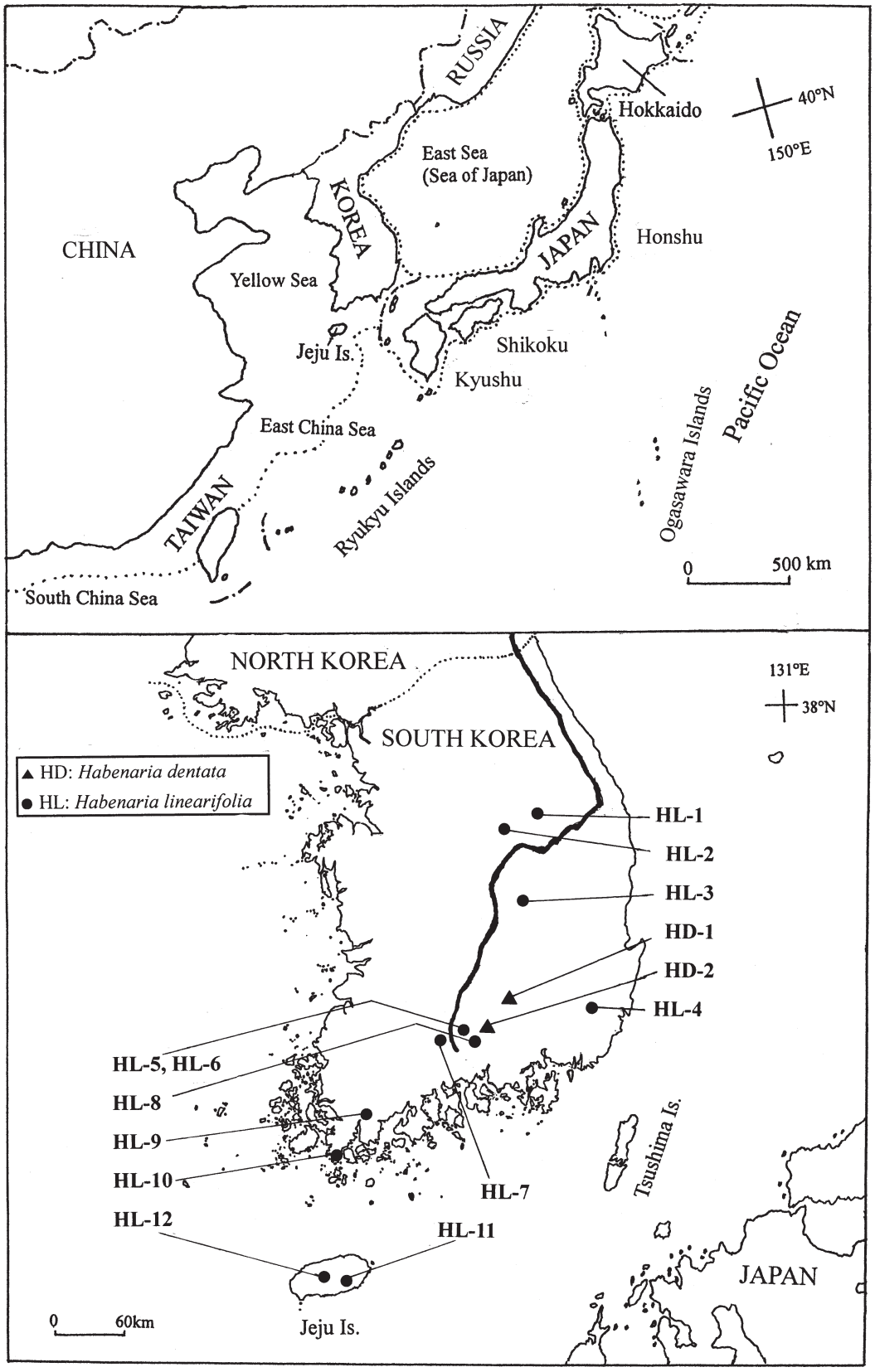

Figure 1 – Top map shows northeastern Asia. Dotted line indicates the LGM paleo-coastline. Bottom map shows locations of sampled populations of *Habenaria dentata* (HD-1 and HD-2) and *H. linearifolia* [HL-1 to HL-12; note that the linear distance between HL-5 (Wangdeungjae) and HL-6 (Oegogae) is c. 1030 m] in South Korea. Thick solid line indicates the location and shape of the main mountain range of the country, the so-called "Baekdudaegan," which runs north to south along the Korean Peninsula.

Table 1 – Summary of genetic diversity measures and mean fixation values (F_{IS}) for two populations of *Habenaria dentata* and 12 populations of *H. linearifolia* from South Korea.

Abbreviations: *Pop* population, *Alt (m)* altitude (m) a.s.l., *n* sample size, *%P* percentage of polymorphic loci, *A* mean number of alleles per locus, *AR* mean allelic richness based on a minimum sample size of 9 individuals (HL-1 and HL-10), *RA* rare allele (allele frequency < 0.05), H_o observed heterozygosity, H_e H–W expected heterozygosity or genetic diversity, SE standard error, F_{IS} fixation index within populations; [a] significance ($P < 0.05$) based on permutation (999 replicates) under the null hypothesis of $F_{IS} = 0$; [b] significant (at the 0.05 level) Weir & Cockerham (1984) estimate of F_{IS} over populations; [c] data from Chung et al. (in press); [d] data from Case (2002).

Species	*Pop*	*Alt (m)*	*n*	*%P*	*A*	*AR*	*RA*	H_o (SE)	H_e (SE)	F_{IS} (95% CI)
H. dentata	HD-1	102	30	0.0	1.00	1.00		0.000 (0.000)	0.000 (0.000)	
	HD-2	187	63	0.0	1.00	1.00		0.000 (0.000)	0.000 (0.000)	
H. linearifolia	HL-1	248	9	17.6	1.18	1.18	0	0.065 (0.047)	0.059 (0.034)	-0.110
	HL-2	206	24	11.8	1.12	1.11	0	0.015 (0.015)	0.036 (0.028)	0.603[a]
	HL-3	238	50	35.3	1.59	1.36	4	0.061 (0.027)	0.078 (0.035)	0.220[a]
	HL-4	700	11	29.4	1.29	1.27	2	0.080 (0.046)	0.085 (0.041)	0.051
	HL-5	973	15	29.4	1.35	1.31	1	0.071 (0.033)	0.081 (0.040)	0.131
	HL-6	729	41	29.4	1.35	1.28	2	0.080 (0.039)	0.078 (0.036)	-0.027
	HL-7	470	14	17.6	1.18	1.17	0	0.050 (0.029)	0.044 (0.025)	-0.155
	HL-8	502	31	11.8	1.12	1.08	1	0.028(0.026)	0.028 (0.026)	-0.016
	HL-9	132	55	29.4	1.35	1.24	2	0.051 (0.026)	0.052 (0.027)	0.008
	HL-10	42	9	17.6	1.18	1.18	0	0.033 (0.023)	0.042 (0.023)	0.233
	HL-11	504	31	41.2	1.53	1.30	6	0.078 (0.038)	0.077 (0.036)	-0.009
	HL-12	1100	30	35.3	1.35	1.26	2	0.049 (0.025)	0.057 (0.024)	0.143
Population average		470	27	25.5	1.30	1.23	2	0.055 (0.006)	0.060(0.006)	0.082[b]
Pooled samples			320	64.7	2.12		20	0.055 (0.021)	0.077 (0.031)	
Mean values for rare orchids in Korea[c]										
Population level ($N = 24$)				18.6	1.22				0.070	
Species level ($N = 24$)				21.8	1.34				0.075	
Mean values for Orchidaceae[d]										
Population level ($N = 36$)				33.7	1.46				0.107	
Species level ($N = 32$)				46.2	1.83				0.119	

spreading, c. 15 mm long, deeply 3-lobed near middle; spur is pendulous, 2.5–3.5 cm long. The flowering period is from July to September (Chen & Cribb 2009, Lee et al. 2013a). Like *H. dentata*, *H. linearifolia* is self-compatible (H.H. Yang, pers. comm.), and nectar-producing flowers seem to be pollinated by hawkmoths and butterflies (K. Suetsugu, pers. comm.). Mature capsules (1.5–2 cm long) contain numerous small seeds. The chromosome number is $2n = 28$ (Chen & Cribb 2009).

Population sampling

We sampled almost all ($n = 93$) individuals of *H. dentata* from the two only known Korean populations: Ssangchaek myeon ($n = 30$, hereafter referred to HD-1) and Gahoe myeon ($n = 63$, hereafter referred to HD-2) (fig. 1 and table 1). For *H. linearifolia*, we collected a total of 320 flowering individuals (ranging from nine in HL-1 and HL-10 to 55 in HL-9 with an average of c. 27 per population) from 12 populations (HL-1 to HL-12; fig. 1 and table 1), which cover the entire distribution of *H. linearifolia* in the Korean Peninsula (fig. 1, table 1). To minimize the damage to these orchids, we only cut 0.5 cm from the tip of a leaf per plant. We kept all sampled leaf materials on ice until they were transported to the laboratory, where they were stored at 4°C until protein extraction.

Allozyme electrophoresis

For enzyme extraction, we ground leaf samples using a pestle and mortar by adding a crushing buffer (Mitton et al. 1979), and we absorbed enzyme extracts onto 4 × 6 mm wicks cut from Whatman 3MM chromatography paper. We then stored them in microtiter plates at -70°C. We used horizontal starch-gel (13%) electrophoresis with two buffer systems. We used a modification (Haufler 1985) of the system 6 of Soltis et al. (1983) to resolve alcohol dehydrogenase (*Adh*), cathodal peroxidase (*Cpx*), diaphorase (*Dia-1*, *Dia-2*), fluorescent esterase (*Fe*), leucine aminopeptidase (*Lap*), malic enzyme (*Me*), phosphoglucoisomerase (*Pgi-1*, *Pgi-2*), phosphoglucomutase (*Pgm*), and triosephosphate isomerase (*Tpi-1,Tpi-2*). We also used the morpholine-citrate buffer system (pH 6.1)

of Clayton & Tretiak (1972) to resolve fructose-1,6-diphosphatase (*F1,6dp*), isocitrate dehydrogenase (*Idh*), malate dehydrogenase (*Mdh-1*, *Mdh-2*), and 6-phosphogluconate dehydrogenase (*6Pgd*). We followed stain recipes from Soltis et al. (1983), except for diaphorase (Cheliak & Pitel 1984). We designated putative loci sequentially, with the most anodally migrating isozyme designated as *1*, the next *2*, and so on. We numbered the different alleles within each locus sequentially, giving the fastest migrating allele the lowest number.

Data analysis

We considered a locus to be polymorphic when two or more alleles were observed, regardless of their frequencies. We estimated the genetic diversity parameters within populations using the programs POPGENE (Yeh et al. 1999) and FSTAT (Goudet 1995). The standard parameters estimated were percent 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 & Petit 1996), mean observed heterozygosity (H_o), and mean Hardy–Weinberg (H–W) expected heterozygosity or Nei's (1978) gene diversity (H_e). Except for *AR* and H_o, we also estimated these parameters for the total samples as a whole (i.e. at the species level).

We conducted linear regression analyses (altitude vs. *%P*, *A*, *AR*, and H_e) to explore the possible relationships between altitude and within-population genetic variation in *H. linearifolia*. Additionally, we also performed linear regressions between the number of rare alleles (*RA*, those occurring at frequencies below 0.05) and altitude.

To test for recent decreases in effective population size (N_e) (i.e. genetic bottlenecks) in *H. linearifolia*, we evaluated differences across loci between the H–W H_e and the equilibrium heterozygosity (H_{eq}) expected assuming mutation–drift equilibrium. Using the program BOTTLENECK (Cornuet & Luikart 1996), we evaluated these differences using a sign test and a Wilcoxon sign-rank test under an infinite allele model. Since allelic diversity is generally lost more rapidly than H_e (Nei et al. 1975), recently bottlenecked populations will exhibit an excess of H–W H_e relative to H_{eq} (Cornuet & Luikart 1996, Luikart et al. 1998).

We used the program SPAGeDi (Hardy & Vekemans 2002) to calculate population-level F_{IS} (inbreeding) and its significance level by 999 permutations under the null hypothesis of $F_{IS} = 0$. To measure deviations from H–W equilibrium at each polymorphic locus, we calculated averages of Wright's (1965) F_{IS} and F_{ST} (deviations from H–W equilibrium of individuals relative to their local populations, and local populations relative to the total population, respectively) following Weir & Cockerham (1984). Using FSTAT, we constructed 95% bootstrap CI (999 replicates) around means of the F_{IS} and F_{ST}, and considered the observed F_{IS} and F_{ST} to be significant when 95% CI did not overlap zero.

We estimated Nei's (1978) genetic identity (*I*) for all pair of populations using POPGENE. In addition, a neighbour-joining tree was drawn from the genetic distance (Nei et al. 1983) matrix with branch support produced by 999 bootstrapping over loci, using Populations v. 1.2.30 (Langella 1999) and TREEVIEW v. 1.6 (Page 1996). We assessed the genetic structure by means of the Bayesian algorithm implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). The program estimates the likelihood of the individuals being structured in a given number of genetic clusters (or genetic populations, *K*). We selected 'admixture' and 'correlated' as appropriate models for ancestry and allele frequencies, respectively, as events of migration and populations with shared ancestry are presumably to occur in *H. linearifolia*. We set the burn-in period and Markov Chain Monte Carlo (MCMC) to 50 000 and 500 000 iterations, respectively, and 10 replicates per *K* were run. We determined the most likely value of *K* by the Δ*K* statistics of Evanno et al. (2005), with the aid of Structure Harvester (Earl & vonHoldt 2012). As the Δ*K* method tends to identify $K = 2$ as the top level of hierarchical structure (Janes et al. 2017), we combined it with the method of choosing the smallest *K* after the log probability of data [ln Pr(*X*|*K*)] values reached a plateau (Pritchard et al. 2010). The results of both methods were visualized with the aid of Structure Harvester (Earl & vonHoldt 2012).

It is common practice to estimate pair-wise F_{ST} values between genetic clusters identified with STRUCTURE, even when occurring in sympatry (Daïnou et al. 2010, Fischer et al. 2015, Torroba-Balmori et al. 2017). We averaged the ancestry proportion (*q*) per individual per cluster over the 10 repetitions per *K* using the program Clumpp v.1.1.2 (Jakobsson & Rosenberg 2007). We further used the program Distruct v.1.1 (Rosenberg 2004) to graphically display the results produced by Clumpp. We chose a threshold of 0.8 to assign all individuals to the two clusters; we excluded individuals from the subsequent F_{ST} analysis which did not pass the threshold. We calculated F_{ST} between the two clusters ($K = 2$, see results) assigned in the total sample across the 12 populations. We also estimated F_{ST} values between the two clusters within populations; however, we could only estimate F_{ST} in HL-6 and HL-8, because only in these populations the number of samples for the less frequent cluster was more than 10.

To grasp the overall pattern of genetic structure at the regional scale (i.e. isolation-by-distance effects), we conducted a linear regression analysis between all pairwise $F_{ST}/(1–F_{ST})$ (F_{ST} was calculated following Weir & Cockerham 1984) and the corresponding logarithm of pairwise geographical distances (Rousset 1997). Using FSTAT, we tested a linear regression model using a Mantel test (by making 999 replicates) under the null hypothesis of no spatial genetic structure (regression slope, $b = 0$).

Finally, to gain insight into the patterns of recent gene flow between individual populations, we estimated migration (*m*) rates using the program BayesAss v. 1.3 (Wilson & Rannala 2003). We ran 3×10^6 Markov chain Monte Carlo iterations, with a burn-in of 999 999 iterations and a sampling frequency of 2000 by setting delta at 0.15 (the default value).

RESULTS

Levels of genetic variation within populations

All 17 loci examined were monomorphic in all the *Habenaria dentata* samples in South Korea (table 1, see electronic appendix 1 for the allozyme data). In contrast, 11 (*Adh*, *Cpx*,

Table 2 – Results of statistical tests for evidence of recent population bottlenecks in Korean populations of *Habenaria linearifolia*.
Numbers reported are *p*-values of sign and Wilcoxon sign-rank tests conducted using the program BOTTLENECK.

Population	Sign test	Wilcoxon sign-rank test
HL-1	0.410	0.812
HL-2	0.706	0.250
HL-3	0.116	0.960
HL-4	0.633	0.890
HL-5	0.519	0.890
HL-6	0.393	0.500
HL-7	0.437	0.812
HL-8	0.679	0.875
HL-9	0.270	0.922
HL-10	0.072	0.945
HL-11	0.298	0.815
HL-12	0.216	0.976

Dia-1, *Dia-2*, *Fe*, *Idh*, *Mdh-1*, *Mdh-2*, *6Pgd*, *Tpi-1*, and *Tpi-2*) out of 17 loci were polymorphic across 12 populations of *H. linearifolia*. However, variation at allozyme loci within populations was low (table 1). At the population level, the average percentage of polymorphic loci (*%P*) was 25.5%, mean number of alleles per locus (*A*) was 1.30, allelic richness (*AR*) was 1.23, and mean observed (H_o) and expected heterozygosity (H_e) were 0.055 and 0.060, respectively (table 1). Higher levels of *%P* (64.7%) and *A* (2.12) were found when samples were treated as a whole, although the H_e estimate (0.077) was similar to population average (table 1) due to 20 rare alleles harboured in eight populations (table 1).

We found no significant correlation between the altitude and the four genetic parameters in *H. linearifolia*; *%P* (r = 0.437, P = 0.155), *A* (r = 0.264, P = 0.406), *AR* (r = 0.321, P = 0.309), and H_e (r = 0.516, P = 0.086) (electronic appendix 2). In addition, there was no significant correlation between the altitude and the number of rare alleles (*RA*) (r = 0.087, P = 0.789; electronic appendix 3). For *%P* and *A* we obtained 20 sets of sub-samples manually by randomly choosing nine individuals in 10 populations by applying rarefaction and bootstrapping to avoid sample size bias. However, sampling size corrected estimates of *%P* and *A* also yielded weak and statistically non-significant correlations with altitude, indicating that our results (correlations with altitude) were not affected by sample size bias. Finally, we found no significant indication of recent bottlenecks in any of the populations of *H. linearifolia* (table 2).

Population genetic structure

We found a significant deficiency of heterozygotes relative to H–W expectations in only two populations of *H. linearifolia* (F_{IS} = 0.603 at HL-2, F_{IS} = 0.220 at HL-3; table 1), and the multi-population-level F_{IS} was significantly greater than zero (0.082; bootstrapped 95% CI: 0.024–0.168). Deviations from H–W expectations due to allele frequency differences between populations were also significant (F_{ST} = 0.237; bootstrapped 95% CI: 0.066–0.334).

We found relatively high pairwise Nei's (1978) *I* values between populations of *H. linearifolia*, ranging from 0.922 to 0.999, with a mean of 0.981 ± 0.016 (data not shown); these were slightly higher than the average of the values reported for conspecific populations of both orchids (average I = 0.955 ± 0.051, N = 84; Chung & Chung 2012) and plants overall (average I = 0.950 ± 0.059, N = 1572; van der Bank et al. 2001). The neighbour-joining tree showed little association between populations of *H. linearifolia* in relation to their geographic location (electronic appendix 4).

The best clustering scheme of STRUCTURE was K = 2 according to the ΔK statistic, as there was a clear peak; however, a small peak was detected at K = 6 (electronic appendix 5). In contrast, when the ln Pr($X|K$) was plotted, the 'plateau' was approximately reached at K = 6 (electronic appendix 5). Thus, we selected both K = 2 and K = 6 as optimal number of clusters. At K =2, several populations presented individuals belonging to different clusters (especially HL-6 to HL-8 and HL-12); such pattern of individuals belonging to different clusters at each population was even more evident at K = 6 (fig. 2). Pair-wise F_{ST} between the two clusters in the total sample was high (F_{ST} = 0.363; bs 95% CI: 0.030–0.603), suggesting that the two gene pools are significantly differen-

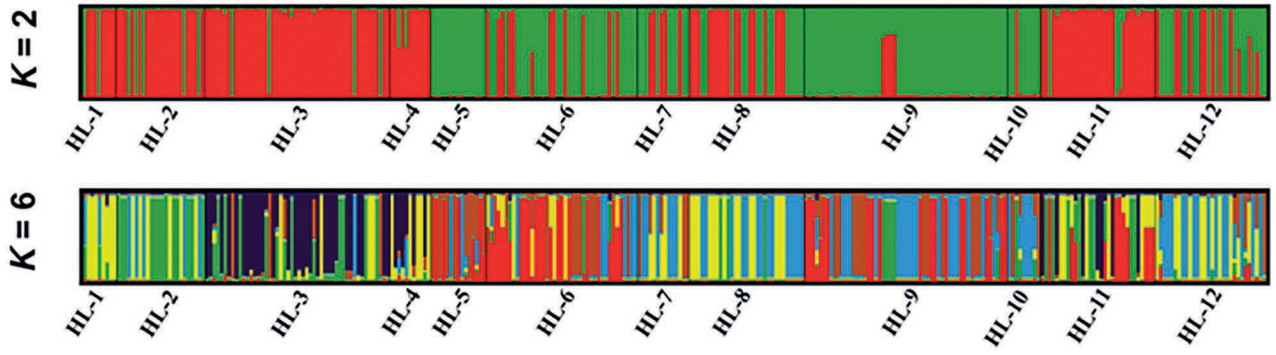

Figure 2 – Results of STRUCTURE for all studied individuals of *Habenaria linearifolia*. Assignation of individuals to genetic clusters is at K = 2 and K = 6.

Table 3 – Mean value of the posterior distribution of the recent migration rates (*m*) of each *Habenaria linearifolia* population pairs estimated from allozyme data using the BayesAss program (Wilson & Rannala 2003).
Note that values on the diagonal in bold italic are the proportions of individuals derived from source populations. Values higher than 0.110 (the 95% CI upper limit) for *H. linearifolia* are presented in bold.

	HL-1	HL-2	HL-3	HL-4	HL-5	HL-6	HL-7	HL-8	HL-9	HL-10	HL-11	HL-12
HL-1	***0.6977***	0.0186	0.0217	0.0107	0.0116	0.0120	0.0108	0.0106	0.0251	0.0115	0.0279	**0.1419**
HL-2	0.0025	***0.9471***	0.0030	0.0021	0.0020	0.0024	0.0020	0.0022	0.0045	0.0023	0.0040	0.0255
HL-3	0.0008	0.0013	***0.9878***	0.0007	0.0008	0.0010	0.0009	0.0009	0.0011	0.0009	0.0020	0.0015
HL-4	0.0091	0.0150	**0.1307**	***0.6935***	0.0101	0.0090	0.0093	0.0084	0.0100	0.0100	0.0336	0.0610
HL-5	0.0070	0.0080	0.0075	0.0072	***0.6867***	0.0616	0.0075	0.0068	**0.1814**	0.0071	0.0076	0.0115
HL-6	0.0027	0.0076	0.0051	0.0029	0.0028	***0.6828***	0.0030	0.0029	0.0625	0.0029	0.0196	**0.2047**
HL-7	0.0075	0.0103	0.0124	0.0074	0.0071	0.0078	***0.6873***	0.0076	0.0464	0.0073	0.0106	**0.1880**
HL-8	0.0030	0.0072	0.0046	0.0029	0.0029	0.0032	0.0032	***0.6771***	0.0051	0.0032	0.0053	**0.2818**
HL-9	0.0006	0.0007	0.0006	0.0006	0.0005	0.0008	0.0006	0.0005	***0.9924***	0.0006	0.0007	0.0010
HL-10	0.0115	0.0207	0.0149	0.0108	0.0126	0.0117	0.0105	0.0111	0.0506	***0.6977***	0.0167	**0.1309**
HL-11	0.0011	0.0037	0.0030	0.0011	0.0014	0.0017	0.0012	0.0013	0.0023	0.0013	***0.9775***	0.0038
HL-12	0.0010	0.0011	0.0010	0.0012	0.0010	0.0010	0.0009	0.0010	0.0012	0.0010	0.0012	***0.9879***

tiated. Similar high F_{ST} values were also observed between the two clusters in the HL-6 (F_{ST} = 0.297; bootstrapped 95% CI was not conducted owing to only two polymorphic loci) and HL-8 (F_{ST} = 0.533) populations.

We found no significant correlation between pairwise genetic differentiation estimates and their corresponding logarithm of pairwise geographical distance values (r = 0.054, P = 0.655; electronic appendix 6), suggesting that most of the variation in genetic differentiation must be attributed to other factors than geographic distance. The BayesAss results revealed low average m rates between populations of *H. linearifolia* (0.0150). Only seven out of 132 pairwise estimates indicated significant evidence of recent gene flow between populations; from HL-3 to HL-4, from HL-9 to HL-5, and other five cases were from HL-12 to HL-1, HL-6, HL-7, HL-8, and HL-10 (table 3). All the other m values fell within the CI expected in instances where there is no information in the data (bootstrapped 95% CI: 1.17×10^{-13}, 0.110).

DISCUSSION

Population establishment history of *Habenaria dentata*

The complete lack of allozyme diversity found in *H. dentata* supports the first hypothesis of a single source (a single glacial refugium) origin for the Korean populations. Such genetic impoverishment across populations may be a consequence of long distance dispersal events from source populations (probably from southern Japan or southern China) and subsequent bottlenecks/founder effects resulting in the loss of genetic variation (Hewitt 1996). In addition, the recently established population might have not yet accumulated genetic diversity by mutation or by further gene flow from source populations (Maki et al. 2008, 2010, Chung et al. 2013d).

As orchids produce tiny numerous seeds, they have the potential for long-distance seed movement once seeds are released and enter the air column (Dressler 1981, Arditti & Ghani 2000, Trapnell et al. 2013). For example, the terrestrial orchid *Oreorchis coreana* Finet was considered endemic to Jeju Island (South Korea; fig. 1) in the past, but also occurs in Nasushiobara City, Tochigi Prefecture, Honshu (Japan; fig. 1) (Takashima et al. 2016). Unlike on Jeju Island, only c. 10 individuals in a single population are known in Honshu. As there is no sequence divergence in the ITS regions between Jeju and Nasushiobara populations, the Japanese population may be the result of recent long-distance dispersal from Jeju Island rather than being an old relict population separated via vicariance (Takashima et al. 2016). Another example is the facultative autogamous terrestrial orchid *Geodorum densiflorum*, of which there is a single population of 108 individuals in the uninhabited Mukoujima, a small island that belongs to the Ogasawara Archipelago (see fig. 1), situated c. 1000 km south of Tokyo, Japan (Yukawa et al. 2012). The authors suggest recent long-distance dispersal as the likely origin of this population; the closest extant populations of *G. densiflorum* from Mukoujima Island are either those of the Ryukyu Islands or those of Micronesia (both are c. 1500 km apart from the Ogasawara Islands). These examples would also support the scenario that the self-compatible *H. dentata* originally established in southern Korea by a single long-distance migrant that carried homozygous alleles at many allozyme loci from the south or the south-west (southern Japan, southern China, Taiwan, or even northern Vietnam). BayesAss results show that gene flow was indirectly detected at long distances between populations of *H. linearifolia*. The relatively high rates of gene flow for *H. linearifolia* (e.g. from population HL-12 in Jeju Island to continental populations such as HL-1, HL-6, HL-7, HL-8, and HL-10)

may indicate that long-distance seed dispersal would also have been possible for *H. dentata* (table 3).

Extremely low or low levels of allozyme diversity have been reported for other warm-temperate plant species from southern Korea; the two terrestrial orchids *Pecteilis radiata* (H_e = 0.000; M.Y. Chung et al., unpubl. data) and *Tipularia japonica* (H_e = 0.000; Chung 2009), the sundew *Drosera peltata* var. *nipponica* (H_e = 0.000; Chung et al. 2013c), the two ferns *Cyrtomium falcatum* (H_e = 0.034; Chung et al. 2012a) and *Selliguea hastata* (H_e = 0.000 for the mainland populations; Chung et al. 2013d), the terrestrial orchid *Bletilla striata* (H_e = 0.049; Chung et al. 2013a), and the broad-leaved evergreen tree *Neolitsea sericea* (H_e = 0.073; Chung et al. 2000). For this latter species, Lee et al. (2013b), using chloroplast DNA sequences, found much higher genetic diversity for Japanese populations (mean estimate of haplotype diversity across 18 populations, h = 0.592) of *N. sericea* compared to Korean ones (h = 0.092), and significant genetic divergence between the two regions (Φ_{ST} = 0.513). Furthermore, the authors found only one haplotype from eight studied southern Korean populations. These results strongly suggest that several warm-temperate or subtropical plant species might have recolonized southern Korea from a single refugium (Chung et al. 2013d, 2017, Lee et al. 2013b).

The scenario for population origin from multiple glacial refugia (the second hypothesis) is still applicable for other warm-temperate species from southern Korea based on several allozyme diversity studies. These include the terrestrial orchid *Cymbidium goeringii* (H_e = 0.240; Chung & Chung 1999), three terrestrial orchids belonging to the genus *Calanthe* (*C. discolor*, *C. sieboldii*, and *C. reflexa*; H_e = 0.227, 0.280, and 0.185, respectively; Chung et al. 2013b), *Cremastra appendiculata* var. *variabilis* (H_e = 0.217; Chung et al. 2013e), and the broad-leaved evergreen tree *Machilus thunbergii* (H_e = 0.185; Chung et al. 2014b). Thus, although we proposed two extreme situations [a single (or a few) refugium vs. multiple refugia], post-glacial recolonization of the warm-temperate flora from its LGM suitable refugia was probably a very complex process, and intermediate or other scenarios would be possible depending on the number of colonization events and the number of propagules arriving at each colonization event.

Inference of population history of *Habenaria linearifolia*

The levels of genetic variation within populations of *H. linearifolia* and within the species (i.e. the samples pooled as a whole) are substantially lower than those expected for terrestrial orchid species (H_e = 0.060 and 0.077 vs. H_e = 0.107 and 0.119; Case 2002). However, *H. linearifolia* harbours comparable levels of genetic variation at population and species levels typically found in rare orchids in Korea (H_e = 0.070 and 0.075; table 1 and Chung et al. in press). Overall, a low, but significant deficiency of heterozygotes relative to H–W expectations (multi-population-level F_{IS} = 0.082) is consistent with its life-history traits (self-compatibility and pollination by hawkmoths and butterflies). *Habenaria linearifolia* exhibits a high degree of genetic divergence among populations (F_{ST} = 0.237), which is comparable to the mean of 13 rare terrestrial orchids (F_{ST} = 0.279; Phillips et al. 2012).

If *H. linearifolia* was able to persist *in situ* along the BDDG during the LGM, we would expect a significant correlation between the altitude and levels of genetic diversity (Chung et al. 2014a, 2014c). However, we did not find a significant correlation for any of the four studied genetic parameters (*%P*, *A*, *AR*, and H_e; electronic appendix 2). The distribution of rare alleles (*RA*) in populations of plant species is often associated with their refuge areas (Konnert & Bergmann 1995, Charrier et al. 2014). A putative refugium would tend to have a greater number of *RA* than recently colonized areas because these alleles can only be found under conditions of stable N_e over time (Nei et al. 1975, Widmer & Lexer 2001). However, we failed to detect a significant correlation between altitude and *RA*. Instead, we found larger numbers of *RA* at populations located between 200 to 800 m above sea level (electronic appendix 3).

Taken all these factors into account, the low levels of within-population genetic diversity in *H. linearifolia* would support the 'microrefugia' hypothesis, a scenario in which the species would have endured the glacial periods in favourable enclaves in the hilly lands and lowlands. Random genetic drift, caused by the rarity of the species (small N_e) together with strong isolation nature of populations, might be involved in the maintenance of low levels of within-population genetic variation and high degree of between-population genetic differentiation. In contrast to this, some boreal orchid species with high levels of within-population genetic diversity would have survived the glacial periods in larger refuge areas ('macrorefugia'), including *Cypripedium macranthos* (H_e = 0.185; Chung et al. 2009), *Galearis cyclochila* (H_e = 0.210; Chung et al. 2005a), *Liparis makinoana* (H_e = 0.317; Chung et al. 2005b), and *Oreorchis patens* (H_e = 0.237; Chung et al. 2012b).

Regarding the STRUCTURE patterns, one might speculate that *H. linearifolia* survived in at least two distinct refugia, which would have caused the partition of individuals into two genetic clusters (F_{ST} = 0.363). HL-3 and HL-5 are among the candidates to be refugia, because they harbour the highest genetic variation (table 1), are relatively genetically homogeneous in terms of genetic clusters (fig. 2), and they have the largest population areas (M.Y. Chung & M.G. Chung, pers. obs.). As *H. linearifolia* reaches 80 cm in height, and flowers are pollinated by hawkmoths and butterflies, gene flow is expected to be high within populations. However, we found considerably high genetic differentiation between two gene pools in sympatry (F_{ST} = 0.297 in HL-6 and F_{ST} = 0.533 in HL-8). Considering this, one may wonder why different clusters are maintained in the same population. Selfing may not be a major factor because F_{IS} was not significantly greater than zero (F_{IS} = -0.027 in HL-6 and F_{IS} = -0.016 in HL-8). Perhaps, some kind of ecotypic differentiation or incompatibilities between the two gene pools might play a role in maintaining this pattern. Alternatively, the sharing of genetic clusters by several populations (fig. 2) would be attributed to ancient shared ancestry and not to extensive gene flow between populations (the latter being expected for a scenario of survival in 'macrorefugia') given the high values of F_{ST} = 0.237.

Conservation implications

The two species studied here are rare at the regional level, and vulnerable to endangered. They, however, were not listed as an endangered species in KNA (2012) and MOE (2014) perhaps because of its recent discovery (*H. dentata*) and/or its abundance relative to extremely rare orchids (*H. linearifolia*). As the only two populations of *H. dentata* have been found in Korea, *in situ* and *ex situ* conservation efforts should be of particular importance for this species. Although the conservation team from Korea National Arboretum (KNA) has tried to install fences in the two known populations, supplementary *in situ* conservation measures for the short-term persistence of *H. dentata* are essential; increasing population sizes and numbers would be critical to buffer against extinction due to demographic stochasticity (Lesica et al. 1988). To do this, population reinforcements from HD-1 to HD-2 and *vice versa* (the linear distance between HD-1 and HD-2 is c. 28 km), provided that the two populations are genetically different by highly variable DNA markers, and founding of new populations near the extant ones from adults made by an asymbiotic seed germination and in vitro seedling development would be suggested (Godt et al. 1996). Fortunately, the KNA conservation team is securing large quantities of adults made from in vitro seedling development (H.H. Yang and S.W. Son, Korea National Arboretum, pers. comm.). Concerning the *ex situ* conservation measures, collections of seeds in populations would be useful for the success of any conservation action (e.g. further reinforcements or reintroductions). For the long-term conservation strategy, increases of genetic diversity are recommendable through the artificial introduction of plants or propagules presumably from their colonization sources (e.g. the former LGM refugium) into genetically depauperate southern Korean populations, if they are genetically similar to the southern Korean populations (to avoid the disruption of coadapted gene complexes; Fenster & Dudash 1994).

In South Korea, *H. linearifolia* usually occurs in bogs and wet grasslands, thus, most populations are highly isolated and of small size (in the order of tens of adults). Fortunately, two of the wetlands where the species is present (HL-4 at Moojechineup and HL-12 at the "1100 Altitude Wetland" on Jeju Island) have been included in the list of Ramsar Wetlands (http://www.ramsar.org/wetland/republic-of-korea). In addition, HL-5 (Wangdeungjae Wetland), HL-6 (Oegogae), and HL-7 (near Baemsagol) are located within Jirisan National Park, and HL-5 has been well managed by the park authority. HL-11 is located near an experimental station of the Warm-Temperate Forest Research Center on Jeju Island. Population HL-1 (Jangreung Wetland) is adjacent to Yeongwol Jangreung (a historic site of the tomb of King Danjong of the Joseon Dynasty) and has been well preserved and managed by the authority (Cultural Heritage Administration, Republic of Korea). In addition, most of these populations are subjected to periodical surveillance. Other populations, however, have been severely disturbed by human activities: collection (at the small population HL-2 at Jaecheon), trampling (the relatively large population HL-3 at Sangju Wetland and HL-6), and park construction (HL-8). In fact, the populations HL-2 and HL-6 are close to extinction (M.Y. Chung & M.G. Chung, pers. obs.). As short-term *in situ* conservation measures, it is essential to minimize the negative human impacts, responsible for the observed decreases in N_e. For example, as the relatively large Hogye Wetland (HL-9) – located near national road 2 – has suffered from human disturbance, special protection measures (e.g. fence installation) should be undertaken to protect this population.

The low levels of genetic diversity found in *H. linearifolia* from South Korea justify the implementation of some conservation measures to ensure its long-term preservation. To do this, an understanding of how genetic diversity is partitioned within and among plant populations may be essential to design adequate conservation plans (Godt & Hamrick 2001, Sun & Wong 2001). Since *H. linearifolia* exhibits a high degree of divergence among populations (F_{ST} = 0.237), a few populations with relatively high levels of within-population genetic variation should be targeted for both *in situ* and *ex situ* conservation. Using the formula proposed by Ceska et al. (1997), $P = 1 - (F_{ST})^n$ (where P is the proportion of genetic variation desired to be preserved and n is the number of populations to be protected/sampled), we should protect/sample at least four populations in order to conserve more than 99% of the genetic diversity found in *H. linearifolia* in South Korea. Populations with high allelic richness could be used as source populations for reintroductions (Godt & Hamrick 2001). In addition, genetically unique populations may deserve the highest priority for protection (Godt et al. 1996). The populations HL-3 to HL-6 and HL-11 are the most genetically diverse (table 1). Among them, HL-3 and HL-6 have one ($Adh^4$and Idh^2, respectively) and HL-11 has three private (those that only occur in a single population) alleles ($Mdh\text{-}1^3$, $Mdh\text{-}2^3$, and $Tpi\text{-}1^3$) with low frequencies (< 0.05). Taken together, we suggest that the populations HL-3, HL-4, HL-6, and HL-11 deserve both *in situ* and *ex situ* conservation in southern Korea and, as an urgent measure, they should be protected by installing fences.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data) and consist of the following: (1) allozyme data for *Habenaria linearifolia*; (2) no significant correlation between altitude and the four genetic parameters; *%P*, *A*, *AR* and H_e; (3) no significant correlation between altitude and the number of rare alleles *RA*; (4) neighbour-joining tree based on Nei et al.'s (1983) genetic distances between populations of *Habenaria linearifolia*; (5) the most likely K was estimated using the log probability of data [ln Pr($X|K$)] values (Pritchard et al. 2010) and the ΔK statistics of Evanno et al. (2005) using Structure Harvester 0.6.94 (Earl & vonHoldt 2012); and (6) differentiation between populations of *Habenaria linearifolia*.

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