Plant Syst Evol (2016) 302:1483–1494
DOI 10.1007/s00606-016-1346-y

ORIGINAL ARTICLE

The establishment history of alpine *Leontopodium japonicum* (Asteraceae) resembles that of warm-temperate plants on the Korean Peninsula

Dong-Hyuk Lee[1] · Jung-Hyun Lee[2] · Won-Bum Cho[1] · Byoung-Hee Choi[1]

Received: 2 November 2015 / Accepted: 9 August 2016 / Published online: 30 August 2016

Abstract Historical migration patterns of boreal and alpine plants have had a narrow focus on the Korean Peninsula, generally being characterized as southern relics from northern-sourced populations. Here, we present another hypothesis for the pattern associated with Korean alpine species. A genetic analysis was conducted with *Leontopodium japonicum* Miq., which grows in high mountain ranges with a disjunct distribution across Korea, China, and Japan. We inferred its phylogeography using sequences of nuclear (ITS) and chloroplast DNA (*trn*L–F, *rps*16, and *rpl*16) for 11 populations from Korea and adjacent regions. Our molecular data (SAMOVA and network analyses) revealed a distinct genetic isolation of Korean populations with the highest genetic differentiation (Korea vs. China and Japan). By comparison, a non-significant level of differentiation, but a high degree of genetic diversity, was detected between Chinese and Japanese populations, resembling that of warm-temperate species. These findings demonstrate that, rather than migrating southward from more northern latitudes, current populations in Korea are distributed due to colonization via East China Sea land bridges, similar to movement by warm-temperate species. Furthermore, geographical isolation because of an oceanic barrier has led to allopatric speciation for Korean populations. This specific scenario for *L. japonicum* is a meaningful example that will enhance our understanding of the history of plants growing in alpine (or subalpine) zones of Korea.

Keywords Allopatric speciation · Alpine plant in eastern Asia · Chloroplast and nuclear ribosomal DNA · Korea · *Leontopodium japonicum* · Phylogeography across East China Sea (ECS)

Introduction

Dramatic oscillations in climatic conditions during the Quaternary profoundly influenced the current distribution and evolution of plant and animal species (Avise 2000; Hewitt 2000, 2003; Hampe and Petit 2005). During glacial periods, the ranges of boreal and alpine plants generally shifted into southern regions but then returned to their present locations during warm, inter-glacial times (Hultén and Fries 1986). The environment associated with high mountains in those southern areas, where the climate is cooler at higher elevations than in the surrounding landscape, also met their biological and environmental requirements (Harrison et al. 2001; Petit et al. 2003; Stewart et al. 2010). Consequently, some of those southern populations remained in isolated patches where habitats continued to be suitable for growth (Huck et al. 2009; Michl et al. 2010). Particularly in countries of East Asia, such as Korea, China, and Japan, the stages of evolutionary history for a species have largely been affected by cyclical fluctuations in sea levels associated with dynamic landscape configurations (Fig. 1a; see also work by Qiu et al.

Handling Editor: Yunpeng Zhao.

Electronic supplementary material The online version of this article (doi:10.1007/s00606-016-1346-y) contains supplementary material, which is available to authorized users.

✉ Byoung-Hee Choi
bhchoi@inha.ac.kr

[1] Department of Biological Sciences, Inha University, 100 Inha-ro, Nam-gu, Incheon 22212, Republic of Korea

[2] Department of Biology Education, Chonnam National University, Gwangju 61186, Republic of Korea

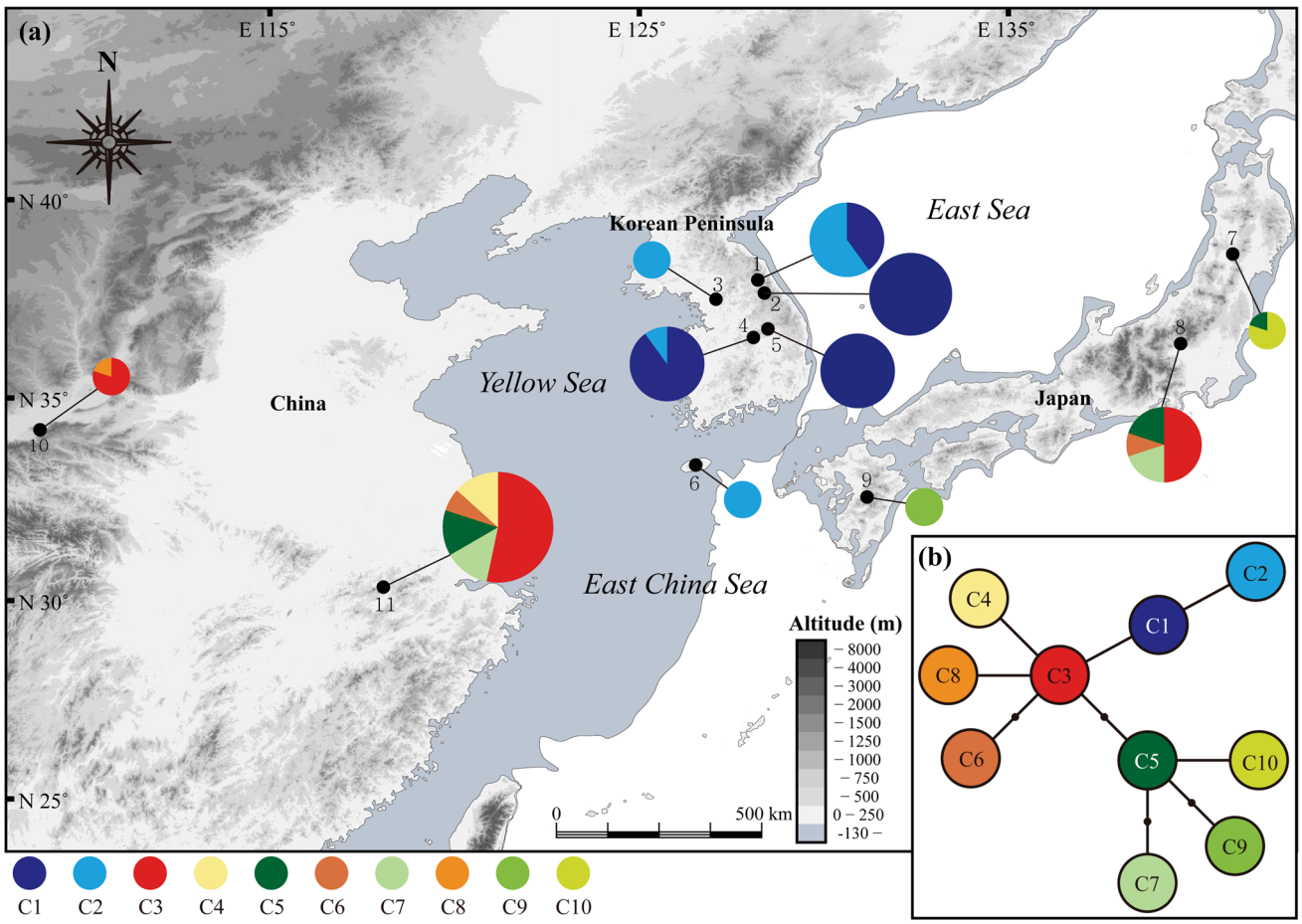

Fig. 1 Geographical distribution of chloroplast DNA haplotypes detected in 11 populations of *Leontopodium japonicum*. **a** Population codes are explained in Table 1. *Bluish shading* indicates assumed exposed coastal areas and sea basins (–130 m) of East Asia during period of glacial-induced alterations in sea level in late Pleistocene. **b** TCS-derived network of genealogical relationships among ten chlorotypes

2009a, b; Sakaguchi et al. 2011, 2012; Zhai et al. 2012; Lee et al. 2013, 2014). Therefore, the historical migration of plants currently isolated on high mountains in the region might also have been largely affected by repeated changes in sea levels along with repetitive migration by lowland vegetation during periods of climatic oscillations.

Korea, where approximately 70 % of the terrain is covered by mountains and hills, is located on the southeastern edge of the Asian continent. Small alpine (or subalpine) zones (e.g., Mt. Seolak: 1707 m; Mt. Halla: 1950 m) are scattered across mountain ranges that are relatively lower in elevation than in adjacent regions of China and Japan. However, the vegetation composition of those Korean zones has been well maintained by abiotic factors such as strong winds, the presence of limestone rock, and heavy winter snowfalls (Kong and Watts 1993). Those areas include a remarkably diverse biota with abundant boreal and alpine species (Kong and Watts 1993), with some of those mountains forming the southernmost boundary of distribution for certain alpine plants, e.g., *Diapensia lapponica* var. *obovata* F.Schmidt and *Empetrum nigrum* var. *japonicum* (R.D.Good) H.Hara. Therefore, those geographical areas have long been of great interest to researchers (Kong and Watts 1993; Chung et al. 2012, 2015). Traditionally, such species were thought to have migrated from more northern ancestral populations, e.g., Amur, Kamchatka, Sakhalin, and Ussuri of Far East Russia (Im 1992; Kong and Watts 1993; Kong 2007). A paleovegetation reconstruction by Harrison et al. (2001) suggested that this landmass was covered by boreal forests during the Last Glacial Maximum (LGM; 20,000 years ago). Recent advances in molecular techniques that take a population genetics approach have supported this current hypothesis on the origin of vegetation in these Korean boreal forests (Aizawa et al. 2012; Chung et al. 2012, 2015). Investigations have shown that the Korean populations have high genetic diversity due to frequent colonization from more northern regions (Chung et al. 2012, 2015). However, those examined species have followed historically similar patterns of regulation, i.e., southward migration for their long-term survival.

Table 1 Description of sampling populations for *Leontopodium japonicum* in East Asia

Population code	Locality	Latitude/ Longitude	*n* (cpDNA/ ITS)	*h* (cpDNA/ ITS)	*R* (cpDNA/ ITS)	$\pi \times 10^2$ (cpDNA/ ITS)	Haplotypes (cpDNA/ITS)
Korea							
1	Mt. Seolak, Gangwon-do	N38°07′, E128°22′	10/8	0.533/0	0.976/0	0.032/0	C1(4), C2(6)/R1(8)
2	Mt. Gariwang, Gangwon-do	N37°25′, E128°29′	11/11	0/0.327	0/0.491	0/0.052	C1(11)/R1(9), R4(2)
3	Mt. Unak, Gyeonggi-do	N37°52′, E127°19′	5/3	0/0	0/0	0/0	C2(5)/R1(3)
4	Mt. Daeya, Gyeongsangbuk-do	N36°40′, E127°55′	10/9	0.200/0	0.500/0	0.012/0	C1(9), C2 (1)/R1(9)
5	Mt. Sobaek, Chungcheongbuk-do	N36°57′, E128°29′	10/8	0/0.429	0/0.643	0/0.068	C1(10)/R1(2), R2(6)
6	Mt. Halla, Isl. Jeju	N33°21′, E126°31′	5/3	0/0	0/0	0/0	C2(5)/R3(3)
Regional mean				0.122/0.126	0.246/0.189	0.007/ 0.020	
Japan							
7	Mt. Fubo, Miyagi Pref.	N38°08′, E140°26′	6/6	0.533/0	1.000/0	0.032/0	C5(2), C10(4)/R6(6)
8	Mt. Akagi, Gunma Pref.	N36°33′, E139°11′	10/9	0.733/0.389	2.052/0.583	0.132/ 0.369	C3(5), C5(2), C6(1), C7(2)/ R5(7), R8(2)
9	Mt. Shiroiwa, Kumamoto Pref.	N32°17′, E130°42′	5/5	0/0/	0/0	0/0	C9(5)/R7(5)
Regional mean				0.422/0.130	1.017/0.194	0.055/ 0.123	
China							
10	Mt. Taibai, Shaanxi Prov.	N33°56′, E107°44′	5/5	0.400/0	1.000/0	0.024/0	C3(4), C8(1)/R9(5)
11	Mt. Huang, Anhui Prov.	N30°08′, E118°09′	15/11	0.705/0.327	2.041/0.491	0.112/ 0.311	C3(8), C4(2), C5(2), C6(1), C7(2)/R5(9), R8(2)
Regional mean				0.553/0.164	1.521/0.246	0.068/ 0.156	
Total mean				0.282/0.134	0.688/0.201	0.031/ 0.624	

n, number of collected individuals for cpDNA/ITS analyses, *h*, haplotype diversity, *R*, haplotype richness, π, nucleotide diversity

Leontopodium japonicum Miq. (Asteraceae), an edelweiss plant, is an herbaceous species growing in parts of East Asia, including China, Korea, and Japan (Handel-Mazzetti 1928). On the Korean Peninsula, this species fragmentarily inhabits the upper regions of high mountains with other alpine plants such as *D. lapponica* var. *obovata*, *Pedicularis spicata* Pall., and *Bupleurum euphorbioides* Nakai. Their existence is reminiscent of the habitat isolation that resulted from the south–north migration model typical of alpine plants. Although regarded as an alpine species, *L. japonicum* shows a geographically inverse distribution pattern when compared with Korean alpine plants. For example, while that country tends to represent the southern limit of geographical distribution for many alpine species since the northward post-glacial retreat, it is instead the northern limit for *L. japonicum*. Indeed, this species covers a wide range southward across the more extensive warm-temperate regions of East Asia (Kim 2007) that are characterized by disjunctive forest patches due to the East China Sea (ECS) barrier (i.e., East China, southern tip of the Korean Peninsula, and southern Japan). Moreover, some Korean populations are located at relatively low elevations, i.e., <1000 m (Mt. Unak: 916 m; Mt. Gariwang: 728 m), where biological and environmental conditions are more favorable for *L. japonicum*.

Given its extraordinary distribution, an historical scenario entirely different from that of other alpine plants on the Korean Peninsula would seem appropriate for this

species. Several recent molecular analyses, including sampling on that Peninsula, have allowed researchers to reconstruct the evolutionary history of warm-temperate forest species in East Asia as it pertains to long-term connections among now-disjunctive populations around Korea (Qiu et al. 2009a; Chung et al. 2013; Lee et al. 2013, 2014). Although some of those results have differed, there has been broad consensus for migration via a submerged continental shelf. Thus, we might suspect that, unlike the spreading habits typical of other alpine plants, *L. japonicum* has been part of historical events similar to those associated with warm-temperate *Croomia* (Li et al. 2008), *Kirengeshoma* (Qiu et al. 2009a), or *Platycrater arguta* Siebold & Zucc. (Qiu et al. 2009b; Qi et al. 2014). Furthermore, *Leontopodium* plants in Korean populations manifest morphological characters, e.g., shape of the leaf and the base of the pappus (Nakai 1917), that differ from plants of that species in other regions. Based on those morphological features, plants on the Korean Peninsula were once treated as *L. coreanum* Nakai, an independent species endemic to the region (Lee et al. 2016).

In this study, we inferred the phylogeographical history of *L. japonicum*, a species disjunctively distributed in East Asian temperate regions. Based on results from our analyses with nuclear and chloroplast DNA, we found that its history resembles that of warm-temperate plants. In contrast to a previous hypothesis describing a southward migration and long-term survival in multiple refugia, we believe that our data present another possibility for the historical pattern of movement by Korean alpine plants, i.e., long-term isolation following a transfer of genetic material from more southern regions via a continental shelf and ECS land bridges. In addition, we evaluated the taxonomic entity of Korean *L. japonicum* and its relatives through a molecular approach. These investigations will help improve our scientific understanding about the history of plants growing in alpine (or subalpine) zones of Korea.

Materials and methods

Sampling materials

Leontopodium japonicum is experiencing a rapid decline in population sizes, especially in Korea and Japan, where plants are managed as endangered (Korea Forest Service 2008; Saitama Prefecture 2011). Therefore, only a few individuals remain in some locations. Because we intended to canvass each population thoroughly, we had to collect an appropriate number of samples that would be representative of each extant population. Here, we regarded "sample size" as sufficiently equivalent to "population size". In all, 92 plants were examined from 11 populations (5–10 individuals per population) in Korea, China, and Japan. To minimize the damage to this study species, we removed only a 2-cm-long tip from each sampled leaf and dried those slices in silica gel. Only one ramet per population was collected to prepare voucher specimens, which were then deposited in the herbarium of Inha University (IUI), Incheon, Korea (Online Resource 1).

Total DNA extraction and amplification

Genomic DNA was extracted with a G-spin™ IIp Kit for plants (iNtRON, Sungnam, Korea) according to the manufacturer's instructions. After preliminary screening of several noncoding regions for cpDNA, we selected three that had the most variation: *trn*L$^{(UAA)}$ –*trn*F$^{(GAA)}$, *rps*16, and *rpl*16. The *trn*L$^{(UAA)}$ 5′-*trn*F $^{(GAA)}$ region was amplified with universal primers "c" and "f" (Taberlet et al. 1991). Primers *rpl*16F, *rpl*16R, *rps*16/1F, and *rps*16/1R, *rps*16/2F, and *rps*16/2R were used to amplify the noncoding regions of the *rpl*16 and *rps*16 intron, respectively (Nishizawa and Watano 2000), while primer sets ITS4 and ITS5 were used to amplify the ITS regions of nrDNA (White et al. 1990). All PCRs were conducted in 50-μL volumes comprising 10 ng of DNA, 1 U of Taq DNA polymerase (TaKaRa, Seoul, Korea), 1 × PCR buffer with 1.5 mM $MgCl_2$, 200 μM dNTPs (GeneCraft, Lüdinghausen, Germany), and 20 μM of the appropriate primer pairs. The PCR amplifications were performed as follows: initial denaturation for 2 min at 94 °C; then 35 cycles of denaturing for 30 s at 94 °C, annealing for 45 s at 52 °C, and extension for 1 min at 72 °C; with a final extension for 10 min at 72 °C. All reactions were conducted with a GeneAmp® PCR System 2700 Thermal Cycler (Applied Biosystems, CA, USA). The PCR products were visualized on 1 % agarose gels, treated with a high pure PCR product Purification Kit (iNtRON), and sequenced with an ABI 3100 Genetic Analyzer, using the ABI Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The haplotype sequences identified in *L. japonicum* were deposited in the GenBank database (KP713350–KP713388).

Phylogeographical analyses of cpDNA and ITS

All cpDNA sequences were aligned by Clustal X version 1.83 (Thompson et al. 1997), with additional, minor adjustments done manually. The sequences of both cpDNA regions were combined into one sequence per individual. Each indel (or simple sequence repeat variation) was treated as a one-point mutation in all analyses. Both cpDNA (*trn*L–*trn*F, *rps*16, and *rpl*16) and ITS (ITS1, 5.8S, and ITS2) haplotypes were determined based on those aligned sequences.

For each dataset (cpDNA or ITS), the genealogical degree of relatedness among haplotypes was represented by a statistical parsimony network, generated by the program TCS version 1.21 (Clement et al. 2000). Each gap was counted as an event and then treated as a fifth state. In these analyses, *L. leontopodioides* Beauverd and *Anaphalis sinica* Hance served as outgroups to determine the ancestral haplotype. The haplotype diversity (Nei 1978) was calculated for each population (h_S) and for the overall range (h_T), using DnaSP version 3.53 (Rozas and Rozas 1999). We also calculated haplotype richness, correcting for differences in sample size by applying the rarefaction method (Petit et al. 1998) as implemented in CONTRIB (https://www6.bordeaux-aquitaine.inra.fr/biogeco_eng/Scientific-Production/Computer-software/Contrib-Permut/Contrib). To provide robust estimates, we used the smallest sample size ($n = 5$ and 3 for cpDNA and nrDNA, respectively) for rarefaction. Two coefficients for gene differentiation—G_{ST} (all populations) and N_{ST} (differentiation influenced by both haplotype frequencies and genetic distances between haplotypes)—were estimated from haplotypes according to the methods of Pons and Petit (1996) and by using the program PERMUT (available at http://www.Pierroton.inra.fr/genetics/labo/Software/Permut/). To test for the presence of a phylogeographical structure, we compared values for G_{ST} and N_{ST} with a permutation test that used 10,000 permutations. If $N_{ST} > G_{ST}$, then the closely related haplotypes occurred in the same population, thereby indicating that such a structure existed (Pons and Petit 1996). Population structure was assessed with a spatial analysis of molecular variance (SAMOVA), using SAMOVA version 1.0 (Dupanloup et al. 2002). For this, SAMOVA iteratively seeks a user-defined number of groups (K) that maximizes the total genetic variance resulting from differences among groups (F_{CT}) while minimizing the genetic variance shared between populations within groups (F_{ST}). We set the number of initial conditions to 100 with $K = 2$ to 10. Although K with the highest F_{CT} represented the optimum number of groups and the best population configuration, group structure could not be derived for a configuration of K with one or more single-population groups.

Finally, we estimated the divergence time of the Korean *L. japonicum* lineage, using the program BEAST version 1.8.1 (Drummond and Rambaut 2007). For this, we downloaded two chloroplast genomes of Asteraceae species—*Aster spathulifolius* Maxim. (KF279514; Choi and Park 2015) and *Artemisia frigida* Willd. (JX293720; Liu et al. 2013)—and extracted three noncoding regions for *trn*L$^{(UAA)}$ –*trn*F$^{(GAA)}$, *rps*16, and *rpl*16. Because no fossil records or substitution rates are available for *L. japonicum*, we used 36 Mya and 1.2 Mya as calibration points, ages that have previously been reported (Blöch et al. 2010; Nie et al. 2016). All Markov chain Monte Carlo (MCMC) runs were performed with ten million generations and sampling of every 1000 generations. The results were summarized and checked by Tracer version 1.6 (Drummond and Rambaut 2007). After discarding the first 1000 trees as burn-in, the samples were summarized in a maximum clade credibility tree, using TreeAnnotator version 1.8.2 (Drummond and Rambaut 2007) with the posterior probability limit set to 0.5, and summarizing mean node heights. For the cpDNA dataset, demographic history was explored by mismatch distribution analysis (MDA) of observed haplotype pairwise differences, following a sudden population expansion model (Rogers and Harpending 1992). This procedure employed 1000 bootstraps, as performed by ARLEQUIN (Excoffier et al. 2005). When expansions were detected, the MDA-derived expansion parameter (τ) was converted to an absolute estimate of time (T) by the equation $T = \tau/2u$ (Rogers and Harpending 1992; Rogers 1995), where u is the mutation rate per generation for the entire analyzed sequence. The value for u was calculated as $u = \mu kg$, where μ is the substitution rate per site per year (s/s/y), k is the average sequence length of the cpDNA region under study, and g is the generation time in years. We used a substitution rate for cpDNA of 1.0 to 3.0×10^{-9} s/s/y (i.e., setting 1.0×10^{-9} s/s/y as the lower limit and 3.0×10^{-9} s/s/y as the upper limit for nucleotide substitution rate; Wolfe et al. 1987), and assumed a generation time of 4 years (Keller and Vittoz 2015). In addition, we conducted tests of selective neutrality (Tajima's D and Fu's F_S) to infer potential population growth and expansion (Tajima 1989; Fu 1997).

Results

Sequence variations and distribution of *Leontopodium japonicum*

We sequenced three cpDNA regions from 92 individuals (11 populations) of *Leontopodium japonicum* collected in East Asia. Most of the variations were detected within *rps*16 (646–647 bp) and included three substitutions and one indel. Three nucleotide substitutions each were found in *trn*L–*trn*F (905 bp) and *rpl*16 (254 bp). When combined, their sequences were aligned for a consensus length of 1806 bp, and ten cpDNA haplotypes (chlorotypes C1–C10) were recognized (Table 1). The geographical distribution and frequencies of all 10 are shown in Fig. 1a. Of those, no single haplotype dominated the entire distributional range of this species. Four of the 10 haplotypes were shared across regions, except for Korean populations. Among them, haplotype C3 occurred at high frequency in the populations from China and Japan, while C5, C6, and

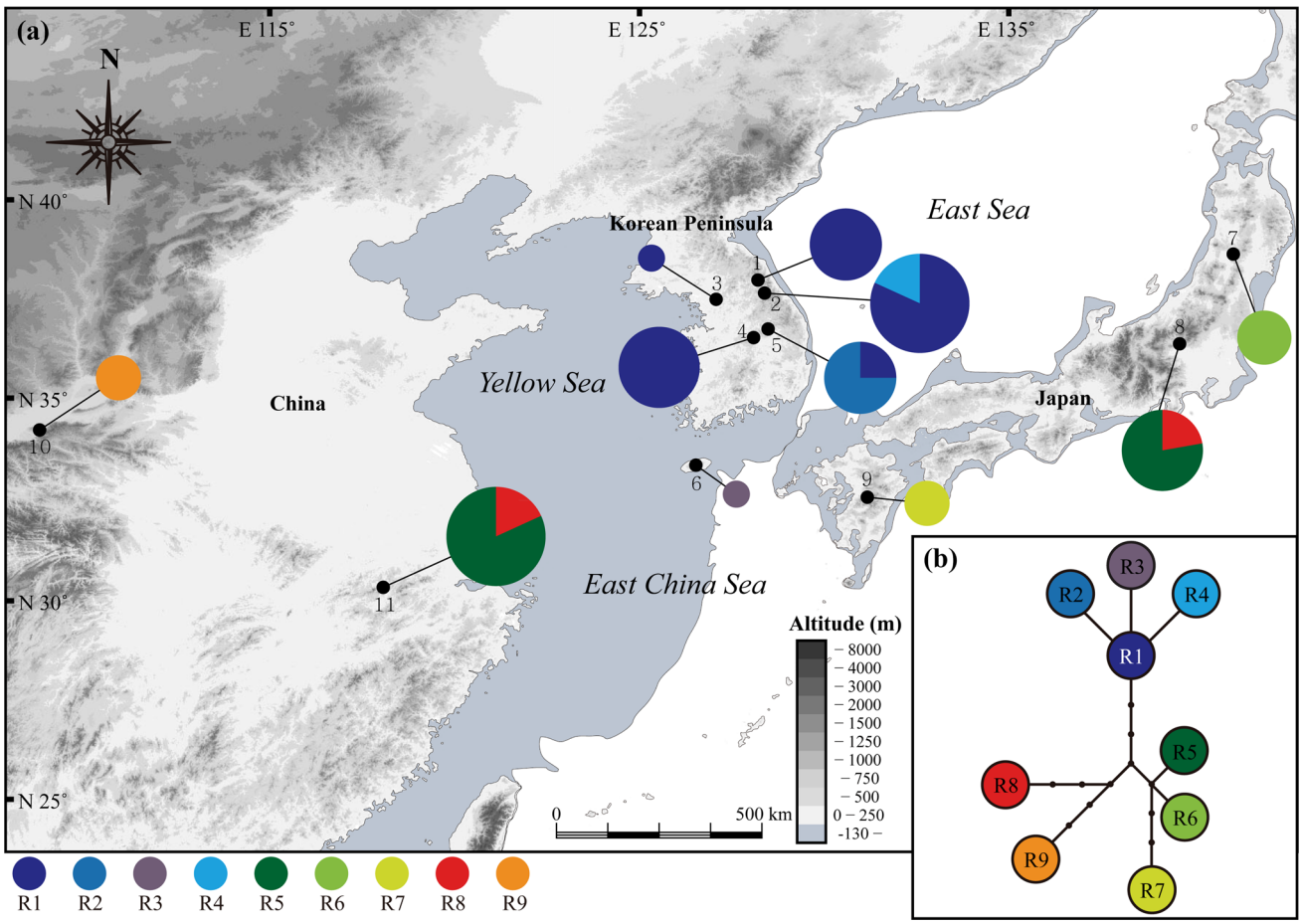

Fig. 2 Geographical distribution of nrDNA haplotypes detected in 11 populations of *Leontopodium japonicum*. **a** Population codes are explained in Table 1. *Bluish shading* indicates assumed exposed coastal areas and sea basins (–130 m) of East Asia during period of glacial-induced alterations in sea level in late Pleistocene. **b** TCS-derived network of genealogical relationships among nine ribotypes

Table 2 AMOVA results for *Leontopodium japonicum* based on two cpDNA population groups defined through SAMOVA

	df	Sum of squares	Variance components	Percentage of variation (%)
Among groups	1	34.017	0.659	47.4
Among populations within groups	9	31.066	0.384	27.6
Within populations	81	28.167	0.348	25.0
Total	91	93.250	1.391	100.0

C7 were distributed in two disjunctive populations (Mt. Huang in China and Mt. Akagi in Japan). The remaining six haplotypes (C1, C2, C4, and C8–C10) were region-specific and not shared.

For nrDNA, the sequences were aligned for a total length of 632 bp from 78 individuals. The sequence alignment presented 19 nucleotide substitutions (ITS1: 8; ITS2: 11) and no indels, and the 5.8S-coding region was monomorphic. With regard to nrDNA, nine ITS haplotypes (ribotypes R1–R9) were identified (Table 1). None occurred over the entire distribution range (Fig. 2a). Although R5 and R8 were shared across regions, with a distinct geographical distribution between two populations in China (Mt. Huang) and Japan (Mt. Akagi), most ribotypes were region-specific and had single populations in China, Korea, or Japan. Moreover, R1 through R4 were found only in populations of Korea. The most common, R1, was observed in all Korean populations except for Jeju, which comprised a region-specific ribotype, R3. Of the five, three were fixed with ribotype R1 while the other two comprised R1/R2 or R4. The polymorphic sites of cpDNA and nrDNA are given in Online Resources 2 and 3, respectively.

Phylogeographical relationships and genetic diversity

To investigate the genetic structure of this species, we applied SAMOVA to define groups among the 11 populations from three disjunctive regions (Table 2). In the SAMOVA of cpDNA haplotypes, two groups ($K = 2$) had the highest F_{CT} value (0.47) that included no single populations. These groups reflected the geographical distribution of the study species. Group I consisted of six populations of Korea while Group II comprised five populations in China and Japan. In the unrooted TCS network of cpDNA (Fig. 1b), the haplotypes were distinguished from each other by one or two mutational steps. With region-specific haplotypes in each lineage (i.e., C4 and C8 for China; C1 and C2 for Korea; C9 and C10 for Japanese Archipelago), this somewhat enabled us to arrange the ten haplotypes for China, Korea, and Japan into three lineages that illustrated geographical distributions even though several haplotypes were shared between China and Japan (i.e., C3, C5–C7; Fig. 1a). The common haplotype C3 was positioned at the center of the network and was assigned by TCS as the highest root probability ($P = 0.308$). Furthermore, the related species *L. leontopodioides* (as the outgroup) was connected with C3 (data not shown). These results suggested that C3 is the ancestral haplotype of *L. japonicum*.

For nrDNA, the statistical parsimony network of ITS (Fig. 2b) also had three lineages and was largely congruent with the network for cpDNA, although the relationships were more complex. Furthermore, features in the network for *L. japonicum* ribotypes were broadly consistent with the strict consensus tree (data not shown). The three lineages had a somewhat geographical basis, i.e., R8 and R9 for China; R1 through R4 for Korea; and R5 through R7 for the Japanese Archipelago. Within the Korea clade, the most abundant ribotype, R1, was identified by one- or two-step mutations away from its ancestral type, while R2 and R4 were considered derivatives. In the Jeju population, plants of R3, once treated as *L. hallaisanense* Hand.–Mazz., also clustered with *L. japonicum* ribotypes of Korea.

Total genetic diversity (h_T) and average within-population diversity (h_s) for the cpDNA data were 0.792 and 0.282, respectively. In addition, total haplotype richness (R_T) and average within-population richness (R_s) for the cpDNA data were 3.419 and 0.688, respectively. The estimates of within-population haplotype diversity and richness varied among populations (Table 1). At the regional level, the highest average value was detected in China ($h_s = 0.553$; $R_s = 1.521$), whereas the Korean populations ($h_s = 0.122$; $R_s = 0.246$) showed comparatively lower-than-average diversity. The permutation test indicated that N_{ST} (0.775) was significantly higher than G_{ST} (0.672), supporting a phylogeographical structuring of the haplotypes in the data. When compared among the three regions, estimated divergences in terms of Φ_{ST} revealed more genetically similar relationships between China and Japan ($\Phi_{ST} = 0.471$). In contrast, the highly significant genetic differentiation between Korea and Japan ($\Phi_{ST} = 0.846$) was slightly higher than the differentiation between Korea and China ($\Phi_{ST} = 0.742$).

For the ITS data set, values for diversity and richness were $h_T = 0.785/h_s = 0.134$ and $R_T = 2.423/R_s = 0.201$, respectively. Although at the regional level both values for Korea ($h_s = 0.126$; $R_s = 0.189$) were similar to those estimated with cpDNA data ($h_s = 0.122$; $R_s = 0.246$), average within-population diversity and richness in the other two regions were comparatively lower for ITS data (China: $h_s = 0.164$; $R_s = 0.246$, Japan: $h_s = 0.130$; $R_s = 0.194$) than for cpDNA (China: $h_s = 0.553$; $R_s = 1.521$, Japan: $h_s = 0.422$; $R_s = 1.017$). Unlike the cpDNA data, the total N_{ST} (0.897) based on ITS haplotype variation was slightly larger than the G_{ST} (0.846).

Estimating lineage divergence and population demographic histories

Using the cpDNA chronogram produced by BEAST, we dated the divergence between groups to approximately 0.30 Mya (95 % HPD: 0.043–0.942; Online Resource 4, 5). We then performed MDA with cpDNA data but failed to infer the long-time demographic history of populations (Table 3; Fig. 3).

The mismatch distribution of Group I and Group II was unimodal and multimodal, respectively, indicating that the latter group did not fit a model of sudden expansion as well as the former did. However, such an expansion model was not supported by the values for SSD and *Hrag* (Harpending's raggedness index; Harpending 1994), i.e., Group I:

Table 3 Results of neutrality tests and mismatch distribution analysis for two population groups

	τ	SSD (P value)	*Hrag* (P value)	Fu,s F_S (P value)	Tajima's D (P value)
Group I	0.781	0.024 (0.021)	0.260 (0.007)	1.995 (0.797)	1.729 (0.973)
Group II	3.447	0.026 (0.184)	0.089 (0.183)	−0.215 (0.502)	0.002 (0.548)

τ time in number of generations elapsed since sudden expansion event, *SSD* sum of squared deviations, *Hrag* Harpending's raggedness index (Harpending 1994)

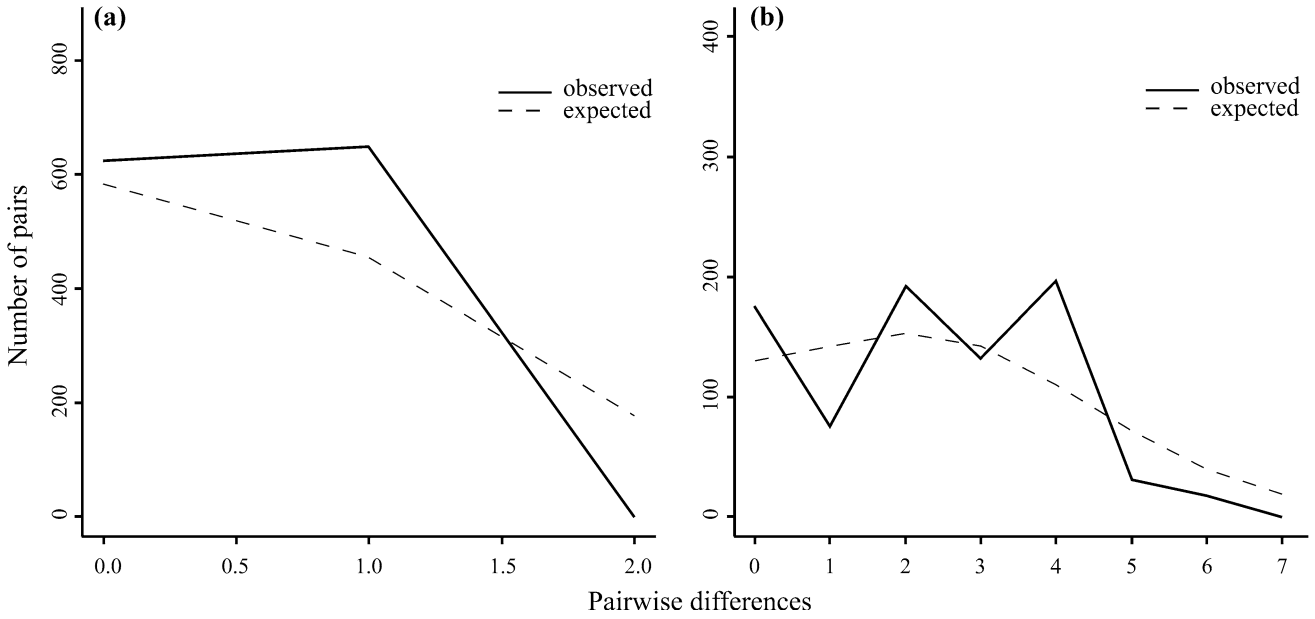

Fig. 3 Mismatch distribution of chloroplast DNA sequence data for *Leontopodium japonicum* within Group I (**a**) and Group II (**b**). *Dotted line* indicates observed values; *solid line* values expected under model of sudden population expansion (Rogers and Harpending 1992)

SSD = 0.026, *Hrag* = 0.089 versus Group II: SSD = 0.024, *Hrag* = 0.260. Nevertheless, after considering the corresponding cpDNA substitution rate, a sequence length of 1806 bp, a four-year generation time, and τ-value of 0.781, we were able to date the expansion of geographical range by Korean populations (i.e., Group I) to the last glacial cycle (ca. 0.02–0.05 Mya).

Discussion

Korean populations of *Leontopodium japonicum* originated from southern regions

The patterns of genetic variation identified here are largely congruent between the cpDNA and nrDNA data (Figs. 1 and 2). Our results with cpDNA reveal a strong phylogeographical structure across the East China Sea ($N_{ST} > G_{ST}$, $P < 0.01$). Despite their considerable geographical distance, China and Japan share two and four haplotypes (ribotype and chlorotype, respectively) that include both ancestral and derived types in two separate lineages. These findings imply a deep genetic affinity between them that might have been caused by frequent gene flow during multiple glacial-interglacial cycles. We find it interesting that these genetic patterns for *L. japonicum*, regardless of geographical distance, seem to resemble the genetic footprint of warm-temperate plant species that have had a primary migration corridor across the ECS. For example, phylogeographical studies of a common warm-temperate deciduous tree, *Cercidiphyllum japonicum* Siebold & Zucc., have presented almost no genetic differentiation on either side of the ECS, apparently following a predicted continuous distribution of that species via land bridges during LGM (Qi et al. 2012).

The genetic isolation of Korean populations further strengthens our hypothesis of a south-to-north historical migration by *L. japonicum* that resembles that of warm-temperate plants. If the current distribution of our test species had resulted from a previously described scenario typical of other Korean alpine plants, i.e., spreading from northern-sourced populations (Ikeda and Setoguchi 2007; Ikeda et al. 2008; Chung et al. 2012, 2015), then one would have expected to see a genetic composition of current Korean populations that is comparable to those in the adjacent two regions of China and Japan. However, our network and haplotype distribution maps present a complete absence of sharing and, instead, our AMOVA and SAMOVA results reveal two well-defined groups corresponding to Korean populations (Group I) and those of China/Japanese main islands (Group II).

These findings largely coincide with the genetic differentiation reported for *Kirengeshoma* (Korea vs. China/Japan), which is evidence of long-term isolation after the genetic material of that genus migrated from more southern regions (Qiu et al. 2009a). In fact, previous phylogeographical studies (Qiu et al. 2009a; Aizawa et al. 2012; Lee et al. 2013) firmly demonstrated that the initial founding event as well as subsequent gene flow by other species from more southern populations onto the Korea Peninsula was fairly restricted, even when linked by a land bridge during glacial periods. This was due to

inhospitable corridor conditions (e.g., locally arid habitats along the continental shelf plus vast non-forested lands). Moreover, the dominance of warm-temperate forests and the retreat to the north again by boreal forests across the continental shelf during glacial periods would have further restrained any range expansion by *L. japonicum* in isolated habitats (Harrison et al. 2001).

Based on this, we can assume that the Korean *L. japonicum* populations migrated northward from southern regions via ECS land bridges. However, considering their few mutational steps in network analysis and divergence estimation (0.30 Mya), this pattern has been shaped only relatively recently, perhaps shortly before the divergence (penultimate glacial period). After those events, individual populations might have become differentiated and then been maintained through long-term isolation within the Korean region, without any subsequent re-colonization during LGM. Nonetheless, because of the sharing of chlorotype C3 between China and Japan, which is directly connected to the lineage for the differentiated Korean chlorotypes, we cannot determine the exact origin of the initial Korean population, whether it be China or Japan. Alternatively, we might hypothesize that the first Korean populations were colonized from a southern source, within a distribution range that stretched across the ECS via land bridges, even though that area is now submerged. Consequently, our molecular data suggest that, as with warm-temperature species, the current disjunctive distribution of *L. japonicum* is a result of migration via a continental shelf during glacial periods. Furthermore, following migration from southern areas, this long-term geological isolation from adjacent regions, presumably occurring since the time of that migration, may have caused these Korean populations to evolve independently, leading to genetic divergence and a specific genetic structure.

Establishment of genetic diversity within Korean populations

Our cpDNA analysis showed that Korean populations are characterized by the lowest level of within-population diversity ($h_S = 0.122$) and have only one or two chlorotypes (C1 and/or C2). These results contrast markedly with previous predictions for Korean alpine plants, which typically have high genetic diversity (Chung et al. 2012, 2015). Our findings also do not correspond to the high genetic diversity reported for the warm-temperate plant *Kirengeshoma koreana* Nakai, which migrated from southern refugia via the continental shelf, although the analyses using ISSR markers have shown slightly less genetic diversity in Korea than in China and Japan (Qiu et al. 2009a). Therefore, this establishment of diversity for Korean *L. japonicum* does not conform well to previous theories that historical migration follows a particular direction. Instead, we can speculate that the period of settlement since the initial migration event onto the Korean Peninsula is most likely the principal factor that explains why within-population diversity is the lowest for *L. japonicum* populations in that region.

As described above, the ancient, initial population would have migrated during the penultimate glacial (Riss) period, at approximately 302,000 years BP, with no subsequent re-colonization from adjacent regions. This would indicate that those Korean populations underwent only one interglacial/glacial cycle in that region (i.e., Riss-Würm interglacial and subsequent Würm glacial period). We would then expect that such a relatively brief establishment history would limit the development of genetic variation, as has also been reported for *Pinus koraiensis* (Aizawa et al. 2012). This conjecture is consistent with the theory that populations with a long evolutionary history exhibit greater genetic diversity (Huang et al. 2001).

In addition, a strong bottleneck or founder effect accompanied by the current fragmented habitat might be another factor associated with decreased and simplified diversity. However, in this case, the similarity of habitats among adjacent regions means that those founder effects are, in fact, not a primary factor. Rather, the homogenous structure, along with our unimodal mismatch distribution for the region (Fig. 3a), indicates that the extant population on that peninsula was derived from a small interglacial refugium through recent range expansion in that region, presumably during LGM, even though our significant SSD and raggedness values do not support this. Together with those traits, the high levels of genetic diversity calculated for China and Japan as well as a large contiguous habitat imply that both regions represented in Group II served as important interglacial refugia in the past. Thus, population sizes remained stable and effective in those regions over a long period.

Taxonomic entity of Korean *L. japonicum*

Because *Leontopodium japonicum* is greatly diversified in its external morphology, its taxonomic boundary can be confusing. For example, several taxa previously regarded as distinct species are now merged into *L. japonicum* as only regional varieties (China: var. *saxatile*, var. *microcephalum*; Japan: var. *perniveum*, var. *shiroumense*, var. *spathulatum*). The populations of Korea were originally described as *Leontopodium coreanum* Nakai (1917), a closely related species, based on morphological characters such as leaf shape and pappus base (Nakai 1917). However, those two taxa have not been clearly distinguished in terms of their morphological traits, and their validity is still

questioned (Lee 1996; Lee et al. 2010). Plants within the southernmost population of this species in Korea have also been treated as a separate species because of their shorter stems and different bract shape (*L. hallaisanense* Hand.-Mazz.; Handel-Mazzetti 1928). In this study, we also found slight morphological differences among Korean populations (i.e., plants once regarded as *L. coreanum* and *L. hallaisanense*), when compared with populations in China and Japan. However, such vegetative character variations were easily observed at the intraspecific level of the genus (Ling 1965), which actually complicated the identification of those species. Thus, it is not an easy task to discern their taxonomic boundary between Korea and other regions.

To explain these subtle morphological differences, our molecular analysis provides further insightful evidence. In the clustering analyses based on nrDNA and cpDNA, the Korean populations represent a separate genetic lineage without any shared haplotypes. Such genetic distinctions are further confirmed by SAMOVA results, which demonstrate a clear-cut geographical division between Korea (Group I) and China/Japanese main islands (Group II). These obvious geographical patterns of genetic and morphological divergence enable us to conclude that the Korean populations have undergone allopatric divergence through geographical isolation. Furthermore, previously reported chromosome counts for *L. japonicum* somewhat coincide with our assumption. Although chromosome numbers for Korean *L. japonicum* are $2n = 28$ (Lee et al. 2010), those for the other two regions are equally counted as $2n = 26$ with the secondary constriction (Arano 1956; Russell et al. 2013). This could be interpreted as an increase in dysploidy that supposedly took place in *Leontopodium* during its adaptation to an unfamiliar environment (Meng et al. 2012), such as the splitting of a secondary constriction since its migration into Korea. In particular, such chromosomal changes also might have functioned as an introgression barrier that inhibited recurrent gene flow between two groups despite the existence of land bridges during glacial periods. Indeed, a similar phenomenon (allopatric divergence as a major precursor leading to speciation) has been reported for other plants distributed around the ECS, including *Croomia* (Li et al. 2008), *Kirengeshoma koreana* (Qiu et al. 2009a), *Neolitsea sericea* (Blume) Koidz. (Lee et al. 2013), and *Platycrater arguta* Siebold & Zucc. (Qiu et al. 2009b; Qi et al. 2014). Consequently, our molecular data definitely prove that the plants in Korean populations are not conspecific with *L. japonicum* and should be treated as an independent species or at least a regional variety of that species. Based on the amount of haplotypes missing in our network analysis, however, we are still concerned about the limited number of populations sampled in China and Japan. This could lead to biased results such as significantly fewer mutational steps between groups as well as a distorted demographic history and genetic diversity in the populations of China and Japan. Therefore, further studies using highly polymorphic markers with broad sampling are needed if we are to gain more comprehensive knowledge about these regions as putative refugial areas.

Acknowledgments The authors thank their colleagues J.Y. Lee, I.S. Choi, and D.P. Jin at the Plant Systematics Laboratory of Inha University for help in collecting plant materials and assisting with statistical analysis. This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. NRF–2015R1D1A1A01059886).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Information on Electronic Supplementary Material

Online resources 1. Collection information for 11 populations of Leontopodium japonicum sampled and analyzed in this study. Voucher specimens were deposited in the herbarium of Inha University (IUI), Incheon, Korea.
Online resources 2. Polymorphic sites and cpDNA haplotypes based on sequences of three noncoding regions from *Leontopodium japonicum.*
Online resources 3. Polymorphic sites of ITS1 and ITS2 (5.8S-excluded) regions and nrDNA haplotypes based on sequences from *Leontopodium japonicum.*
Online resources 4. BEAST Bayesian divergence time estimates of the *Leontopodium japonicum* based on three non-coding regions of cpDNA. The values above the branching points represent divergence time (Mya). Arrows indicate each calibration point (36 Mya and 1.2 Mya).
Online resources 5. Alignment data for BEAST Bayesian divergence time estimates of the *Leontopodium japonicum* based on three non-coding regions of cpDNA. (NXS 38 kb).

References

Aizawa M, Kim ZS, Yoshimaru H (2012) Phylogeography of the Korean pine (*Pinus koraiensis*) in northeast Asia: inferences from organelle gene sequences. J Pl Res 125:713–723

Arano H (1956) Karyotaxonomic studies in tribe Tubuliflorae of Compositae. I. The karyotype analysis and its phylogenic considerations in subtribe Gnaphaliineae. Jap J Genet 31:137–143

Avise JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge

Blöch C, Dickoré WB, Samuel R, Stuessy TF (2010) Molecular phylogeny of the edelweiss (*Leontopodium*, Asteraceae–Gnaphalieae). Edinburgh J Bot 67:235–264

Choi KS, Park S (2015) The complete chloroplast genome sequence of *Aster spathulifolius* (Asteraceae); genomic features and relationship with Asteraceae. Gene 572:214–221

Chung MY, López-Pujol J, Maki M, Kim KJ, Chung JM, Sun BY, Chung MG (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. J Heredity 103:692–702

Chung MY, López-Pujol J, Moon MO, Chung JM, Kim CS, Sun BY, Kim KJ, Chung MG (2013) Comparison of genetic diversity in the two arctic–alpine plants *Diapensia lapponica* var. *obovata* (Diapensiaceae) and *Empetrum nigrum* var. *japonicum* (Empetraceae) between Sakhalin in Russian Far East and Jeju Island in Korea, the southernmost edge of their distribution range. Popul Ecol 55:159–172

Chung MY, López-Pujol J, Lee YM, Oh SH, Chung MG (2015) Clonal and genetic structure of *Iris odaesanensis* and *Iris rossii* (Iridaceae): insights of the Baekdudaegan Mountains as a glacial refugium for boreal and temperate plants. Pl Syst Evol 301:1397–1409

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Molec Ecol 9:1657–1659

Drummond AJ, Rambaut A (2007) BEAST: bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214

Dupanloup I, Schneider S, Excoffier L (2002) A simulated annealing approach to define the genetic structure of populations. Molec Ecol 11:2571–2581

Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform Online 1:47–50

Fu X-Y (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915–925

Hampe A, Petit RJ (2005) Conserving biodiversity under climate change: the rear edge matters. Ecol Lett 8:461–467

Handel-Mazzetti H (1928) Systematische monographie der gattung *Leontopodium*. Beih Bot Centralbl 44:1–178

Harpending H (1994) Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. Hum Biol 66:591–600

Harrison SP, Yu G, Takahara H, Prentice IC (2001) Palaeovegetation: diversity of temperate plants in East Asia. Nature 413:129–130

Hewitt GM (2000) The genetic legacy of the Quaternary ice ages. Nature 405:907–913

Hewitt GM (2003) Ice ages: their impact on species distributions and evolution. In: Rothschild LJ, Lister AM (eds) Evolution on planet earth. Academic Press, Oxford, pp 339–361

Huang S, Chiang YC, Schaal BA, Chou CH, Chiang TY (2001) Organelle DNA phylogeography of *Cycas taitungensis*, a relict species in Taiwan. Molec Ecol 10:2669–2681

Huck S, Büdel B, Kadereit JW, Printzen C (2009) Range-wide phylogeography of the European temperate-montane herbaceous plant *Meum athamanticum* Jacq.: evidence for periglacial persistence. J Biogeogr 36:1588–1599

Hultén E, Fries M (1986) Atlas of North European Vascular Plants: North of the Tropic of Cancer, I–III. Koeltz Scientific Books, Königstein

Ikeda H, Setoguchi H (2007) Phylogeography and refugia of the Japanese endemic alpine plant *Phyllodoce nipponica* Makino (Ericaceae). J Biogeogr 34:169–176

Ikeda H, Senni K, Fujii N, Setoguchi H (2008) Survival and genetic divergence of an arctic-alpine plant, *Diapensia lapponica* subsp. *obovata* (Fr. Schm.) Hultén (Diapensiaceae), in the high mountains of central Japan during climatic oscillations. Pl Syst Evol 272:197–210

Im HT (1992) Plant geographical study for the plant of Cheju. Korean J Pl Taxon 22:219–234

Keller R, Vittoz P (2015) Clonal growth and demography of a hemicryptophyte alpine plant: *leontopodium alpinum* Cassini. Alp Bot 125:31–40

Kim KJ (2007) *Leontopodium*. In: Flora of Korea Editorial Committee (eds) The Genera of vascular plants of Korea, Academy Publishing Co., Seoul, pp 1003–1005

Kong WS (2007) Biogeography of Korean plants. Geobook, Seoul

Kong WS, Watts D (1993) The plant geography of Korea with an emphasis on the alpine zones. Kluwer Academic Publishers, Boston

Korea Forest Service (2008) Rare plants data book in Korea. Korea National Arboretum, Pocheon

Lee WT (1996) Lineamenta florae Koreae. Academy Publishing Co., Seoul

Lee DH, Chung GY, Choi BH (2010) Chromosome number of four Korean species of *Leontopodium* (Asteraceae). Korean J Pl Taxon 40:153–156

Lee JH, Lee DH, Choi BH (2013) Phylogeography and genetic diversity of East Asian *Neolitsea sericea* (Lauraceae) based on variations in chloroplast DNA sequences. J Pl Res 126:193–202

Lee JH, Lee DH, Choi IS, Choi BH (2014) Genetic diversity and historical migration patterns of an endemic evergreen oak, *Quercus acuta*, across Korea and Japan, inferred from nuclear microsatellites. Pl Syst Evol 300:1913–1923

Lee DH, Park JS, Choi BH (2016) A taxonomic review of Korean *Leontopodium* R. Br. ex Cassini (Asteraceae). Korean J Pl Taxon 46:149–162

Li EX, Yi S, Qiu YX, Guo JT, Comes HP, Fu CX (2008) Phylogeography of two East Asian species in *Croomia* (Stemonaceae) inferred from chloroplast DNA and ISSR fingerprinting variation. Molec Phylogen Evol 49:702–714

Ling Y (1965) Notulae de nonnulis generibus tribus Inulearum familiae Compositarum florae Sinicae. Acta Phytotax Sin 10:167–181

Liu Y, Huo N, Dong L, Wang Y, Zhang S, Young HA, Feng X, Gu YQ (2013) Complete chloroplast genome sequences of Mongolia medicine *Artemisia frigida* and phylogenetic relationships with other plants. PLoS ONE 8:e57533

Meng Y, Nie ZL, Sun H, Yang Y-P (2012) Chromosome numbers and polyploidy in *Leontopodium* (Asteraceae: gnaphalieae) from the Qinghai-Tibet Plateau of S.W. China. Caryologia 65:87–93

Michl T, Huck S, Schmitt T, Liebrich A, Haase P, Buedel B (2010) The molecular population structure of the tall forb *Cicerbita alpina* (Asteraceae) supports the idea of cryptic glacial refugia in central Europe. Bot J Linn Soc 164:142–154

Nakai T (1917) Notulae ad plants Japonicae et Koreae, XIV. Bot Mag (Tokyo) 31:97–112

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–590

Nie ZL, Funk VA, Meng Y, Deng T, Sun H, Wen J (2016) Recent assembly of the global herbaceous flora: evidence from the paper daisies (Asteraceae: Gnaphalieae). New Phytol 209:1795–1806

Nishizawa T, Watano Y (2000) Primer pairs suitable for PCR-SSCP analysis of chloroplast DNA in angiosperms. J Phytogeogr Taxon 48:63–66

Petit RJ, Mousadik AE, Pons O (1998) Identifying populations for conservation on the basis of genetic markers. Conserv Biol 12:844–855

Petit RJ, Aguinagalde I, de Beaulieu IL, Bittkau C, Brewer S, Cheddadi R, Ennos R, Fineschi S, Grivet D, Lascoux M, Mohanty A, Müller-Starck G, Demesure-Musch B, Palme A, Martin JP, Rendell S, Vendramin GG (2003) Glacial refugia: hotspots but not melting pots of genetic diversity. Science 300:1563–1565

Pons O, Petit R (1996) Measuring and testing genetic differentiation with ordered versus unordered alleles. Genetics 144:1237–1245

Prefecture Saitama (2011) Saitama red data book 2011. Saitama Prefecture, Saitama

Qi XS, Chen C, Comes HP, Sakaguchi S, Liu YH, Tanaka N, Sakio H, Qiu YX (2012) Molecular data and ecological niche modelling reveal a highly dynamic evolutionary history of the East Asian Tertiary relict *Cercidiphyllum* (Cercidiphyllaceae). New Phytol 196:617–630

Qi XS, Yuan N, Comes HP, Sakaguchi S, Qiu YX (2014) A strong 'filter' effect of the East China Sea land bridge for East Asia's temperate plant species: inferences from molecular phylogeography and ecological niche modelling of *Platycrater arguta* (Hydrangeaceae). BMC Evol Biol 14:41

Qiu YX, Sun Y, Zhang XP, Lee J, Fu CX, Comes HP (2009a) Molecular phylogeography of East Asian *Kirengeshoma* (Hydrangeaceae) in relation to Quaternary climate change and landbridge configurations. New Phytol 183:480–495

Qiu YX, Qi XS, Jin XF, Tao XY, Fu CX, Naiki A, Comes HP (2009b) Population genetic structure, phylogeography, and demographic history of *Platycrater arguta* (Hydrangeaceae) endemic to East China and South Japan, inferred from chloroplast DNA sequence variation. Taxon 58:1226–1241

Rogers AR (1995) Genetic evidence for a Pleistocene population explosion. Evolution 49:608–615

Rogers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. Molec Biol Evol 9:552–569

Rozas J, Rozas R (1999) DNASP, version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics 15:174–175

Russell A, Safer S, Weiss-Schneeweiss H, Temsch E, Stuppner H, Stuessy EF, Samuel R (2013) Chromosome counts and genome size of *Leontopodium* species (Asteraceae: Gnaphalieae) from south-western China. Bot J Linn Soc 171:627–636

Sakaguchi S, Takeuchi Y, Yamasaki M, Sakurai S, Isagi Y (2011) Lineage admixture during postglacial range expansion is responsible for the increased gene diversity of *Kalopanax septemlobus* in a recently colonised territory. Heredity 107:338–348

Sakaguchi S, Qiu YX, Liu YH, Qi XS, Kim SH, Han J, Takeuchi Y, Worth JRP, Yamasaki M, Sakurai S, Isagi Y (2012) Climate oscillation during the Quaternary associated with landscape heterogeneity promoted allopatric lineage divergence of a temperate tree *Kalopanax septemlobus* (Araliaceae) in East Asia. Molec Ecol 21:3823–3838

Stewart JR, Lister AM, Barnes I, Dalén L (2010) Refugia revisited: individualistic responses of species in space and time. Proc Roy Soc London, SerB, Biol Sci 277:661–671

Taberlet P, Gielly L, Patou G, Bouvet J (1991) Universal primers for amplification of three noncoding regions of chloroplast DNA. Pl Molec Biol 17:1105–1109

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acids Res 25:4876–4882

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, California, pp 315–322

Wolfe KH, Li WH, Sharp PM (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proc Natl Acad Sci USA 84:9054–9058

Zhai SN, Comes HP, Nakamura K, Yan HF, Qiu YX (2012) Late Pleistocene lineage divergence among populations of *Neolitsea sericea* (Lauraceae) across a deep sea-barrier in the Ryukyu Islands. J Biogeogr 39:1347–1360