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[Dear Editor-in-Chief, revision based on Reviewer# 1 is **in purple**; revision by Reviewer# 2 is **in blue**]

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

Mi Yoon Chung · Jordi López-Pujol · You Mi Lee · Seung Hwan Oh · Myong Gi Chung

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Right running head: M. Chung et al.
Left running head: Clonal and genetic structure in two *Iris* species

M. Y. Chung
Department of Biology Education
Seowon University
Cheongju 361-742, Republic of Korea
e-mail: miyoon71@seowon.ac.kr

J. López-Pujol
BioC-GReB, Laboratori de Botànica, Facultat de Farmàcia, Universitat de Barcelona, Barcelona 08028, Spain
(current address: BioC-GReB, Botanic Institute of Barcelona (IBB-CSIC-ICUB), Barcelona08038, Spain)

Y. M. Lee
Office of Director, Korea National Arboretum, Pocheon 487-821, Republic of Korea

S. H. Oh
Division of Forest Biodiversity, Korea National Arboretum, Pocheon 487-821, Republic of Korea

M. G. Chung (corresponding author)
Department of Biology and the Research Institute of Natural Science
Gyeongsang National University
Jinju 660-701, Republic of Korea
e-mail: mgchung@gnu.ac.kr

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Abstract The main Korean mountain range that stretches from north to south (the Baekdudaegan) has been suggested to harbor an important glacial refugium for boreal and temperate plant species. Under this scenario, we expect high levels of within-population genetic variation and low or moderate degree of among-population differentiation within these species. To test this hypothesis, we examined clonal diversity and levels of allozyme diversity in the boreal *Iris odaesanensis* and in its temperate congener *I. rossii*. In addition, we compiled data on boreal and temperate species whose distribution in the Peninsula is mostly centered in the Baekdudaegan to determine if there is a common pattern. We found lower clonal diversity in *I. odaesanensis* compared to *I. rossii*. Both studied species maintained high levels of genetic variation as well as a moderate genetic differentiation (*%P* = 52.5 and 47.5, *A* = 1.70 and 1.58, H_e = 0.158 and 0.150, and F_{ST} = 0.196 and 0.189 for *I. odaesanensis* and *I. rossii*, respectively), in line with what occurs for the species distributed on the Baekdudaegan (*n* = 14, *%P* = 46.7, *A* = 1.73, H_e = 0.161, and F_{ST} = 0.190). This study strongly suggests that the Baekdudaegan may have acted as a refugium for boreal and temperate species, in a similar way to the southern Appalachians in the southeastern United States.

Keywords Allozymes · Congener · Conservation · Genetic diversity · Glacial refugium · Historical factor · *Iris* · Korean Peninsula.

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Introduction

On the Korean Peninsula, its main mountain range, the "Baekdudaegan Mountains" (the Baekdudaegan from now on) — which is sometimes regarded as the backbone of the Peninsula because it runs north to south with over 1600 km long (Choi 2004; Fig. 1) — has been proposed as harboring multiple refugia for the boreal and temperate flora (Chung et al. 2012, 2013a,b, 2014) on the basis of a series of population-genetics studies conducted on species native to this mountain range (see table 3 in Chung et al. 2014). Using allozymes, a common pattern of high/moderate within-population and low/moderate between-population genetic variation seems to be emerging for many boreal and temperate elements native to this mountain system, both widespread and range-restricted. According to these authors (Chung et al. 2012, 2013a,b, 2014), this pattern would be attributable to the existence of large refugial areas ("macrorefugia" sensu Rull 2009) throughout the Baekdudaegan. The varied topography of these mountains (peaking over 1500–2000 m), coupled with a north-south orientation, the close proximity to the sea, and the fact that the Baekdudaegan remained totally unglaciated (with the exception of high-elevation mountains of over 2300 m on its north tip; Fig. 1) would have allowed plant species to have persisted there throughout the glacial/interglacial cycles, presumably maintaining large effective population sizes and high rates of recurrent gene flow (Kong and Watts 1993). Paleoecological data of the Korean Peninsula are mostly in agreement with this proposed scenario; pollen records from localities within or nearby the Baekdudaegan suggest the existence of boreal and/or temperate forests during or around the Last Glacial Maximum (LGM) instead of steppe or desert vegetation (Choi 1998; Chung et al. 2006), as most

palaeovegetation reconstructions for the Peninsula do (Harrison et al. 2001; Hope et al. 2004; Prentice et al. 2011; but see Adams and Faure 1997; Zheng et al. 2007).

To gain further insights into the validity of this scenario, we chose two congeneric herbs native to the Baekdudaegan, *Iris odaesanensis* Y. N. Lee, a boreal species, and *I. rossii* Baker, a temperate one. *Iris odaesanensis* is distributed from Jilin Province in northeastern China to the Korean Peninsula. In Jilin, *I. odaesanensis* occurs on forest margins, meadows, and damp hillsides along ditches in medium-elevation mountains (ca. 1500 m) (Zhao et al. 2000). On the Korean Peninsula, *I. odaesanensis* occurs largely under *Quercus mongolica*-dominated temperate deciduous forests at 1000–1500 m (Table 1), both in the Baekdudaegan (IO-1 to IO-5, and IO-7; Fig. 1) and in one of its branches, the so-called "Nakdongjeongmaek" (IO-6 and IO-8; Fig. 1). Although the species is listed as endangered in the Wildlife Protection Act of Korea (Ministry of Environment 2005), it is relatively common along its distributional range, with the number of shoots per population—we use the term "shoot" (see definition below) instead of "individual" because the species can propagate via rhizomes—usually ranging from hundreds to thousands (rarely tens, such as in IO-1 and IO-2; Table 1). The congener *Iris rossii* has a somewhat wider distribution, occurring in eastern Liaoning Province in northeastern China, in the Korean Peninsula, and in Japan (Zhao et al. 2000). In China, *I rossii* occurs at low-elevation (ca. 100 m) meadows at forest margins or on sunny hillsides (Zhao et al. 2000). On the Korean Peninsula, *I. rossii* can occur from low hills (300–500m; Table 1) in the peripheral areas of the Baekdudaegan (under pines and deciduous forests) to the medium-elevation mountains of the main ridge of the Baekdudaegan, under deciduous forests (such as the IR-1 population, located at 980 m; Table 1). Like *I. odaesanensis*, *I. rossii* is locally abundant across the Korean Peninsula.

The life-history traits and ecology of the two *Iris* species show some striking differences, such as stoloniferous rhizomes in *I. odaesanensis* (7–35 cm long) vs. short ones in *I. rossii* (2–10 cm long), and mat-like shoots within populations of *I. odaesanensis* vs. a few shoots within populations of *I. rossii*. Based on these differences, we expect lower clonal (genotypic) diversity in *I. odaesanensis* compared to *I. rossii*. Considering that the Baekdudaegan and its adjacent mountain ranges likely harbored multiple, large refugia ("macrorefugia") for boreal and temperate plant species during the Pleistocene, then we would find high genetic diversity within populations and low differentiation among populations for both species. To test these predictions, we used allozymes as suitable genetic markers to identify clones and to estimate clonal diversity and levels and partitioning of genetic diversity with a representative sample of populations of the two *Iris* species.

Materials and methods

Study plants

Inflorescences of *I. odaesanensis* are 9–13 cm long and bear two white flowers (3–4 cm in diameter) with the outer segments with a central, yellow signal patch. Flowers are open from April to May. Strongly 3-angled fruit (capsule) matures from June to July and is ovoid (2.3–2.7 cm long) at maturity (Zhao et al. 2000; M. Y. Chung and M. G. Chung pers. obs.). Inflorescences of *I. rossii*, barely emerging above ground, bear violet solitary flowers (3.5–4.0 cm in diameter); flowers are open from April to May, and capsules mature from June to August, being globose at maturity (Zhao et al. 2000; M. Y. Chung and M. G. Chung pers.

obs.). The breeding systems and pollinators for the two *Iris* species are unknown. Many species of *Iris* are predominantly outcrossers and self-compatible (Kron et al. 1993; Hannan and Orick 2000; Liu et al. 2011), although the species within the section *Oncocyclus* are known to be self-incompatible (Sapir et al. 2005). Members of *Iris* are usually pollinated by a variety of bees and flies (Uno 1982; Sutherland 1990; Liu et al. 2011; Watts et al. 2013). Whereas gravity (barochory) seems to be a main dispersal mechanism on the terrestrial irises, hydrochory has been described for several *Iris* species (e.g., *I. pseudacorus*) occurring on wetlands, floodplains, or riparian plant communities (Whitehead 1971). Both species studied here can also propagate via rhizomes.

Population sampling

From June to July in 2009, we collected 555 leaf samples from eight populations of *I. odaesanensis* (IO-1 to IO-8; Table 1 and Fig. 1), and 141 shoots from six populations of *I. rossii* (IR-1 to IR-6; Table 1 and Fig. 1). As rhizomes of *I. odaesanensis* are relatively long and branched, we collected leaf samples at 30 cm intervals from mat-like shoots; for *I. rossii*, we collected samples from all visually identified shoots (except for closely located shoots, for which we collected only one shoot) because many shoots are scattered within the populations. In our study system, a “shoot” is the aerial part of a ramet, as the term “ramet” also includes the rhizome connecting it with other ramets of a given genet (the genetic individual).To minimize the damage to these irises, we collected only one leaf per shoot.

Enzyme electrophoresis

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

Data analysis

To identify clones and to conduct further genetic analyses, we considered a locus to be polymorphic when two or more alleles were observed, regardless of their frequencies. As

multiple ramets (N_T) representing allozyme-based identical multilocus genotypes (MLG) could result either from clonal propagation or distinct sexual reproduction events, it is important to discriminate these cases to correctly identify clonal ramets. Using the program GenClone v. 2.0 (Arnaud-Haond and Belkhir 2007), we calculated $P_{gen}F_{IS}$, the probability of identical MLG to occur by chance due to sexual reproduction by taking into account departures from Hardy-Weinberg (H-W) equilibrium (Parks and Werth 1993; Arnaud-Haond et al. 2007). We averaged $P_{gen}F_{IS}$ estimates generated from one such value for each MLG in each population, and used a probability $P_{gen} < 0.05$ cut off for the discrimination of ramets versus genets. Under this criterion, we prepared a second data set (N_G) in which all but one clonal ramets per genet were excluded (that is, each distinct MLG was only represented once).

Arnaud-Haond et al. (2007) and Becheler et al. (2010) recommend the use of four parameters to describe clonal diversity and distribution: genotypic richness [$R = (N_G - 1)/(N_T - 1)$; Dorken and Eckert 2001], the Simpson diversity index (Pielou 1969) of clonal heterogeneity (D, the probability of encountering distinct MLGs when randomly taking two units in a population) and its equitability (ED, Simpson evenness; Hurlbert 1971), and the Pareto index β (Arnaud-Haond et al. 2007). To characterize the genet size (N_R, the number of ramets belonging to each genet), we fitted a cumulative function of the Pareto distribution to the data following the method described by Arnaud-Haond et al. (2007). This function takes the following form: $N_{\geq X} = a\,X^{-\beta}$, where $N_{\geq X}$ is the number of genets containing X or more ramets and a is a constant. For each population per species we obtained the shape parameter β by multiplying –1 by the linear regression slope (b_P) of $\log_{10}$ (reverse cumulative frequency of $N_{\geq X}$) vs. $\log_{10}$ (X), and to check the quality of the Pareto approximation we estimated its associated coefficient of determination (R^2). To test

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whether each b_P was statistically significant under the null hypothesis ($b_P = 0$), we estimated the 95% confidence intervals (CIs) around b_P using the classical least-squares regression theory. The estimated b_P (and thus, β) was considered significant when its 95% CIs did not overlap zero. Both *R* and *ED* influence the Pareto index β value. High *R* and *ED* (i.e., clonal ramets all having approximately equal sizes) will result in a high β value (a steep slope), whereas low *R* and *ED* (i.e., a skewed clonal distribution with very few, large clonal lineages and many small ones) will result in a shallow slope (a low β value). For all these calculations, we used GenClone v. 2.0 (Arnaud-Haond and Belkhir 2007). Finally, a contingency χ^2-test was conducted to determine whether distribution of clone sizes was significantly different between populations of each species and between species.

Using the trimmed data set excluding replicate clonal ramets (N_G), we estimated the following genetic diversity parameters using the programs POPGENE (Yeh et al. 1999) and FSTAT (Goudet 1995): 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 and Petit 1996), observed heterozygosity (H_o), and Nei's (1978) unbiased gene diversity or Hardy-Weinberg (H-W) expected heterozygosity (H_e). Hereafter, the subscript "s" indicates species' (or pooled samples) values, while the subscript "p" indicates population means.

To test for recent decreases in effective population size (bottlenecks), we evaluated for individual loci the difference between the H-W H_e and the equilibrium heterozygosity (H_{eq}) expected assuming mutation–drift equilibrium. These differences were evaluated using a sign test and a Wilcoxon sign-rank test conducted across loci under an infinite allele model using the program BOTTLENECK (Cornuet and Luikart 1996). Since allelic diversity is generally lost more rapidly than H_e (Nei et al. 1975), recently bottlenecked

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populations are expected to exhibit an excess of H-W equilibrium H_e relative to H_{eq} (Cornuet and Luikart 1996; Luikart et al. 1998).

We estimated population-level F_{IS} (inbreeding) and calculated its significance level (P values) by gene permutation tests (999 replicates) under the null hypothesis ($F_{IS} = 0$) using the program SPAGeDi (Hardy and Vekemans 2002). We also calculated Wright's (1965) F_{IS} and F_{ST} over loci following Weir and Cockerham (1984). These fixation indices measure the average deviation from H-W equilibrium of individuals relative to their local populations (F_{IS}, a measure of local inbreeding) and local populations relative to the total population (F_{ST}, also a measure of differentiation between local populations). The significance of multi-population F_{IS} and F_{ST} estimates was determined by a permutation test (999 randomizations of alleles between individuals within samples and 999 randomizations of genotypes between populations, respectively). These calculations were performed using FSTAT (Goudet 1995).

To determine the degree of genetic divergence between populations of each taxon, we calculated Nei's (1978) unbiased genetic identity (I) between pairs of populations. In addition, a UPGMA (unweighted pair-group method using arithmetic averages) phenogram was generated from Nei et al. (1983) genetic distance (D_A) matrix with branch support produced by 1000 bootstrapping over loci, utilizing Populations v. 1.2.30 (Langella 1999) and TreeView v. 1.6 (Page 1996). 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 and Cockerham 1984) and the corresponding logarithm of pairwise geographical distances (Rousset 1997). Using the program Permute! (Legendre et al. 1994), 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, $b = 0$). 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 and 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

Identification of clones

For *I. odaesanensis*, 16 (*Dia-1*, *Dia-2*, *Dia-3*, *Fe-1*, *Fe-2*, *Idh-1*, *Mdh-1*, *Mdh-2*, *Me*, *6Pgd-1*, *6Pgd-2*, *Pgi-2*, *Pgi-3*, *Pgm-2*, *Pgm-3*, and *Tpi*) of the 20 putative loci were polymorphic across eight populations and consistent with Mendelian inheritance. Populations IO-1 and IO-2, composed by one MLG (uniclonal; Table 1), had two heterozygous loci each ('*cd*' at *Mdh-2* and '*cd*' at *Tpi* for IO-1; '*ab*' at *Dia-1* and '*bd*' at *Idh-1* for IO-2). If sexual reproduction occurred within these populations, then we would expect homozygotes at these loci (formed through recombination). The occurrence of a unique MLG with two heterozygous loci strongly suggests that these two populations consist of single clones (that is, they are likely uniclonal). Except for the two uniclonal populations, the power to discriminate clonal genotypes from sexually produced genotypes in the other six (multiclonal) populations was about 1.0 (P_{gen} F_{IS} was 0.008; Table 1). We identified a total of 192 (N_G, the number of individuals excluding clonal ramets) MLG out of 510 total samples (N_T) across the six multiclonal populations (IO-3 to IO-8; Table 1).

For *I. rossii*, 13 (*Adh*, *Dia-1*, *Dia-2*, *Fe-1*, *Fe-2*, *Idh-1*, *Me*, *6Pgd-2*, *Pgi-2*, *Pgi-3*,

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Pgm-1, *Pgm-3*, and *Tpi*) of the 20 putative loci were polymorphic. Accordingly, the power to discriminate clonal genotypes from sexually produced genotypes was also about 1.0 (average P_{gen} F_{IS} was 0.005; Table 1). Thus, it is safe to consider for all subsequent analyses that ramets sharing identical MLG in a population were members of the same clone. We identified a total of 123 (N_G) distinct MLG out of 141 total samples (N_T) across six populations (Table 1). In three populations (IR-1, IR-3, and IR-6) all the individuals showed different (unique) MLG (thus, $N_T = N_G$; Table 1).

Clonal diversity (genotypic diversity)

Estimates of genotypic richness (R) varied greatly among populations of *I. odaesanensis*, ranging from 0.000 (IO-1 and IO-2) to 0.504 (IO-8), with a mean of 0.267 (Table 1). Accordingly, Simpson diversity indices (D) were also very variable, ranging from 0.000 (IO-1 and IO-2) to 0.976 (IO-8), with a mean of 0.682 (Table 1). All values of the Simpson evenness index (ED) were greater than 0.7, with a mean of 0.877 (Table 1). The $\log_{10}$ of the cumulative distribution of ramets among genets was linearly related to the $\log_{10}$ of N_R (the genet size), thus supporting the Pareto distribution [index β ($-1 \times$ regression slope, b_P)] hypothesis in all the populations ($R^2 = 0.608$ to 0.970, $P < 0.05$). Also, 95% CIs for b_P for the six multiclonal populations did not overlap zero (Table 1). The values of the Pareto index β were highly variable, ranging from 0.246 (IO-7) to 1.009 (IO-4) with a mean of 0.669 (Table 1).

Estimates of R were high for *I. rossii* populations, ranging from 0.679 (IR-2) to 1.000 (IR-1, IR-3, and IR-6), with a mean of 0.878 (Table 1). Accordingly, Simpson diversity indices (D) were also high, ranging from 0.968 (IR-2) to 1.000 (IR-1, IR-3, and IR-6), with

a mean of 0.989 (Table 1). When applicable, all values of the Simpson evenness index (*ED*) were greater than 0.6, with a mean of 0.776 (Table 1). The regression slope (b_P) of the $\log_{10}$ of the cumulative distribution of ramets among genets to the $\log_{10}$ of N_R was significantly negative in all populations ($R^2 = 0.976$ to 0.999, $P < 0.05$). Also, 95% CIs for b_P did not overlap zero (Table 1). The values of the Pareto index β were high with a mean of 1.825 (Table 1). The Mann-Whitney *U*-test revealed that populations of *I. rossii* exhibit a significantly higher clonal diversity (β) than those of *I. odaesanensis* ($U = 18$, $P < 0.05$).

Except for IO-1 and IO-2 populations, the genet size (N_R) was skewed to small genets in *I. odaesanensis*, and clones ranged in size from one to 39 ramets, with a large majority of genets (139 out of 194) being composed by one or two ramets (Table 2). We found significant differences in the distribution of clone sizes (or number of ramets per genet, Table 2) among the eight populations (contingency χ^2-test, $\chi^2 = 495.6$, d.f. = 112, $P = 0.000$). For *I. rossii*, N_R was highly skewed to small genets, and clones ranged in size from one to four ramets, with most of them (62 out of 75) of just one ramet (Table 2). We found no significant differences in the distribution of clone sizes among the three populations (contingency χ^2-test, $\chi^2 = 4.939$, d.f. = 6, $P = 0.552$). Finally, we found no significant differences in the distribution of clone sizes between the two studied species (contingency χ^2-test, $\chi^2 = 22.71$, d.f. = 16, $P = 0.122$).

Genetic diversity in *Iris odaesanensis* and *I. rossii*

The two populations IO-1 and IO-2 consisted of a single genet and had to be excluded from most statistical analyses to avoid bias (e.g., Gustafson et al. 2013). The rest of the populations of *Iris odaesanensis* were rich in MLGs, with N_G averaging 24 and ranging

from 11 to 62 (Table 1). The uniclonal nature of IO-1 and IO-2 can be easily attributed to their very small size (with just a few tens of shoots compared to the thousands of shoots for most of the populations throughout Korea).

Iris odaesanensis maintained high levels of genetic variation in pooled samples (n = 192, $\%P_s$ = 80.0, A_s = 2.60, and H_{es} = 0.176; Table 3). Lower, but still high, levels of genetic variation were found within populations: average n = 32, $\%P_p$ = 52.5, AR = 1.57, A_p = 1.70, and H_{ep} = 0.158 (Table 3). Comparable levels of genetic variation were found in *I. rossii* both at the total sample level and at the population level: n = 123, $\%P_s$ = 65.0, A_s = 1.95, and H_{es} = 0.177 (Table 3); average n = 21, $\%P_p$ = 47.5, AR = 1.52, A_p = 1.58, and H_{ep} = 0.150 (Table 3). Among six populations for each species, two populations (IO-7 in *I. odaesanensis* and IR-5 in *I. rossii*) displayed significant P values for both sign and Wilcoxon sign-rank test (Table 4), suggesting the occurrence of recent bottlenecks in these populations.

Inbreeding and population genetic structure

Of the population-level F_{IS} estimates in the six populations of *I. odaesanensis*, three out of five positive values were significant at the 0.05 level (F_{IS} = 0.141 to 0.198; Table 3). The significantly negative estimate (F_{IS} = –0.427) found in IO-3 is unusual; perhaps may be artifact due to small sample size. Multi-population-level F_{IS} was low but significantly positive (F_{IS} = 0.084, P = 0.001; Table 3), suggesting an overall deficit of heterozygotes within populations. If we exclude IO-3, multi-population-level F_{IS} rose to 0.188 (P = 0.001). All but one populations of *I. rossi* showed significantly positive F_{IS} estimates, with a considerably high multi-population-level F_{IS} (0.331). Deviation from H-W expectations

due to allele frequency differences between populations were significantly different from zero and similar between the two species (F_{ST} = 0.196 in *I. odaesanensis* and F_{ST} = 0.189 in *I. rossii*, for both cases P = 0.001).

Nei's (1978) unbiased genetic identity (I) between populations of *I. odaesanensis* and between populations of *I. rossi* were high [mean I = 0.943 ± 0.030 (SD) and mean I = 0.958 ± 0.031 (SD), respectively]. These means are comparable to that expected for conspecific plant populations (mean I = 0.950 ± 0.059, n = 1572; van der Bank et al., 2001). The UPGMA phenogram (Fig. 2) revealed no clear genetic patterns between populations of each species in relation to their geographic location. There was no significant positive linear relationship between pairwise $F_{ST}/(1 - F_{ST})$ and logarithm of pairwise linear geographic distances for both *I. odaesanensis* (r = 0.092, P = 0.777) and *I. rossii* (r = –0.096, P = 0.724).

BayesAss results indicated a similar intensity of recent gene flow between species (Table 5). For *I. odaesanensis*, only two out of 30 cases (from IO-4 to IO-7 and from IO-7 to IO-3, Table 5) indicated evidence of recent gene flow between populations [all the other m values fell within the confidence intervals (CI) expected in instances where there is no information in the data (95% CI: 1.58×10^{-6}, 0.160; Table 5]. Similarly, there were three cases for *I. rossii* on the basis of this criterion (Table 5). On average, m rates between populations of *I. odaesanensis* (n = 30, mean m rate = 0.0213) did not significantly differed from those between populations of *I. rossii* (n = 30, mean m rate = 0.0420; t = –1.181, two-sided P-value = 0.242).

Discussion

Clonal diversity, inference of seedling recruitment strategy, and inbreeding

As predicted, our results for clonal diversity are consistent with the life-history traits and ecology of the two *Iris* species. Levels of clonal diversity found in *I. odaesanensis* populations were considerably lower—with the only exception of *ED* (Table 2)—than those for *I. rossii*. The Pareto index β is, nevertheless, the best suited for summarizing clonal diversity and for making comparisons among different studies (Arnaud-Haond et al. 2007; Ohsako 2010). The mean value of β (0.668) for *I. odaesanensis* is lower than the mean obtained for 15 populations belonging to 11 terrestrial and marine plant species compiled by Ohsako (2010; $\beta = 0.930$). In contrast, the mean value of β (1.825) for *I. rossii* is considerably higher than the Ohsako's (2010) average. Unlike *I. odaesanensis*, the high value of β found for *I. rossii* indicates that their populations have a tendency to be formed by several small clones with no large ones (Table 2).

The architecture and the extent of clonal growth in plant populations have crucial effects on their genetic diversity and demographic structure (Eriksson 1989). The studies of Eriksson (1989, 1993) have demonstrated how genetic diversity is modulated depending on the seedling recruitment strategy of clonal plants. Under the "initial seedling recruitment" (ISR) strategy, no recruitment occurs after the establishment of the initial cohort, which could result in a decrease of genetic diversity over time, and ultimately populations would be composed of a small number of large, old, and even-aged clones. At the other extreme, in the "repeated seedling recruitment" (RSR) strategy, a steady recruitment of genets occurs and populations will contain clones of variable age and size, largely maintaining local genetic variability (Eriksson 1989). Because of this linkage between seedling recruitment strategy and clonal structure, it is possible to infer the mode of recruitment of a given

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species (Parker and Hamrick 1992; Kudoh et al. 1999). Except for IO-1 and IO-2 of *I. odaesanensis*, the skewed distribution of ramet numbers per genet (with a clear predominance of small clones; Table 2) suggests RSR as the main recruitment strategy operating within the multiclonal populations of the two *Iris* species. Obviously, the IO-1 and IO-2 populations fit better the ISR model.

In *I. odaesanensis*, we found a low but significant deficit of heterozygotes in three populations, with a multi-population-level F_{IS} of 0.084, suggesting a predominantly outcrossing breeding system for this species. For *I. rossii*, the higher value of multi-population-level F_{IS} (0.331) suggests, instead, a mixed mating system. Factors such as biparental inbreeding (mating with relatives), Wahlund effect (population subdivision) or geitonogamous selfing between clonal ramets (if the species are self-compatible) might account for the observed heterozygote deficiency at several populations of both species.

Genetic diversity in *Iris odaesanensis* and *I. rossii*: the role of the Baekdudaegan as glacial refugium for boreal and temperate species

As predicted, populations of *I. odaesanensis* and *I. rossii* maintain substantial levels of genetic variation. Within-population genetic estimates are higher than those averaged for populations of short-lived herbaceous perennials, plants with a narrow distribution, plants with outcrossing-animal breeding system, rare plants in the southeastern United States, endemic plants, and all plants (Table 6). Also, as predicted, populations of *I. odaesanensis* and *I. rossii* exhibit a moderate degree of among-population genetic differentiation, comparable to those averaged for plant species with the traits mentioned above (Table 6). The mean migration rates (m) for *I. odaesanensis* (0.0213) and *I. rossii* (0.0420) are

comparable or higher to the average for other plant species occurring in northeastern Asia (m = 0.0209 averaged from 15 entries; M. Y. Chung et al. unpubl. data), which indicates that gene flow between populations is also occurring at a moderate scale at present (and perhaps during glacial times).

It is worth noting that levels of genetic diversity found in *I. odaesanensis* and *I. rossii* both at the population and the species levels are similar to those reported for the congeneric *I. cristata*, a widespread species of unglaciated regions of the southeastern United States primarily distributed in the southern Appalachians (Table 6). However, the considerably low degree of among-population differentiation exhibited by *I. cristata* (F_{ST} = 0.018) might be ascribed to exclusion of two variable loci (*6Pgd* and *Mdh*) for the calculation of this parameter, due to difficulty of interpretation of the banding patterns for these loci (Hannan and Orick 2000). A complete lack of allozyme variation in 18 isozyme loci was found for the range-restricted *I. lacustris* which occurs on LGM-glaciated habitats of Great Lakes shorelines (Hannan and Orick 2000). Assuming that they have similar breeding systems, any contrasting difference in the genetic diversity patterns between these two North American *Iris* species would be attributable to population history (that is, long-term population stability due to survival in the Appalachian glacial refugium for *I. cristata* vs. a dynamics of population extinction and recolonization for *I. lacustris*) in addition to the marked difference in geographic range.

The high levels of genetic diversity and the moderate levels of genetic differentiation among populations of *I. odaesanensis* and *I. rossii* are in agreement with Chung et al. (2012, 2013a,b, 2014) hypothesis that the Baekdudaegan mountain range harbored important refugial areas for boreal and temperate vegetation during the LGM. If we compile all the species studied with allozymes whose distribution in Korea is mostly centered in the

Baekdudaegan (Table 6), then a common pattern consisting of high within-population and low among-population genetic variability is envisaged, with a very few exceptions. Only two species from this list have values of H_{ep} below 0.100, and only one is clearly genetically depauperate (*Leontice microrhyncha*; Table 6), although it should be taken into account that this species is autogamous (Chang et al. 2004). A series of features of the Baekdudaegan should have enhanced its role as a Quaternary refugium, with its topography playing a central role. First, the north-south orientation of the Baekdudaegan favored latitudinal migrations of plants to track the climate shifts (Hewitt 2000); second, its relatively wide altitudinal gradient—of up to 2000 m—allowed altitudinal migrations to track warm interglacials/cold glacials (Hewitt 2000; Nieto Feliner 2011); third, its marked ruggedness (with numerous valleys, ravines, canyons and gorges) would have provided many sheltered habitats from the cold winds (Birks and Willis 2008; Kaltenrieder et al. 2009). The more or less continuous supply of moisture even during the LGM (due to the orographic rain and also to the close proximity to the East Sea/Sea of Japan), and the fact that the Baekdudaegan remained nearly totally unglaciated even during the coldest episodes of the Pleistocene were also key factors for the species persistence along the Quaternary.

The Baekdudaegan may constitute a sort of 'eastern counterpart' of the southern Appalachians on the basis of its role as Quaternary refugium for boreal and temperate plants. The southern Appalachians have been considered as a prime refugial area for the flora of North America, capable to sustain a rich assemblage of boreal and temperate forests even during the coldest periods of the Pleistocene (Delcourt and Delcourt 1981; Graham 1999; Williams et al. 2000; Soltis et al. 2006; Prentice et al. 2011). This mountain range approximately runs north to south in an analogous way to the Baekdudaegan, with a similar floristic richness [ca. 1,400 taxa (Highlands Biological Station 2013) versus over

1,500 for the Baekdudaegan (Lim 2003)], length (ca. 1,500 km), and average elevation. As for the Baekdudaegan, the Appalachians are also a "hotspot" of genetic diversity, with several examples of high levels of genetic diversity in the Appalachians compared to more northern conspecific or congeneric populations (e.g., Broyles 1998; Hannan and Orick, 2000). Moreover, the meta-analysis of Godt and Hamrick (2001), although not exclusively circumscribed to the Appalachians, constitutes another proof of the role of these North American mountains as a refuge of genetic diversity.

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Table 1 Summary of clonal diversity measures observed in eight populations of *Iris odaesanensis* and six populations of *I. rossii*

Species / Population	Area (m^2)	Altitude (m)	N_T	N_G	$P_{gen}F_{IS}$	R	D	ED	β (95% CIs for b_P[a])	R^2
Iris odaesanensis										
IO-1	2 × 5	540	29	1	0.923	0.000	0.000	na	na	na
IO-2	1 × 3	1180	16	1	0.887	0.000	0.000	na	na	na
IO-3	10 × 20	1012	65	11	0.010	0.156	0.853	0.890	0.383 (–0.691, –0.076)	0.608
IO-4	10 × 20	1280	66	32	0.004	0.477	0.945	0.927	1.009 (–1.623, –0.395)	0.839
IO-5	20 × 20	1480	93	37	0.001	0.391	0.932	0.855	0.516 (–0.606, –0.426)	0.970
IO-6	5 × 20	980	75	31	0.011	0.405	0.961	0.942	0.974 (–1.437, –0.510)	0.853
IO-7	20 × 20	1390	89	19	0.007	0.205	0.791	0.717	0.246 (–0.341, –0.151)	0.870
IO-8	30 × 70	401	122	62	0.017	0.504	0.976	0.932	0.887 (–1.185, –0.588)	0.898
Average			69	24	0.008[b]	0.267	0.682	0.877	0.669	
Iris rossii										
IR-1	20 × 20	980	23	23	na	1.000	1.000	na	na	na
IR-2	20 × 20	480	29	20	0.005	0.679	0.968	0.932	1.418 (–2.214, –0.623)	0.976
IR-3	30 × 30	319	9	9	na	1.000	1.000	na	na	na
IR-4	20 × 20	385	31	27	0.010	0.867	0.989	0.649	2.128 (–2.319, –1.937)	0.999
IR-5	50 × 20	380	33	28	0.002	0.844	0.989	0.747	1.930 (–3.053, –0.816)	0.998
IR-6	20 × 20	390	16	16	na	1.000	1.000	na	na	na
Average			24	20	0.005	0.878	0.989	0.776	1.825	

N_T, the total number of ramets sampled; N_G, the number of genets; $P_{gen}F_{IS}$, probability of the identical multilocus genotypes (MLG) occurring by chance due to sexual reproduction by taking into account departures from Hardy-Weinberg (H-W) equilibrium; R, genotypic richness; D, Simpson diversity index of clonal heterogeneity; ED, Simpson evenness index; β, the Pareto index describing the Pareto distribution ($\beta = -1 \times b_P$); b_P, the linear regression slope between $\log_{10}$ (reverse cumulative frequency of the number of genets containing X or more ramets, $N_{\geq X}$) on $\log_{10}$ (number of replicates, X); 95% CIs, 95% confidence intervals;

R^2, square of correlation coefficient of each b_P.; and na, not applicable

[a] All b_P indicated significance with $P < 0.05$

[b] Mean from six populations (from IO-3 to IO-8)

PLSY-D-14-00116R1

Table 2 Distribution of clones found in eight populations of *Iris odaesanensis* and three populations of *I. rossii*. The other three populations of *I. rossii* (IR-1, IR-3, and IR-6) are not included here because they show $N_T = N_G$

Species/population	Number of ramets per genet (N_R)																	N_G
	1	2	3	4	5	6	7	8	9	11	12	14	15	16	19	29	39	
Iris odaesanensis																		
IO-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
IO-2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
IO-3	3	2	0	1	1	0	0	0	1	1	0	1	1	0	0	0	0	11
IO-4	20	1	5	4	0	1	1	0	0	0	0	0	0	0	0	0	0	32
IO-5	22	8	1	1	2	0	0	1	0	1	0	0	0	0	1	0	0	37
IO-6	14	7	2	4	2	1	0	0	1	0	0	0	0	0	0	0	0	31
IO-7	8	2	1	3	2	1	1	0	0	0	0	0	0	0	0	0	1	19
IO-8	40	12	1	2	2	3	1	0	0	0	1	0	0	0	0	0	0	62
Average	18	5	2	3	2	1	1	0	0	0	0	0	0	0	0	0	0	32
Total	107	32	10	15	9	6	3	1	2	2	1	1	1	1	1	1	1	194
Iris rossii																		
IR-2	14	4	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	20
IR-4	24	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	27
IR-5	24	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	28
Average	21	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25
Total	62	9	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	75

Table 3 Levels of genetic diversity in six populations of *Iris odaesanensis* and in six populations of *I. rossii* in South Korea. Note that the two uniclonal populations IO-1 and IO-2 are excluded from data analysis

Species/ population	N_G	*%P*	*AR*	*A*	H_o (SE)	H_e (SE)	F_{IS}
Iris odaesanensis							
IO-3	11	40.0	1.50	1.50	0.264 (0.078)	0.166 (0.052)	–0.427[a]
IO-4	32	55.0	1.58	1.75	0.127 (0.047)	0.147 (0.047)	0.141[a]
IO-5	37	60.0	1.59	1.70	0.157 (0.040)	0.167 (0.040)	0.061
IO-6	31	45.0	1.55	1.70	0.107 (0.042)	0.133 (0.046)	0.198[a]
IO-7	19	50.0	1.67	1.70	0.203 (0.052)	0.215 (0.055)	0.057
IO-8	62	65.0	1.55	1.85	0.102 (0.028)	0.120 (0.038)	0.145[a]
Average	32	52.5	1.57	1.70	0.160 (0.026)	0.158 (0.013)	0.084[b]
Pooled samples	192	80.0		2.60		0.176 (0.025)	
Iris rossii							
IR-1	23	50.0	1.48	1.55	0.141 (0.045)	0.143 (0.039)	0.015
IR-2	20	45.0	1.48	1.55	0.100 (0.032)	0.127 (0.042)	0.211[a]
IR-3	9	45.0	1.65	1.65	0.100 (0.031)	0.178 (0.049)	0.438[a]
IR-4	27	50.0	1.45	1.55	0.102 (0.036)	0.132 (0.042)	0.226[a]
IR-5	28	50.0	1.56	1.60	0.075 (0.021)	0.184 (0.047)	0.592[a]
IR-6	16	45.0	1.48	1.60	0.078 (0.029)	0.137 (0.047)	0.427[a]
Average	21	47.5	1.52	1.58	0.099 (0.010)	0.150 (0.010)	0.331[b]
Pooled samples	123	65.0		1.95		0.177 (0.026)	

%P percentage of polymorphic loci, *AR* mean allelic richness (adjusted for a sample size of 11 and nine individuals for *I. odaesanensis* and *I. rossii*, respectively), *A* mean number of alleles per locus, 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 $F_{IS} = 0$

PLSY-D-14-00116R1

1 [b] Significant (at the 0.05 level) Weir and Cockerham (1984) estimate of F_{IS} over populations

PLSY-D-14-00116R1

Table 4 Results of statistical tests for evidence of recent population bottlenecks in *Iris odaesanensis* and *I. rossii*

Species/population	Sign test	Wilcoxon sign-rank test
Iris odaesanensis		
IO-3	0.258	0.125
IO-4	0.159	0.711
IO-5	0.321	0.285
IO-6	0.162	0.715
IO-7	**0.011**	**0.007**
IO-8	0.102	0.966
Iris rossii		
IR-1	0.497	0.385
IR-2	0.369	0.787
IR-3	0.193	0.990
IR-4	0.536	0.539
IR-5	**0.040**	**0.053**
IR-6	0.515	0.715

Tests were not conducted in two uniclonal populations of *I. odaesanensis* (IO-1 and IO-2). Numbers reported are *P* values of sign and Wilcoxon sign-rank tests conducted using the program BOTTLENECK, and significant *P* values (at the 0.05 level) are boldfaced

PLSY-D-14-00116R1

Table 5 Mean value of the posterior distribution of the recent migration rates (m) of each *Iris odaesanensis* and *I. rossii* population pairs estimated from allozyme data using the BayesAss program. Note that values on the diagonal in bold underlined are the proportions of individuals derived from source populations. Values higher than 0.160 (the 95% CI upper limit) are presented in bold.

Iris odaesanensis

	From					
To	IO-3	IO-4	IO-5	IO-6	IO-7	IO-8
IO-3	**0.6926**	0.0129	0.0130	0.0122	**0.2569**	0.0123
IO-4	0.0023	**0.9826**	0.0033	0.0029	0.0026	0.0061
IO-5	0.0020	0.0026	**0.9882**	0.0024	0.0022	0.0025
IO-6	0.0023	0.0027	0.0027	**0.9857**	0.0023	0.0043
IO-7	0.0072	**0.2106**	0.0155	0.0140	**0.7380**	0.0147
IO-8	0.0017	0.0135	0.0063	0.0023	0.0023	**0.9740**

Iris rossii

	From					
To	IR-1	IR-2	IR-3	IR-4	IR-5	IR-6
IR-1	**0.6800**	0.0061	0.0067	**0.2928**	0.0071	0.0072
IR-2	0.0080	**0.6819**	**0.2408**	0.0389	0.0154	0.0150
IR-3	0.0170	0.0145	**0.7516**	**0.1754**	0.0194	0.0222
IR-4	0.0026	0.0028	0.0026	**0.9858**	0.0028	0.0035
IR-5	0.0053	0.0052	0.0056	0.1463	**0.8306**	0.0071
IR-6	0.0090	0.0080	0.0093	0.1469	0.0166	**0.8104**

PLSY-D-14-00116R1

Table 6 Comparisons of allozyme-based genetic diversity and genetic differentiation [F_{ST} or Nei's (1973) G_{ST}] between *Iris odaesanensis*, species occurring mainly on the Baekdudaegan[a] (Fig. 1), and species having similar life history traits. This table was modified from table 3 of Chung et al. (2014)

Species	Family	Ecol. affinity[b]/ Range[c]	$\%P_s$	$\%P_p$	A_s	A_p	H_{es}	H_{ep}	F_{ST} or G_{ST}	Ref.[d]
Species occurring mainly in the Baekdudaegan										
Adenophora grandiflora	Campanulaceae	B/KE	62.5	59.4	2.88	2.56	0.266	0.259	0.027	1
Bupleurum euphorbioides	Apiaceae	B/NEC, K	58.8	46.1	2.24	1.64	0.151	na	0.297	2
Cypripedium macranthos	Orchidaceae	B/C, J, K, R	71.4	46.7	1.79	1.47	0.291	0.185	0.077	3
Forsythia ovata	Oleaceae	T/KE	71.4	48.6	2.07	1.63	0.220	0.200	0.144	4
Hanabusaya asiatica	Campanulaceae	T/KE	76.9	67.7	2.77	2.06	0.217	0.182	0.132	5
Iris odaesanensis	**Iridaceae**	**B/NEC, K**	**80.0**	**52.5**	**2.60**	**1.70**	**0.176**	**0.158**	**0.196**	**6**
Iris rossii	**Iridaceae**	**T/NEC, J, K**	**65.0**	**47.5**	**1.95**	**1.58**	**0.177**	**0.150**	**0.189**	**6**
Leontice microryncha	Berberidaceae	B/NEC, K	35.7	10.1	1.50	1.10	0.120	0.022	0.627	7
Lilium cernuum	Liliaceae	B/NEC, K, RFE	71.4	49.1	2.29	1.71	0.178	0.159	0.119	8
Megaleranthis saniculifolia	Ranunculaceae	T/KE	78.6	31.6	2.57	1.40	0.151	0.083	0.450	9
Oreorchis patens	Orchidaceae	B/C, J, K, RFE	76.5	62.8	2.53	1.96	0.258	0.236	0.075	10
Parasenecio pseudotaimingasa	Asteraceae	T/KE	66.7	40.2	1.92	1.61	0.157	0.120	0.215	11
Pinus koraiensis	Pinaceae	B/NEC, J, K, RFE	na	45.8	na	2.00	na	0.181	0.059	12, 13
Taxus cuspidata	Taxaceae	B/NEC, J, K, RFE	52.0	45.0	2.09	1.78	0.200	0.192	0.059	14
Average			66.7	46.7	2.25	1.73	0.197	0.161	0.190	
Iris species in the eastern United States										
Iris cristata	Iridaceae	T/SEUS	73.3	51.4	3.00	1.87	0.231	0.199	0.018	15
Iris lacustris	Iridaceae	B/NAE, CA	0.00	0.00	1.00	1.00	0.000	0.000	na	15,16
Plants with a narrow distribution			45.1	30.6	1.83	1.45	0.137	0.105	na	17
Short-lived herbaceous perennials			41.3	28.0	1.70	1.40	0.116	0.096	0.233	18
Plants with outcrossing-animal breeding system			51.1	35.9	1.99	1.54	0.167	0.124	0.197	18
Rare plants in the southeastern United States			46.7	33.3	1.87	1.53	0.123	0.100	0.187	19
Endemics			40.0	26.3	1.80	1.39	0.096	0.063	0.248	18
All plants			52.2	35.1	1.99	1.53	0.153	0.116	0.225	20

%P, percentage of polymorphic loci; *A*, mean number of alleles per locus; H_e, H-W

expected heterozygosity or genetic diversity; F_{ST} (G_{ST}), measures of among-population differentiation; and –, not available. The subscript 's' indicates species' (or pooled samples) values, while the subscript 'p' indicates population means

[a] Only species with most of their populations in Korea (more than half) occur in the Baekdudaegan (on its main ridge or in the peripheral areas to the main ridge)

[b] Ecological affinity: B, boreal; T, temperate

[c] Range: C, China; CA, Canada; J, Japan; K, Korea; KE, Korean endemic; NAE, North American endemic; NEC, northeastern China; R, Russia; RFE, Russian Far East; SEUS, southeastern United States

[d] Source references: 1, Chung and Epperson (1999); 2, Chang et al. (2003); 3, Chung et al. (2009); 4, Chung et al. (2013a); 5, Chung et al. (2001); 6, present study; 7, Chang et al. (2004); 8, Chung et al. (2014); 9, Jeong et al. (2010); 10, Chung et al. (2012); 11, Chung et al. (2013b); 12, Kim et al. (1994); 13, Kim et al. (2005); 14, Chung et al. (1999); 15, Hannan and Orick (2000); 16, Simonich and Morgan (1994); 17, Godt and Hamrick (1998a) and updated in Wang et al. (2004); 18, Hamrick and Godt (1990); 19, Godt and Hamrick (2001); 20, Godt and Hamrick (1998b)

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PLSY-D-14-00116R1

Figure legends

Fig. 1 Locations of sampled populations of *Iris odaesanensis* (IO-1 to IO-8) and *I. rossii* (IR-1 to IR-6) in South Korea. Dotted line indicates the location and shape of the main mountain range of the country, the Baekdudaegan, which runs north to south along the Korean Peninsula, and solid line represents the so-called "Nakdongjeongmaek", one of the 13 mountainous branches of the Baekdudaegan. White triangles indicate the Pleistocene-glaciated high mountains in the Korean Peninsula (Kong and Watts, 1993): Mountains Baekdu (2744 m), Kwanmo (2541 m), Seolryeong (2442 m), Nampodae (2435 m), and Bukpodae (2289 m)

Fig. 2 UPGMA phenogram based on Nei et al.'s (1983) genetic distances (D_A) between populations of *Iris odaesanensis* (IO-3 to IO-8) and *I. rossii* (IR-1 to IR-6) in South Korea. Numbers above branches represent bootstrap support for 1,000 replicates, and values greater than 50% are shown above the branches

PLSY-D-14-00116R1

1

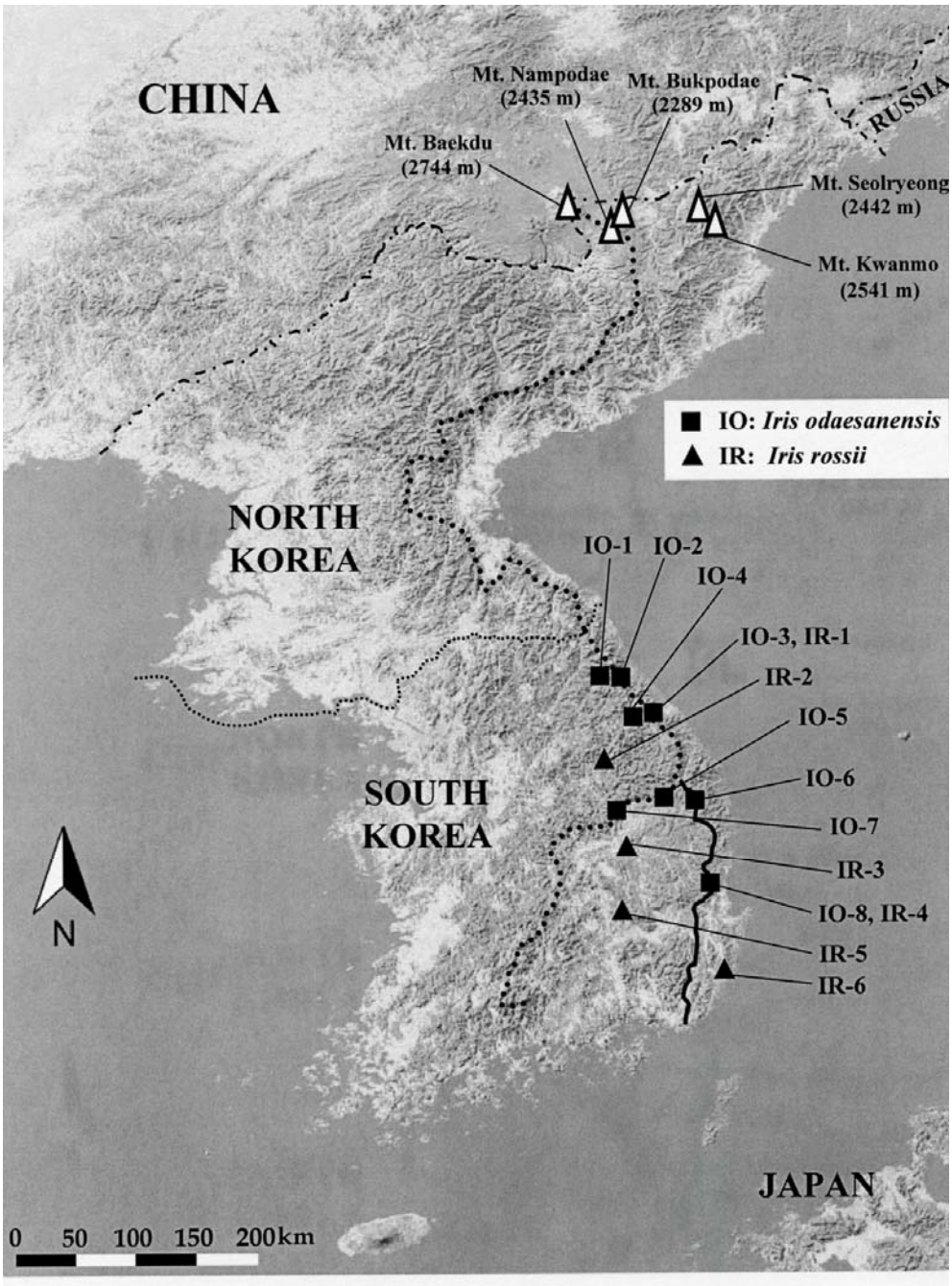

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Fig. 1

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PLSY-D-14-00116R1

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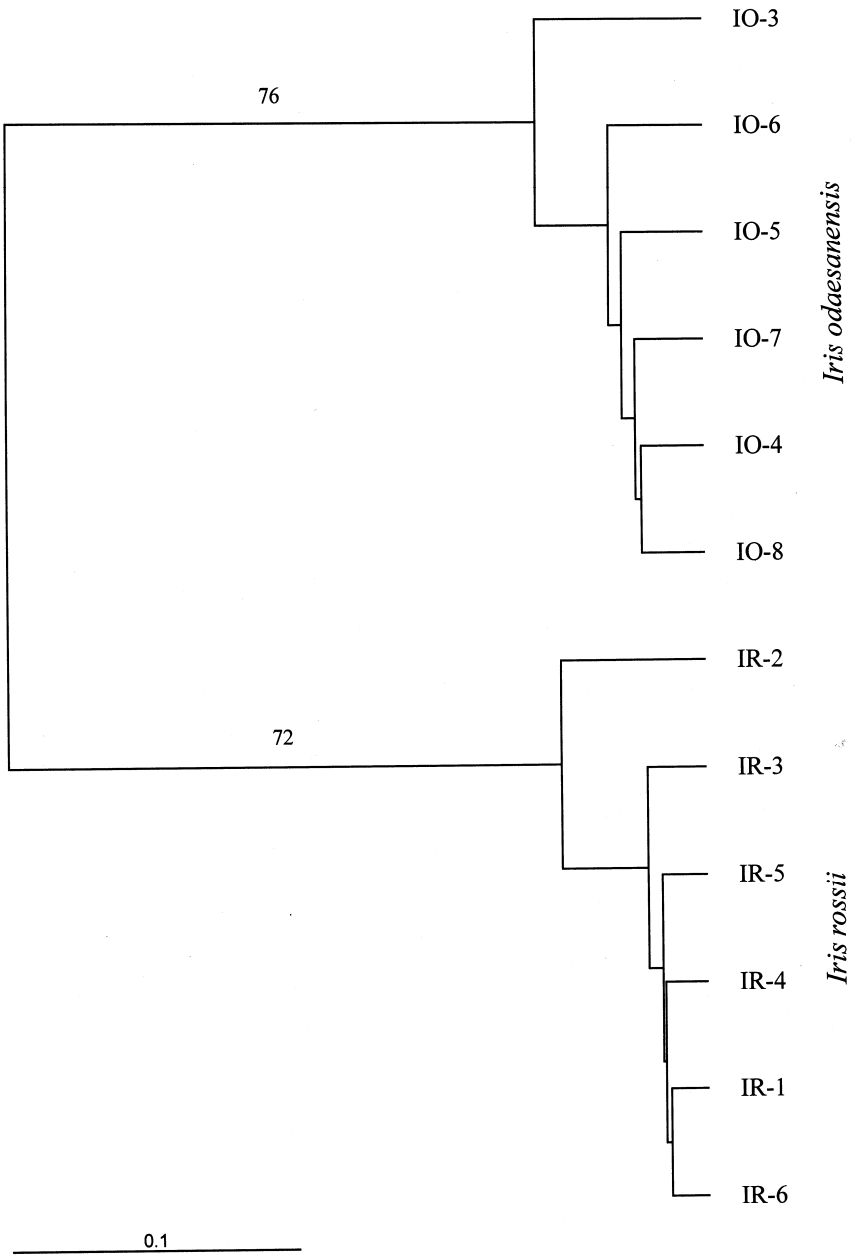

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Fig. 2