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Contrasting Levels of Clonal and Within-Population Genetic Diversity between the 2 Ecologically Different Herbs *Polygonatum stenophyllum* and *Polygonatum inflatum* (Liliaceae)

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Abstract

Comparative studies on clonal and genetic structure between ecologically contrasting congeners may provide valuable insights into the mechanisms promoting the maintenance of genetic diversity in clonal plant species. *Polygonatum stenophyllum* has long rhizomes (ca. 30–40 cm long) and largely occurs on sandy soils in open river banks, whereas its congener *Polygonatum inflatum* has short ones (ca. 5–10 cm long) and occurs on humic soils under deciduous forests. Using 21 allozyme loci, we comparatively assessed levels of clonal and genetic diversity in the 2 clonal species. Seven populations of *P. stenophyllum* consisted of single clones, and levels of within-population clonal and genetic variation were considerably lower than those of *P. inflatum*. However, when samples were pooled, *P. stenophyllum* harbored higher genetic variation than *P. inflatum*, which is due to higher among-population genetic differentiation in the former species compared with the latter (F_{ST} = 0.636 vs. F_{ST} = 0.165). Our data suggest that populations of *P. stenophyllum* have been mainly founded by a single seed or rhizome (through river water) or by a few seeds, whereas populations of *P. inflatum* would have been established through multiple, repeated seedling recruitment. Moderate levels of genetic diversity in a population of *P. stenophyllum* located at the foot of the Baekdudaegan Mountains and in all the populations of *P. inflatum* are consistent with the previous hypothesis that these mountains served as a glacial refugium for many boreal species of the Korean Peninsula.

Subject areas: *Conservation genetics and biodiversity; Population structure and phylogeography*

Key words: *clonal diversity, conservation, ecological traits, genetic diversity,* Polygonatum, *population history*

The extent of clonal growth in plant populations (the proportion of clonal vs. sexual reproduction) has crucial effects on their genetic diversity and demographic structure (Eriksson 1989). A certain level of genetic recombination may enhance clonal diversity and, thus, the potential to adapt to a changing environment (Balloux et al. 2003). 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). The RSR seems to predominate among species that propagate above ground (e.g., by stolons or creeping aerial stems) with long-distance seed dispersal, such as

many species inhabiting grasslands. The ISR, instead, is more common in species that propagate below ground (e.g., by rhizomes, corms, or bulbs) with short-distance seed dispersal, such as those inhabiting woodlands (Eriksson 1989). Because of this linkage between seedling recruitment strategy and clonal structure, it would be possible to retrospectively infer seedling recruitment of a given species by analyzing the clonal and genetic structure of its populations.

As Amat et al. (2013) noted, clonal growth could be "a double-edged sword" for rare and endangered species because the short-term insurance against extinction might result in a long-term hazard of random genetic drift in small inbred populations with low fecundity. On the one hand, for many clonal plant species inhabiting in heterogeneous habitats or depending on disturbance, asexual reproduction might be the best strategy to ensure survival. The clonal nature of many species represents an advantage for colonizing and competing successfully in a range of habitats. On the other hand, sexual reproduction can be (almost permanently) often suppressed by natural and human-mediated artificial factors such as canopy closure, flooding, mowing, and grazing (Chung et al. 1991; Kerley et al. 1993; Schaal and Leverich 1996; Kudoh et al. 1999; Amat et al. 2013). This situation may lead to lowered genetic diversity or inbreeding, especially in small populations (Charpentier et al. 2000; Honnay and Bossuyt 2005). For self-incompatible clonal species, they may not be able to reproduce sexually if their populations contain a single clone (Godt et al. 1997).

As plant congeners usually share similar life-history traits, in particular breeding systems and seed dispersal mechanisms (due to the "phylogenetic signal"; Losos 2008), comparisons of congeneric species pairs allow to partially control the variation that is attributable to the phylogeny (Karron 1987; reviewed in Godt and Hamrick 2001). This comparative approach could lead to less biased interpretations of genetic results between species pairs, in which 1 species is the target and the other is the reference. With this in mind, we selected 2 congeneric boreal herbaceous perennials, *Polygonatum stenophyllum* Maxim. (as the target species) and *Polygonatum inflatum* Kom. (as the reference species) to comparatively investigate clonal/genetic diversity and structure. *Polygonatum stenophyllum* is distributed from Primorsky Krai in Russian Far East to the Korean Peninsula, including 3 northeastern provinces (Heilongjiang, Jilin, and Liaoning) in China (Chen and Tamura 2000). In northeastern China, *P. stenophyllum* occurs under forests and in thickets. Except for PS-9 to PS-11 (Figure 1), all populations of *P. stenophyllum* in central Korea are located west of the so-called "Baekdudaegan" (the main mountain system of the Peninsula, which runs north to south with ca. 1625 km long; Figure 1). *Polygonatum inflatum* is more widely distributed (occurring in the same 3 northeastern provinces in China, the Korean Peninsula, and Japan) and inhabits forests or forest margins from near sea level to 1000 m above sea level (a.s.l.).

On central Korea, *P. stenophyllum* mainly grows on exposed sandy soils with low nutrient levels in river banks where frequent disturbance occurs (because of flooding; Song et al. 2009). In such highly disturbed habitats, new populations along the same river systems are likely to be established by vegetative propagules rather than by seeds. To adapt to nutrient-poor sandy soils in open, unstable river banks, *P. stenophyllum* appears to have a highly clonal growth form with extensive underground rhizomes (ca. 3–7 mm wide, 30–40 cm long) that are easily broken. This particular type of life-history strategy is also found in its congener *P. humile* (which grows in sunny grasslands and nutrient-poor sand dunes; Hasegawa and Kudo 2005) as well as in coastal plants including the widespread *Calystegia soldanella* and *Lathyrus japonicus* and the eastern Asian *Carex pumila* and *C. kobomugi* (Ohsako 2010). In contrast, *P. inflatum* has relatively thick and short rhizomes (ca. 7–10 mm wide, 5–10 cm long) and grows in stable and fertile soils of deciduous forests in the Baekdudaegan Mountains (PI-1 to PI-3; Figure 1) and its mountainous branches (PI-4; Figure 1). During our preliminary field surveys, we found that shoots in 3 populations (PS-1, PS-5, and PS-11; Figure 1) of *P. stenophyllum* did not bear any mature fruit, and we have never found seedlings within those relatively small populations. Another liliaceous herb, *Hosta clausa*, often co-occurring with *P. stenophyllum*, reproduces mainly vegetatively by rhizomes. Chung et al. (1991) suggested that extensive clonal growth compensates for the inefficiency of sexual reproduction under unstable habitat conditions. Frequent flooding due to high precipitation brought by typhoons or rainy spells in summer would have negatively affected the fruit maturation of *P. stenophyllum* and *H. clausa* (Chung et al. 1991). Unlike *P. stenophyllum*, however, we have observed both mature fruits and seedlings of *P. inflatum* (in populations PI-3 and PI-4).

These observations (no mature fruits and predominantly asexual reproduction) for *P. stenophyllum* may lead us to speculate that shoots—that occur on the order of 10s to several 1000s within certain populations—belong to a single or to a few clones (genets). If this prediction is true, we expect that ISR model would operate within *P. stenophyllum* populations, whereas RSR would be prevalent for *P. inflatum* populations. Directly related to this second prediction, we also expect that within-population clonal (genotypic) diversity and genetic variation found in *P. stenophyllum* would be considerably lower than those harbored by *P. inflatum*, whereas among-population genetic divergence would be higher in *P. stenophyllum* compared with *P. inflatum*. In this study, we conduct an allozyme study throughout the distributional range of *P. stenophyllum* and *P. inflatum* in South Korea to evaluate these predictions. As *P. stenophyllum* has been listed as "critically endangered" by the Ministry of Environment of South Korea due to its rarity and limited distribution in the country (Ministry of Environment 2005), we use genetic data obtained from this study to provide guidelines for the management and recovery of this species.

Materials and Methods

Study Species

The stem of *P. stenophyllum* is erect, 40–120 cm tall, with leaves in whorls of 4–6 on each node. In June, each flowering shoot produces 2 reflexed peduncles on each node from which 2 white cylindric flowers (0.8–1.3 cm long) hung down on each

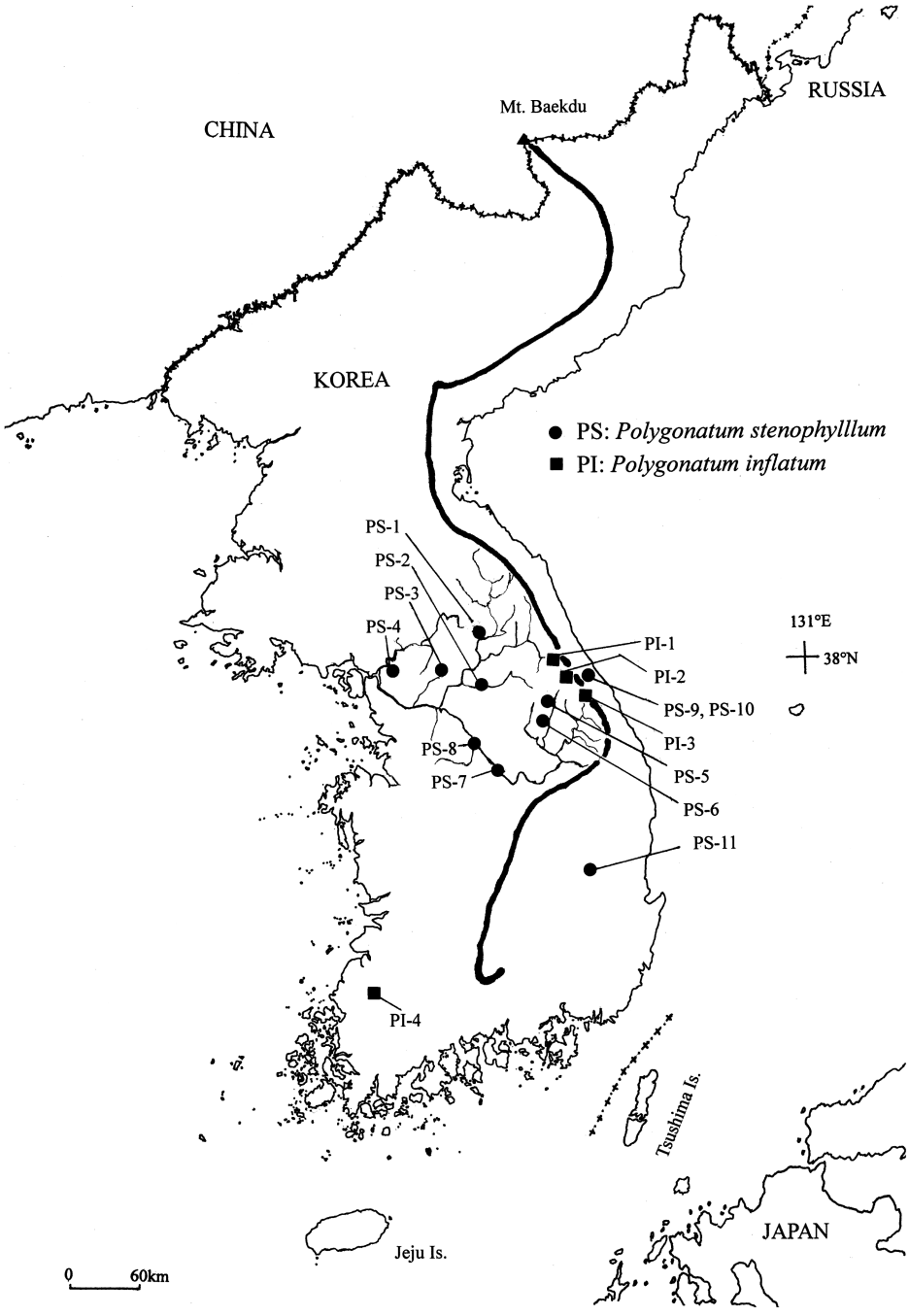


Figure 1. Locations of sampled populations of *Polygonatum stenophyllum* (PS-1 to PS-11) and *Polygonatum inflatum* (PI-1 to PI-4) in the Korean Peninsula. Thick solid line indicates the location and shape of the main mountain range of the country, the so-called "Baekdudaegan," which runs north to south along the Korean Peninsula. The thin curved line represents river systems in central Korea. Nine populations occur along rivers or streams, or on river banks: PS-1 on riverbank of Bukhan River; PS-2 on riverside of Hongcheon River, a branch of Bukhan River; PS-3 on stream bank of Bongcheonsa Stream, a branch of Han River; PS-4 on stream bank of Munsan Stream, a branch of Imjin River; PS-5 and PS-6 on riverside and on bank of Han River (Dong River); PS-7 and PS-8 on riverbank of Namhan River; and PS-11 on riverside of Gilan Stream.

peduncle. Fruit (berry, 7–8 mm in diameter) is globose, black at maturity, and contains 4–8 seeds. The stem of *P. inflatum* is arching and relatively short (30–60 cm long) and stout, with 5–9 alternative leaves. In June, each flowering shoot produces 1 peduncle on each node from which 3–7 pale green campanulate-cylindric, pendulous flowers (2.2–2.5 cm long) hung down. Fruit (berry, 1.0–1.2 cm in diameter) is globose, bluish-black, and 9–13 seeded.

Although breeding systems of *P. stenophyllum* and *P. inflatum* are not known, many *Polygonatum* species are self-incompatible or have very low selfing ability, with their flowers mainly pollinated by bumblebees (Yasaka et al. 1994; Guitián et al. 2001, 2004; Hasegawa and Kudo 2005; Ohara et al. 2007; Kosiński 2012). The seeds contained in the fruits (black to bluish-black) of *Polygonatum* species are dispersed by birds (DeFilipps 1980; Thompson 1981).

Population Sampling

For *P. stenophyllum*, we collected leaf samples from 470 shoots (with a mean of total samples [N_T] = 43 and a range of 13–91 samples per population; Table 1) from 11 populations (PS-1 to PS-11; Figure 1). The area occupied by these populations varied widely, from 10 (5 × 2 m, PS-9) to 4000 m^2 (200 × 20 m, PS-8), with density also varying (Table 1). Except for PS-9 and PS-10, populations occur along rivers or streams, or on river banks (Figure 1). Interestingly, PS-9 and PS-10 are located on the foot of a mountain hill and are separated by 87 m in linear distance. As *P. stenophyllum* propagates extensively by branching rhizomes (and thus, it forms mat-like clusters of shoots), we collected samples at least at 2-m intervals to avoid duplicate samples with the exception of relatively small populations (PS-3, PS-9 to PS-11; Table 1). For these small populations, we collected samples at about 30-cm intervals to get sample sizes over 10 shoots.

For *P. inflatum*, we collected samples from all visually identified shoots because populations are very small (<50 shoots) and individuals are scatteredly distributed. The area occupied by populations is relatively homogeneous, from 500 (50 × 10 m, PI-2) to 800 m^2 (40 × 20 m, PI-3); thus, we got similar sample sizes (30–33 shoots; Table 1). To minimize the damage to these plants, we collected only 1 leaf per individual.

Enzyme Electrophoresis

Leaf samples were wrapped in wet paper towels, placed in plastic bags, returned to the laboratory, and then stored at 4 °C until protein extraction. We extracted enzymes from individual leaf samples as described in Chung and Kang (1994). We conducted electrophoresis on 13% starch gels, with 2 buffer systems. We used a modification (Haufler 1985) of the system 6 of Soltis et al. (1983) to resolve alcohol dehydrogenase (*Adh-1* and *Adh-2*), diaphorase (*Dia-1*, *Dia-2*, *Dia-3*, and *Dia-4*), fluorescent esterase (*Fe-1* and *Fe-2*), phosphoglucoisomerase (*Pgi-1*, *Pgi-2*, and *Pgi-3*), phosphoglucomutase (*Pgm-1* and *Pgm-2*), and triosephosphate isomerase (*Tpi-1* and *Tpi-2*). We also used the morpholine-citrate buffer system (pH 6.1) of Clayton and Tretiak (1972) to resolve isocitrate dehydrogenase (*Idh-1* and *Idh-2*), malate dehydrogenase (*Mdh-1* and *Mdh-2*), and 6-phosphogluconate dehydrogenase (*6Pgd-1* and *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).

Identification of Clones, Clonal Diversity, Genetic Diversity, and Genetic Structure

To identify clones and to conduct further genetic analyses, we considered a locus to be polymorphic when 2 or more alleles were observed, regardless of their frequencies. As multiple

Table 1 Summary of clonal diversity measures examined in 11 populations of *Polygonatum stenophyllum* and 4 populations of *Polygonatum inflatum*

Species/ population	Area (m^2)	N_T	N_G	P_{gen} F_{IS}	*R*	*D*	*ED*	β (95% CIs for b_P[a])
Polygonatum stenophyllum								
PS-1	20 × 10	36	1	0.778	0.000	0.000	na	na
PS-2	70 × 10	46	3	0.643	0.044	0.371	0.478	0.104 (–0.374, 0.166)
PS-3	10 × 10	21	1	0.905	0.000	0.000	na	na
PS-4	100 × 10	74	13	0.234	0.164	0.691	0.616	0.181 (–0.234, –0.128)
PS-5	50 × 10	31	1	0.953	0.000	0.000	na	na
PS-6	100 × 10	68	1	0.974	0.000	0.000	na	na
PS-7	30 × 20	33	1	1.000	0.000	0.000	na	na
PS-8	200 × 20	91	5	0.424	0.044	0.456	0.511	0.097 (–0.129, –0.064)
PS-9	5 × 2	13	1	0.926	0.000	0.000	na	na
PS-10	20 × 5	42	5	0.186	0.098	0.611	0.672	0.214 (–0.271, –0.157)
PS-11	6 × 2	15	1	0.946	0.000	0.000	na	na
Average		43	3	0.724	0.032	0.194	0.569	0.148
Polygonatum inflatum								
PI-1	30 × 20	31	20	0.017	0.633	0.959	0.818	1.117 (–1.626, –0.608)
PI-2	50 × 10	30	20	0.025	0.655	0.968	0.863	1.455 (–2.503, –0.406)
PI-3	40 × 20	33	18	0.039	0.531	0.951	0.885	1.116 (–1.807, –0.425)
PI-4	20 × 30	31	17	0.042	0.533	0.948	0.878	1.091 (–1.674, –0.509)
Average		31	18	0.031	0.588	0.957	0.861	1.195

na, not applicable; N_T, number of individuals sampled (including clonal ramets); N_G, number of individuals excluding clonal ramets; P_{gen} F_{IS}, probability of the identical MLG occurring by chance due to sexual reproduction by taking into account departures from H-W equilibrium; *R*, genotypic richness; *D*, Simpson diversity index of clonal heterogeneity; *ED*, Simpson evenness index; β, parameter for Pareto distribution (–1 × regression slope, b_P); CIs, confidence intervals.

[a]Except for PS-2 ($P = 0.128$), all log–log regressions between $N_{\geq X}$ on *X* indicated significance with $P < 0.05$.

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 2.0 (Arnaud-Haond and Belkhir 2007), we calculated P_{gen} F_{IS}, the probability of identical MLG occurring 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 excluding all but 1 clonal ramet per genet, resulting in each distinct MLG only represented once per population (N_G, the number of individuals excluding clonal ramets).

Arnaud-Haond et al. (2007) and Becheler et al. (2010) recommend the use of 4 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 MLG when randomly taking 2 units in a population) and its equitability (ED, Simpson evenness index; 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 of 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 $N_{\geq X}$) versus $\log_{10}$ (X), and to check the quality of the Pareto approximation, we estimated its associated coefficient of determination (R^2). To test 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 0. 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 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 N_G, we estimated the following genetic diversity parameters with the programs POPGENE (Yeh et al. 1999) and FSTAT (Goudet 1995): percentage of polymorphic loci (*%P*), mean number of alleles per locus (*A*), allelic richness (*AR*) using a rarefaction method that compensates uneven population sample sizes (Hurlbert 1971; El Mousadik and Petit 1996), observed heterozygosity (H_o), and Nei's (1978) unbiased gene diversity (H_e).

To test for evidence of recent bottleneck events, for individual loci we evaluated the differences between the H–W expected heterozygosity (H_e) and the equilibrium heterozygosity (H_{eq}) expected assuming that populations are at 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 with the program BOTTLENECK (Piry et al. 1999). Recently bottlenecked 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) because loss of allelic diversity under genetic drift is expected to more rapidly reduce H_{eq} than H_e (Nei et al. 1975).

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 the 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 multipopulation 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. F_{IS} and F_{ST} were only estimated for those populations that had sample sizes (i.e., number of MLG) ≥ 5.

Results

Identification of Clones

For *P. stenophyllum*, 11 (*Adh-1*, *Adh-2*, *Dia-1*, *Dia-3*, *Idh-2*, *Mdh-1*, *Mdh-2*, *6Pgd-1*, *Pgi-2*, *Pgm-2*, and *Tpi-1*) of the 21 putative loci were polymorphic across 11 populations. Except for PS-10, number of polymorphic loci ranged from 0 to 3. Thus, the power to discriminate clonal genotypes from sexually produced genotypes was very low, ranging from 0 (no allozyme variation in PS-7) to 0.814 (PS-10) with an average of 0.276 (Table 1). Although the power was very low, we consider for all subsequent analyses that samples sharing identical MLG within populations were members of the same clone (see Justification for Single Clones and Clonal Diversity: Inference of Population History in the Discussion for further explanation of this consideration). At the population level, we identified a total of 33 N_G (MLG) out of 470 N_T (Table 1). N_G was very variable, with populations showing 1 (PS-1, PS-3, PS-5, PS-6, PS-7, PS-9, and PS-11), 3 (PS-2), 5 (PS-8 and PS-10), and 13 MLG (PS-4; Table 1). A careful inspection of Table 2 indicates, however, that the total number of different MLG was 31 instead of 33 (the MLG no. 3 in PS-2 is the same as the MLG no. 24 in PS-8, and the MLG in PS-5 is the same as that found in PS-6 (Table 2).

For *P. inflatum*, 7 (*Adh-3*, *Fe-1*, *Pgi-2*, *Pgi-3*, *6Pgd-1*, *6Pgd-2*, and *Tpi-2*) of the 21 putative loci were polymorphic. The power

Table 2 MLG based on 11 polymorphic loci found in 11 populations of *Polygonatum stenophyllum*

Population	MLG	N_R	*Adh-1*	*Adh-2*	*Dia-1*	*Dia-3*	*Idh-2*	*Mdh-1*	*Mdh-2*	*6Pgd-1*	*Pgi-2*	*Pgm-2*	*Tpi-1*
PS-1	1	36	*ab*	*aa*	*cc*	*cc*	*aa*	*aa*	*aa*	*aa*	*bc*	*cc*	*aa*
PS-2	2	36	*aa*	*bb*	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*bb*	*bc*	*aa*
	3	6	*aa*	*bb*	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*bb*	*cc*	*aa*
	4	4	*aa*	*bb*	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*bb*	*bb*	*aa*
PS-3	5	21	*aa*	*ab*	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*bc*	*bb*	*aa*
PS-4	6	40	*aa*	*ab*	*aa*	*aa*	*aa*	*aa*	*aa*	*ac*	*bb*	*bb*	*ab*
	7	8	*aa*	*ab*	*aa*	*aa*	*aa*	*aa*	*aa*	*ac*	*bb*	*bb*	*bb*
	8	5	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*ac*	*bb*	*bb*	*ab*
	9	4	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*bb*	*bb*	*aa*
	10	3	*aa*	*ab*	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*bb*	*bb*	*ab*
	11	2	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*bb*	*bb*	*bb*
	12	2	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*aa*	*ac*	*bb*	*bb*	*bb*
	13	2	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*aa*	*ac*	*bb*	*bb*	*ab*
	14	2	*aa*	*ab*	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*bb*	*bb*	*aa*
	15	2	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*ac*	*bb*	*bb*	*aa*
	16	2	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*aa*	*ac*	*bb*	*bb*	*aa*
	17	1	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*	*bb*	*bb*	*ab*
	18	1	*aa*	*ab*	*aa*	*aa*	*aa*	*aa*	*aa*	*ac*	*bb*	*bb*	*aa*
PS-5	19	31	*aa*	*aa*	*aa*	*bb*	*bb*	*aa*	*aa*	*aa*	*bb*	*bc*	*aa*
PS-6	20	68	*aa*	*aa*	*aa*	*bb*	*bb*	*aa*	*aa*	*aa*	*bb*	*bc*	*aa*
PS-7	21	33	*aa*	*aa*	*aa*	*bb*	*bb*	*aa*	*aa*	*aa*	*bb*	*cc*	*aa*
PS-8	22	66	*aa*	*ab*	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*bb*	*cc*	*aa*
	23	11	*ab*	*aa*	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*bb*	*cc*	*aa*
	24	6	*aa*	*bb*	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*bb*	*cc*	*aa*
	25	5	*ab*	*aa*	*aa*	*bb*	*aa*	*aa*	*aa*	*ac*	*bb*	*cc*	*aa*
	26	3	*aa*	*aa*	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*	*bb*	*cc*	*aa*
PS-9	27	13	*aa*	*aa*	*bb*	*bb*	*bb*	*aa*	*aa*	*aa*	*bb*	*bc*	*bb*
PS-10	28	25	*aa*	*bb*	*bb*	*aa*	*bb*	*cc*	*cc*	*aa*	*bb*	*bb*	*ab*
	29	7	*aa*	*aa*	*bb*	*aa*	*bb*	*cc*	*cc*	*aa*	*bb*	*bb*	*ab*
	30	5	*aa*	*aa*	*cc*	*bb*	*bb*	*aa*	*aa*	*aa*	*bb*	*bc*	*bb*
	31	3	*aa*	*bb*	*cc*	*aa*	*bb*	*cc*	*cc*	*aa*	*bb*	*bb*	*ab*
	32	2	*aa*	*bb*	*bb*	*bb*	*bb*	*cc*	*cc*	*aa*	*bb*	*bb*	*ab*
PS-11	33	15	*aa*	*aa*	*bb*	*bb*	*bb*	*aa*	*aa*	*aa*	*bc*	*cc*	*aa*

Heterozygous genotypes are indicated by boxes. Note that the MLG #3 (PS-2) is the same as the MLG #24 (PS-8), and the MLG in PS-5 is the same as that in PS-6.

to discriminate clonal genotypes from sexually produced genotypes was greater than 0.95 (average P_{gen} F_{IS} was 0.031; Table 1). Thus, it was safe to consider for all subsequent analyses that neighboring ramets sharing identical MLG were members of the same clone. We identified a total of 75 (N_G) distinct MLG out of 125 total samples (N_T) across 4 populations (Table 1).

Clonal Diversity

For 7 populations (PS-1, PS-3, PS-5, PS-6, PS-7, PS-9, and PS-11) of *P. stenophyllum*, estimates of genotypic richness (*R*) and Simpson diversity indices (*D*) were 0 because multiple ramets belonged to single genets (Table 1; Supplementary Table S1). Furthermore, the other 4 populations possessing 3 (PS-2), 5 (PS-8 and PS-10), and 13 (PS-4) genets exhibited low estimates of *R* (0.044–0.164), *D* (0.371–0.691), and Simpson *ED* (0.478–0.672; Table 1). Accordingly, average estimates of these parameters across 11 populations were very low (averages of *R*, *D*, and *ED* were 0.032, 0.194, and 0.569, respectively; Table 1). Analyses of the Pareto index β were conducted only in 4 populations (PS-2, PS-4, PS-8, and PS-10). With the exception of PS-2, the $\log_{10}$ of the cumulative distribution of ramets among genets was linearly related to the $\log_{10}$ of N_R (the genet size or the number of ramets belonging to each genet), thus supporting the Pareto distribution hypothesis in a highly significant manner (R^2 = 0.939–0.979, $P < 0.05$; Figure 2). The correlation for PS-2 was not significant probably due to the low number of points (only 3) in the scatter graph (i.e., with insufficient power to reject the null hypothesis, $b_P = 0$; $R^2 = 0.960$, $P = 0.128$; Figure 2). However, the values of the Pareto index β (–1 × regression slope, b_P) were low for the 3 populations, ranging from 0.097 (PS-8) to 0.214 (PS-10) with a mean of 0.148 (Table 1). The b_P of PS-10 was significantly steeper than that of PS-8, as 95% CIs of b_P did not overlap (Table 1). We found significant differences in the distribution of clone sizes among 11 populations (contingency χ^2 test, $\chi^2 = 290$, df = 180, $P = 0.000$; Supplementary Table S1). It is worthy to note that in all of the *P. stenophyllum* populations with multiple MLG, a single common MLG makes up more than half of the ramets that were sampled (Table 2).

Relative to *P. stenophyllum*, the 4 populations of *P. inflatum* maintained higher levels of genotypic diversity, with averages for *R*, *D*, and *ED* of 0.588, 0.957, and 0.861, respectively (Table 1). The b_P estimates for all 4 populations of *P. inflatum* were significantly lower than the null hypothesis ($b_P = 0$; R^2 = 0.898–0.978, P = 0.009–0.027; Figure 2). The values of the Pareto index β (with a mean of 1.195, ranging from 1.091 to 1.455; Table 1) were similar among them (as 95% CIs of b_P did overlap) and were substantially higher than those calculated in *P. stenophyllum* (as 95% CIs of b_P did not overlap; Table 1). Unlike *P. stenophyllum*, there were no significant differences in the distribution of clone sizes among the 4 populations of *P. inflatum* (contingency χ^2 test, $\chi^2 = 4.35$, df = 12, $P = 0.976$; Supplementary Table S1). Accordingly, we found significant differences in the distribution of clone sizes between the 2 studied species (contingency χ^2 test, $\chi^2 = 51$, df = 18, $P = 0.000$; Supplementary Table S1).

Genetic Diversity and Structure

Populations of *P. stenophyllum* maintained low levels of allozyme variation (*%P* = 9.5, *AR* = 1.06, *A* = 1.10, and H_e = 0.045; Table 3). However, we found high levels of genetic variation in pooled samples (*%P* = 52.4, *A* = 1.62, and H_e = 0.171; Table 3). In contrast, populations of *P. inflatum* harbored moderate levels of genetic variation (*%P* = 25.0, *AR* = 1.31, *A* = 1.31, and H_e = 0.092; Table 3). When samples were pooled, we found slightly higher levels of genetic variation (*%P* = 33.3, *A* = 1.48, and H_e = 0.106; Table 3).

Except for PS-4, the sample size of the remaining populations of *P. stenophyllum* was less than 10, and thus, we only conducted the BOTTLENECK test in this population. We found a marginally significant signal of recent bottleneck (both with sign and Wilcoxon sign-rank tests; Table 4). Regarding *P. inflatum*, populations PI-1 and PI-3 displayed significant *P* values for both sign (although only marginally significant for PI-3) and Wilcoxon sign-rank test (Table 4).

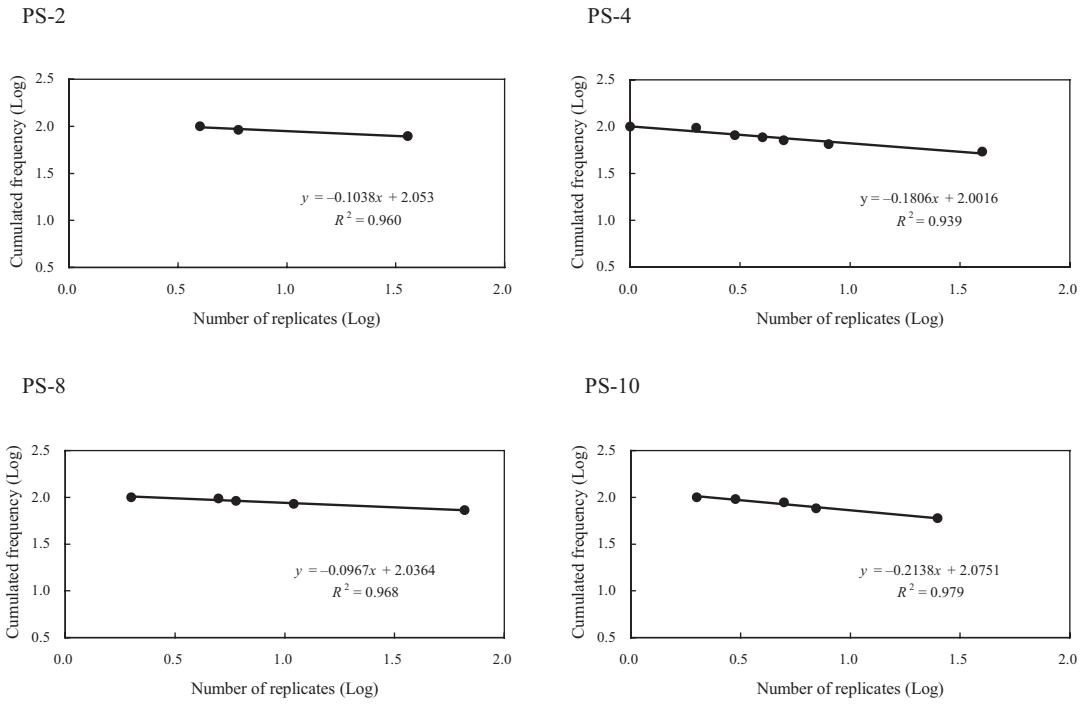
Of the population-level F_{IS} estimates for the 4 populations of *P. stenophyllum* in which this parameter can be calculated, only that of PS-10 was significantly positive at the 0.05 level (F_{IS} = 0.697; Table 3). This result, as well as the significant multipopulation level F_{IS} (F_{IS} = 0.316, P = 0.001; Table 3), indicates a substantial deficit of heterozygotes within populations. Among the 4 populations studied of *P. inflatum*, 3 populations (PI-1 to PI-3; Table 3) exhibited significant deficits of heterozygotes, and multipopulation level F_{IS} was also significant (F_{IS} = 0.285, P = 0.001; Table 3). *Polygonatum stenophyllum* showed a considerably higher F_{ST} value than *P. inflatum* (F_{ST} = 0.636 vs. F_{ST} = 0.165).

Discussion

Justification for Single Clones and Clonal Diversity: Inference of Population History

We found that only 4 populations (PS-2, PS-4, PS-8, and PS-10) of *P. stenophyllum* were multiclonal, with the remaining 7 populations consisting of a single MLG (Table 1). Six out of 7 populations composed by 1 MLG had at least 1 heterozygous locus (1 in PS-5, PS-6, PS-9, and PS-11; 2 in PS-1 and PS-3; Table 2). If sexual reproduction occurred within these populations, homozygotes at these loci should be formed through recombination. In fact, there is evidence for segregation at those loci that are heterozygous for the most common MLG in the 4 populations with ≥3 MLG (*Adh-2*, *6Pgd-1*, *Pgm-2*, and *Tpi-1*; Table 2): the 2 homozygotes for these loci are present in less frequent MLG, indicating that the most common MLG may have been the founding genotype and the homozygotes are its progeny. Except for PS-7, predominance of genets having at least 1 heterozygous locus in the uniclonal populations suggests that selection may have acted in favor of these clones at 1 or several stages of the life cycle (Lee and Chung 1999; Pappert et al. 2000). Assuming linkage equilibrium between allozyme loci and that they are located at different chromosomes, we expect a maximum of 708 588 MLG in the study samples (among 11 polymorphic

Polygonatum stenophyllum



Polygonatum inflatum

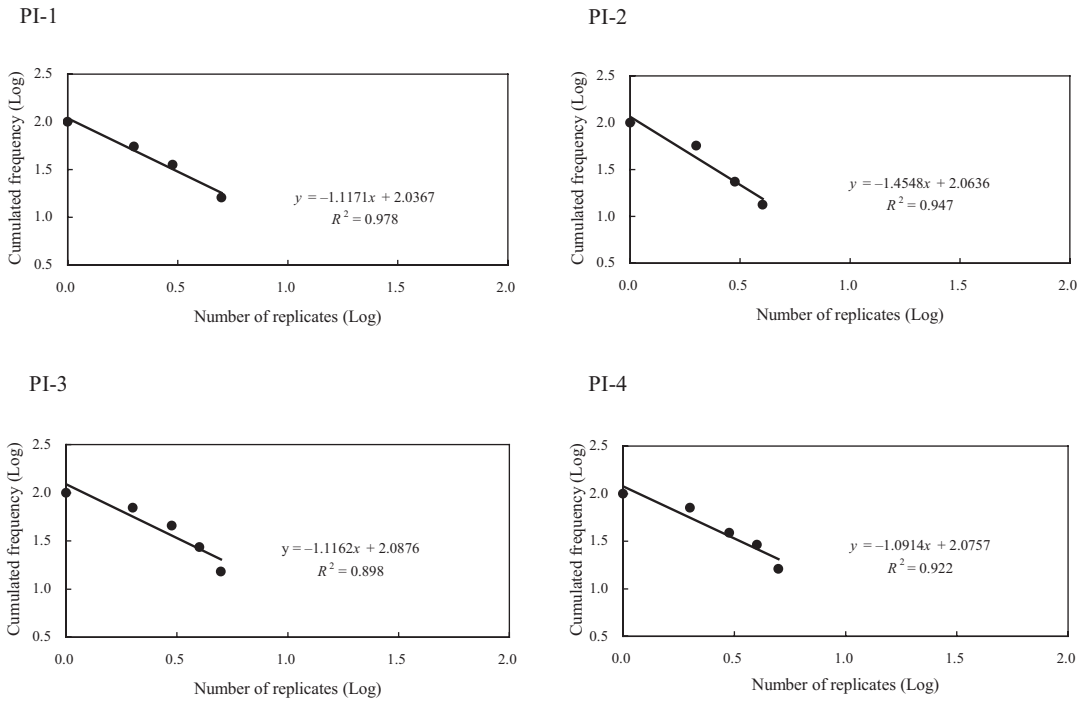


Figure 2. Pareto plots showing the distribution of putative clonal ramets in 4 populations of *Polygonatum stenophyllum* and in 4 populations of *P. inflatum*. Regression slope (b_P) was obtained by plotting the double logarithmic linear regression of reverse cumulative frequency of the number of genets containing X or more ramets ($N_{\geq X}$) on the number of sampled ramets belonging to a genet (X) and R^2 represents square of correlation coefficient (coefficient of determination) between $N_{\geq X}$ on X.

Table 3 Levels of genetic diversity in 11 populations of *Polygonatum stenophyllum* and in 4 populations of *Polygonatum inflatum*

Species/population	Altitude (m)	N_G	%*P*	*AR*	*A*	H_o (SE)	H_e (SE)	F_{IS}
Polygonatum stenophyllum								
PS-1	145	1	9.5	1.10	1.10	0.095 (0.066)	0.048 (0.033)	nc[a]
PS-2	96	3	4.8	1.03	1.05	0.016 (0.016)	0.032 (0.032)	nc
PS-3	86	1	9.5	1.10	1.10	0.095 (0.066)	0.048 (0.033)	nc
PS-4	11	13	14.3	1.07	1.14	0.066 (0.037)	0.068 (0.037)	0.027
PS-5	280	1	4.8	1.05	1.05	0.048 (0.048)	0.024 (0.024)	nc
PS-6	204	1	4.8	1.05	1.05	0.048 (0.048)	0.024 (0.024)	nc
PS-7	71	1	0.0	1.00	1.00	0.000 (0.000)	0.000 (0.000)	nc
PS-8	45	5	14.3	1.05	1.14	0.038 (0.022)	0.050 (0.029)	0.238
PS-9	135	1	4.8	1.05	1.05	0.048 (0.048)	0.024 (0.024)	nc
PS-10	140	5	33.3	1.14	1.33	0.048 (0.039)	0.157 (0.053)	0.697*
PS-11	190	1	4.8	1.05	1.05	0.048 (0.048)	0.024 (0.024)	nc
Average	128	3	9.5	1.06	1.10	0.050 (0.009)	0.045 (0.013)	0.316[b]
Pooled samples		33	52.4		1.62		0.171 (0.044)	
Polygonatum inflatum								
PI-1	1240	20	23.8	1.29	1.29	0.069 (0.030)	0.099 (0.042)	0.304*
PI-2	1180	20	28.6	1.38	1.38	0.062 (0.023)	0.096 (0.037)	0.357*
PI-3	980	18	19.1	1.24	1.24	0.053 (0.027)	0.086 (0.041)	0.386*
PI-4	580	17	28.6	1.33	1.33	0.081 (0.032)	0.086 (0.033)	0.057
Average	995	19	25.0	1.31	1.31	0.066 (0.006)	0.092 (0.003)	0.285[b]
Pooled samples		75	33.3		1.48		0.106 (0.038)	

%*P*, percentage of polymorphic loci; *AR*, mean allelic richness; *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.

AR is adjusted for a sample size of 1 and 17 plants for *P. stenophyllum* and *P. inflatum*, respectively.

[a]nc, not conducted (analysis of F_{IS} within populations was only conducted for populations with $N_G \geq 5$).

[b]Significant Weir and Cockerham (1984) estimate of F_{IS} over 3 populations.

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

Table 4 Results of statistical tests for evidence of recent population bottlenecks in PS-4 of *Polygonatum stenophyllum* and in 4 populations of *Polygonatum inflatum*

Species/population	Sign test	Wilcoxon sign-rank test
Polygonatum stenophyllum		
PS-4	0.094	0.063
Polygonatum inflatum		
PI-1	**0.032**	**0.016**
PI-2	0.442	0.500
PI-3	0.068	**0.031**
PI-4	0.326	0.656

Note that tests were conducted only in the population PS-4 of *P. stenophyllum* because the number of the different MLG (putative genets) was less than 10 in the remaining 10 populations. 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.

loci, 9 loci can have 3 different genotypes and 2 loci can have 6 genotypes, thus, $3^9 \times 6^2$). However, we only found 31 different MLG (Table 2). These results strongly suggest that the 7 populations consist of single clones (uniclonal) and support the first hypothesis (shoots belong to a single or a few clones). Limitation of sexual reproduction also suggests that *P. stenophyllum* is self-incompatible, although this should be confirmed by further studies on the breeding system.

As the probability of finding an identical MLG of *P. stenophyllum* is high, 2 MLG can be identical by chance alone. This might be the case of the identical MLG found in PS-2 and PS-8 (no. 3 and no. 24, respectively) because there is no obvious connection between PS-2 and PS-8 (there is no possibility of rhizome[s] transportation by river water between PS-2 and PS-8; Figure 1). In contrast, the uniclonal populations PS-5 (280 m a.s.l.) and PS-6 (204 m a.s.l.) show an identical MLG because the latter was probably founded through rhizome(s) transportation by water from the former (PS-5 is located upstream on the same river; Figure 1). Based on uniqueness of MLG composition and location and topographic traits of populations (Figure 1; Table 3), the other uniclonal populations of *P. stenophyllum* in addition to PS-5 (PS-1, PS-3, PS-7, PS-9, and PS-11) were probably founded by single seeds from genetically diverse source populations (perhaps transported by birds), suggesting that the ISR model fits for these populations. It is highly likely that PS-9 (135 m a.s.l.) was established by a single seed from PS-10 (140 m), as they are closely located (separated by 87 m in linear distance). The 4 multiclonal populations of *P. stenophyllum* (PS-2, PS-4, PS-8, and PS-10) were likely established by a few seeds from multiclonal sources, suggesting that they fit better a RSR model. Thus, our results do not fully support the second hypothesis, as RSR seems to have been operating within the multiclonal populations of *P. stenophyllum* instead of the ISR model. For *P. inflatum*, the skewed distribution of ramet numbers per genet (Supplementary Table

S1) strongly suggests that the RSR model is characteristic of their populations, in agreement with the second hypothesis.

Inference of the RSR model is further supported by levels of genotypic diversity. Levels of clonal (genotypic) diversity (*R*, *D*, *ED*, and the Pareto index β) found in *P. stenophyllum* populations were considerably lower than those for *P. inflatum*. The Pareto index β is well suited for summarizing clonal diversity and for making comparisons among different studies (Arnaud-Haond et al. 2007; Ohsako 2010). The mean value of β (0.148) for *P. stenophyllum* is substantially lower than the average value from 15 populations belonging to 11 terrestrial and marine plant species compiled by Ohsako (2010; β = 0.930). In contrast, the mean value of β (1.195) for *P. inflatum* is higher than the Ohsako's (2010) average. Low values of β indicate that populations of *P. stenophyllum*, unlike other clonal plants (including *P. inflatum*), have a tendency towards being formed by a few large clones and several small ones (Supplementary Table S1). In agreement with this distribution, genotypic richness (*R*) values found in the 11 populations of *P. stenophyllum* (mean *R* = 0.032) are substantially lower than the average (0.294) for the 11 clonal plant species reported by Ohsako (2010). Like β value, the mean value of *R* (0.588) for *P. inflatum* is higher than the Ohsako's average. This pattern of genotypic diversity exhibited by *P. stenophyllum* and *P. inflatum* support the third hypothesis: within-population clonal (genotypic) diversity found in *P. stenophyllum* is considerably lower than that of *P. inflatum*. A high level of within-population genotypic diversity in *P. inflatum* further suggests that the RSR model is fitted for this species.

Genetic Diversity and Structure: Mountains as a Glacial Refugium

As predicted by the third hypothesis, within-population genetic variation and among-population differentiation found in *P. stenophyllum* are lower and higher than those exhibited by *P. inflatum*. Populations of *P. stenophyllum* maintain lower levels of genetic variation than those averaged for short-lived herbaceous perennials (%*P* = 28.0, *A* = 1.40, and H_e = 0.096; Hamrick and Godt 1990), whereas populations of *P. inflatum* show comparable levels to Hamrick and Godt's (1990) averages. The genetic differentiation between populations of *P. stenophyllum* (F_{ST} = 0.636) is substantially higher than that of *P. inflatum* (F_{ST} = 0.165) and also than that averaged for short-lived herbaceous perennials and monocots (G_{ST} = 0.233 and 0.231, respectively; Hamrick and Godt 1990). The most likely explanation of why contemporary populations of *P. stenophyllum* (except for PS-10) maintain extremely low levels of within-population genetic variation (as well as low clonal diversity) and very high among-population differentiation is that they were established by a single or by a few seeds. Founder effects usually lead to decreased within-population genetic variation and increased among-population genetic divergence (e.g., Westerbergh and Saura 1994). When samples are pooled as a whole, *P. stenophyllum* is more variable than *P. inflatum* (%*P* = 52.4 vs. 33.3, *A* = 1.62 vs. 1.48, and H_e = 0.171 vs. 0.106; Table 3) and short-lived herbaceous perennials (%P_s = 41.3, A_s = 1.70, and H_{es} = 0.116; Hamrick and Godt 1990). This result reflects population genetics theory that founder effects should affect the levels and distribution of genetic diversity within and among populations of plant species, but it should have relatively little effect on the level of genetic diversity within species as a whole (Halliburton 2004). That is, the high levels of genetic diversity at the species level found in *P. stenophyllum* should be attributed to the high among-population genetic differentiation (F_{ST} = 0.636).

Why does the population PS-10 of *P. stenophyllum* maintain moderate levels of genetic variation? Chung et al. (2012) hypothesized that the Baekdudaegan mountain range served as a refugium for the boreal montane vegetation during the Last Glacial Maximum (LGM). In this regard, we should acknowledge the location of the genetically highest diverse population PS-10, at the foot of the Baekdudaegan mountain range (the other *P. stenophyllum* population located in this mountains, PS-9, is probably the result of a founder effect from the nearby PS-10; see section Justification for Single Clones and Clonal Diversity: Inference of Population History). PS-10 harbored 7 polymorphic loci (*Adh-2*, *Dia-1*, *Dia-3*, *Mdh-1*, *Mdh-2*, *Pgm-2*, and *Tpi-1*) and maintained moderate levels of within-population variation (%*P* = 33.3, *A* = 1.33, and H_e = 0.157; Table 3). In addition, we found that PS-10 has a more even distribution of MLG (Supplementary Table S1). Levels of genetic variation for the population PS-10 of *P. stenophyllum* and for all the populations of *P. inflatum* (that also occurs on the Baekdudaegan) are comparable to the values reported for other species growing in this mountain system, including the herbaceous perennials *Adenophora grandiflora*, *Bupleurum euphorbioides*, *Cypripedium macranthos*, *Hanabusaya asiatica*, *Megaleranthis saniculifolia*, *Oreorchis patens*, and *Parasenecio pseudotaimingasa*, the shrub *Forsythia ovata*, and the coniferous tree *Taxus cuspidata* (Table 3 in Chung et al. 2013). The common genetic diversity patterns for these species suggest the existence of multiple, continuous refugia along the Baekdudaegan during the LGM, which would have enabled plant species to maintain relatively large population sizes and high rates of recurrent gene flow (Chung and Chung 2014). This scenario would be favored by the topography, as it allowed tracking the quaternary climatic oscillations by altitudinal migrations (Chung et al. 2012) and by the local environmental conditions (with a continuous water supply through the orographic rain and the closely related Pacific Ocean; Yi 2011).

Conservation Implications

Polygonatum stenophyllum is a species of interest in conservation, as it is included in the Wildlife Protection Act of Korea (Ministry of Environment 2005). Preservation of its genetic diversity in South Korea should be ensured, as Korean populations might harbor some unique genetic traits (e.g., private alleles) compared with the other populations within the species range. Thus, we propose that efforts toward conservation management should be aimed at preserving (and ideally increasing) the number and size of current populations (i.e., number of genetically distinct individuals, genets). It should be noted that the 7 uniclonal populations might have only a single sexual individual (i.e., effective number of

population size is equal to 1); one may expect, thus, that the 7 uniclonal populations of *P. stenophyllum* would almost certainly become extinct without the introduction of genotypic diversity. However, a dynamics of extinction-recolonization would occur relatively frequently in highly disturbed populations such as those that characterize this species; in this regard, it is necessary to carry out a long-term study on population dynamics prior to proceed with any reinforcement.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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