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Genetic evidence of a progenitor–derivative species pair in East Asian *Lilium*

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Boreal *Lilium distichum* and temperate *L. tsingtauense* are morphologically very similar, thus they have been placed in the section *Martagon*. Recent molecular phylogenetic analyses revealed that *L. distichum* and *L. tsingtauense* are indeed the most closely related species within that section. *Lilium distichum* has a wider geographic range and a broader niche than *L. tsingtauense*. We hypothesized that *L. distichum*–*L. tsingtauense* might be a classical "progenitor–derivative" (P–D) species pair and examined the levels of allozyme diversity in the two species in South Korea. Whereas the allelic composition of *L. tsingtauense* represented a subset of *L. distichum*, the former had significantly lower allozyme variability at both the population and the species levels than the latter. Except for the locus *Fe* (fluorescent esterase), allele frequencies of *L. distichum* were very similar to those of *L. tsingtauense*. Accordingly, pairwise genetic identities between populations of *L. distichum* and *L. tsingtauense* were very high, with a mean of 0.919. Our allozyme results support the hypothesis that *L. tsingtauense* is a derivative species of the progenitor *L. distichum*.

Introduction

Lilium consists of approximately 100 species that are widely distributed throughout boreal and temperate regions of the northern hemisphere (MacRae 1998). Among them, *L. distichum* is a boreal species usually occurring on forested slopes, forest margins, and hillsides along streams (200–1800 m), distributed in Russian Far East (Primorsky Krai), northeastern China (Heilongjiang, Jilin, and Liaoning provinces) and the Korean Peninsula (Liang & Tamura 2000; Fig. 1). In Korea, *L. distichum* occurs in grasslands or gaps of *Quercus mongolica*-dominated temperate deciduous forests, at altitudes of 900–1600 m a.s.l. along the Baekdudaegan (the main mountain system of Korea, which runs through most of the length of the Korean Peninsula) and in one of its main branches, the Nakdongjeongmaek (Fig. 1). The species is relatively common where it occurs, with the number of individuals per population on the order of hundreds. Its congener *L. tsingtauense* is a temperate species with a narrower range, distributed in eastern

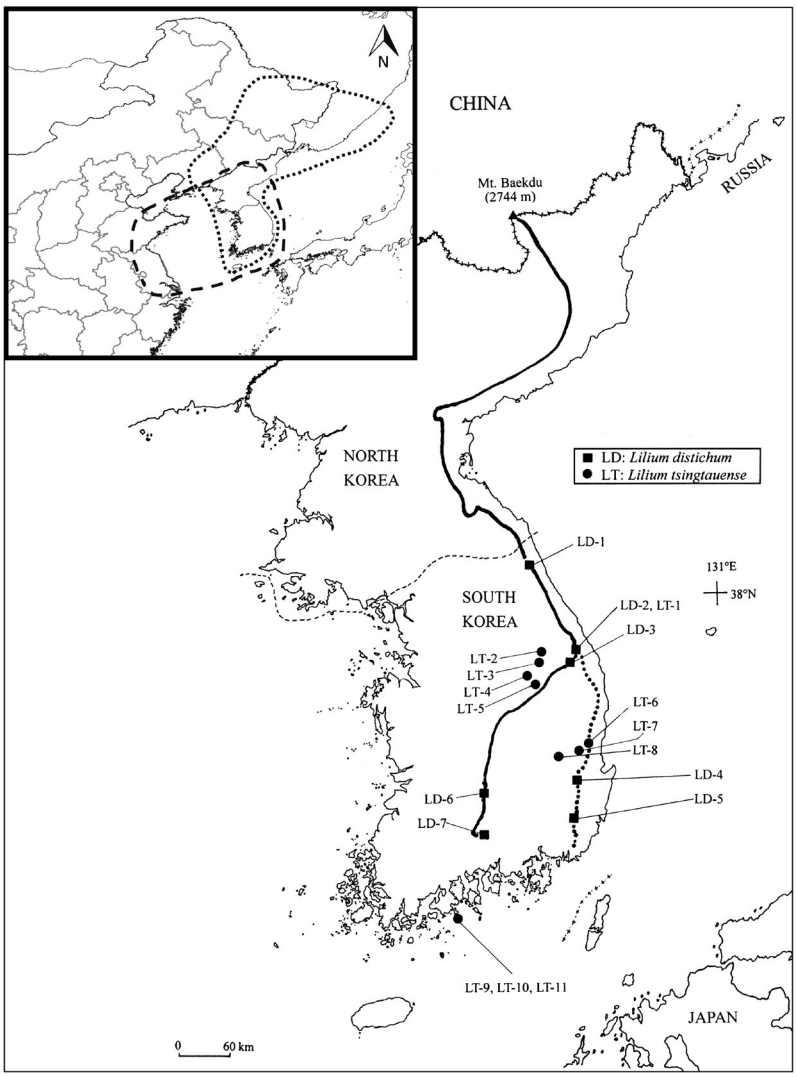

Fig. 1. Locations of sampled populations of *Lilium distichum* (LD-1 to LD-7) and *L. tsingtauense* (LT-1 to LT-11) in South Korea. Sample sizes: LD-1 = 47, LD-2 = 24, LD-3 = 44, LD-4 = 21, LD-5 = 27, LD-6 = 45, LD-7 = 37; LT-1 = 18, LT-2 = 48, LT-3 = 41, LT-4 = 29, LT-5 = 37, LT-6 = 22, LT-7 = 36, LT-8 = 38, LT-9 = 42, LT-10 = 58, LT-11= 74. The solid line indicates the location and shape of the main mountain range of the country, the Baekdudaegan, which runs from north to south along the Korean Peninsula (spanning over 1600 km). The dotted line represents the Nakdongjeongmaek, one of the 13 mountainous branches of the Baekdudaegan. The approximate geographic ranges of both *Lilium* species are shown in the inset map (dotted line, *L. distichum*; dashed line, *L. tsingtauense*).

China (Shandong and Anhui provinces) and in the Korean Peninsula (Liang & Tamura 2000; Fig. 1). In China, it occurs on sunny forested slopes or in bushy or grassy places, at a wide range of elevations (100–1000 m a.s.l.; Liang & Tamura 2000, Guo *et al*. 2011). In Korea, *L. tsingtauense* is mainly found in relatively open habitats in low-altitude mountains (0–500 m; hereafter we define low elevation as < 500 m, mid elevation as 500–1000 m, and high elevation as > 1000 m) across the Peninsula, including peripheral regions of the Baekdudaegan and the islands. Isolated individuals, however, can be found at mid to high elevations in montane habitats on the main ridge of the Baekdudaegan. Like *L. distichum*, *L. tsingtauense* is locally common, with populations of hundreds of individuals.

Because of their similar morphology, *L. distichum* and *L. tsingtauense* were placed in the same section *Martagon*, one of the seven sections in *Lilium* proposed by Comber (1949). A close relationship has been further corroborated by molecular phylogenetic analyses, which clearly point out that *L. distichum* and *L. tsingtauense* are the most closely related species within this section (Sultana *et al*. 2010, 2011, Lee *et al*. 2011). They are sympatric in some locations in mid-elevation mountains in Korea (e.g. LD-2, 902 m, and LT-1, 889 m, at Mt. Deokhang; Fig. 1). However, *L. distichum* has a much wider geographic range, with

a broader niche (as estimated from ecological niche modelling analyses in Chung *et al*. 2014a) than *L. tsingtauense*. *Lilium distichum* thrives in places with temperatures ranging from –21.5 °C to 24.8 °C (mean temperatures in winter and summer, respectively) and precipitation ranging from 550 mm to 1500 mm, whereas *L. tsingtauense* has narrower temperature and precipitation ranges (from –7 °C to 24.6 °C, and from 700 mm to 1500 mm, respectively). Based on phylogenetic information, ecological traits, and distribution data, we hypothesize that *L. distichum*–*L. tsingtauense* might constitute a "progenitor–derivative" (hereafter P–D) species pair; that is, instead of true sister species pair, a D or "derived" species budded off and acquired new traits while the P or "parental" species remained largely unchanged (Gottlieb 1984, 2003, Crawford 1985, 1990, 2010). Under this scenario, we can expect three genetic outcomes. First, the spectrum of alleles observed in *L. tsingtauense* should be a subset of those found in *L. distichum*, with few, if any, unique alleles. Second, levels of within-population genetic variation observed in *L. tsingtauense* should be lower than in *L. distichum*. Third, interspecific genetic identities would be similar to or slightly lower than intraspecific identities, as a result of the recent divergence of the D species (Gottlieb 1973, 2003, Crawford 1983, 2010).

Material and methods

Study plants

Lilium distichum is a herb 60–120 cm tall, with a papillose stem and leaves in a whorl of 7–9 near the middle of stem. Two to seven orange to pale vermilion flowers with purple-red spots (tepals are ca. 5.0 cm long) are arranged in racemes, nodding to horizontal, and open from July to August. Fruits (capsules) are obovoid, 1.5 cm long. *Lilium tsingtauense* is a somewhat shorter plant (40–85 cm tall), with leaves in one or two whorls of 5–14. Orange to vermilion flowers with purple-red spots are solitary or in racemes of up to seven, of similar size to those of *L. distichum*, and open from June to July. Capsules are also ca. 1.5 cm long. The breeding systems and pollinators for the two *Lilium* species are unknown, although many lilies are known to be insect-pollinated and self-compatible (Yang & Sun 2005, Arzate-Fernández *et al*. 2007, Rodger *et al*. 2010) with few exceptions (e.g. *L. longiflorum* is generally regarded as self-incompatible; Miller 1993).

Population sampling

For *L. distichum*, we collected 245 individuals from seven populations (Table 1 and Fig. 1), five from the main ridge of the Baekdudaegan (LD-1 to LD-3, LD-6, and LD-7) and two from the Nakdongjeongmaek (LD-4 and LD-5). For *L. tsingtauense*, we sampled a total of 443 individuals from 11 populations (Table 1 and Fig. 1), mostly located on the low- to mid-elevation peripheral areas of the Baekdudaegan, although three populations (LT-9, LT-10, and LT-11) were located on Oenaro Island, on the southern coast of the Korean Peninsula (Fig. 1). As populations

Table 1. Levels of genetic diversity of *Lilium distichum* and *L. tsingtauense* in South Korea estimated from 7 and 11 populations, respectively. Statistics for levels of within-population genetic variation are provided in Chung *et al.* (2014a: table 1). Abbreviations: *n* = number of individuals sampled, %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.

Species	*n*	%P	AR	*A*	H_o (SE)	H_e (SE)
Lilium distichum						
population average	35	65.3	1.90	1.94	0.151 (0.012)	0.190 (0.012)
pooled samples	245	85.7		2.57		0.204 (0.046)
Lilium tsingtauense						
population average	40	56.5	1.68	1.81	0.090 (0.009)	0.113 (0.007)
pooled samples	443	78.6		2.57		0.114 (0.037)

of both species are of large size, we collected samples (one leaf per individual) as random as possible, from well-separated individuals. Samples were the same as those used by Chung *et al*. (2014a), in a study that was aimed to infer past history and palaeodistribution (at the Last Glacial Maximum, LGM) of the two *Lilium* species on the Korean Peninsula. 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.

Enzyme electrophoresis

For enzyme 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*), fluorescent esterase (*Fe*), phosphoglucoisomerase (*Pgi-1*, *Pgi-2*), phosphoglucomutase (*Pgm*), and triosephosphate isomerase (*Tpi-1*, *Tpi-2*). We also used the morpholine–citrate buffer system (pH 6.1) of Clayton and Tretiak (1972) to resolve isocitrate dehydrogenase (*Idh*), 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 & 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 & Wendel 1990).

Data analysis

To estimate genetic diversity and structure of the two *Lilium* species, we considered that a locus was polymorphic when two or more alleles were observed, regardless of their frequencies. 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), observed heterozygosity (H_o), and Nei's (1978) unbiased gene diversity or Hardy-Weinberg (H-W) expected heterozygosity (H_e).

To determine the degree of genetic divergence among populations of each species and between the two species, we calculated Nei's (1978) unbiased genetic identity (*I*) between all pairs of studied populations.

Results

For *L. distichum*, 12 (*Adh*, *Dia-1*, *Dia-2*, *Fe*, *Idh*, *Mdh-1*, *Mdh-2*, *6Pgd-1*, *6Pgd-2*, *Pgi-2*, *Pgm*, and *Tpi-1*) out of 14 putative loci were polymorphic across the seven populations. High levels of genetic variation were found within populations: %P = 65.3, AR = 1.90, *A* = 1.94, and H_e = 0.190 (Table 1). For *L. tsingtauense*, 11 (*Adh*, *Dia-1*, *Dia-2*, *Fe*, *Idh*, *Mdh-1*, *Mdh-2*, *6Pgd-2*, *Pgi-2*, *Pgm*, and *Tpi-1*) of the 14 surveyed loci were polymorphic across the 11 populations. All within-population genetic parameters showed moderate values: %P = 56.5, AR = 1.68, *A* = 1.81, and H_e = 0.113 (Table 1). For the two *Lilium* species, slightly higher levels of genetic variation were observed for total samples (Table 1). On average, populations of *L. distichum* harboured significantly higher levels of genetic variation than those of *L. tsingtauense* (Wilcoxon rank sum test: p = 0.058 for %P, p = 0.008 for AR, and p = 0.001 for H_e). Details on genetic estimates at the population level are available in Chung *et al*. (2014a: Table 1).

Except for *Fe*, allele frequencies of *L. distichum* were very similar to those of *L. tsingtauense* (Table 2). In *L. distichum*, the most common allele at the *Fe* locus was Fe^b (occurring at a frequency of 0.700; Table 2), which was absent from *L. tsingtauense* (that is, it can be regarded as a diagnostic allele for *L. distichum*). In *L. tsingtauense*, the most common allele at

the *Fe* locus was, instead, Fe^e (occurring at a frequency as high as 0.897; Table 2), absent from *L. distichum* (i.e. a diagnostic allele for *L. tsingtauense*).

Discarding the alleles Fe^b and Fe^e, all the alleles that were species-specific (five in each species) were present at very low frequencies (0.001–0.190; Table 2). Accordingly, pairwise Nei's (1978) *I* values between congeneric populations of *L. distichum* and *L. tsingtauense* were high, ranging from 0.876 (LD-4 *vs*. LT-4) to 0.943 (LD-2 *vs*. LT-7), and with a mean ± SD of 0.919 ± 0.017 (Table 3). Pairwise Nei's (1978) *I* values between conspecific populations of *L. distichum* were high, ranging from 0.946 (LD-1 *vs*. LD-5) to 0.998 (LD-6 *vs*. LD-7), and with a mean ± SD of 0.971 ± 0.014 (Table 3). High *I* values were also found for *L. tsingtauense*, ranging from 0.977 (LT-4 *vs*. LT-6) to 1.000 (LT-10 *vs*. LT-11), and with a mean ± SD of 0.990 ± 0.005 (Table 3).

Discussion

Information on allele distributions and levels of genetic variation between closely related species has provided valuable insights into the identification of P–D species pairs (Gottlieb 1973, 2003, Crawford & Smith 1982, Crawford 1983, 2010, Loveless & Hamrick 1988, Pleasants & Wendel 1989, Chung *et al*. 1991, Maki *et al*. 1999, Hiramatsu *et al*. 2001, López-Pujol *et al*. 2001). In accordance with those previous studies, our predictions concerning the origin of *L. tsingtauense* as a D species from *L. distichum* are supported by genetic data.

First, *L. tsingtauense* exhibits significantly lower allozyme variability at both the population and the species levels than *L. distichum* (Table 1). Second, to some extent, the allelic composition of *L. tsingtauense* represents a subset of *L. distichum*. Across the 12 polymorphic loci, 34 alleles were detected in *L. distichum*; 28 (82%) of these were also detected in *L. tsingtauense* (Table 2). Except for Fe^e (allele frequency = 0.897), all the species-specific alleles of *L. tsingtauense* ($Dia\text{-}1^d$, $Dia\text{-}2^a$, $Mdh\text{-}2^c$, $Pgi\text{-}2^e$, and $Tpi\text{-}1^a$) were detected at very low frequencies (0.002–0.032; Table 2). Thus, we could speculate that these rare alleles may have remained undetected in *L. distichum*. The differences in allele composition and frequency (e.g. the two species have different major alleles at the *Fe* locus, and five additional alleles in each species are taxon-specific) might be, instead, attributable to interspecific divergence between

Table 2. Allele frequencies of 12 and 11 polymorphic loci for *Lilium distichum* and *L. tsingtauense* in Korea, respectively. Frequencies indicating most common alleles at each locus are set in boldface, diagnostic alleles are set in italics, and species-specific alleles are underlined.

Locus	Allele	*L. distichum*	*L. tsingtauense*
Adh	*b*	**0.986**	**0.995**
	c	0.014	0.005
Dia-1	*a*	0.012	0.001
	b	**0.982**	**0.988**
	c	0.006	0.007
	d	0	0.005
Dia-2	*a*	0	0.002
	b	**0.949**	**0.976**
	c	0.051	0.021
Fe	*a*	0.035	0
	b	*0.700*	0
	c	0.249	0.077
	d	0.016	0.026
	e	0	*0.897*
Idh	*a*	0.218	0.035
	b	**0.761**	**0.938**
	d	0.020	0.027
Mdh-1	*a*	0.055	0.026
	b	**0.945**	**0.974**
Mdh-2	*b*	**0.941**	**0.936**
	c	0	0.032
	d	0.059	0.033
6Pgd-1	*a*	0.002	0
	b	0.190	0
	c	**0.808**	**1**
6Pgd-2	*a*	0.067	0.019
	b	**0.869**	**0.881**
	c	0.063	0.099
Pgi-2	*b*	0.163	0.067
	c	**0.818**	**0.827**
	d	0.018	0.096
	e	0	0.010
Pgm	*a*	0.059	0
	b	0.227	0.164
	c	**0.665**	**0.694**
	d	0.049	0.142
Tpi-1	*a*	0	0.007
	b	**0.814**	**0.973**
	c	0.171	0.020
	d	0.014	0

sister species (that is, some of the observed isozyme diversity might have arisen — or have been lost — since they diverged). Anyway, such numbers of unique alleles are not uncommon in recently-derived species (0–8 in the 11 P–D species pairs compiled by Pleasants and Wendel 1989). Third, mean interspecific genetic identity for the pair *L. distichum–L. tsingtauense* (I = 0.919; Table 3) is only slightly lower than the mean intraspecific (i.e. inter-population) identities (I = 0.971 for *L. distichum* and I = 0.990 for *L. tsingtauense*; Table 3), and almost the same as that (I = 0.915) averaged from 11 progenitor-derivative P–D species pairs compiled by Pleasants and Wendel (1989).

The value of genetic identity for the pair *L. distichum–L. tsingtauense* is much higher than those estimated for pairs of six species of *Lilium* [*L. distichum*, *L. tsingtauense*, *L. cernuum*, *L. amabile*, *L. callosum*, and *L. lancifolium* (= *L. tigrinum*)] studied by our research group (n = 14, I = 0.436 to 0.704 with a mean of 0.575; Table 3). Also notably, the mean I (0.919) between *L. distichum* and *L. tsingtauense* is slightly higher than that for *L. longiflorum–L. formosanum* (I = 0.816; Hiramatsu *et al*. 2001; *see* below), which is a well-known P–D species pair within East Asia.

The distribution patterns of *L. distichum* and *L. tsingtauense* also suggest that they may constitute a P–D species pair. Although *L. tsingtauense* occurs mainly in forests in low mountains and *L. distichum* is usually found at high elevations, these species grow together in some locations at mid-elevation mountains in South Korea (Lee *et al*. 2011; M.Y. Chung and M.G. Chung pers. obs.). Also, *L. distichum* has a much wider geographic range than *L. tsingtauense*. In general, P species are more widely distributed than their D species (with D species occurring in small areas at the edges of widely distributed species), a common feature shown by many P–D species pairs (Crawford 2010). Despite the sympatric distribution of *L. distichum* and *L. tsingtauense* in the central ranges of the Baekdudaegan (Fig. 1), no hybridization events have been reported between them; thus, their high allele similarity should not be attributed to introgression and/or hybridization.

Our genetic results for the *L. distichum–L. tsingtauense* pair are similar to those for *L. longiflorum–L. formosanum*, the only P–D species pair detected to date within the genus (Hiramatsu *et al*. 2001). *Lilium longiflorum* (P species) and *L. formosanum* (D species) have been classified into the section *Leucolirion* (Comber 1949), and a series of molecular phylogenetic studies have revealed a very close relationship between them (Nishikawa *et al*. 1999, Hayashi & Kawano 2000, Lee *et al*. 2011). In addition, *L. longiflorum* has a much wider geographic range than *L. formosanum*. The former is distributed from the northernmost islands of the Ryukyu Archipelago to the northern tip of Taiwan (and to small islands in the eastern part of Taiwan), whereas the latter is restricted to main-

Table 3. Average Nei's (1978) genetic identity values, standard deviations and ranges (in parentheses) for all intraspecific (along the diagonal) and interspecific (above the diagonal) population pairwise comparisons.

	L. distichum	*L. tsingtauense*	*L. cernuum*[a]	*L. amabile*[a]	*L. callosum*[b]	*L. lancifolium*[b]
L. distichum	0.971 ± 0.014 (0.946–0.998)	0.919 ± 0.017 (0.876–0.943)	0.625 ± 0.031 (0.548–0.673)	0.704 ± 0.028 (0.669–0.774)	0.584 ± 0.016 (0.559–0.610)	0.595 ± 0.019 (0.571–0.622)
L. tsingtauense		0.990 ± 0.005 (0.977–1.000)	0.626 ± 0.027 (0.573–0.682)	0.671 ± 0.013 (0.637–0.698)	0.529 ± 0.009 (0.510–0.542)	0.534 ± 0.008 (0.533–0.541)
L. cernuum[a]			0.973 ± 0.014 (0.942–0.993)	0.505 ± 0.030 (0.454–0.564)	0.494 ± 0.022 (0.459–0.538)	0.647 ± 0.024 (0.613–0.671)
L. amabile[a]				0.999 ± 0.001 (0.997–1.000)	0.657 ± 0.005 (0.651–0.667)	0.440 ± 0.007 (0.433–0.451)
L. callosum[b]					1.000	0.436
L. lancifolium[b]						1.000

[a] Values for eight populations from Chung *et al.* (2014b).
[b] Value from one population from M.Y. Chung *et al.* (unpubl. data).

land Taiwan (Hiramatsu *et al*. 2001). As seen in many P–D species pairs, *L. formosanum* possesses a subset (85%) of *L. longiflorum* alleles for 11 polymorphic loci. In addition, *L. formosanum* exhibits lower allozyme variability than *L. longiflorum* (at the population level, %P = 48.2 *vs*. 31.7, A = 1.72 *vs*. 1.43, and H_e = 0.187 *vs*. 0.121; at the species level, %P = 100 *vs*. 76.9, A = 3.46 *vs*. 2.46, and H_e = 0.312 *vs*. 0.142; Hiramatsu *et al*. 2001). Similar to *L. tsingtauense*, the recently derived species *L. formosanum* harbors a small number of species-specific alleles (three).

However, the mean I value (0.816) between *L. formosanum* and *L. longiflorum* is lower than that between *L. distichum* and *L. tsingtauense* (I = 0.919), which can be attributed to several causes. These are (i) a longer divergence between *L. longiflorum* and *L. formosanum* compared with that of the *L. distichum–L. tsingtauense* pair, or (ii) the fewer opportunities for gene flow for the pair *L. longiflorum–L. formosanum* given that they occur on islands. The mean intraspecific genetic identity for *L. longiflorum* (I = 0.850; Hiramatsu *et al*. 2001), whose populations occur on isolated islands along over 1300 km, is even lower than that for the pair *L. distichum–L. tsingtauense* (I = 0.919).

The present study suggests that *L. tsingtauense* was probably (recently) derived from southern Korean populations of *L. distichum* when it migrated from more northern areas during one of the cold phases of the Pleistocene (Im 1992, Kong & Watts 1993). Based on the study of Guo *et al*. (2011) with ISSR (which revealed higher genetic variability for the South Korean populations compared to the Chinese populations of Shandong), one can hypothesize that *L. tsingtauense* expanded to China from Korea through the Yellow/East China Sea shelf, which was periodically exposed at each glacial period (and during the LGM, until ca. 16000 years BP; Chung 2007). This hypothesis could be tested by employing molecular phylogeography approaches and by sampling the whole region.

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