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Strong genetic differentiation of the East-Himalayan *Megacodon stylophorus* (Gentianaceae) detected by Inter-Simple Sequence Repeats (ISSR)

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Abstract. *Megacodon stylophorus* (Clarke) Smith is a perennial alpine herb endemic to the species-rich eastern Himalayan region. Its populations are locally scattered as isolated patches throughout this region. Genetic variation within and among six populations of this species was assessed using ISSR fingerprinting with 13 primers. High levels of genetic diversity exist within species ($P = 69.83\%$, $H_T = 0.1949$ and $H_{sp} = 0.3047$), while the within-population diversity is low ($P = 11.21\%$, $H_E = 0.0532$ and $H_{pop} = 0.0792$). Extraordinarily high levels of genetic differentiation were detected among populations based on various statistics, including Nei's genetic diversity analysis (72.7%), Shannon's diversity index (74.01%) and AMOVA (80.70%). That is, populations shared low levels of genetic identity ($I = 0.8203 \pm 0.0430$). This genetic structure was probably due to severe genetic drift of the small-sized patchy populations resulting from postglacial habitat fragmentations. The observed genetic structure of the populations implies that as many populations as possible should be considered for any *in situ* and *ex situ* conservation practice on this species.

Introduction

The Eastern Himalaya is one of the 25 global (one of the two in the northern hemisphere) biodiversity 'hotspots' (Myers 1988; Wilson 1992; Myers et al. 2000). The area comprises Nepal, Bhutan, and neighbouring states of northern India along a continuous sector of the southeast Tibet, southwest Sichuan, and northwest Yunnan provinces of China. This region is the meeting ground of the Indo-Malayan, Indo-Chinese, and eastern Asian biogeographical realms following the formation of the Himalayas, which resulted from the collision of the India plate with the Laurasia landmass (Behera et al. 2002). Studies have shown that this region was a centre of active speciation and a refugium for various flowering plants during glacial maxima (Takhtajan 1969; Rao 1994). A conservative estimate suggests that over 3000 dicot and 1000 monocot species are endemic to the Himalayas (Mani 1978). Being a part of the eastern Himalayan area, Hengduan Mountains lie at the eastern end of this area and extend from west Sichuan and north Yunnan provinces to east Tibet. The Hengduan Mts. comprise a series of spectacular north–south trending ridges along four major rivers of Asia, that is, Brahmaputra, Salween,

Mekong, and Changjiang (Yangtze) Rivers, and coincide in large part with the eastern Himalayan biodiversity hotspot. The biota of the Hengduan Mts. is renowned for its typical examples of phyletic radiations and concomitant high levels of endemism as well as species and generic richness (Raven and Axelrod 1978; Wilson 1992; Ying et al. 1993). This region was a centre of species diversification during the Tertiary and a refugium for many Laurasian angiosperms, such as *Rhododendron*, *Primula*, and *Gentiana* (Wu 1988). The extraordinarily high diversity appears to be a function of the extreme topographic variation and complexities with deep valleys coupled with recently uplifted mountains. However, despite the richness, the species diversity in this region is facing serious environmental threats due to human over exploitation, including forest destruction and subsequent soil erosion. The high potential instability and inherent vulnerability of mountain ecosystems render the Himalaya region one of the ecologically fragile biogeographic zones (Rodges and Panwar 1988). Loss of habitat by deforestation and excessive grazing pressure in high altitude pastures now threaten the survival of endemic and rare plants in the Hengduan Mts. (Kala 2000; Rai et al. 2000). It is urgent to initiate conservation of the endemic plants in this region.

Genetic diversity, species richness and assemblages are the three main levels for conservation of biodiversity (Secretariat of the Convention on Biological Diversity 2001). Levels of genetic variation within and among natural populations provide fundamental information on the evolutionary processes that bring about divergence within species and lead to speciation ultimately (Krauss 1997) and for the establishment of effective and efficient conservation practices (Hamrick and Godt 1996). Due to the complicated topography of the areas and the low accessibility, biodiversity studies in this region are limited and mostly focus on taxonomic treatment and botanical inventory. No study has been conducted on genetic structure of the endemic species from this region. A genetic study at the population level for an endemic genus will provide insights into both historical processes and ongoing evolutionary mechanisms in maintaining the extraordinarily high biodiversity in this region.

Megacodon is a genus of the Gentianaceae. It is a relatively primitive element within the tribe *Gentianeae* (Yuan and Küpfer 1995; Chassot et al. 2001), and consists of only two species. While *M. venosus* is limited to a small area in central China (Chongqing and western Hubei), *M. stylophorus* is endemic to the eastern Himalayan regions ranging from northwest Yunnan and southwest Sichuan to southwest Tibet in China, extending into Sikkim, Bhutan, Nepal, and northeast India. Plants usually grow in montane habitats beside streams, shrubs or forest margins at an altitude of 3000–4400 m. Although this species is locally abundant, its populations are scattered as isolated patches across mountains throughout this region. With pendant and campanulate corolla, the height of the perennial herb can be more than 100 cm. Its flowers are large and pale yellow-green, ca. 5 cm in diameter with a short (ca. 1 cm long) corolla tube. Seeds are oblong, 3–4 mm long and 2.2–2.5 mm in diameter, with rugate seedcoat. The plant flowers in June and July, and sets fruit from July to September (Ho and Pringle 1995). Except for its taxonomic and phylogenetic relationships, this species has been so far poorly

studied. Nothing is known about its breeding system, population genetic structure, or intraspecific differentiation across its distribution.

Among the various molecular methods for detecting genetic diversity, a technique amplifying inter-simple sequence repeats (ISSR) is a powerful tool for investigating genetic variation within species (Gupta et al. 1994; Zietkiewicz et al. 1994; Wolfe and Liston 1998). Recent ISSR studies of natural populations have demonstrated a hypervariable nature of the markers and their potential use for population-level studies (Culley and Wolfe 2001). Technically, the ISSR reaction is more specific than RAPD amplification due to the longer SSR-based primers, thus enabling higher-stringency DNA amplifications (Wolfe et al. 1998). The high-stringency results in very few problems with reproducibility, a common criticism against the low-stringency RAPD assay (Yang et al. 1996). Limitations of the ISSR technique, as with RAPDs, are that bands are scored as dominant markers and that genetic diversity estimates are based on diallelic characters.

As the primary step in investigating the genetic diversity of *M. stylophorus*, the present study addresses the following questions by using the ISSR molecular markers: (i) What are the levels of ISSR variation in populations of *M. stylophorus*? (ii) What is the degree of among-population differentiation in this species? (iii) How does this information relate to that measured in other species which have the same patterns of geographical distribution? (iv) What are the applications of the population genetic information for an effective and efficient conservation of this species?

Material and methods

Plant material

Leaves of 136 individuals of *M. stylophorus* were collected from six populations in southwest China (Figure 1, Table 1). Individuals 5–10 m apart from one another were sampled randomly. The leaves were dried with silica-gel. The sampled populations represent the major mountains (with altitudes all over 4000 m a.s.l.) that are isolated by either surrounding relatively low plateaus (altitude around 2000 m) or the major valleys in southwest Sichuan and northwest Yunnan provinces of China. These populations grow in the alpine forest or bushes of mixed *Abies, Picea*, *Rhododendron, Sorbus*, etc. between 3300 and 4000 m a.s.l. Their habitat represents the richly endowed yet rather stable primitive environment in the sampled regions.

DNA extraction and PCR amplification

Genomic DNA was extracted from 0.5 to 1.0 g of dried leaf tissue using the CTAB method described by Doyle (1991). DNA extractions were quantified by comparing band intensities with known standards of lambda DNA on 1% (w/v) agarose gels. In a preliminary study, 100 primers (Biotechnology Laboratory, University of

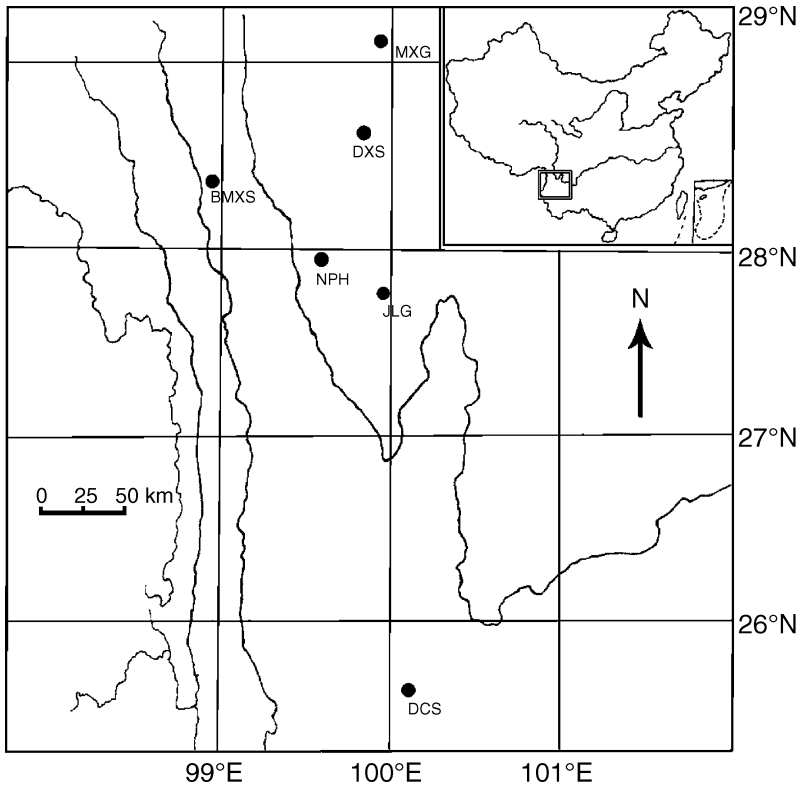

Figure 1. Map showing the locations of the populations of *M. stylophorus* sampled.

Table 1. Sites of the populations surveyed.

Population	Pop. code	Location	Latitude (N)	Longitude (E)	Elevation (m)	Sample size (*N*)
Northwest Yunnan						
Bai-Mang-Xue-Shan	BMXS	Deqen	28°23′	98°58′	3800	22
Dian-Cang-Shan	DCS	Dali	25°40′	100°07′	3900	23
Ji-Li-Gu	JLG	Zhongdian	27°44′	99°58′	3300	23
Na-Pa-Hai	NPH	Zhongdian	27°53′	99°38′	3650	21
Southwest Sichuan						
Da-Xue-Shan	DXS	Xiangcheng	28°38′	99°50′	4000	23
Ma-Xiong-Gou	MXG	Xiangcheng	29°09′	99°55′	3850	24

British Columbia, primer set # 9, Vancouver, BC, Canada: http://www.biotech.ubc.ca/services/naps/primers/Primers.pdf) were screened for PCR amplification. Thirteen of these ISSR primers (UBC # 807, 808, 810, 811, 827, 830, 836, 842,

846, 857, 859, 864 and 888) that gave clear, reproducible banding patterns were chosen for final analysis. Polymerase chain reactions (PCR) were carried out in a volume of 20 μL consisting of ca. 20 ng of template DNA, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 2% formamide, 0.2 μM primer, 1.5 units of *Taq* polymerase (Shanghai Sangon Biological Engineering Technology and Service Co. Ltd., Shanghai, China) and double distilled water on a MJ Research 96-well thermal cycler with hot bonnet following the conditions of Ge and Sun (1999). The amplification products were separated via electrophoresis on 2.0% (w/v) agarose gels (migration distance: 10 cm) with 0.5 × TBE buffer and visualized using ethidium bromide staining (0.1 μg/mL). The amplified DNA fragments were documented by using image analysis software *LabWorks Software* Version 3.0 (UVP, Upland, CA 91786, USA). Negative controls, lacking template DNA, were included in each PCR set to test for the possibility of contamination.

Data analysis

Only bands that could be unambiguously scored were used in the analysis. ISSR profiles were scored for each individual as present (1) or absent (0) of a specific band. A set of measures of intra- and inter-population genetic statistics were generated using the program POPGENE 1.31 (Yeh et al. 1999), including Nei's (1973) gene diversity, the percentage of polymorphic loci (*P*), expected heterozygosity (H_E), total genetic diversity (H_T), genetic diversity within population (H_S), genetic diversity between populations (D_{ST}) and the relative magnitude of genetic differentiation among populations ($G_{ST} = D_{ST}/H_T$). Based on the island model, gene flow was inferred indirectly using Wright's (1931) formula: $N_m = 0.25(1 - F_{ST})/F_{ST}$. Nei's (1972) genetic identity (*I*) and genetic distance (*D*) were calculated for all pairwise combinations of populations.

Shannon's index was also employed to characterize the gene diversity and distribution of the variation. Shannon's index of gene diversity was calculated as $H_o = -\Sigma\, p_i \log_2 p_i$ (Lewontin 1972), in which p_i is the frequency of a given ISSR fragment. H_o was calculated at two levels: the average diversity within populations (H_{pop}), and the total diversity (H_{sp}). The proportion of diversity among populations was estimated as $(H_{sp} - H_{pop})/H_{sp}$.

In addition, an analysis of molecular variance (AMOVA) was applied to estimate variance components for ISSR phenotypes, partitioning the variation among populations and among individuals. Input data files for the AMOVA v. 1.55 program (Excoffier et al. 1992) were generated using AMOVA-PREP (Miller 1998). The variance components were tested statistically by nonparametric randomization tests using 1000 permutations.

A UPGMA (unweighted pair-group method using arithmetic average) dendrogram was constructed based on the matrix of Nei's genetic distance using the SAHN – clustering and TREE programs from NTSYS-pc 2.0 (Rohlf 1998). In order to test for a correlation between genetic (*D*) and geographical distances (in km) among

Table 2. Genetic variation in populations of *M. stylophorus* shown by ISSR. *P*, percentage of polymorphic loci; H_E, expected heterozygosity. H_{pop}: Shannon's diversity index.

Population	*P* (%)	H_E	H_{pop}
DCS	16.38	0.0649	0.0957
JLG	14.66	0.0517	0.0774
NPH	11.21	0.0420	0.0628
BMXS	18.10	0.0688	0.1011
DXS	10.34	0.0372	0.0562
MXG	14.66	0.0548	0.0819
Mean	14.23	0.0532	0.0792

Table 3. AMOVA on 136 individuals of 6 populations of *M. stylophorus* using 116 ISSR markers.

Source of variation	d.f.	SSD	MSD	Variance component	% total variance	*p*-value[a]
Among populations	5	1019.4801	203.896	8.90	80.70	<0.001
Within populations	130	276.7699	2.129	2.13	19.30	<0.001

d.f.: Degrees of freedom; SSD: Sum of squares; MSD: Mean squared deviation. [a]Significance tests after 1000 permutation.

populations, a Mantel test was performed using Tools for Population Genetic Analysis (TFPGA; Miller 1997) (computing 1000 permutations).

Result

Genetic diversity

In this study, we scored totally 116 unambiguous and reproducible electrophoretic bands (loci), among them 81 (69.83%) were polymorphic. The percentages of polymorphic loci (*P*) for a single population ranged from 10.34% (Da-Xue-Shan) to 18.10% (Bai-Mang-Xue-Shan) with an average of $14.23 \pm 2.97\%$. The average genetic diversity was estimated to be 0.0532 at population level (H_E) and 0.1949 at the species level (H_T). The Shannon's indices (H_o) were 0.0792 at the population level (H_{pop}), and 0.3047 at the species level (H_{sp}), respectively (Table 2).

Of the total 116 bands scored, 51% were found in more than 90% of the sampled individuals, 4% of the bands were found in less than 30% of the individuals, 17% of the bands in 30–69% individuals, and 28% of the bands in 70–89% individuals. Apparently, the ISSR divergence among populations of *M. stylophorus* was mainly due to frequency differences among the populations rather than local fixation of ISSR fragments in a specific population.

Table 4. Nei's (1972) genetic distance and geographical distance among the populations of *M. stylophorus* (upper right: geographical distance in kilometers; Lower left: Nei's genetic distance).

Pop ID	DCS	JLG	NPH	BMXS	DXS	MXG
DCS	–	228	250	328	330	386
JLG	0.2589	–	37	121	102	158
NPH	0.2846	0.1927	–	85	86	144
BMXS	0.1901	0.1929	0.2203	–	89	126
DXS	0.2665	0.1105	0.1762	0.2116	–	57
MXG	0.2352	0.1424	0.1496	0.2343	0.1250	–

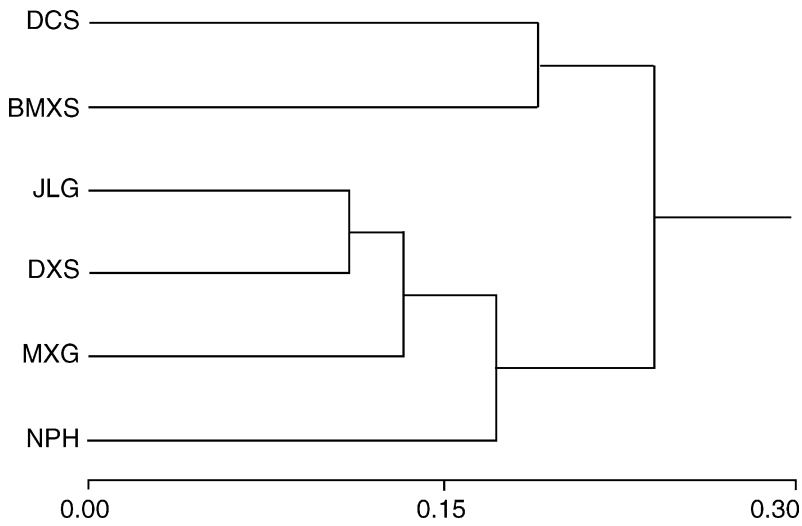

Figure 2. UPGMA dendrogram based on Nei's (1972) genetic distance.

Genetic structure

Most of the total gene diversity (H_T) in *M. stylophorus* was distributed among populations (D_{ST}). G_{ST} was estimated as 0.727, indicating that 72.7% of the genetic variability was distributed among populations, or as 74.01% based on the Shannon's index. Nei's genetic identities (I) among pairs of populations also suggested a high level of genetic differentiation among populations. The average genetic identity was 0.8203 ± 0.0430 (ranging between 0.7523 and 0.8954). The overall level of inferred gene flow among populations was extremely low ($N_m = 0.0939$), suggesting limited pollen and seed dispersal between populations.

Significant genetic differences ($P < 0.001$) among populations were detected based on the AMOVA (Table 3). Of the total molecular variance, 80.70% was attributable to among-populations diversity and the rest (19.30%) to differences

within populations. The result of a Mantel test with 1000 permutations revealed that the genetic divergence of populations was not significantly correlated with geographic distance (Mantel test, $r = 0.5307$; $P = 0.1460$) (Table 4). That is, an 'isolation by distance' model was not supported. The UPGMA tree (Figure 2) based on Nei's (1972) genetic distance revealed a similar pattern that the genetic distances among the populations do not show a spatial pattern corresponding to their geographic locations (Figure 1). For example, the geographically distant Dian-Cang-Shan and Bai-Mang-Xue-Shan populations show a closer genetic distance to each other (Figure 2).

Discussion

Genetic variation of ISSR in M. stylophorus and its possible causes

Analyses of the ISSR markers using various statistics (Nei's genetic diversity analysis, Shannon's diversity measure and AMOVA) all revealed similar patterns of genetic structure of populations of *M. stylophorus*: low genetic variation within populations, and remarkable genetic differentiation among populations. While the ISSR markers were shown to be powerful for investigating genetic variations within species (e.g., Gupta et al. 1994; Zietkiewicz et al. 1994; Wolfe and Liston 1998; Culley and Wolfe 2001), they may give biased estimations on genetic structure of a species due to a possible uneven distribution of the binding sites of the ISSR primers in a genome, such as revealed in *Arabidopsis thaliana* (Barth et al. 2002). Such a biased inference of ISSR markers in *M. stylophorus* is yet to be confirmed by using different markers. Nevertheless, similar pattern of genetic variation was found in other rare gentians such as *Gentiana pneumonanthe* L. in the Gentianaceae family by applying different markers (Raijmann et al. 1994; Oostermeijer et al. 1998).

Megacodon stylophorus is a perennial herb with patchy distribution. The populations are often located in distant mountains, and are strongly isolated from each other by plateaus or valleys. To date there have been no comprehensive studies on its pollination biology and seed dispersal. Our field trips sporadically encountered bumblebees visiting its flowers, indicating the bumblebee as a potential pollinator. Seeds are released when the capsules are dry and dehiscent. Large amounts of seeds are often produced from a single plant, and are numerous from each capsule. Seeds are small sized (3–4 mm long and 2–3 mm in diameter) and relatively light (ca. 1.5 g/1000 pcs when dried). The seedcoat is slightly rugate. Without any apparent epizoic adaptations, its seeds are probably dispersed over short distances via gravity. Based on its regional distribution and considerations on dispersal of its pollen and seeds, high levels of diversity and differentiation were expected. At the species level, *M. stylophorus* maintains relatively high levels of genetic variation compared to other species with a scattered distribution (H_E: 0.150, based on allozyme data; Hamrick and Godt 1989) but lower than widespread species (H_E: 0.202). However, at the population level, *M. stylophorus* has a lower level of genetic variation than regionally (H_E: 0.118) and narrowly distributed species

(H_E: 0.105), but is close to endemic species (H_E: 0.063). A low level of polymorphism was detected in all the six sampled populations of *M. stylophorus*, ranging from 10.34 to 18.10% (Table 2).

The genetic structure of plant populations reflects the interactions of various evolutionary processes including the long-term evolutionary history, such as shifts in distribution, habitat fragmentation, and population isolation, mutation, genetic drift, mating system, gene flow, and selection (Schaal et al. 1998). A high level of population differentiation may be explained by several factors, including the species' breeding system, genetic drift or genetic isolation of populations (Hogbin and Peakall 1999).

The scattered distribution of this species throughout the huge eastern Himalayas implies such complicated evolutionary processes. Other molecular phylogenetic studies suggested that *Megacodon* is a basal genus within the tribe Gentianeae of the family Gentianaceae (Yuan and Küpfer 1995; Chassot et al. 2001; von Hagen and Kadereit 2002). The divergence of *Megacodon* and its sister groups was dated to the Miocene based on a molecular clock, about 15 million years before present (von Hagen and Kadereit 2002). Considering the large distribution range, *M. stylophorus* might have been widespread before the Quaternary glaciation, and the present patchy distribution of the populations was probably the results of historical fragmentation due to mountain glaciation and subsequent climatic oscillation. As a matter of fact, well-developed mountain glaciers are still active in this region; for example, the Mingyong glacier in Deqen runs down to 2900 m a.s.l. into forests. Past fragmentation leading to geographical isolation and limited pollen/seed dispersal between relict populations resulted in extraordinarily high diversity among populations and low diversity within populations in this species. Several consequences of the population structure of this relict species after past fragmentation can be examined.

First, *M. stylophorus* populations in the Hengduan Mts. should have experienced severe extinction and recolonization processes following fragmentation/vicariance events. Frequent extinctions and recolonizations often cause low levels of genetic diversity within species (Qiu and Parks 1994). These are usually linked to changing climatic conditions associated with glacial/interglacial periods during the Pleistocene. Hengduan Mts. consist of a range of south–north running mountains and deep valleys. In response to the cooling–warming cycles of the climate, *M. stylophorus* populations may have experienced frequent retreat/expansion along the altitude and latitude gradient of the mountains. During the harsh period, *M. stylophorus* populations retreated downward and southward, and survived in refugia. During favourable periods, they expanded upward and northward. Repeated northward and upward re-colonization of small populations from the valley refugia may account for the loss of alleles in the extant alpine populations. In addition, colonizing populations are more susceptible to founder events and bottlenecks due to fluctuations in the effective breeding population (Nei et al. 1975). Founder events during postglacial re-colonizations and/or bottlenecks may also contributed to the low levels of genetic diversity within populations and high genetic differentiation among patchy populations of *M. stylophorus*. Similar patterns were found in other plants. Bauert et al. (1998) detected no genetic variation within and among three

isolated relict populations of *Saxifraga cernua*, but considerable differences were found among regions.

Second, due to the fragmentation and the subsequent climatic fluctuation in the ice age, the population sizes of *M. stylophorus* may have been remarkably reduced. These small-sized populations were likely subjected to strong genetic drift, especially after a long-time isolation from one another. Genetic drift changes the distribution of genetic variation in two ways: (i) decrease of variation within populations, and (ii) increase of differentiation among populations (Ellstrand and Elam 1993). Populations with continually small effective population sizes are especially susceptible to the loss and reorganization of variation by genetic drift. Genetic drift could particularly result in the loss of low-frequency alleles in populations. In *M. stylophorus*, we observed only five electrophoretic bands (4%) with a frequency lower than 30%, suggesting a possible stochastic process of genetic drift. The Mantel test and the UPGMA dendrogram further clarified that the genetic differentiation does not show any spatial pattern and there is no significant correlation between genetic distance and geographic distance, providing further evidence of the action of genetic drift (Dodd and Helenurm 2002).

Third, the identities of long isolated populations have been maintained by limited between-population gene flow. In this study, the patchy habitats of *M. stylophorus* and the specific topology of the region, mountains separated by deep valleys or intermountain plateaus, have reduced gene flow among populations. Although no explicit study has been done on seed dispersal, the morphology of seeds suggests they are mostly dispersed via gravity, and thus are constrained within short distance. The pollination biology of this species is also unknown. Effective long-distance pollen movement among populations has yet to be confirmed. The observed diversity pattern suggests that gene flow among the populations is limited. The pollen and/or seed-mediated gene flow among populations is probably too low to alleviate genetic drift within populations. Similar situation was also revealed in the rare perennial *Gentiana pneumonanthe*: the isolation of its populations in nature reserves resulted in relatively high levels of genetic differentiation among populations (Raijmann et al. 1994).

Fourth, genetic structure of a species is dramatically influenced by its breeding system, and selfing can result in low genetic diversity within populations. According to Hamrick et al. (1991), selfing species can have five folds more genetic diversity among populations and only half less diversity within populations than a wind-pollinated outcrossing species. As mentioned, the breeding system of *M. stylophorus* is not known, and possible selfing due to limitation of the pollinators is yet to be confirmed. Therefore, we cannot rule out the possibility that the observed low within-population diversity was due to a certain degree of selfing.

Implication of the genetic information for conservation

Habitat-restricted species, occurring in isolated populations, usually tend to be genetically homogeneous at population level, as shown in this study, in *Magnolia*

sieboldii ssp. *japonica* (Kikuchi and Isagi 2002), and the Hawaiian *Brighamia insignis* (Gemmill et al. 1998). These findings have important implications for conservation of biodiversity. Fragmentation of populations and subsequent genetic drift certainly occur naturally. The natural-history related fragmentation and the induced genetic drift of relict and ancient taxa calls attention for measurement of interpopulational genetic diversity when considering diversity conservation in an area of biodiversity hotspots. Maintenance of a limited number of populations is in such cases not sufficient to preserve the major diversity at the species level. To conserve the genetic diversity of *M. stylophorus,* it is necessary to preserve as many populations as possible in the wild. Given that most populations are genetically unique, loss of any population will lead to dramatic loss of genetic variation. Concerning *ex situ* conservation in gardens, introduction should be performed to also include as many populations as possible. Further studies on breeding system and pollination biology of the species are urgently needed to better understand the genetic structure and perform effective conservation of the species.

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