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ORIGINAL ARTICLE

High RAPD but no cpDNA sequence variation in the endemic and endangered plant, *Heptacodium miconioides* Rehd. (Caprifoliaceae)

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Abstract *Heptacodium miconioides* Rehd. is an endangered species endemic to China and has suffered rapid decrease of distribution range and population size. This species has been disappeared in central China where the modal specimen was collected. We analyzed the genetic variation of the remaining populations to reveal whether the genetic diversity also suffered decrease and to provide some suggestions for conservation. All the nine known remaining populations were sampled. Genetic variation was analyzed based on RAPD markers and two fragments of cpDNA sequence, intergenic spacers of *pet*G-*trn*P and *trn*S-*trn*G. No variation was observed in the two fragments of cpDNA sequence. However, the species exhibited high level of RAPD variation compared to other threatened or rare plants. Measures of genetic diversity within populations were strongly related to the log of estimated population size, indicating that large populations usually have more genetic diversity than that of small ones. About 25% of the variation was partitioned among populations. Significant relationship was observed between differentiation and geographical distance, indicating a pattern of isolation-by-distance. Given for few populations remaining, all the populations should be protected and urgent efforts be paid on the small populations to avoid their local extinction.

Keywords Genetic diversity · Genetic differentiation · *Heptacodium miconioides* · RAPD markers · Chloroplast DNA

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Introduction

Heptacodium is a monotypic genus endemic to China. However, its taxonomic position is problematic (Tang and Li 1994). Recent molecular evidence suggests that it is the sister group of Caprifoliaceae (Pyck and Smets 2000; Bell et al. 2001), but with limited confidence (Bell et al. 2001). The sole species of the genus, *H. miconioides*, locally named seven-son-flower, was listed as nationally endangered in China (Fu and Jin 1992). This small-sized deciduous tree previously occurred in central and eastern China. Due to human's destruction, its distribution range has been decreased at an alarming speed. This species was first described according to the specimen collected from Xingshan County of Hubei Province, locating in central China. However, no wild individual has been observed again in central China in recent decades. About nine natural populations were remained in remote mountains of Zhejiang Province and Anhui Province, but they all still suffered rapid decrease in population size (Li et al. 2004). Until now, three of them are located in natural reserves, and among them one was established aiming to protect *H. miconioides.*

Throughout its present range, *H. miconioides* is found in rocky valleys of remote mountains. It occurs in deciduous forests characterized by *Lindera rubronervia*, *Alangium chinense*, *Fraxinus relusa*, *Albizia kalkora*, some sites

having evergreen species, such as *Cyclobalanopsis glauca*, *C. gracilis* (Jin 1996). *H. miconioides* is an insect-pollinated species. It has a low level of seed set (Bian et al. 2002), and low seed germination, limiting its dispersal and sexual regeneration, but its ability of coppicing from older stems is strong, which might play a key role in population persistence. For example, though there are only about 400 individuals remaining—and simultaneously facing the threat of destruction, such as cutting—*H. miconioides* in Dapan Mountain of Zhejiang Province may regenerate naturally and persist for long-term via coppicing (Li et al. 2004).

It is widely appreciated that understanding patterns of genetic structure is of critical importance to the conservation of threatened species (Ellstrand and Elam 1993; O'Brien 1994; Alvarez-Buylla et al. 1996; Chen 2000). However, no information on genetic variation of this species was available. The purpose of this study was to investigate the genetic diversity that present within and among populations of seven-son-flower using RAPDs and cpDNA sequences.

Although RAPDs have some limitations, such as dominant allelic expression, occasionally low reproducibility, they have advantages in investigating genetic variation, such as random sampling in the whole genome, high levels of polymorphism, and easy performance (Sun and Wong 2001; Nybom 2004). Therefore, if using reproducible loci, RAPDs can provide valuable information in genetic variation. CpDNA is maternally inherited in most plant species (Mogensen 1996), and has been widely used to infer population history (Newton et al. 1999). The main objectives were to (1) assess the level of genetic variation in the species; (2) determine if there is a relationship between population size and the level of variation; and (3) provide suggestions for conservation.

Materials and methods

Plant materials

Heptacodium miconioides is a diploid ($2n = 28$), multi-stemmed, deciduous small tree. Leaves are shiny, ovate-oblong on top with entire margins and a long drip tip. They have 3 veins parallel to the margins. Flowers are followed in fall by an equally showy display: small, purplish-red fruits (1/2-inch-long drupes) crowned by five very showy, sepal-like rose calyces which elongate after bloom and last into late fall.

Based on recent surveys, 9 nature populations of *H. miconioides* were found in Zhejiang Province and Anhui Province. Population sizes were estimated from about 10 to several hundred (Table 1). Samples were collected from the 9 populations throughout the natural range of the species (Table 1 and Fig. 1). Leaf samples were obtained from a total of 241 trees. Trees were selected randomly for sampling within each medium to large population. For small populations, as possible as more trees were collected with a minimum distance of 2 m in order to avoid sampling genetically identical individuals. Five to 10 fresh leaves (depending on leaf size) were collected from each tree and placed into plastic sealable bags containing about 40 g of silica gel for rapid drying and preservation of leaves. Before silica gel-dried, fresh leaves were simply cleaned to decrease the pollution of fungi and bacteria.

DNA extraction

DNA was isolated with the CTAB extraction method modified from Doyle and Doyle (1987). 0.05 g dried leaves

Table 1 Location of sampling sites and sampling sizes

Localities	Abbr.	Latitude	Longitude	Altitude (m)	Estimated population size	Sampling size
Banqiao of Ningguo County, Anhui Province	BQ	30°33.35′ N	118°41.38′ E	860–1130	<20	11
Qingliangfeng of Lin'an City, Zhejiang Province	QL	30°16.09′ N	119°07.42′ E	700–1000	~80	34
Guanyinping of Lin' an City, Zhejiang Province	GY	30°12.57′ N	119°03.21′ E	650–950	~150	23
Dongbai Mountain of Dongyang City, Zhejiang Province	DB	29°28.70′ N	120°27.00′ E	800–825	~10	7
Beishan Mountain of Enhua City, Zhejiang Province	BS	29°12.54′ N	119°38.22′ E	800–880	~50	26
Dapan Mountain of Pan'an County, Zhejiang Province	DP	28°58.81′ N	120°31.88′ E	500–1070	~400	49
Siming Mountain of Fenhua County, Zhejiang Province	SM	29°45.47′ N	121°04.95′ E	500–750	~100	24
Kuocang Mountain of Linhai City, Zhejiang Province	KC	28°47.83′ N	120°54.43′ E	800–900	~150	25
Tiantai Mountain of Tiantai County, Zhejiang Province	TT	29°15.49′ N	121°05.82′ E	600–1000	~300	42

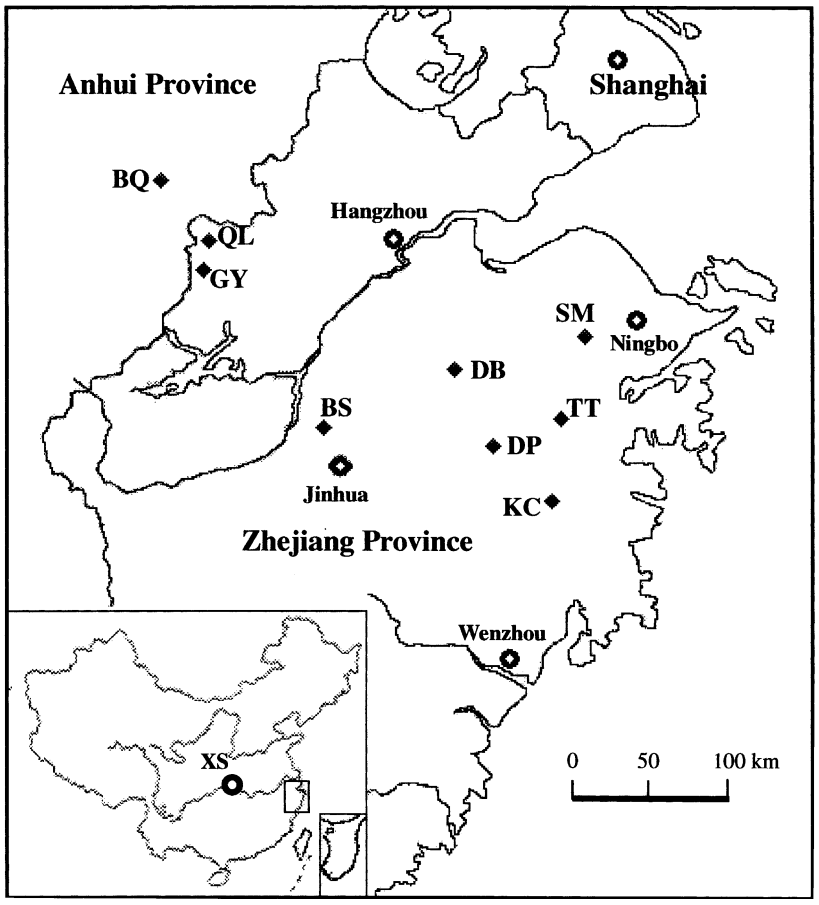

Fig. 1 Locations of the study populations of *H. miconioides*. BQ, Banqiao; QL, Qingliangfeng; GY, Guanyinping; DB, Dongbai Mountain; SM, Shiming Mountain; BS, Beishan Mountain; DP, Dapan Mountain; TT, Tiantai Mountain; KC, Kuocang Mountain. XS in the small figure indicates position of Xingshan County of Hubei Province, where the type specimen was collected but no naturally regenerated individuals were found again

were ground to powder with cold grinder, suspended it with 750 μl homogenate buffer (100 mmol/l Tris–HCl (pH 8.0), 20 mmol/l EDTA (pH 8.0), 0.3% β-mercaptoethanol) (V/V) and 3% PVP (W/V)), and then centrifugated at 8,000 rpm for 5 min at 4°C. After repeated, the sediments were suspended with 750 μl 65°C extraction buffer (100 mmol/l Tris–HCl (pH 8.0), 1.4 mol/l NaCl, 20 mmol/l EDTA (pH 8.0), 2% CTAB (W/V)) and incubated for 90 min. The mixture was treated with equal volumes of chloroform/isoamyl alcohol, followed by centrifugation to separate phases and removal of the aqueous layer. Isopropanol was added to the final aqueous extract and gently mixed to precipitate the DNA. The precipitate was then centrifuged to pellet DNA and the sediments were dissolved in TE buffer. After adding 2 μl 10 μg/ml RNaseA and incubating at 37°C for 30 min, the mixture was treated several times with 75 μl of chloroform/isoamyl alcohol, followed by centrifugating at 14,000 rpm under 4°C for 10 min. We added 1/10 volume of NaAc and 2.5 volumes of 100% ethanol to the aqueous layer, incubated for 30 min at −20°C and centrifugated at 12,000 rpm. The sediment was washed and precipitated with cold 70% ethanol and finally the DNA was dissolved in 50 μl TE buffer and stored at 4°C.

RAPD analysis

RAPD assays were performed using the conditions described in a previous paper (Fan et al. 2004). PCR reactions were carried out using an Engine DYAD^{TM} (MJ Research Inc., USA). Negative controls were included in each thermo-cycler run to check for contamination. To ensure reproducibility, two replicates were performed for each reaction. PCR amplification products were separated on 1.6% agarose gel in 0.5× TBE buffer and visualized by staining with ethidium bromide and photographed over UV-light with Gel Doc2000^{TM} system (Bio-Rad Laboratories, Australia).

A set of 150 random 10-mer primers was purchased from Sagon Inc., Shanghai. After screening, 10 primers that amplified clear, reproducible banding patterns were chosen for further studies. The presence of bands was scored by eye and Quantity One$^{@}$ system and only unequivocal bands were scored, with weak and spurious bands not being included. Unfortunately PCR amplification was unsuccessful for several individuals, or the bands were too weak for scoring. Therefore, these individuals were excluded from further analysis. In total, 75 reliable bands were found, and scored for 241 individuals.

Each PCR product was assumed to represent a single locus and was scored for presence and absence. Gene diversity (*H*) at population and at the species level were calculated based on Lynch and Milligan's Taylor expansion estimate (Lynch and Milligan 1994) using TFPGA (Tools for Population Genetic Analyses) version 1.3 (Miller 1997). Shannon diversity index (Lewontin 1972) was calculated to provide a relative estimate of the degree of variation within each population using Popgene 1.31 (Yeh et al. 1999).

Genetic variation within and among populations was analyzed using analysis of molecular variance (AMOVA) (Excoffier 1993). AMOVA-PREP version 1.01 (Miller 1998) was used to construct a data matrix for AMOVA version 1.55 (Excoffier 1993). Φ_{ST} statistics, analogous to Wright's F_{ST}, were generated. AMOVA is the least biased method for partitioning variation among and within populations for RAPD data. The significance level for Φ_{ST} was determined using 1,000 bootstrap replicates. A Mantel type matrix randomization test (Mantel 1967) was performed to evaluate the relationship between the Φ_{ST} value matrix and the matrix of geographic distances using TFPGA (Miller 1997).

CpDNA amplification and sequencing

Polymerase chain reaction amplification was achieved for *pet*G-*trn*P and *trn*S-*trn*G, using the primers proposed by Hamilton (1999). We sequenced these two fragments because they had been reported to be polymorphic in other plant species (Hamilton 1999; Huang et al. 2002). The PCR mixture (50 µl) contained 2.5 mM $MgCl_2$, 1 PCR reaction buffer, 200 µM dNTPs, 0.2 µM primer, 15 ng template DNA and 2 U *Taq* polymerase. Amplifications were performed with an initial denaturing of 5 min at 96°C followed by 35 cycles of 45 s at 96°C, 60 s at 54°C (*pet*G-*trn*P) or 60°C (*trn*S-*trn*G), 90 s at 72°C and ending with a 5-min extension at 72°C. PCR products were purified and sequenced in both directions using Taq Dye Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and Model ABI377 automated sequencer (Applied Biosystems).

Results

The 10 primers used for analysis were assumed to be a random sample of the genome and generated a total of 75 bands, among which polymorphic bands were 69 (or 92.0%) and 65 (or 87.7%) based on 99% and 95% criteria, respectively (Table 2). The number of scored bands ranged from 4 for primer S1219 to 12 for primer S27. Each of the 241 individuals from the 9 populations was found to have a unique multi-locus genotype. Only one band was present and fixed in one population (TT) and absent in all others.

The relative degree of diversity in each population as measured by Shannon's index varied from 0.1844 (DB) to 0.3608 (QL) (Table 3). The mean diversity for all populations was 0.2925 and the pooled species-level value was 0.4112. Percent polymorphic RAPD loci varied from 33.3 (DB) to 74.7% (QL), based on 99% criterion, with a mean of 57.9% (Table 3). The Nei's gene diversity (*H*) ranged from 0.1350 to 0.2462 with a mean of 0.2027 and the pooled species-level value was 0.2954. Among the nine populations, population QL exhibited the highest level of genetic variability (*P*, *H* and SI) and population TT was the next, whereas population DB was the lowest (Table 3). Regression analysis indicated significant positive relationships between all parameters (*P*, *H* and SI) of within-population genetic diversity and the log of estimated population size (Fig. 2).

The partitioning of genetic variation was further examined by AMOVA. Although most of the variation (75.14%)

Table 2 RAPD primers used in the survey of *H. miconioides*, number of amplified products, and number of polymorphic bands

Primer	Sequence 5′–3′	Amplified bands	Polymorphic bands
S1018	GGGCTAGTCA	8	7
S1071	CAGTGTGCTC	8	8
S1125	GGGTGCAGTT	8	8
S1200	GTGAACGCTC	5	5
S1219	CTGATCGCGG	4	4
S1348	AGGCTTCCCT	8	5
S1444	GTAGGCCTCA	8	7
S2025	GGGCCGAACA	5	4
S27	GAAACGGGTG	12	12
S420	AGGTCTTGGG	9	9
Total		75	69

Table 3 Patterns of genetic diversity for *H. miconioides* populations

Population	P_{95}	P_{99}	*H*	SI
BQ	37.33	44.00	0.1665	0.2309
QL	65.33	74.67	0.2462	0.3608
GY	46.67	52.00	0.1941	0.2757
DB	33.33	33.33	0.1350	0.1844
BS	56.00	58.67	0.2004	0.2922
DP	61.33	68.00	0.2194	0.3250
SM	53.33	64.00	0.2109	0.3078
KC	54.67	62.67	0.2181	0.3160
TT	57.33	64.00	0.2336	0.3397
Mean	51.70	57.93	0.2027	0.2925
Species level	86.67	92.00	0.2954	0.4112

H denotes mean Nei's gene diversity based on Nei's (1978) unbiased estimates; SI, Shannon's diversity index

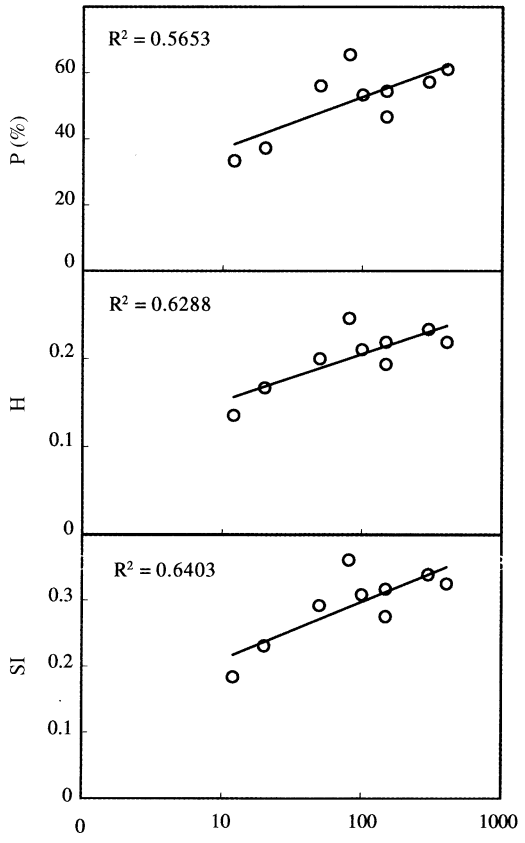

Fig. 2 Relationship between genetic diversity and population size in *H. miconioides*. *P*: percentage of polymorphic loci based on 95% criterion, *H*: Nei's gene diversity, SI: Shannon diversity index

was observed within populations, a significant proportion was attributable to differences among populations (24.86%) ($P < 0.001$, tested using a 1,000 replication bootstrap) (Table 4). All pair-wise Φ_{ST} values derived from AMOVA were significant when individual pairs of populations were compared (Table 5). Significant correlation was found between Φ_{ST} value and geographical distance ($r = 0.5041$, $P = 0.003$) based on the Mantel test (Fig. 3). Based on the Φ_{ST} value, the calculated gene flow, i.e., historical gene flow, was moderate (0.756).

CpDNA sequence of *H. miconioides* was amplified by primers of *pet*G-*trn*P and *trn*S-*trn*G (Hamilton 1999). Sequence of *pet*G-*trn*P is 480 bp (AY723293), including 153 A, 88 C, 94 G and 145 T, and that of *trn*S-*trn*G is 802 bp (AY723294), including 268 A, 143 C, 110 G, and 281 T. All the analyzed individuals had the same sequence and no variation was found in two fragments of cpDNA.

Discussion

Genetic diversity

Heptacodium miconioides is a long-lived woody perennial. As expected, its populations harbored moderate to high-level nuclear genetic variation (Nybom 2004). It exhibited a high level of genetic diversity in comparison to other endangered or rare plant taxa that had been examined using RAPD markers. The percentage of polymorphic loci at species level of *H. miconioides* (92% at the 99% criterion) was higher than that of most endangered or threatened tree or shrub species based on the same or 100% criterion, such as *Aldrovanda vesiculosa* (14%, Martin et al. 2003), *Pinus chiapensis* (24.5%, Newton et al. 2002), *Pilgerodendron uviferum* (35.7%, Allnutt et al. 2003), *Haplostachys haplostachya* (42%, Morden and Loeffler 1999), and

Table 4 AMOVA of RAPD variation for *H. miconioides* populations

	df	Sum of squares	Mean squares	Variance	Percentage of total variance	*P*
Among populations	8	511.7275	63.966	2.2018	24.86	< 0.001
Within populations	232	1544.1397	6.656	6.6558	75.14	
Total	240	2055.8672				

The *P*-value was calculated by 1000 replication bootstrap between populations

Table 5 Pair-wise Φ_{ST} values calculated by AMOVA illustrating differences between populations of *H. miconioides*

	PQ	QL	GY	DB	BS	DP	SM	KC	TT
BQ	–								
QL	0.2839	–							
GY	0.2801	0.2529	–						
DB	0.3404	0.2588	0.2969	–					
BS	0.3166	0.2535	0.2533	0.1885	–				
DP	0.3082	0.3102	0.3377	0.1437	0.1920	–			
SM	0.3362	0.3225	0.4085	0.3203	0.2861	0.2157	–		
KC	0.2817	0.2509	0.2879	0.1808	0.1935	0.1236	0.1936	–	
TT	0.2454	0.1865	0.2411	0.1265	0.2162	0.1940	0.2661	0.2057	–

All the Φ_{ST} values were significant at $P < 0.001$

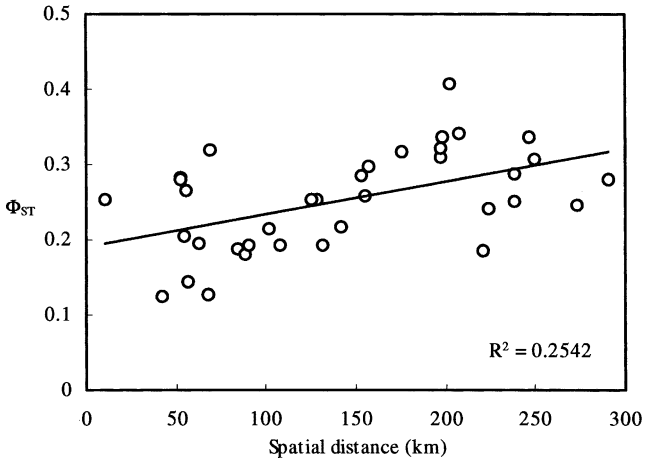

Fig. 3 Relationship between Φ_{ST} value and geographical distance in km of pair-wise populations of *H. miconioides* (Mantel test of correlation, $r = 0.5041$, $P = 0.003$)

Colubrina oppositifolia (47%, Kwon and Morden 2002), but slightly lower than that found in *Metasequoia glyptostroboides* (94.8%, Li et al. 2005) and *Caesalpinia echinata* (96%, Cardoso et al. 1998). Other measures of genetic diversity (0.2954 and 0.4112 for gene diversity and Shannon diversity index, respectively) indicated similar situation (Prathepha and Baimai 1999).

Although suffered serious destruction, *H. miconioides* has relatively high genetic variation, probably due to its strong ability of coppicing from older stems. It is observed that, if its original habitats have not been converted to tea gardens or other commercial plantations, *H. miconioides* could sprout out after destruction (Li et al. 2004). For example, in Kuocang Mountain of Zhejiang Province (population KC), *H. miconioides* coppiced from remnant stems in the next year after being clear-cut and burnt, and was among the earliest of plants appearing (Li et al. 2004). This coppicing ability might delay the negative effects of drift and inbreeding on genetic diversity in small populations for several generations. Another possible explanation of high variation comes from the experiments. During screening proper primers, primers that amplified 1 or 2 bands had not been used in further study. Discarding these primers might lead to over-estimated percentages of polymorphic loci.

Although *H. miconioides* has strong ability of coppicing, an asexual reproductive system, each of the 241 individuals has a unique multi-locus genotype. Unlike other asexual reproduction, such as producing stolons, rhizomes or bulbs, dispersal of coppicing is very restricted and all sprouts were observed to cluster on the stems. Furthermore, samples were collected from individuals with at least of 2 m between each other, decreasing the possibility to sample ramets of an old clone.

Contrary to the high RAPD diversity, no variation was found in two fragments of cpDNA sequence. The cpDNA of most angiosperms is maternal inheritance (Mogensen 1996) and with no recombination. No cpDNA variation indicated that these individuals shared the same maternity and could be interpreted that these populations originated from a large population after glaciations. This species is distributed in rocky valleys in mixed forests zone of deciduous and evergreen broad-leaved species or in deciduous forests zone (usually >800 m above sea level) in subtropical areas of eastern China. Only a few mountains meet the needs of this species. However, during the last glaciations, temperature in eastern China was about 5–13°C lower than at present (Shi et al. 1989). The lower limit of its vertical distribution was expected to decrease, thus the distribution range was expected to be much wider and continuous than the current one. After the glaciations, the distribution range was fragmented and restricted to a few mountains. In recent decades, populations have became more fragmented due to heavy human activities and some populations have even gone extinction, as seen in the local extinction in Xingshan County, Hubei Province, where the type specimen was collected in 1907. This hypothesis was also supported by low gene differentiation ($\Phi_{ST} = 0.25$, 74% of the mean of plants using RAPD markers (Nybom 2004)) though populations were seriously fragmented and contemporary gene flow was limited.

Population size and variation

Genetic diversity within populations was strongly related to the log of estimated population size in *H. miconioides* (Fig. 2). As expected, low genetic variation was observed in small populations, due to small sampling sizes. Similarly, reduced genetic variation in smaller populations has been reported for 11 of 16 plant taxa mentioned in a review by Frankham (1996) and in some species studied since then, such as *Senecio vulgaris* (Mueller-Scharer and Fischer 2001). These relationships were usually interpreted as results of genetic drift (Chen 2000). It might take several generations for drift to have significant impacts. However, the studied populations of *H. miconioides* suffered serious population size decline in short time. Due to the low seed set rate (Bian et al. 2002) and low germination rate of seeds of *H. miconioides*, sexual reproduction might play a minor role in population persistence. Therefore, drift might have insufficient time to reduce the genetic diversity. A strong relationship between Φ_{ST} value and geographical distance also suggests that drift played a minor role.

Besides effects on genetic diversity via drift and inbreeding, a dramatic decrease in population size also has an instantaneous effect, i.e., stochastic loss of rare alleles because only a small portion of the original gene pool

remains after the decrease. Buchert et al. (1997) had estimated the effect of population size decrease in eastern white pine (*Pinus strobus*) by harvesting. They found total and mean number of alleles were reduced by 25% after tree density reductions of 75%. About 40% of the low frequency alleles and 80% of the rare alleles were lost because of harvesting (Buchert et al. 1997). The instantaneous effect of population size decrease may explain the low genetic diversity in small populations of *H. miconioides.* For example, population DB, whose genetic diversity was least, had about 10 individuals remaining. The population has been decreased in recent years due to natural habitats being converted into tea gardens. The remnant individuals are adjacent to tea gardens, and the population is facing the risk to extinction.

Genetic differentiation and gene flow

The majority of the variation in *H. miconioides* was found within rather than among populations as estimated by both gene differentiation and AMOVA analysis. This is attributed to the majority of markers present in all populations. Only one locus was found exclusively in a single population. Relatively low differentiation demonstrated a close relationship among populations.

The calculated gene flow (0.756) was moderate and enough to prevent differentiation led by drift. This gene flow was calculated based on the differentiation among populations, reflecting historical gene flow (Sork et al. 1999). However, the contemporary gene flow among *H. miconioides* populations is expected to be limited. Although the remaining elongated calyces may do benefit to dispersal, the seeds usually disperse to a short distance off the maternal individuals (unpublished observation). *H. miconioides* is an insect-pollinated species (Bian et al. 2002). The individuals are spatially distributed in valleys and insect pollinators tend to forage inside each valley, restricting gene flow. Hence, spatial isolation of present populations may keep these insects to each mountain, and lead to a restricted contemporary gene flow.

Conservation implications

Although *H. miconioides* maintains high genetic variation at species level, reduction in population size associated with habitat destruction has resulted in the erosion of polymorphism and gene diversity. Early work suggested that the minimum effective size of a population needed to maintain sufficient genetic richness is 50 individuals, and 500 for a given population to counteract the effects of genetic drift (Franklin 1980), i.e., 50/500 rule. However, the rule has been challenged and effect sizes on the order of 1000–5000 have been suggested to maintain adaptive variation and avoid accumulation of deleterious mutations (Lande 1995; Lynch and Lande 1998). Given that all population sizes were estimated lower than 1,000 and several populations contained less than 50 individuals, further decrease of genetic variability within populations should be expected. Coppicing might delay the processes, but might also compromise the ability to respond to changing environments.

For endangered species with a few populations remaining, such as *H. miconioides*, all the remnant populations should be protected. However, due to limited financial support and social and institutional considerations (Johnson 1995), we should identify populations for priority conservation (Petit et al. 1998), Positive relationship between population size and genetic variation indicated that population size is an indicator for genetic variation in *H. miconioides*, and large populations have priority of conservation for effectively preserving genetic variation (Kark et al. 1999; Chen et al. 2002). Population DP was largest and had abundant genetic variation. Fortunately, a province-level reserve had been set up several years after being found by the scientific field and one of the objectives is to protect *H. miconioides.* Our results indicated that some moderate populations also maintained high genetic diversity and had high priority for conservation. For instance, although population QL, which is located in a national reserve, had less than 100 individuals, it had the most abundant genetic variability (Table 3), and also should receive high priority for conservation because one primary aim is to conserve genetic diversity (Kark et al. 1999). However, this suggestion was based on RAPDs variation and might be biased because RAPDs are dominant markers. More convincing data should be utilized in the future.

Threat is another criterion in considering worthy of conservation. Populations facing the greatest imminent danger or harm should also have high priority of conservation (Johnson 1995). Most populations of *H. miconioides* are located in reserves, forest parks or scenic areas suffering relatively slight threat of conversion utilization of habitats, which is the largest threat to this species. However, habitats in DB and KC are facing the threat to be converted into tea gardens. Therefore, urgent conservation efforts should be undertaken to avoid the local extinction of *H. miconioides*.

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