植 物 分 类 学 报 45 (4): 488–496(2007) doi:10.1360/aps06214
Acta Phytotaxonomica Sinica http://www.plantsystematics.com

Phylogeographic structure of *Primula obconica* (Primulaceae) inferred from chloroplast microsatellites (cpSSRs) markers

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Abstract *Primula obconica* has been cultivated widely as a popular garden plant. In order to discover the pattern of genetic diversity and the evolutionary process, a total of 278 individuals from 17 populations throughout its distribution in China were analyzed using chloroplast microsatellites (cpSSRs) markers. Four loci and a total of 14 haplotypes were identified by our data set. The total gene diversity (H_T=0.971) is high, while gene diversity within populations (H_S=0.028) is low. Analysis of molecular variance (AMOVA) shows that about 98% variation is among populations. The results suggest that past fragmentation and limited dispersal ability of seeds might play important roles in forming the present genetic structure. A significantly higher value of N_{st} than that of G_{st} indicates that closely related haplotypes are often found in the same area, and we found two different groups in the minimum spanning tree (MST), which occupy different geographic regions. Furthermore, older haplotypes were detected in the two groups, respectively. Possible refugia are inferred in western Hubei Province and SW China during the glacial period.

Key words *Primula obconica*, genetic diversity, chloroplast microsatellite, glacial period, refugia.

Primula obconica Hance is a perennial herb first introduced to Britain from China in 1880 (Connolly et al., 2004). Since then it has been cultivated by horticulturist as popular houseplants. So far, *P. obconica* has been widely accepted as an ornamental primrose plant. However, it was also claimed as one of the most allergenic species among primroses and known as a significant source of allergic contact dermatitis in Europe, especially in England, Germany, and the Scandinavian countries (Christensen & Larsen, 2000). Surprisingly, allergic compounds were not found in wild plants of this species, suggesting the wild ones will be ideal resource for horticultural use (Nan et al., 2002, 2003).

Primula obconica is widely distributed in the area south of Yangtze River in China, mainly growing at 500–3000 m elevation in moist thickets, forests and rocks in mountain woods (Hu, 1990; Hu & Kelso, 1996). In contrast to its behavior in cultivation, this species shows exceptional variations in morphological traits in the wild (Richards, 2002). Six subspecies had been taxonomically recognized, viz., ssp. *obconica*, ssp. *begoniiformis* (Petitm.) W. W. Smith & Forr., ssp. *parva* (I. B. Balfour) W. W. Smith & Forr., ssp. *werringtonensis* (Forr.) W. W. Smith & Forr., ssp. *nigroglandulosa* (W. W. Smith & Fletcher) C. M. Hu, and ssp. *fujianensis* C. M. Hu & G. S. He (Hu, 1990; Hu & Kelso, 1996; He & Hu,

Received: 28 December 2006 Accepted: 6 March 2007
Supported by the National Natural Science Foundation of China, Grant Nos. 30470125, 40671066, and the Knowledge Innovation Project of Chinese Academy of Sciences, Grant No. KSCX2-SW-132.
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2002). Little was known about the genetic diversity of this species in the wild despite its important horticultural value, until Nan et al. (2003) conducted a genetic variation study for a small number of populations in central and southwest China using inter-simple sequence repeats (ISSR) markers.

The organelle genome based molecular markers are useful tools for evolutionary studies since they are nonrecombinant and uniparentally inherited (Provan et al., 2001; Zhang et al., 2005a). The chloroplast genome is always inherited maternally in angiosperms (Palmé et al., 2003). A half/quarter effective population size of the chloroplast genome of that for the nuclear genome makes it more sensitive to the effect of historic events (Schaal et al., 1998; Freeland, 2005). Additionally, high mutation rates in simple sequence repeats give a good chance to address the issue of genetic diversity (Hardy et al., 2003). These features make chloroplast microsatellites (cpSSRs) a useful tool for studies in plant ecology and evolution, especially as several conserved primers have been developed. To date, cpSSRs has been widely used to detect genetic diversity, to understand crop plant evolution and domestication, and even to elucidate the phylogeny of species with taxonomic problem (Echt et al., 1998; Chaïr et al., 2005; Cubas et al., 2005).

Here we employed cpSSRs to survey intraspecific polymorphisms of *P. obconica* in China. We aimed to infer the evolutionary history of this species based on the recovered distribution of genetic patterns.

1 Material and methods

1.1 Sampling of plant materials

A total of 17 populations of *P. obconica* were sampled for the present study. Fresh leaves were collected and dried in silica gel immediately in the field. The sampling strategy was to cover the natural range of the species, and to include all the subspecies. Unfortunately, we were unable to collect *P. obconica* ssp. *parva* and *P. obconica* ssp. *nigroglandulosa*, which are believed very rare now in the wild. The sampling information is indicated in Table 1.

1.2 DNA extraction, amplification, and screening

Total DNA was isolated with a modified CTAB protocol (Doyle, 1991). Polymerase chain reaction (PCR) assays were performed in a total volume of 20 μL, containing 10 ng of genomic DNA, 0.15 μmol/L of each primer, 100 μmol/L of dNTPs, 2.0 mmol/L of $MgCl_2$, and 1 U *Taq* polymerase. PCR amplification was performed on a MJ Research PTC-100 Thermocycler programmed with an initial denaturation step at 94 ℃ for 3 min, 30 cycles denaturation at 94 ℃ for 30 s, the annealing temperature indicated in Table 2 for 30 s, and extension at 72 ℃ for 30 s, followed by a final extension step at 72 ℃ for 7 min.

A total of twenty sets of primers (ccmp and ccSSR) were used in a preliminary study (Weising & Gardner, 1999; Chung et al., 2003). One individual per population was selected randomly, and amplified using these primers. PCR products stained with ethidium bromide were checked in 1% (w/v) agarose gel electrophoresis. When the amplified fragments were detected, the products mixed with loading buffer (7 μL) were loaded on vertical polyacrylamide gel, and electrophoresed in 1×TBE buffer at constant voltage 125 V for approximately 2 h 30 min. Bands with the help of fragment size standards were discriminated visually in silver stained gel. The primers with polymorphic bands were used in whole populations for further analysis. Presence or absence of polymorphic bands was scored with 1 or 0, respectively.

1.3 Data analysis

Because of the haploid characteristic of chloroplast genomes, the chloroplast haplotype is defined as the unique combination of polymorphic scores obtained from each individual.

Table 1 Sampling subspecies, populations, and individuals of *Primula obconica* Hance

Code	Subspecies	Location	Voucher	Sample size	Haplotype
NP	ssp. *fujianensis* C. M. Hu & G. S. He (福建报春)	Nanping, Fujian, China (福建南平)	F. Y. Wang (王凤英) s.n. (IBSC)	20	H6
LC	ssp. *obconica* (鄂报春)	Lechang, Guangdong, China (广东乐昌)	H. G. Ye (叶华谷) s.n. (IBSC)	3	H7
RY	ssp. *obconica* (鄂报春)	Ruyuan, Guangdong, China (广东乳源)	G. Hao (郝刚) 392 (IBSC)	20	H1, H14
SZ-1	ssp. *obconica* (鄂报春)	Sangzhi, Hunan, China (湖南桑植)	G. Hao (郝刚) 297 (IBSC)	15	H2
SZ-2	ssp. *obconica* (鄂报春)	Sangzhi, Hunan, China (湖南桑植)	L. C. Tian (田连成) 17987 (IBSC)	8	H6
YC	ssp. *obconica* (鄂报春)	Yichang, Hubei, China (湖北宜昌)	G. Hao (郝刚) 295 (IBSC)	17	H1, H5
EMS	ssp. *obconica* (鄂报春)	Emeishan, Sichuan, China (四川峨眉山)	G. Hao (郝刚) 432 (IBSC)	22	H1, H5
LD	ssp. *obconica* (鄂报春)	Luding, Sichuan, China (四川泸定)	G. Hao (郝刚) 448 (IBSC)	14	H8
DJY-1	ssp. *Obconica* (鄂报春)	Dujiangyan, Sichuan, China (四川都江堰)	G. Hao (郝刚) 422 (IBSC)	20	H4
DJY-2	ssp. *obconica* (鄂报春)	Dujiangyan, Sichuan, China (四川都江堰)	G. Hao (郝刚) 443 (IBSC)	6	H4
ML-1	ssp. *werringtonensis* (Forr.) W. W. Smith & Forr. (波叶鄂报春)	Muli, Sichuan, China (四川木里)	G. Hao (郝刚) 540 (IBSC)	21	H11
ML-2	ssp. *werringtonensis* (波叶鄂报春)	Muli, Sichuan, China (四川木里)	G. Hao (郝刚) 565 (IBSC)	20	H12
LQ	ssp. *begoniiformis* (Petitm.) W. W. Smith & Forr. (海棠叶鄂报春)	Luquan, Yunnan, China (云南禄劝)	G. Hao (郝刚) 378 (IBSC)	20	H3
DL	ssp. *obconica* (鄂报春)	Dali, Yunnan, China (云南大理)	G. Hao (郝刚) 524 (IBSC)	21	H10
WX	ssp. *obconica* (鄂报春)	Weixi, Yunnan, China (云南维西)	G. Hao (郝刚) 516 (IBSC)	20	H9
BS-1	ssp. *Obconica* (鄂报春)	Baoshan, Yunnan, China (云南保山)	G. Hao & H. F. Yan (郝刚, 颜海飞) 580 (IBSC)	19	H13
BS-2	ssp. *begoniiformis* (海棠叶鄂报春)	Baoshan, Yunnan, China (云南保山)	G. Hao & H. F. Yan (郝刚, 颜海飞) 584 (IBSC)	12	H13

Several population genetic parameters were computed for each population: haplotype frequency in all individuals (P), the effective number of haplotypes (N_e) computed as $N_e=(\sum p_i^2)^{-1}$, and unbiased haplotype diversity (H_E) according to the formula: $H_E=[n(n-1)^{-1}](1-\sum p_i^2)$, where n is the number of individuals analyzed and p is the frequency of the i-th haplotype in a population (Nei, 1987).

In addition, estimates of average diversity over populations (H_S), the average total gene diversity (H_T), and the value of genetic differentiation among populations (G_{st} and N_{st}) were calculated by PERMUT 1.0 (developed by R. J. Petit, available at http://www.pierroton.inra.fr/genetics/labo/Software/Permut/)(Pons & Petit, 1996). Genetic variance components within and among populations between and within groups were inferred by a hierarchical analysis of molecular variance (AMOVA) using Arlequin Ver. 3.0 (Excoffier et al., 2005). A minimum spanning tree (MST) computed from the matrix of pairwise distances calculated between all pairs of haplotypes using a modification of the algorithm described in Rohlf

(1973) was estimated by the Arlequin package.

2 Results

By analyzing twenty primer pairs, only 13 loci were successfully amplified (10 loci were ccmp series, 3 belonged to ccSSR series). Four loci (ccmp1, ccmp4, ccSSR-5, ccSSR-7) were polymorphic and used for further analyses. All of four fragments presented a bright band by silver staining. We analyzed 278 individuals using these four primer pairs (Table 2). Consequently, 16 alleles were identified, and a total of 14 haplotypes was detected throughout all individuals identified as H1–14 (Table 1).

Table 2 The list of chloroplast microsatellite primers used in the present study

Loci	Location	Sequence (5′-3′)	T_m (℃)
ccmp1	*trnK* intron	F: CAGGTAAACTTCTCAACGGA R: CCGAAGTCAAAAGAGCGATT	50
ccmp4	*atpF* intron	F: AATGCTGAATCGAYGACCTA R: CCAAAATATTBGGAGGACTCT	58
ccSSR-5	*rps2-rpoC2*	F: TCTGATAAAAAACGAGCAGTTCT R: GAGAAGGTTCCATCGGAACAA	55
ccSSR-7	*psbC-trnS*	F: CGGGAAGGGCTCGKGCAG R: GTTCGAATCCCTCTCTCTCCTTTT	58

The genetic diversity of *P. obconica* is shown in Table 3. Haplotype frequencies (*P*) for all individuals range from 0.011 to 0.122. Populations EMS, YC, and RY are the divergent populations revealed by all parameters, where H1 is shared with the highest haplotype frequency (0.122). In contrast, H7 and H14 are the lowest frequency haplotypes, which are only found in three samples, respectively. Five of the 14 haplotypes (H1, H4, H5, H6, and H13) are shared by two or three populations, while the rest of them are population-specific. The "private" haplotypes are about 60% in all haplotypes, and most of them are located in SW China (Sichuan and Yunnan provinces).

Table 3 Haplotype frequencies and diversity assessed by cpSSRs in *Primula obconica*

Haplotype	Population code																	*P*
	YC	SZ-1	LQ	RY	DJY-1	EMS	SZ-2	LC	LD	DJY-2	NP	WX	DL	ML-1	ML-2	BS-1	BS-2	
H1	16			17		1												0.122
H2		15																0.054
H3			20															0.072
H4					20					6								0.094
H5	1					21												0.079
H6							8				20							0.101
H7								3										0.011
H8									14									0.050
H9												20						0.072
H10													21					0.076
H11														21				0.076
H12															20			0.072
H13																19	12	0.112
H14				3														0.011
n	17	15	20	20	20	22	8	3	14	6	20	20	21	21	20	19	12	
N_e	1.125	1	1	1.342	1	1.094	1	1	1	1	1	1	1	1	1	1	1	
H_E	0.118	0	0	0.268	0	0.090	0	0	0	0	0	0	0	0	0	0	0	

n, the number of sampling per population; N_e, the effective number of haplotypes; H_E, unbiased haplotype diversity; *P*, the frequency of haplotypes in all individuals.

The mean total gene diversity, H_T=0.971, is extraordinarily high, while the average diversity within populations (H_S=0.028) is very low. Only three populations (YC, RY, and EMS) are composed of different haplotypes and present higher polymorphism than average located in central and southeast China. AMOVA analysis shows that 98.55% variation is among populations, only 1.45% is within population. When AMOVA analysis is conducted for two groups (Fig. 1), the apportionment of the variation is that: 39.48% is due to differences between two groups, 59.35% is within groups, and only 1.07% to difference within populations (Table 4).

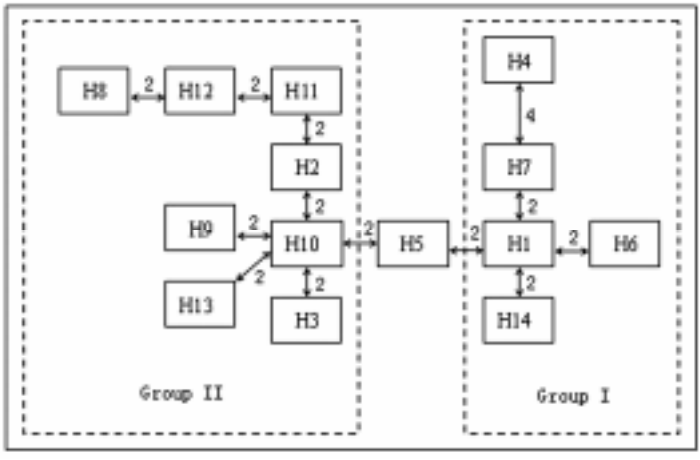

Fig. 1. A minimum spanning tree (MST) for 14 haplotypes based on cpSSRs data. The haplotype names are given in pane. Two groups are indicated. Numbers between panes represent mutational step.

Table 4 Results of molecular variance analysis (AMOVA) for populations of *Primula obconica*

Source of variation	df	Variance components	Variation (%)	P
Group I				
Among populations	7	1.90291	95.85	0
Within populations	108	0.08233	4.15	0
Group II				
Among populations	8	1.59873	100	0
Within populations	153	0	0	0
Two groups				
Among groups	1	1.14570	39.48	0
Within groups	15	1.72251	59.35	0
Within populations	261	0.03407	1.07	0
All the populations				
Among populations	16	2.31896	98.55	0
Within populations	261	0.03407	1.45	

A high level of genetic differentiation among populations (G_{st}=0.971) was found, while the value of N_{st} (0.988) was significantly higher than that of G_{st} (P<0.05). This indicates a high degree of geographic structure of genetic distribution. Phylogenetic relationship of the 14 haplotypes constructed by a minimum spanning tree (MST) is shown in Fig. 1. Most of the haplotypes differ from one another by two mutational steps. A tip haplotype H4, however, is separated by four mutation steps from H7. The MST shows that the haplotypes of *P. obconica* are clustered into two groups. H5 shows the connection between two groups. Haplotypes from the group II are mainly restricted to the SW China, whereas central and southeast China is

occupied by the other, except for the haplotype H4 (from the north of Sichuan province). The central position of the two groups is occupied by H10 and H1, respectively, while other tips enclose them, forming a star-like topology.

3 Discussion

In the present study, low average diversity within populations (H_S=0.028), and high diversity among populations (H_T=0.971), are recovered in *P. obconica*. In addition, the level of differentiation among populations (G_{st}=0.971) is remarkably high as compared to the value (G_{st}=0.5197) assessed by ISSR (Nan, 2002). The unique genetic structure (high population differentiation but low within-population diversity) of this species as well as the distinct discrepancy with ISSR measure was mainly caused by the following four reasons. Firstly, the uniparentally inherited chloroplast genome has a twofold smaller effective population size than biparentally nuclear genomes. Therefore, this chloroplast marker is more susceptible to historical events, such as genetic drift, bottlenecks, and founder effects, and the differentiation based on its variation is obviously larger than those from bi-parental nuclear markers (Cruzan & Templeton, 2000; Powell et al., 1995; Petit et al., 1997). Secondly, the limited ability of seed dispersal of this species may similarly lead to increased differentiation between populations, as also revealed in other species, such as *Saxifraga hirculus* (Oliver et al., 2006) and *Carpinus betulus* (Grivet & Petit, 2003). By our observation in the field, the seeds of this species were dispersed mainly by the opening power of the ripened capsules. Furthermore, the patch-like populations separated by high mountains and valleys undoubtedly reduce the opportunity for gene exchange through seeds. This geographical isolation was also proposed to account for the high differentiation in *Amentotaxus argotaenia* that has a similar distribution pattern with *P. obconica* (Ge et al., 2005). Finally, climate oscillations could affect the distribution of plants and animals, especially in the ice age, which became increasingly severe through the Pleistocene (Hewitt, 1999). Glacial-induced downward migrations of some alpine species occurred in central and west of Yunnan Province during the Pleistocene (Li, 1998). Given the small size of population and the unstable temperature during the Pleistocene ice-age, these repeatedly occurred expansion-contraction processes, as well as geographical barriers, may have reduced the polymorphism within populations as well increasing the population differentiation.

A higher N_{st} than G_{st} indicates a significant geographic structure in this species (Zhang et al., 2005b) and our results further suggest that the closely related haplotypes are often found in the same area (Fig. 1; Table 1). Two groups with special geographic regions in the MST were detected. Based on the coalescent theory, ancestral alleles with high frequency and broad distribution have a greater probability of becoming interior haplotypes (Posada & Crandall, 2001). Notably, the haplotype H1 with highest frequency and wide distribution occupies the central position of group I in the MST. This result implies that haplotype H1 may be one of the ancestral haplotypes of *P. obconica*. In addition, western Hubei and the adjacent Sichuan might be one of the predicted refugia of tertiary flora with many of the primitive temperate genera and endemic relicts (Ying et al., 1979). As expected, the population YC located in this region is mainly occupied by H1. Thus, a rational prediction is that the ancestral haplotype might be preserved in this region during the glacial periods. The downward movement of alpine species will expand to previously isolated habitats during glacial periods, despite that such an expansion may be slow due to habitat constraints (the phalanx model; cf. Chiang & Schaal, 2006). The fact that the old haplotype H1 is located in the north, and tip haplotypes are scattered in the south China suggests that a migration may have occurred from the North during cold times. The average temperature in SE China during the LGM (13000 a BP) was

lower about 4–6 ℃ than that of the present (Pu, 1991). It afforded an opportunity for *P. obconica* to migrate southward, and retreat to mountains following the temperature arising, and finally forming the present distribution.

The other group is mainly restricted to SW China. This region is the assumed center of origin of Primulaceae, and includes the mountain regions of Yunnan, the south of Guizhou, the west of Guangxi of China, and Vietnam, Myanmar, and the northern part of Thailand (Hu, 1994). Because of the special geographic condition, SW China was affected slightly during the Pleistocene glaciations (Ying, 2001; Shen et al., 2005), and was considered as a key area for resolving the issue of the origin of the flora of northern temperate zone (Wang, 1992). Additionally, its climate was warm and humid during the rigor time of glaciations (Li, 1998). Those special environments give the chances for species in refugia evolving rapidly and spreading along the high mountain ranges (Hu, 1994). Therefore, it is assumed that this region may also be the origin place or at least the evolutionary center of *P. obconica*. Populations of *P. obconica* were saved in different refugia when the glacial periods advanced, since many private haplotypes were fixed.

H4, remarkably, in the north of Sichuan Basin, is isolated from H7 by four unique mutations. Uncertain relationships between the two haplotypes (H4 and H7) should be given more attention, since there is a distance of more than 1000 km between the two sampling sites. Another ambiguity occurs in Hunan Province, with two close populations (SZ-1 and SZ-2) belonging to two different groups in MST respectively (Fig. 1). It is plausible that this region may be the potential contact area of the species *P. obconica* derived from different ancestors. More sampling sites included in this region or the adjacent areas are required for further phylogeographic study of *P. obconica*.

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基于叶绿体微卫星研究鄂报春谱系遗传结构

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摘要 鄂报春*Primula obconica*作为一种广泛栽培的园艺植物, 其野生居群的遗传多样性及遗传结构的研究还少见报道。本文通过叶绿体微卫星分析了17个鄂报春野生居群(共278个个体), 共发现4个多态性位点(16个等位基因), 得到14个单倍型。结果表明鄂报春具有很高的总基因多样性(H_T=0.971)和极低的居群内基因多样性(H_S=0.028); 分子方差分析(AMOVA)显示98%的变异存在于居群间。这些结果说明早期的生境片断化及有限的种子传播能力是造成当前遗传结构的重要原因。N_{st}值显著大于G_{st}值, 表明关系相近的单倍型会出现在相同的地区内, 同时最小生成树(MST)的分析结果证实了这样的结论。我们在最小生成树的两个组中推断出一些古老单倍型, 并推测在冰期时湖北和我国的西南地区可能是该物种的避难所。

关键词 鄂报春; 遗传多样性; 叶绿体微卫星; 冰期; 避难所