Annals of Botany **91**: 329–333, 2003
doi:10.1093/aob/mcg018, available online at www.aob.oupjournals.org

Genetic Diversity in *Primula obconica* (Primulaceae) from Central and South-west China as Revealed by ISSR Markers

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Received: 7 March 2002 Returned for revision: 24 August 2002 Accepted: 26 October 2002 Published electronically: 12 December 2002

Genetic diversity was investigated in 60 individuals of *Primula obconica* from four natural populations (from Hubei, central China, and Sichuan, south-west China) and from cultivated material. Inter-simple sequence repeat (ISSR) techniques produced 249 polymorphic bands and identified 60 ISSR genotypes. Based on Shannon's index and Nei's genetic diversity, the genetic diversity detected in all natural populations of *P. obconica* was much higher than that in the cultivated plants, and that in the three Dalaoling (Hubei) populations was higher than that in the Wawushan (Sichuan) population. UPGMA cluster analysis showed that there was no distinct genetic differentiation between populations from the Mt Dalaoling area and the Mt Wawushan area. This study provides a population-level genetic profile of *P. obconica*, which was previously poorly known but which is important for *Primula* breeding and cultivation.

Key words: *Primula obconica*, genetic diversity, ISSR, genotype, cluster analysis, China.

INTRODUCTION

Primula obconica Hance (Primulaceae), the poison primrose, is mainly distributed in central and south-west China where it grows in calcareous soils at altitudes of 800–2000 m (Zheng, 1993; Hu and Kelso, 1996). It was first introduced to Britain from Yichang (Ichang) County, Hubei (Hupeh) Province, China, in 1880 and rapidly became established as a popular ornamental house-plant worldwide (Hausen, 1978; Richards, 1993). However, *P. obconica* is reported to be a significant cause of allergic contact dermatitis, particularly in Europe and the United States (Rook and Wilson, 1965; Aplin and Lovell, 2001) owing to the allergic compounds primin (2-methoxy-6-pentyl-1,4-benzoquinone) and miconidin (2-methoxy-6-pentyl-1,4-dihydroxybenzene) that can be isolated from leaves, stems and flowers (including pedicel and calyx) of many cultivars (Horper and Marner, 1995, 1996; Krebs and Christensen, 1995; Christensen and Larsen, 2000*a*). Breeding of primin-free cultivars of *P. obconica* has therefore become a focus of attention within the horticultural industry (Christensen and Larsen, 2000*b*). Recently, the volatile oil was extracted and analysed from wild *P. obconica* from Yichang, Hubei (central China) but the two allergic compounds were not found (Nan *et al*., 2002*a*; L. P. Christensen, pers. comm.). This research suggested that wild *P. obconica* in China is a potential genetic resource for horticultural use since the allergic compounds do not appear to be present. Thus, further comparative studies of the genetic and chemical diversity among natural populations and cultivars of *P. obconica* are needed.

In recent years a number of molecular markers such as RAPD (random amplified polymorphic DNA; Hu and Quiros, 1991; Munthali *et al*., 1992), AFLP (amplified fragment length polymorphism; Vos *et al*., 1995), SSR (simple sequence repeats; Zietkiewicz *et al*., 1994) and ISSR (inter-simple sequence repeats; Salimath *et al*., 1995; Wolfe and Randle, 2001) have been widely used to detect genetic diversity in plants (Karp *et al*., 1996; Wolfe and Liston, 1998). In particular, ISSR markers can be highly variable within a species and have the advantage over RAPDs in utilizing longer primers that allow more stringent annealing temperatures (Tsumura *et al*., 1996; Wolfe and Liston, 1998; Camacho and Liston, 2001) and revealing many more polymorphic fragments (Fang and Roose, 1997). In the present study the ISSR technique was used to examine natural populations of *P. obconica* from central and south-west China as well as some glasshouse-grown plants to evaluate the population-level genetic diversity of *P. obconica*.

MATERIALS AND METHODS

Sampling

During July and August 2001, four natural populations of *P. obconica* in central (Hubei) and south-west (Sichuan) China were sampled: three natural populations (D1–D3) were from the Mt Dalaoling area of Yichang, Hubei, and one (W) was from the Mt Wawushan area, Sichuan. A few living plants of *P. obconica* (G) were introduced from

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TABLE 1. *Sampling for ISSR survey and genetic diversity of* P. obconica

Population	No. of individuals	Latitude	Longitude	Altitude (m)	PPB	*I*	*I**	*h*	*h**
D1	20	31°04′	110°52′	850	84·5	0·309	0·209	0·202	0·141
D2	13	31°06′	110°57′	930	82·3	0·260	0·192	0·167	0·129
D3	18	31°03′	110°55′	800	84·8	0·294	0·197	0·193	0·133
W	6	29°41′	102°52′	1360	68·9	0·196	0·117	0·131	0·079
G	3	30°33′	114°23′	35	18·2	0·045	0·045	0·031	0·031

Populations D1–D3 sampled from Mt Dalaoling area, Hubei, central China; W from Mt Wawushan area, Sichuan, south-west China. G, plants grown in a glasshouse at the Wuhan Botanical Garden, Hubei.

PPB, Percentage of polymorphic bands; *I*, Shannon index (all individuals); *I**, Shannon index calculated using mean values for three randomly selected individuals; *h*, Nei's genetic diversity (all individuals); *h**, Nei's genetic diversity calculated using mean values for three randomly selected individuals.

Yunnan and had been cultivated in a glasshouse at the Wuhan Botanical Garden, Academia Sinica for over 10 years. They were also sampled in September 2001 for comparison with the natural populations (Table 1).

Total DNA extraction

Fresh leaves of individual plants were collected and preserved in silica gel until required for DNA isolation. Total DNA was extracted using a modified CTAB protocol (Doyle and Dolye, 1987). Dried leaf material was ground in liquid nitrogen, transferred to a 1·5 ml Eppendorf tube holding 800 µl preheated 2 × CTAB extraction buffer containing 0·2 % mercaptoethanol and incubated at 64 °C for 60 min. Subsequently, 600 µl cold chloroform : isoamylalcohol (24 : 1, v/v) was added, and the mixture was shaken gently for 2 min and spun down at 10 000 r.p.m. for 10 min at room temperature. The supernatant was reserved and mixed with 2/3 volume ice-cold isopropanol. DNA was then recovered as a pellet by centrifugation at 12 000 r.p.m. for 8 min, washed in 70 % ethanol, dried, and resuspended in 100 µl TE buffer. DNA quality and quantity were checked on 1·0 % agarose gels.

ISSR PCR amplification

Ninety primers from the UBC set 9 of ISSR primers (http://www.biotech.ubc.ca/) were tested for PCR. Reaction volumes were 10 µl, and consisted of 1 µl 10 × reaction buffer, 3 mM $MgCl_2$, 300 µM dNTPs, 0·25 µM primer, 2 % formamide, 10 ng DNA template and 1 U *Taq* DNA polymerase (Genda Tech. Corp., Toronto, Canada). The thermocycler programme for PCR was set for 1·5 min at 94 °C, followed by 45 cycles of 45 s at 94 °C, 45 s annealing at 51 or 52 °C (Table 2) and 1·5 min extension at 72 °C, and a final extension cycle of 7 min at 72 °C.

Amplification products were resolved electrophoretically on 1·5 % agarose gels in 1 × TAE buffer by loading the entire reaction volumes into prepared wells. Gels were run until a bromophenol blue indicator dye ran 10 cm from the well. Gels were stained with ethidium bromide and bands were visualized and photographed under UV light. Molecular weights were estimated using a 100 bp DNA ladder (Shengong Inc., Shanghai, China).

TABLE 2. *Primers used for ISSR amplification*

Primer	Sequence	Annealing temperature (°C)
807	$(AG)_8T$	51
811	$(GA)_8C$	52
814	$(CT)_8A$	52
827	$(AC)_8G$	52
834	$(AG)_8CTT$	52
845	$(CT)_8AGG$	52
846	$(CA)_8AGT$	52
848	$(CA)_8AGC$	52
852	$(TC)_8AGA$	51
856	$(AC)_8CTA$	52
858	$(TG)_8AGT$	52
868	$(GAA)_5GAA$	52
888	$CGTAGTCGT(CA)_7$	52
890	$ACGACTACG(GT)_7$	51

Data analysis

All ISSR bands were scored as present (1) or absent (0). The number of bands, the percentage of polymorphic bands (PPB) and the number of ISSR genotypes were calculated manually, and the Shannon index of diversity (*I*) and Nei (1973) genetic diversity (*h*) were calculated using POPGENE version 1.31 (Yeh *et al*., 1999) (available free from http://www.ualberta.ca/~fyeh/). Two separate analyses of genetic diversity were conducted, one including all the individuals and the other using average scores of three individuals for each population (randomly selected about ten times).

The Nei and Li (1979) coefficient for measuring pairwise band similarities between individuals was calculated using NTSYSpc version 2.02 (Rohlf, 1998). A clustering analysis of all individuals was performed using the unweighted pair-group method with an arithmetic average (UPGMA) using NTSYSpc 2.02.

RESULTS AND DISCUSSION

By comparing the effects of magnesium concentration and annealing temperature during amplification, 14 of the 90 primers were chosen for further analysis (Table 2). For the

TABLE 3. *Number of bands per primer and number of ISSR genotypes determined for each population per primer*

Primer	No. of bands	No. of polymorphic bands	ISSR genotypes				
			D1(20)	D2(13)	D3(18)	W(6)	G(3)
807	20	14	19	**13**	**18**	**6**	2
811	22	18	18	**13**	**18**	**6**	2
814	19	14	**20**	**13**	15	**6**	2
827	21	17	**20**	10	**18**	**6**	2
834	24	16	**20**	11	17	**6**	2
845	23	20	19	12	17	5	2
846	24	17	19	**13**	**18**	**6**	**3**
848	22	18	**20**	**13**	16	**6**	2
852	21	20	12	**13**	15	**6**	**3**
856	22	19	**20**	**13**	**18**	**6**	2
858	21	19	**20**	**13**	11	4	2
868	23	17	**20**	**13**	**18**	**6**	**3**
888	24	20	**20**	**13**	16	**6**	2
890	22	20	**20**	**13**	**18**	**6**	1
Total	308	249	**20**	**13**	**18**	**6**	**3**

Sample sizes in parentheses. Bold type indicates that each individual in a population was found to have a unique banding pattern.

60 *P. obconica* samples the total number of bands scored for the 14 primers was 308, with the PPB being 68–84 % among natural populations and 18·2 % for the cultivated population (Table 1). The size range of PCR fragments was 300–2200 bp. The average number of bands per population per primer ranged from 9·2 to 17·1, and the average number of bands per primer for the species was 22·0. The gene diversity (h) in all populations of *P. obconica* ranged from 0·031 to 0·202 (Table 1). Among the four natural populations, the gene diversity of the three Dalaoling populations (h = 0·193–0·202) was higher than that of the Wawushan population (h = 0·131), and the values of Shannon's index (I) also showed the same trend (Table 1). Since the genetic diversity results may correlate with sample size of a population, an equal number of individuals (three) randomly selected from each population was analysed. Results showed that the values of gene diversity (h*) and Shannon's index (I*) obtained from three random individuals of each population were lower than the original values of h and I, but the trend was same for each index (Table 1). In particular, among the three Dalaoling populations the smallest number of individuals was sampled from population D2, which also had the lowest genetic diversity. Human activity in the Mt Wawushan and Mt Dalaoling areas is believed to be a major factor in the dramatic decrease in population size of *P. obconica* (Shen *et al*., 2000; Yuan, pers. comm.).

The number of bands and polymorphic bands produced by each primer varied. The highest number of bands (24) was produced by primers 834, 846 and 888, and the highest number of polymorphic bands (20) by primers 845, 852, 888 and 890 (Table 3). The number of ISSR genotypes within each population distinguished by each primer is also shown in Table 3. Since the maximum number of genotypes within each population is equal to the number of individuals in the same population, the genotypic diversity (number of genotypes/number of individuals; Wolfe and Randle, 2001) for each population is 1·0.

A dendrogram of all 60 individuals of *P. obconica* based on the ISSR markers was generated using the Nei–Li similarity coefficient matrix and the UPGMA cluster method (Fig. 1). In this dendrogram all individuals of each population form a distinct cluster suggesting that there is genetic differentiation between populations. However, the Wawushan population cluster is shown to be more closely related to the cluster consisting of two Dalaoling populations (D1 and D3) than the other Dalaoling population (D2), indicating that there is no distinct genetic differentiation between populations from the Mt Daloling area and the Mt Wawushan area. In contrast, the cluster of cultivated plants is distinct from that of the four natural populations. The genetic diversity of *P. ovalifolia*, which grows alongside *P. obconica*, has also been investigated recently using ISSR markers (Nan *et al*., 2002*b*). As for *P. obconica*, results showed that there is no distinct genetic differentiation between populations of *P. ovalifolia* from the Mt Dalaoling area (two populations) and those from the Mt Wawushan area (three populations).

This study has increased the understanding of population-level genetic diversity of *P. obconica*, which was previously poorly known but which is important for *Primula* breeding and cultivation. Volatile oils from populations of *P. obconica* in central and south-west China have been analysed. Further comparative studies are being undertaken including an assessment of phytochemical variation among and within natural populations and of the relationship between genetic and chemical diversity of cultivars of this species.

ACKNOWLEDGEMENTS

We thank Professors Chi-Ming Hu and Gang Hao for their help in specimen identification, Zien Zhao, Yafu Yuan and Yinying Shi for fieldwork, and Shuguang Jian, Fengxiao Tan, Yuguo Wang, Yalin Peng, Yaqing Du and Tian Tang for technical assistance. This work was supported by the

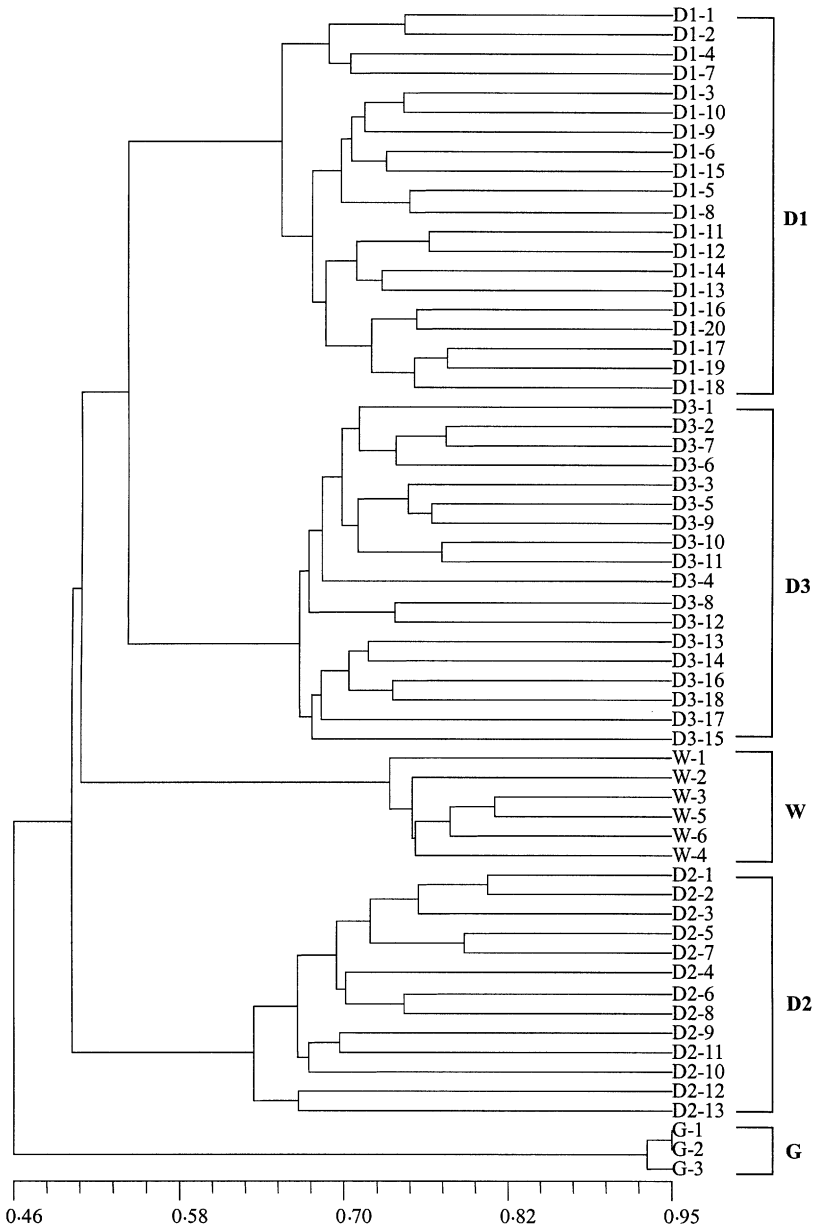

FIG. 1. Dendrogram of *P. obconica* individuals based on ISSR markers generated using the Nei and Li (1979) similarity coefficient and the UPGMA clustering method. Populations D1–D3 sampled from Mt Dalaoling area, Hubei, central China; W from Mt Wawushan area, Sichuan, south-west China; G, plants grown in a glasshouse at the Wuhan Botanical Garden, Hubei.

National Science Foundation of China (39899370), Academia Sinica (STZ-01–36) and the High-Tech Research and Development Programme (863) of China (2002 AA231041).

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