Interpopulation Congruence in Chinese *Primula ovalifolia* Revealed by Chemical and Molecular Markers Using Essential Oils and ISSRs

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The chemical composition of the essential oils of five natural populations of *P. ovalifolia* from central and southwest China and their interpopulation variability were first analyzed by using GC-MS. Twenty-two essential oil compounds were obtained, in which eighteen ones were identified and characterized representing 95%–96% of the oil composition. Three main chemotypes, *i.e.*, the methyl-acetyl-hydroquinone-rich, hydroquinone-rich, and acetyl-hydroquinone-rich chemotypes, were then differentiated, corresponding to the three groups obtained from the cluster analysis based on the essential oil composition percentages. Genetic variations among the five populations were also investigated using the Inter-Simple Sequence Repeats (ISSR) markers. Finally, the Mantel test showed that there was a significant correlation between two distance matrices based on the chemical compounds of essential oils and ISSR markers, confirming the congruence of interpopulation relationships in the *P. ovalifolia* revealed by the chemical and molecular markers.

Key words: Primula ovalifolia, Essential Oil, ISSR Markers

Introduction

The primrose genus *Primula* L., the largest genus in the family Primulaceae, comprises more than 400 species distributed mainly outside of the Asian highland center of diversity and occupies the mountains or high latitudes of North America, Europe, and Asia (Richards, 1993). Approximately 75% of these species are concentrated in the Himalayan mountain chain and western China (Richards, 1993; Hu, 1994). For example, *P. ovalifolia* Franchet distributed mainly in a band through central China to southwest China grows in moist shady places with the altitude between 1200–2500 m, and its natural populations co-occur with those of other primroses such as *P. obconica* (Hu and Kelso, 1996). Up to now, there are more than 1000 cultivars of *Primula* as popular ornamental house plants in the world. However, a variety of cutaneous reactions have also been described among many cultivars, particularly in Europe and the United States (Aplin and Lovell, 2001). Therefore, both the chemical compounds and genetic variations of *Primula* have attracted the attention of phytochemists, horticulturists and geneticists for a long time.

The chemical compounds including the allergic primin (2 -methoxy-6-pentyl-1, 4-benzoquinone) and miconidin (2-methoxy-6-pentyl-1, 4-dihydroxybenzene) were isolated from leaves, stems and flowers (including pedicel and calyx) of many cultivars of *Primula*, especially *P. obconica* (Horper and Marner, 1995, 1996; Krebs and Christensen, 1995; Christensen and Larsen, 2000a). More recently, the volatile oil of wild *P. obconica* collected from central China was analyzed and compared with that of some European cultivars (Nan *et al.*, 2002a). A total of 43 compounds including methyl 2,4-dihydroxy-6-methyl benzoate, methyl 2,6-dihydroxy-4-methyl benzoate and hypnone as major compounds were identified, and the wild *P. obconica* in China seems to be allergen-free due to the absence of primin and miconidin (Nan *et al.*, 2002a). The population-level genetic variations of *P. obconia* and *P. ovalifolia* in central and southwest China were also detected by using the Inter-Simple Sequence Repeats (ISSR) markers (Nan *et al.*, 2002b; Nan *et al.*, in press).

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Since the chemical composition of *P. ovalifolia* is still poorly known, here we initially investigated the population-level variability of the essential oils in *P. ovalifolia* from central and southwest China, and further analyzed the genetic relationships among the populations based on the ISSR markers. Finally, we examined the congruence of interpopulation relationships in the *P. ovalifolia* based on chemical and molecular markers.

Materials and Methods

Sampling

During July and August of 2001, five natural populations of *P. ovalifolia* in central (Hubei Province) and southwest (Sichuan Province) China were found and sampled: two populations D1 (31 °05′ N, 110 °52′ E, 1700 m) and D2 (31 °06′ N, 110 °57′ E, 1750 m) from Mt. Dalaoling of Yichang, Hubei, and three populations W1 (29 °41′ N, 102 °52′ E, 1360 m), W2 (29 °43′ N, 102 °57′ E, 1520 m) and W3 (29 °41′ N, 102 °58′ E, 1250 m) from Mt. Wawushan, Sichuan. The locations of the populations are shown in Fig. 1. The vouchers including seeds of the samples are deposited in South China Institute of Botany, Academia Sinica.

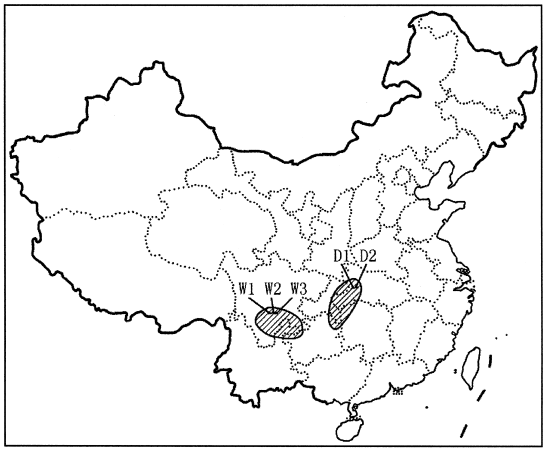

Fig. 1. Distribution (shadow) and sampled populations of *P. ovalifolia* in China. W1–W3 represent three populations at Mt. Wawushan, Sichuan; D1 and D2 represent two populations at Mt. Dalaoling, Hubei. For latitudes, longitudes and altitudes of the five populations see text.

Analysis of essential oils

The essential compounds were extracted from dried leaves/stems samples (0.5–1 g) 2 × with CH_2Cl_2 (1.5–3.0 ml) for 25 min, and filtrated (Christensen and Larsen, 2000a). Then, the extracts were stored in a 2-ml glass vial at −20 °C for gas chromatography-mass spectrometer (GC-MS) analysis.

The GC-MS analysis was performed on a combined GC-MS instrument (Finnigan Voyager, San Jose, CA, USA) using a HP-INNOWax (bondable polyethylene glycol) fused silica capillary column (30 m length, 0.25 mm diameter, 0.25 μm film thickness). A 1 μl aliquot of oil was injected into the column using a 15:1 split injection, which temperature was set up at 250 °C. The GC program was initiated by a column temperature set at 60 °C for 2 min, increased to 250 °C at a rate of 10 °C/min, held for 10 min. Helium was used as the carrier gas (1.0 ml/min). The mass spectrometer was operated in the 70 eV EI mode with scanning from 41 to 450 amu at 0.5 s, and mass source was set up 200 °C. The compounds were identified by matching their mass spectral fragmentation patterns with those stored in the spectrometer database using the National Institute of Standards and Technology Mass Spectral database (NIST-MS).

ISSR-PCR amplification

Total DNA extraction method used was modified CTAB protocol from Doyle and Doyle (1987). Fourteen ISSR primers were used for PCR amplification. Amplifications were performed in 10 μl volume of PCR mixture containing 10 ng of DNA template, 1 μl of the 10 × reaction buffer, 3 mM $MgCl_2$, 300 μM dNTPs, 0.25 μM primer, 2% formamide, and 1U *Taq* DNA polymerase (Genda Tech. Corp., Toronto, Canada). All reaction were overlaid with 1 drop of mineral oil and amplified in the thermocycler program for 45 cycles of denaturing for 45s at 94 °C; annealing for 45s at 51 °C or 52 °C; extension for 1.5 min at 72 °C. PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized and photographed under UV light. Molecular weights were estimated using a 100 bp DNA ladder (Shengong Inc., Shanghai, China) (Nan *et al.*, 2002b).

Data analysis

The Euclidean distance matrix based on the composition percentages of the essential oils from the five *P. ovalifolia* populations was constructed. The presence of a specific band of the ISSR markers was scored as 1 or 0 if absent. Nei (1972)'s index was used for calculating the genetic distances among the five populations and the average genetic differentiation (G_{st}) values were estimated for detecting the genetic diversity among populations and population groups, both with POPGENE 1.31 (Yeh *et al.*, 1999). Cluster analysis of the five populations based on each distance matrix was then performed using the unweighted pair-group method with arithmetic average (UPGMA) (Sneath and Sokal, 1973) with PHYLIP 3.57 (Felsenstein, 1997). The correlation between the two distance matrices was investigated by the Mantel test of matrix correspondence (Mantel, 1967; Sokal and Rohlf, 1995) with NTSYSpc 2.0 (Rohlf, 1998). Statistical significance of the Mantel test was determined by random permutations, with the number of permutation set to 1000.

Results and Discussion

The essential oils from five natural populations of *P. ovalifolia* in China were analyzed for quality and quantity using GC-MS (Table I). Twenty-two essential oil compounds were obtained, in which eighteen ones were identified and characterized on the basis of the mass spectra fragmentation pattern. The identified compounds including homologous series of phenols, hydroquinones, phytols, fatty acides and others ranged from 95% to 96% in the total of 22 compounds.

The composition analysis of the essential oils shows that phenolics have significant implications. Among the five populations, the percentages of phenolics over the total compounds in the five populations were 29%, 35%, 57%, 57%, and 62%, respectively (Table I). In particular, methyl acetyl hydroquinone varied from 0.2% in W3 to 58% in W1, diisobutyl phenol from 0.2% in W1 to 0.3% in W3, hydroquinone from 30% in W2 to trace quantities (< 0.1% detected) in W1, and acetyl hydroquinone from 0.2% in W3 to 55% in D2. However, dimethoxy phenol and methoxy phenol were

Table I. Chemical composition (%) of the essential oils of five populations of *P. ovalifolia* in China.

Compound	Population*				
	W1	W2	W3	D1	D2
Tetramethyl hexadecen-1-ol	9.91	17.44	7.91	14.61	11.85
Trimethyl pentadecanone	0.28	0.36	0.27	0.17	0.30
Methyl acetyl hydroquinone	58.21	3.86	0.20	0.39	0.36
Dimethoxy phenol	0.96	tr	tr	tr	tr
Diisobutyl phenol	0.19	0.25	0.29	0.20	0.28
$C_{11}H_{16}O_2$	0.36	0.18	0.20	0.28	0.17
Methoxy phenol	1.42	tr	tr	tr	tr
Dodecanoic acid	0.18	0.45	0.55	0.28	0.32
$C_{18}H_{28}O_3$	0.17	0.29	0.10	0.13	0.20
Phytol	1.52	3.34	2.79	1.89	2.37
Myristic acid	0.40	0.67	0.69	0.44	0.55
Palmitic acid	5.49	8.56	9.47	6.55	6.41
Hexadecenoic acid	tr	0.87	3.28	0.87	0.81
Hydroquinone	tr	30.37	28.29	6.21	1.43
Squalene	5.39	13.58	24.51	5.23	3.54
Acetyl hydroquinone	0.74	0.53	0.20	46.88	55.02
Octadecanoic acid	1.62	2.20	2.67	1.38	1.34
Octadecenoic acid	2.46	2.56	2.72	2.86	3.41
Octadecadienoic acid	4.55	4.32	5.58	3.28	3.42
$C_{18}H_{36}O_2$	1.12	2.19	1.27	1.16	2.97
Octadecatrienoic acid methyl ester	3.14	5.26	7.03	4.06	3.48
$C_{14}H_{22}$	1.89	2.70	1.97	3.20	1.77
Phenolices	61.51	35.03	28.97	53.69	57.09

* tr: trace quantities (< 0.1% detected).

	W1	W2	W3	D1	D2
W1	0	0.1921	0.2081	0.2049	0.1976
W2	0.1275	0	0.0806	0.1851	0.2025
W3	0.1356	0.0392	0	0.1986	0.1965
D1	0.1506	0.1087	0.1141	0	0.0937
D2	0.1605	0.1285	0.1323	0.0211	0

Table II. Two distance matrices of five populations of *P. ovalifolia* in China. The above diagonal shows Nei's genetic distances based on the ISSR markers; the diagonal shows below Euclidean distances based on the essential oil percentages.

found only in W1. When using the essential oil profiles in the five populations of *P. ovalifolia*, there appeared to may be three chemotypes: the methyl-acetyl-hydroquinone-rich (W1), hydroquinone-rich (W2 and W3), and acetyl-hydroquinone-rich (D1 and D 2) chemotypes. In addition, primin and related phenols such as miconidin and miconidin methyl ether (3,4-dimethoxy –5-pentylphenol) have been detected in many cultivars of at least 20 *Primula* species and reported as a cause of allergic contact dermatitis (Christensen, 2000a, 2000b; Aplin and Lovell, 2001). These allergic compounds, however, were not found in this study, implying the wild *P. ovalifolia* in China might provide a potential genetic resource for horticultural uses of primin-free *Primula.* Since the primin secretions of *Primula*, *e.g.*, *P. obconica*, were reported to be related with the morphological characters and the growing temperature of some cultivars (Higuchi *et al.*, 1999, 2000), further studies on biochemical diversity within *P. ovalifolia* are also needed.

The Euclidean distances among the five populations of *P. ovalifolia* ranged from 0.0211 to 0.1605 (Table II). The dendrogram of the populations based on the Euclidean distances and hierarchal cluster analysis further reveal the interpopulation relationships of *P. ovalifolia* (Fig. 2). Two pairs of populations, *i.e.*, D1 and D2, and W2 and W3, formed two groups, and W1 is distinctly related to them. More importantly, the three groups exactly correspond to the above-mentioned three chemotypes, marked as M, H, and A, respectively. This is compatible with that the secondary products of plants, *e.g.*, essential oils, can be a type of useful markers for inferring interpopulation relationships of plants (Egerton-Warburton *et al.*, 1998; Demetzos *et al.*, 2002).

On the other hand, the Nei's genetic distances among the five populations of *P. ovalifolia* based on ISSR markers ranged from 0.0806 to 0.2081 (Table II). The ISSR dendrogram constructed using Nei's genetic distances and UPGMA cluster analysis is likely congruent with that based on the chemical compounds (Fig. 2). The estimate of G_{st} was 0.5733, indicating that 57% of the total genetic variation existed among the five populations of *P. ovalifolia.* Considering two population groups according to their locations, *i.e.*, Hubei -group (D1 and D2) and Sichuan-group (W1, W2, and W3), the estimate of G_{st} was 0.2325. The higher level of genetic differentiation among the populations than that between the population groups might explain why W1 is distinctly related to the other four populations, regardless of their locations.

The Mantel test showed that there was a significant correlation between the two distance matrices based on the chemical compounds of essential oils and ISSR markers ($r = 0.9455$, $p < 0.030$). It confirms that there is a congruence of interpopulation relationships of *P. ovalifolia* in China revealed by the chemical and molecular markers. Usually, molecular markers that show differences in the whole plant genome are not necessarily related to a specific plant secondary compound. Our study suggests that ISSR markers can be found linked to the

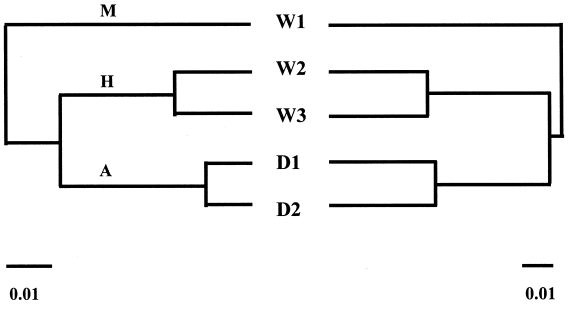

Fig. 2 Two congruent dendrograms of five populations of *P. ovalifolia* in China generated using the unweighted pair-group method with arithmetic average (UPGMA). The left one is based on the chemical compounds of essential oils. M, H, and A represent the methyl-acetyl-hydroquinone-rich chemotype, hydroquinone-rich chemotype, and acetyl-hydroquinone-rich chemotype, respectively; the right one is based on the ISSR markers.

essential oil compounds of *P. ovalifolia*. Obviously, further examination on the actual heritability of some essential oil compound is necessary to determine the actual linkage (Skoula *et al.*, 1999; Vieira *et al.*, 2001).

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