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Genetic relationships and molecular authentication of plant origins and the commercial medicinal herbs in peony using RAPD markers

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SUMMARY

Genetic polymorphism and molecular authentication were investigated with the commercial medicinal herb, Peony (*Paeonia spp.*), using random amplified polymorphic DNA (RAPD) markers. To identify the polymorphism of the RAPD patterns among plant origins, 20 different random primers were applied to the genomic DNA extracted from *Paeonia spp.* plants such as *Paeonia (P.) lactiflora, P. officinale* and *P. japonica*. Ten primers out of 20 primers could be used to discriminate the plant species in the same genus and 72 out of 81 scored DNA fragments (88.9%) generated with these primers were polymorphic. Especially, four primers, such as OPA1, OPA3, OP9, and OPA13, were useful to discriminate the plant origins among the species of Peony. In the results of cluster analysis using RAPD data obtained from the 10 primers, Peony (*Paeonia spp.*) plants used in this study were grouped into the two distinctive clusters, genetically. Herb medicine, especially *P. lactiflora*, were easily identified, when species-specific primers were applied to the investigation for discriminating herb medicine currently traded in domestic herb market, Kyungdongmart. Consequently, RAPD analysis was useful method to discriminate plant origins and the commercial medicinal herbs, *Paeonia spp.*.

Key words: Polymorphism; *Paeonia spp.*; Random amplified polymorphic DNA

INTRODUCTION

Peonies (*Paeonia spp.*) have been provided as useful medicine and attractive ornamental flower for over 3,000 years in East Asia and at least for 500 years in Europe. There are four species of this Ranunculaceae family plant that are utilized in traditional Chinese medicine under the general rubric of peony such as *Paeonia (P.) suffruticosa, P. lactiflora, P. veitchii*, and *P. obovata*.

All peony species are perennial and achieve heights up to nine meters (*P. suffruticasa* is somewhat larger). They have alternate, elliptical, smooth-edged leaves growing on smooth stems bearing two or more flowers. The large blossoms of peony can have a range of color and are generally 4 - 6 cm in diameter (Foster and Yue, 1992). The roots of all peonies are large, straight, and firm with easily peeled bark. Peonies contain glycosides (most notably paeoniflorin), flavonoids, proanthocyanidins, tannins, terpenoids, triterpenoids, and complex polysaccharides that may all contribute to its

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medicinal effects (Bensky *et al.*, 1993). It is commonly used in nourishing blood, activating circulation, alleviating pain, regulating menstruation, treating liver disease and cancer. The extract from *Paeonia* Radix can improve blood flow through its endothelium-dependent vasodilator action on aorta (Goto *et al.*, 1996) and inhibitory effect on thrombosis and platelet aggregation (Wang and Ma, 1990).

Peony is mainly used as a source of medicine together with *Panax ginseng* and *Angelica* Radix in Korea, but it is impossible to distinguish theses Chinese medicine when the rhizomes were manufactured as slices. Therefore, in herbal markets, the Chinese medicinal herbs have been illegally traded by either without the correct label or mixing with domestic medicinal herbs, resulting in big social problems. Finding a means to discriminate Chinese medicines when they were dried and sliced is a very important and imminent project in Korea.

Especially, in Korean pharmacopoeia, Peony is currently defined as the rhizome of either *P. lactiflora* Palls or same genus plants (The Korean Phamacopeia 8th ed., 2002). In the Chinese pharmacopoeia, however, both *P. lactiflora* and *P. veitchii* are defined as white peony and red peony, respectively. This discrepancy caused difficulties in the use of these herbal medicines.

Traditionally, histological and morphological inspections have been the usual methods of authentication, but they are not applicable to most forms of modern herbal drugs, herbal extracts, dried medicinal herbs and pills. Recently, genetic makeup inspection provides a definite answer to the botanical identity of the traditional Chinese medicine, as the genetic makeup of herbal species does not vary with their physical form, physiological and external conditions (Hon *et al.*, 2003).

During the last decade several novel DNA-markers have emerged, which have been rapidly used to common routine laboratory tools available for genome analysis. Random amplified polymorphic DNA technique (Williams *et al.*, 1990) has been utilized a useful means of investigating genetic diversity within and between populations and has been applied to many plants (Jover *et al.*, 2003; Ulloa *et al.*, 2003). RAPD requires very small quantities of DNA, and no cloning, sequencing or hybridization are necessary, for these reasons, it has a distinct advantage over other molecular techniques generally used for genomic characterization. Also once established RAPD-polymerase chain reaction (PCR) has the advantage of being quick and easy, requiring little plant material, and having a high resolution (Gugerli *et al.*, 1999; Bronzini *et al.*, 2002). Because of the efficiency and the convenience of this technique, we decided to analyze our peony accessions using RAPD technique.

The aims of this study are (1) to investigate the genetic variation and to select species-specific primers for differentiating plant origins and (2) to differentiate the commercial medicinal herbs of *Paeonia* species at DNA level.

MATERIALS AND METHODS

Materials and DNA extraction

Plant samples and the commercial medicinal herbs of peony collected from Uiseong Research Institute of Natural Product, Uiseong in Keongbuk province, and Kyungdongmart, domestic herbal market in Seoul, were used for RAPD analysis (Table 1). The plant samples (100 mg of fresh roots and 20 mg dried roots) were frozen in liquid nitrogen and ground in a mortar to become a fine powder. DNA was extracted using QIAGEN DNeasy Plant Kit according to the manufacturer instructions. The relative and concentration of extracted DNA was estimated with the help of Nano Drop ND-1000 (Dupont Agricultural Genomics Laboratory, America), and the final DNA concentration was adjusted to 10 ng/μl.

RAPD analysis

The RAPD analysis was carried out using the following mixture: genomic DNA (10 ng/ml) 2.0 μl,

Table 1. Information of the plant samples used for RAPD analysis

Entry No.	Species	Sites information	Note
1	P. lactiflora	Uiseong Research Institute of Natural Product	Cultivar
2	P. lactiflora	Uiseong Research Institute of Natural Product	Cultivar
3	P. lactiflora	Uiseong Research Institute of Natural Product	Collection lines
4	P. lactiflora	Uiseong Research Institute of Natural Product	Collection lines
5	P. officinale	Uiseong Research Institute of Natural Product	Collection lines
6	P. officinale	Uiseong Research Institute of Natural Product	Collection lines
7	P. officinale	Uiseong Research Institute of Natural Product	Collection lines
8	P. officinale	Uiseong Research Institute of Natural Product	Collection lines
9	P. japonica	Uiseong Research Institute of Natural Product	Collection lines
10	P. japonica	Uiseong Research Institute of Natural Product	Collection lines
11 - 30	Unknown	Kyungdongmart	Commercial medicinal herbs

primer (5 μM) 2.0 μl, dNTPs (250 μM total) 2.0 μl, *Taq*-polymerase (5/μl) 0.2 μl, 10 × buffer 2.5 μl, distilled water 16.3 μl, for a total of 25 μl reaction mixture. The *Taq*-polymerase and buffer were purchased from SOLGENT Inc. (Republic of Korea).

Twenty random primers supplied by OPERON Technologies Inc. (Alameda, CA) were used for the analysis. Amplification reactions were carried out on the DNA Thermal Cycler (PTC-200, MJ Research) subjected to 35 cycles of PCR as follows: 94°C, 1 min; 42°C, 30 s; 72°C, 1 min.

Amplification products were analyzed by electrophoresis on 1.5% agarose gel in 1 × TBE buffer and detected by ethidium bromide staining under UV lights. Only clear and distinct bands were scored both in agarose gels.

Statistical analysis

The RAPD bands were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity. Genetic similarities were calculated based on the Dice method (Nei and Li, 1979). Based on genetic similarity matrix, dendrograms were constructed using the cluster analysis of the unweighted pair group method with arithmetic averages (UPGMA). All analyses were performed with NTSYS-pc (version 2.11a).

RESULTS AND DISCUSSION

In order to select the species specific primer for identification and classification of *Paeonia* accessions at DNA level, RAPD techniques have been applied for those molecular biological researches.

To identify the variation of the RAPD patterns among *Paeonia* accessions, 20 different random primers were applied to each genomic DNAs of ten cultivar and accessions of *P. lactiflora*, *P. officinale*, and *P. japonica*.

To establish the optimum annealing temperature, ten annealing temperatures ranging from 36°C to 45°C, with one degree increase were tested with the PCR condition of 10 ng templet DNA, 4 μM primer, 1.0 unit *Taq* DNA Polymerase in a 25 μl total reaction volume. The optimum PCR condition obtained was as follow: two minutes at 94°C for the initial denaturation; and 35 cycles of 30 s at 94°C, 30 s at 42°C, 60 s at 72°C for amplification reaction; and a final five minutes at 72°C for extension (data not shown).

The percentage of polymorphic bands according to the application of selected primers was shown in Table 2. Ten primers out of 20 primers could be used to discriminate ten accessions of the Peonies. Total 72 polymorphic bands were determined from 81 scored DNA fragments amplified with

Table 2. The primer sequences and number of polymorphic bands generated with each primer

Primer	Nucleotide sequence (5' to 3')	No. of polymorphic bands	No. of PCR products
OPA01[a]	CAGGCCCTTC	8	9
OPA02	TGCCGAGCTG	10	10
OPA03	AGTCAGCCAC	7	9
OPA04	AATCGGGCTG	7	7
OPA09	GGGTAACGCC	13	13
OPA10	GTGATCGCAG	3	7
OPA13	CAGCACCCAC	7	7
OPA17	GACCGCTTGT	7	7
OPA18	AGGTGACCGT	6	7
OPA19	CAAACGTCGG	4	5
	Total	72	81

[a]Primers that reveal polymorphic fragments among *Paeonia* species.

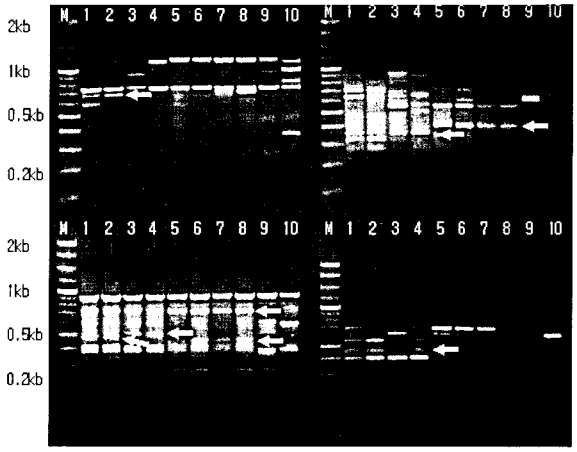

Fig. 1. Profiles of PCR products obtained from genomic DNA using the 10-based OPERON primers, OPA1, OPA2, OPA3 and OPA4. Lane M, 100 bp DNA ladder; Lane 1 - 2, Uiseong peony cultivar (*P. lactiflola*); Lane 3 - 4 ; regional collected line (*P. lactiflola*); Lane 5 - 8, regional collected line (*P. officinale*); Lane 9 - 10, regional collected line (*P. japonica*).

these primers, 88.9% of which were polymorphic. The number of polymorphic bands ranged from five to thirteen per primer and the size of amplified products varied between 0.2 kbp and 1.4 kbp.

Fig. 1 showed an example of the polymorphic bands amplified with primer OPA1, OPA2, OPA3 and OPA4 within *Paeonia* plant species. Especially, OPA1 and OPA3 primers showed specific bands for Uiseong cultivar, which could be useful for discriminating from other *Paeonia* accessions. The molecular weights of specific bands were 0.7 kb, and 0.4 kb, respectively. Also, the result generated by OPA9 and OPA13 primers showed *P. lactiflora* species specific band at 0.2 kb.

Therefore, RAPD markers derived from this study will be useful tool for differentiating the Uiseong cultivar and plant origin of *P. lactiflora* from other accessions, such as *P. officinale* and *P. japonica*, at DNA level.

The RAPD technique is sensitive to reaction conditions, which results in poor reproducibility. To overcome the problems associated with RAPD markers and to improve their utility in marker-assisted selection (MAS), longer primers have been developed from RAPD fragments (Paran and Michelmore, 1993). These longer primers generate a sequence-characterized amplified region (SCAR), which can be particularly useful to follow the inheritance of the marked region of the genome. SCAR markers are preferred over RAPD markers as they detect only a single locus, their amplification is less sensitive to reaction condition, and they can potentially be converted into allele-specific markers. SCAR markers have been developed for many crops (Deng *et al.*, 1997; Hernandez *et al.*, 1999; Ardiel *et al.*, 2002).

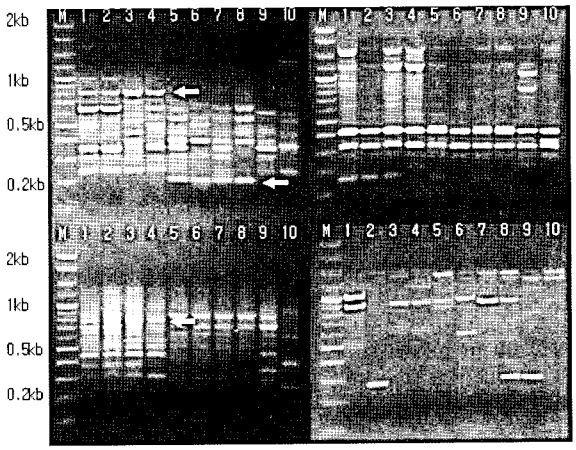

Fig. 2. Profiles of PCR products obtained from genomic DNA using the 10-based OPERON primers, OPA9, OPA10, OPA13 and OPA17. Lane M, 100bp DNA ladder; Lane 1 - 2, Uiseong peony cultivar (*P. lactiflora*); Lane 3 - 4 ; regional collected line (*P. lactiflora*); Lane 5 - 8, regional collected line (*P. officinale*); Lane 9 - 10, regional collected line (*P. japonica*).

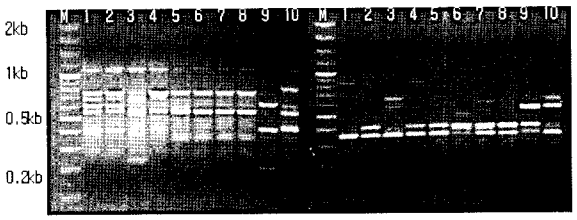

Fig. 3. Profiles of PCR products obtained from genomic DNA using the 10-based OPERON primers, OPA18 and OPA19. Lane M, 100bp DNA ladder; Lane 1 - 2, Uiseong Jakyark peony (*P. lactiflora*); Lane 3 - 4; regional collected line (*P. lactiflora*); Lane 5 - 8, regional collected line (*P. officinale*); Lane 9 - 10, regional collected line (*P. japonica*).

Bang (2003) developed SCAR markers from RAPD clones and these two markers were enough to discriminate between *Atractylodes (A.) japonica* and *A. macrocephala*, as well as between Korean and Chinese herbal medicines obtained from Korean herbal markets.

According to our previous and current results of RAPD analysis, new SCAR markers will be created with specific DNA bands for Uiseng cultivar through cloning and sequencing process. The SCAR markers could be powerful tools to breed cultivar as the selection marker. Also, in preparing for liberalization of trade, it will be able to contribute to the security of foreign intellectual property right for domestic breeding cultivar.

Total 81 reproducible band types were scored and analyzed for computation of similarity matrix and genetic distance/relatedness. Genetic distances were evaluated ranging from 0.92 to 0.06 (Table 3). The smallest genetic distance was observed between *P. officinale*-derived accessions Po5 and Po7 and the largest between *P. japonica*-derived accession Pj10 and *P. officinale*-derived accession Po6.

Genetic distance values were used to construct a dendrogram. Cluster analysis of RAPD markers showed that *P. lactiflora* and *P. officinale* were obviously

Table 3. Similarity indices of ten *Paeonia* accessions based on RAPD data

Accessions	Similarity indices (S.I)									
	Pl 1	Pl 2	Pl 3	Pl 4	Po 5	Po 6	Po 7	Po 8	Pj 9	Pj 10
Pl 1	1.000									
Pl 2	0.868	1.000								
Pl 3	0.520	0.490	1.000							
Pl 4	0.652	0.711	0.619	1.000						
Po 5	0.348	0.356	0.381	0.474	1.000					
Po 6	0.227	0.279	0.293	0.389	0.778	1.000				
Po 7	0.340	0.348	0.326	0.462	0.923	0.757	1.000			
Po 8	0.292	0.340	0.364	0.400	0.900	0.737	0.878	1.000		
Pj 9	0.341	0.400	0.316	0.303	0.242	0.194	0.235	0.286	1.000	
Pj 10	0.140	0.190	0.150	0.114	0.114	0.061	0.111	0.162	0.200	1.000

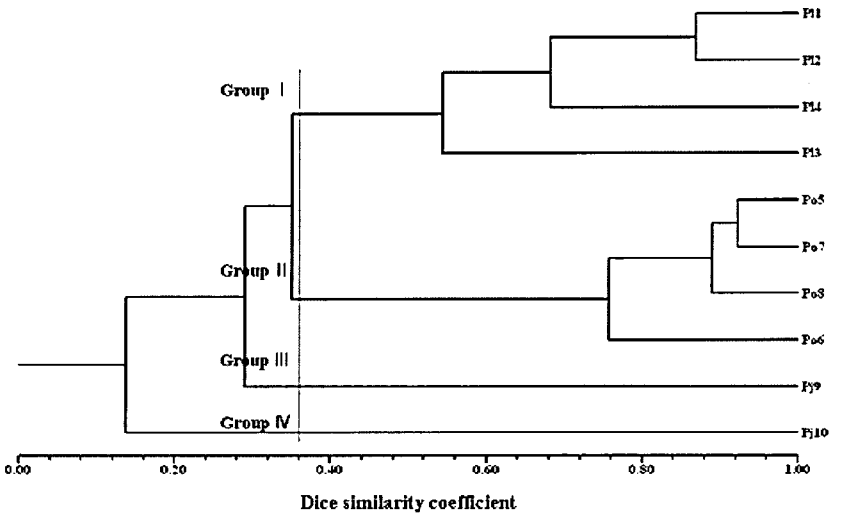

Fig. 4. Dendrogram of ten *Paeonia* accessions based on Dice similarity coefficient by UPGMA analysis.

classified into two different groups, while accessions from *P. japonica* did not cluster as a clade. Consequently, the RAPD technique was a useful method for discriminating the plant origins *P. lactiflore* and *P. officinale* of plant origins from *P. japonica* (Fig. 4).

There were limited number of studies conducted with molecular markers in the peonies compared with other major crops and medicinal herbs. Kim *et al.* (1997) only reported that RAPD is an efficient tool for phylogenetic grouping of the peonies as shown in our current results in this study.

Recently, as the importing amounts of cheap Chinese medicines have been increased, the distribution of inferior and mixed herbal medicines at Korean herbal market is expected. Thus, it is indispensably important issue to establish the distribution system on the basis of discrimination of the herbal medicines and quality control.

In order to differentiate the commercial medicinal herbs, called white peony, peeled peony or great peony, compared with *Paeonia* plant origins at DNA level, RAPD based selective primers have been applied for those molecular biological researches.

The PCR results of the commercial medicinal herbs by primer OPA9 and OPA4 were shown in Figs. 5 and 6. The result generated by OPA9 primer

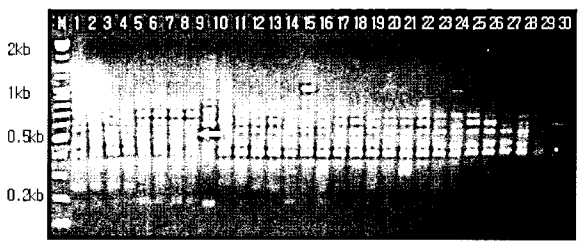

Fig. 5. Profiles of PCR products obtained from genomic DNA using the 10-based OPERON primer, OPA04. Lane M, 100bp DNA ladder; Lane 1 - 2, Uiseong peony cultivar (*P. lactiflora*); Lane 3 - 4; regional collected line (*P. lactiflora*); Lane 5 - 8, regional collected line (*P. officinale*); Lane 9 - 10, regional collected line (*P. japonica*); Lane 11 - 30, commercial medicinal herbs.

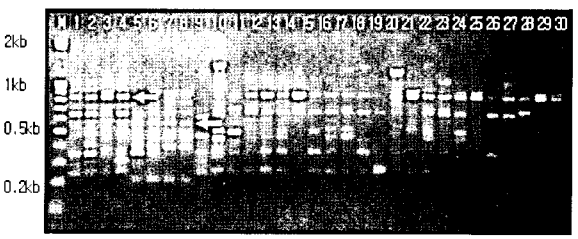

Fig. 6. Profiles of PCR products obtained from genomic DNA using the 10-based OPERON primer, OPA09. Lane M, 100bp DNA ladder; Lane 1 - 2, Uiseong peony cultivar (*P. lactiflora*); Lane 3 - 4; regional collected line (*P. lactiflora*); Lane 5 - 8, regional collected line (*P. officinale*); Lane 9 - 10, regional collected line (*P. japonica*); Lane 11 - 30, commercial medicinal herbs.

showed a specific band for *P. lactiflora* in all Chinese medicine at 0.8 kb, whereas no products were detected specific bands for *P. officinale* at 0.2 kb and 0.7 kb. Also, the result by OPA4 primer showed in all Chinese medicine, which could be useful for discriminating from other *Paeonia* accessions, *P. officinale* and *P. japonica*. The molecular sizes of specific bands were at 0.4 kb, 0.45 kb, 0.55 kb and 0.7 kb, respectively. Meanwhile the PCR results for *P. japonica* showed different banding pattern compared with other accessions, *P. latiflora* and *P. officinale*.

Consequently, the result of application of the species specific primers to investigate current status in domestic herb market, Kyungdongmart, it was identified to be current herb medicines of *P. lactiflora*.

Consequently, RAPD analysis using selective primers was useful method to discriminate plant species of origins in the commercial medicinal herbs. However, for the purpose of overcoming a weak point of RAPD analysis, which occasionally results in poor reproducibility, further studies will be necessary using precise and up-to-date techniques such as SCAR marker or single nucleotide polymorphism (SNP) marker for the application to gene level. In the mean time, it is impossible to distinguish the origins and species of medicinal herbs when the medicinal herbs in the same genus are cultivated at different cultivation area, resulting in another social problem. Thus, it will be also important and imminent project to develop the efficient method for provenance discrimination.

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