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Genetic Relationships of *Panax* Species by RAPD and ISSR Analyses

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ABSTRACT : This study was carried out to develop convenient and reproducible methods for identifying the genetic relationship among germplasms of *Panax* species based on molecular genetics. Using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analyses, genetic polymorphism of the *Panax* species was investigated with following cultivars and accessions, such as Chunpoong, Yunpoong, Kopoong, Sunpoong, and Kumpoong in domestic cultivars, Hwangsuk, Jakyung and Suckju in domestic accessions, and *Panax quinquefolius* L. and *Panax japonicus* C.A. Meyer in foreign introduced accessions, respectively. Specific DNA fragments ranging from 200 to 3,000 base pairs in size could be obtained with various ISSR and RAPD primers under the optimized PCR conditions. The dissimilarity coefficients among the genetic polymorphisms of ginseng cultivars and accessions were calculated from 0.26 to 0.90 in RAPD and from 0.12 to 0.89 in ISSR analysis, respectively. Eleven plant samples were grouped siblings together with cultivars and parents based on cluster analysis of genetic distance depending on genetic property such as origin of the species. In results, both RAPD and ISSR analyses were useful for identifying the genetic relationship among cultivars and accessions of *Panax* species at DNA level.

Key words : Panax ginseng, genetic relationships, genetic polymorphism, ISSR, RAPD

INTRODUCTION

The therapeutic value of traditional Oriental medicines, especially on chronic diseases, has won wide acknowledgement in the West in recent decades. Especially, because of their medicinal effects, several medicinal substances contained in the *Panax* species were highly treasured in the Orient. Both Asian Ginseng (*Panax ginseng* C.A. Meyer) and American Ginseng (*P. quinquefolium* L.) have been used as traditional medicine in Asian for thousands of years and in western countries for recent decades. In order to identify the differences of Korean ginseng, morphological and histological examinations have been traditionally used. But in many cases, such an approach is far from reliable in the authentication of different *Panax* species. The roots of the *Panax* species are very similar in morphological appearance. Furthermore, because many commercial ginseng products are traded as powder or as shredded material, it is difficult or impossible to distinguish the origin of the source by traditional discrimination method.

Practically, various DNA manipulation techniques, such as RAPD, AFLP, SSR, ISSR, and sequence based SNP, have been developed according to the advances in molecular biotechnology. These techniques can potentially be used to overcome the difficulties for authentication of many oriental herbal medicines. Genetic authentication provide a definite answer to the botanical identity of the oriental herbal medicines, because the genetic marker of a herbal species does not vary depending on their physical form, physiological, and external conditions. Authentication of herbal plants is prerequisite to classify oriental herbal drugs for preventing their misapplication.

In genetic analysis of plant species, random amplified polymorphic DNA (RAPD) technique has several advantages over restriction fragment length polymorphism (RFLP) analysis, namely speed, low cost, and the ability to analyze small amounts of samples (Tochika-Komatsu *et al.*, 2001). RAPD analysis in genus *Panax* has become a general method for estimating genetic diversity and variation among plant and cultivars (Wang *et al.*, 2001; Shaw and But., 1995; Shim *et al.*, 2003).

On the other side, ISSR markers involve the DNA segments amplified by PCR using single primers which composed of microsatellite sequences. These primers target microsatellite regions which are abundant throughout the eukaryotic genome (Tautz and Renz, 1984; Zietkiewicz *et al.*, 1994). Furthermore, this technique is as simple as RAPD analysis, since ISSR marker is based on PCR. Thus, ISSR technique has been recently used to genetic analysis of many important crop

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plants as follows ; identification of the cultivars in potato (Prevost and Wilkinson. 1999), determination of genetic diversity and phylogenetic relationships in *Oryza* (Joshi *et al.*, 2000), and identification of the markers related with seed size in wheat (Ammiraju *et al.*, 2001), respectively. But in *Panax* species, ISSR technique was tried only once for estimating genetic diversity and variation among Korean cultivars (Bang *et al.*, 2004).

In the present study, we report the convenient and reproducible methods for RAPD and ISSR analyses followed by identification of the genetic relationship among cultivars and accessions of *Panax* species originated from Korea and foreign countries with the techniques at DNA level.

MATERIALS AND METHODS

Plant materials

In oder to identify the genetic relationships among the *Panax* species in DNA level, 9 Korean cultivars and accessions of *P. ginseng* C.A. Meyer and 2 foreign introduced *Panax* species, which were improved at the preservation field of medicinal herb plant in National Institute of Crop Science, Suwon, in Korea, were used as follows; Chunpoong, Yunpoong, Kopoong, Sunpoong, Kumpoong, Hwangsuk, Jakyung, Suckju and Mimakhi were included in Korean cultivars and accessions of *P. ginseng* C.A. Meyer and *P. quinquefolius* L. and *P. japonicus* C.A. Meyer were foreign introduced accessions.

DNA extraction

Fresh leaf samples (100 ㎎) 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 (Dellaporta, 1983; Doyle and Doyle, 1987).

Optimization of PCR conditions in RAPD and ISSR

For optimizing PCR condition, various concentrations of *dNPT, Taq*-polymerase, and DNA were tested in combination with analyzing annealing temperature with gradient PCR ranging from 32 ℃ to 52 ℃ for RAPD and from 42 ℃ to 64 ℃ for ISSR, respectively.

RAPD analysis

Twenty RAPD primers (OPA series) supplied by Operon Co. Ltd. were used for analysis. The RAPD analysis was conducted with total 20 ㎕ of reaction mixture as follows : 2 ㎕ of genomic DNA (0.1 ng/ ㎕), 2 ㎕ of primer (10 pmole), 2 ㎕ of *dNTP* (10 mM), 0.1 ㎕ of *Taq*-polymerase (5 Unit/ ㎕), 2 ㎕ of 10×buffer, 11.9 ㎕ of distilled water. The *Taq*-polymerase and other reagents were purchased from BIONEER (Korea). Amplification reactions were carried out on the PTC 200 thermal Cycler (MJ research) subjected to an initial five minutes at 94℃, followed by 35 reaction cycles of 60 seconds at 94 ℃, 60 seconds at 42 ℃, 60 seconds at 72 ℃, and a final 7 minutes at 72 ℃. Amplification products were analyzed by electrophoresis on 1.8% agarose gel in 0.5×TBE buffer and detected by ethidium bromide staining under UV-lights.

ISSR analysis

Thirty-five ISSR primers supplied by NAPS Unit standard primers (UBC primer set #9) were used for the analysis. The ISSR analysis was conducted with total 20 ㎕ of reaction mixture as follows : 2.5 ㎕ of genomic DNA (0.1 ng/㎕), 2.5 ㎕ of primer (10 pmole), 0.5 ㎕ of *dNTP* (10 mM), 0.1 ㎕ *Taq*-polymerase (5 Unit/㎕), 2 ㎕ of 10×buffer, 12.4 ㎕ of distilled water. Amplification reactions were carried out on the PTC 200 thermal Cycler (MJ research) subjected to an initial five minutes at 94℃, followed by 44 reaction cycles of 30 seconds at 94 ℃, 45 seconds at 54 ℃, 60 seconds at 72 ℃, and a final 7 minutes at 72℃. Amplification products were analyzed by electrophoresis on 2.0% agarose gel in 0.5×TBE buffer and detected by ethidium bromide staining under UV-lights.

Statistics analysis

Hierarchical analysis was performed with average linkage method and binary distance measures using package '*hclust*' in R language ver 2.1.1. The binary measures, *aka* asymmetric binary, regarded the vectors as binary bits, so non-zero elements are 'on' and zero elements are 'off'. The distance regards as the proportion of bits in which only one is on amongst those in which at least one is on.

RESULTS AND DISCUSSION

Optimization of RAPD and ISSR Conditions

The conditions for polymerase chain reactions were established prior to identify the genetic relationship among plant species by RAPD and ISSR, since it affects the pattern of PCR products. The optimized conditions were established as follows: 0.25 ng of DNA, 200 mM of dNTP, 0.5 units of *Taq*-polymerase, and 45 cycles of reactions (data not shown). Gradient PCR technique made simple for optimization PCR conditions. Annealing temperatures established in the experiment led to achieve more sensitive and clearer bands than in other temperature ranges. Optimized annealing temperatures of RAPD and ISSR were 42℃ and 54℃, respectively. Annealing temperatures for RAPD reported by many of other researchers were ranged from 35 ℃ to 55 ℃ (Kim and Choi, 2003; Lim *et al.*, 1993; Shoyama et al., 1997). In general, annealing temper-

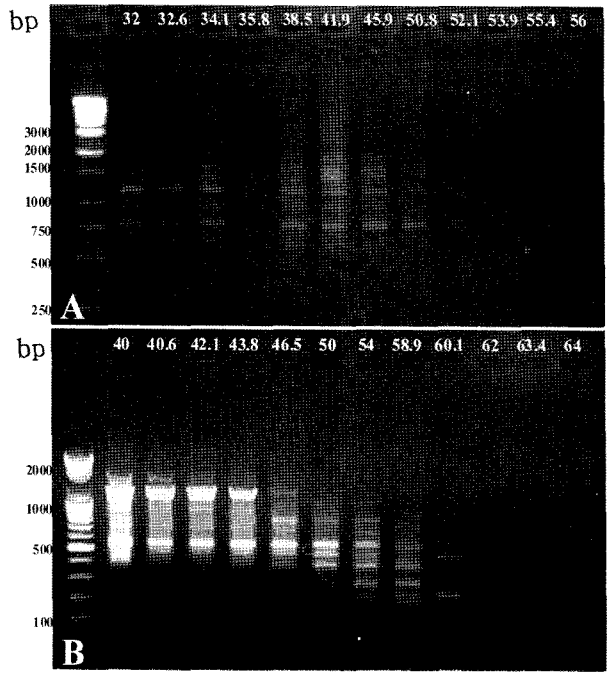

Fig. 1. Gradient PCR Profiles of RAPD and ISSR generated by Operon primers A1(A) and UBC809 (B), using total genomic DNA of *P. ginseng* cultivar, Yunpoong. Numbers indicate annealing temperature (℃).

ature for ISSR analysis was generally higher than for RAPD analysis. It has been known that high annealing temperature lead to more conserved and clearer band patterns. High annealing temperature led to more conserved and clearer band patterns as shown in Fig. 1.

RAPD polymorphisms

Eleven ginseng plants were tested with the twenty random primers. Amplified DNA fragments ranging in size from 100 to about 3,000 base pairs were scored and average of 8 fragments per primer were represented as shown in Fig. 2.

With various primers, clear specific bands were produced in each cultivar and/or accession of *Panax* species and the results were reproducible in repeated experiment. As shown in Fig. 2, specific bands of *P. japonicus* C.A. Meyer and *P. ginseng* Suckju were produced with RAPD primers, such as A1 and A10. In recent years, randomly sequenced primer has been considered to be less important for genetic studies due to its discrimination ability. However, when optimum primers are selectively used, this technique is still useful for investigation of genetic relationship among the *Panax* species.

ISSR polymorphisms

In oder to identify efficient primers, total genomic DNA of the plant samples were amplified with 35 ISSR primers. When

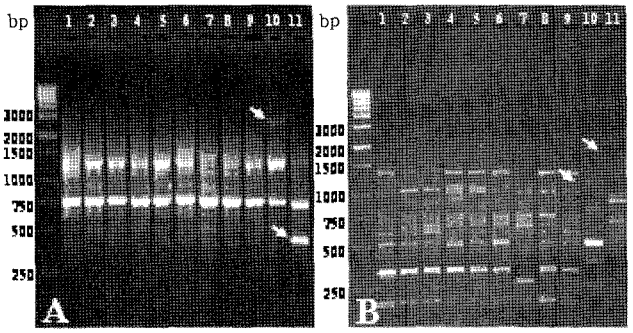

Fig. 2. RAPD polymorphisms of plant samples, *Panax* species, amplified by Operon primers. A: Operon A1, B: Operon A10, 1: *P. ginseng* Chunpoong, 2: *P. ginseng* Yunpoong, 3: *P. ginseng* Kopoong, 4: *P. ginseng* Sunpoong, 5: *P. ginseng* Kumpoong, 6: *P. ginseng* Mimakhi, 7: *P. quinquefolius* L., 8: *P. ginseng* Hwangsuk, 9: *P. ginseng* Jakyung, 10: *P. japonicus* C.A. Meyer, 11: *P. ginseng* Suckju. Arrows indicate specific DNA bands of cultivars or accessions mentioned.

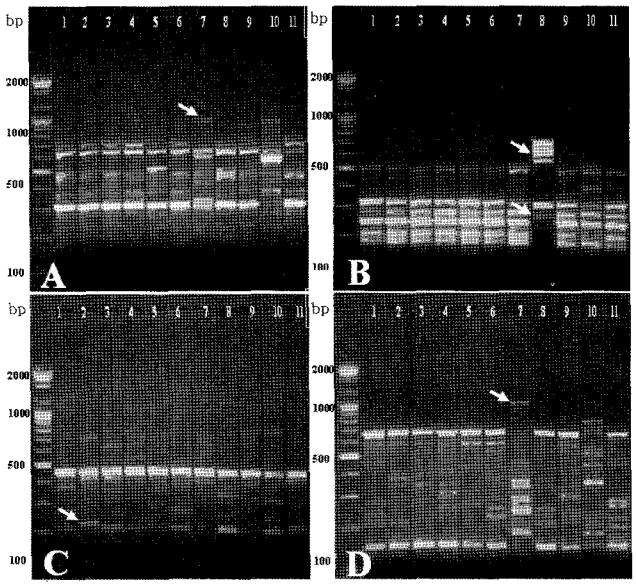

Fig. 3. ISSR polymorphisms of plant samples, *Panax* species, amplified by UBC808 (A), UBC810 (B), UBC848 (C) and UBC850 (D) primers. 1: *P. ginseng* Chunpoong, 2: *P. ginseng* Yunpoong, 3: *P. ginseng* Kopoong, 4: *P. ginseng* Sunpoong, 5: *P. ginseng* Kumpoong, 6: *P. ginseng* Mimakhi, 7: *P. quinquefolius* L., 8: *P. ginseng* Hwangsuk, 9: *P. ginseng* Jakyung, 10: *P. japonicus* C.A. Meyer, 11: *P. ginseng* Suckju. Arrows indicate specific DNA bands of cultivars or accessions mentioned.

DNA fragments ranging in size from 100 to about 3000 base pairs were scored, average 7 fragments per primer were represented as shown in Fig. 3. But polymorphisms of amplified DNA with ISSR primers were more distinguishable than those in RAPD analysis. Furthermore, in repeated experiment, many specific band patterns of *P. ginseng* could represent constantly

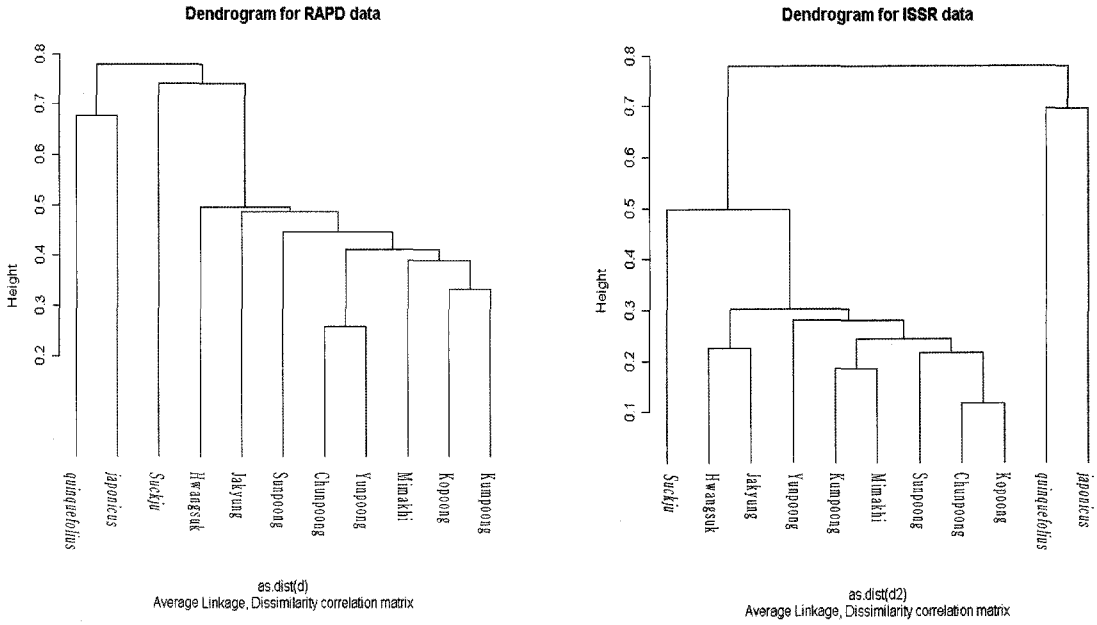

Fig. 4. Total dissimilarity dendrogram in *Panax* species including different cultivars and accessions based on RAPD(A) and ISSR(B) analysis. Korean domestic cultivars and accessions are classified into *P. ginseng* C.A. Mayer. Chunpoong, Yunpoong, Kopoong, Sunpoong, and Kumpoong are included in domestic cultivars and Hwangsuk, Jakyung, and Suckju are included in domestic accessions. *P. quinquefolius*. L. and *P. japonicus* C.A. Meyer are foreign introduced accessions of *Panax* species.

their own cultivars. In recent, Bang *et al.*, (2004) reported the effectiveness of specific ISSR primers to authenticate the Korean improved cultivars of *P. ginseng* with a few of Korean accession. However, the results obtained here was more reliable, since more numerous germplasms of *Panax* species were tested in the present study.

Genetic relationships of *Panax* species

Genetic relationships among cultivars and accessions of *Panax* species were investigated by RAPD and ISSR analyses based on amplified DNA polymorphism. Many ISSR and RAPD primers were selected as the optimum primers, with which clear and reproducible DNA fragments could be produced. The dissimilarity coefficients among the DNA of ginseng cultivars and accessions were calculated from 0.26 to 0.90 in RAPD and 0.12 to 0.89 in ISSR analysis. Cultivars and accessions of *Panax* species having similar genetic properties were grouped siblings together by Hierarchical Cluster analysis based on genetic distance estimates (Fig. 4.).

Genetic cluster of outer group of *Panax* species, such as *P. japonicus* C.A. Meyer and *P. quinquefolius* L., were distinctive comparing those of cultivars and accessions of *P. ginseng*. Cluster analysis based on RAPD and ISSR profiles revealed that the three species were almost equidistant from each other. Our results represented in this study were similar to the results of genetic authentication of *Panax* species reported by Shaw and But (1995). Different *Panax* species could be clearly identified by RAPD analysis. However, it is difficult to identify the genetic relationships among the cultivars or to authenticate the source within inner group of *P. ginseng*, since relatively low resolution or reproducibility of the method might be acted as limited factors (Hon et al., 2003). Hwangsuk and Jakyung, korean accessions of *P. ginseng*, were known as parental group of *P. ginseng* cultivars. Thus, genetic distances among the accessions and/or cultivars within the species, *P. ginseng*, were relatively closer than those between the different *Panax* species. As shown in Fig. 4, genetic dissimilarity coefficients among cultivars and accessions of *P. ginseng* by ISSR analysis was lower than those by RAPD analysis. Since the results might be slightly changed depending on the choice of plant species and PCR methods, further studies for understanding principles of each method of PCR with different primers should be needed prior to conducting molecular genetic studies.

Many scientists have tried to distinguish the molecular genetic properties of *Panax* species. Although some researchers reported that the genetic properties of the plants were very conserved (Kim and Choi, 2003; Zhuravlev et al., 1998). Seo *et al.*, (2003) reported that genetic diversities were existed within genus *Panax*. However, it is hard to mention that which results was more reliable, since their results were obtained under the different experiment condition. Thus, further studies should be conducted to overcome these difficulties.

As results in the present study, although genetic differences were rarely existed among the individual plants within the cultivars or accessions, it could be considered as a minor problem, since clear specific DNA bands were generally reproduced with optimum primers in repeated experiments (data not shown). Also, we certainly established the efficient methods for identifying genetic relationships among the cultivars and accessions of genus *Panax* by RAPD and ISSR analyses and, even for authentication of the cultivars of *P. ginseng* in further studies. Furthermore, these specific DNA fragments obtained with optimum primers will be sequenced followed by specific primers will be designed. These will be possible to develop identification system of ginseng cultivars and accessions. RAPD and ISSR analyses derived from this study will be useful for the identification of cultivars and accessions of *Panax* species at DNA level. Based on the present results, SSR, AFLP, and sequence based SNP are carrying out to establish the marker system for the authentication system of *P. ginseng*.

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