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Molecular discrimination of *Panax ginseng* species

Jae-Young Um[1,2], Hwan-Suck Chung[1], Hyun-Ju Kim[3], Dae-Ki Kim[4], Kyung-Shik Shim[1], Kang-Yong Lee[1], Jeong-Sook Kim[1], Tae-Jin Choi[3], Nam-Song Kim[5], Nyeon-Hyoung An[1], Kang-Min Lee[2], Young-Mi Lee[1] and Jeong-Joong Kim[3*]

[1]College of Pharmacy, Wonkwang University, Iksan, Chonbuk 570-749, South Korea, [2]Division of Biological Sciences, College of Natural Science, Chonju, Chonbuk 561-756, South Korea, [3]Department of Anatomy, Wonkwang University School of Medicine, Iksan, Chonbuk 570-749, South Korea, [4]Laboratory of Allergic Diseases, NIAID, NIH, Bethesda, MA 20852, USA, [5]Department of Preventive Medicine, Wonkwang University School of Medicine, Iksan, Chonbuk 570-749, South Korea.

SUMMARY

In order to develop convenient and reproducible methods for identification of ginseng drugs at a DNA level, RAPD (randomly amplified polymorphic DNA) and PCR-RFLP (PCR-Restriction fragment length polymorphism) analysis were applied within *Panax* species. To authenticate *Panax ginseng* betvyeen Chinese and Korean ginseng population, RAPD analysis were carried out using 20 mer-random primer. The similarity coefficients among the DNA of ginseng plants analyzed were low, ranging from 0.197 to 0.491. In addition, using PCR-RFLP analysis, very different fingerprints were obtained within Korean ginseng plants. These results suggest that these methods are able to authenticate the concerned *Panax* species. Broader application of this approach to authenticate other morphologically similar medicinal materials is rationalized.

INTRODUCTION

The genus *Panax ginseng* belongs to the family Araliaceae. Three of the species are important herbal medicines in the Orient. *P. ginseng* C. A. Meyer (oriental ginseng) and *P. quinquefolius* L. (American ginseng) are used as a tonic for anti-stress, anti-fatigue, and anti-aging purposes. *P. notoginseng* (Burkill) Chen (Sanchi) are used as hematostatic for hemorrhage (Chang *et al.*, 1986). The ginseng trade is a big business. The illegal practice of disguising Korean ginseng as Chinese ginseng has become a common problem in recent years. Obviously an effective program of authentication of Korean herbs including ginsengs is essential to the healthy development of the herbal industry. Traditionally, the authentication of Korean herbs relies upon morphological and histological inspection (Eames, 1961; Radford *et al.*, 1974). In many cases, as in authentication of different *Panax* species, such an approach is far from reliable. The roots of Chinese and Korean ginseng are

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*Correspondence : Prof. Jeong-Joong Kim, Department of Anatomy, Wonkwang University School of Medicine, Iksan, Chonbuk 570-749 South Korea, TEL : 82-63-850-6760; Fax : 82-63 -850-6760; E-mail: jjkim@wonkwang.ac.kr

very similar in morphological appearance. Furthermore, many commercial ginseng products are in the form of powder or shredded slice, rendering their authentication by morphological and histological methods very difficult. Methods have been developed to authenticate ginseng samples by examination of their ginsenoside profiles (Yip *et al.*, 1985 a; Yip *et al.*, 1985 b). However, the application of chemical analysis may be limited as the amount and the profiles of ginsenosides are affected significantly by growth conditions as well as many other variables such as the storage condition, the freshness of the samples and the different post-harvest processing. In addition the chemical method demands a large quantity of material for proper analysis.

In recent years, RAPD (randomly amplified polymorphic DNA) analysis has become a popular method for estimating genetic diversity in plant populations or cultivars (Williams *et al.*, 1990; Halward *et al.*, 1992; Jain *et al.*, 1994; He *et al.*, 1995; López-Braña *et al.*, 1996; Ramser *et al.*, 1996). Furthermore, the RAPD technique has several advantages over isozyme and RFLP (restriction fragment length polymor-phism) analyses, such as speed, low cost, and the use of small amounts of plant material (Jain *et al.*, 1994; Heun *et al.*, 1994; Becerra Velàsques *et al.*, 1994). Lin, *et al.* had reported that RAPD markers were used to assess genetic relationships and variation among ecotypes of the turfgrass seashore paspalum (*Paspalum vaginatum Swartz*) (Lin . *et al.*, 1994).

The 18S rRNA genes of their original plants, *P. ginseng*, *P. japonicus* and *P. quinquefolius* of the family Araliaceae, were found to be of 1,809 base pairs, possessing unique sequences, respectively (Fushimi et al., 1996 a; Fushimi et al., 1997 b). Hence, to ensure the completion of the authentication, PCR-RFLP was performed in Korean ginseng plants.

MATERIALS AND METHODS

Plant materials

Dried roots of four *Panax* ginseng were obtained from China, and the others from Keum-san, Jin-an and Kang-hwa in Korea, respectively.

Extraction of plant DNA

Genomic DNA was extracted from the ginseng plants using the protocol modified from Fushimi *et al.* (1997). Ground sample of dried roots was washed in PBS-EDTA solution consisting of 140mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 and 5.4mM EDTA (pH 7.0). To this precipitate were added 10 vol. of extraction buffer consisting of 100mM Tris-HCl (pH 8.0), 40mM EDTA and 250mM NaCl, 1 vol. of 10% sodium N-lauroyl sarcosine and 1mg of proteinase K. The mixed solution was incubated for 12h at 37℃ with shaking. The resulting clear lysate was extracted by water-saturated Phenol-$CHCl_3$ (1:1), followed by the addition of 0.1vol. of 10% cetyl trimethylammonium bromide (CTAB) and 0.1vol. of 5M NaCl, and was further extracted by $CHCl_3$. Then, 0.1vol. of 3M NaOAC and 2.5vol. of absolute ethanol were added into the isolated aqueous phase The resulting pellet composed of the nucleic acid was resuspended in TE-buffer consisting of 10mM Tris-HCl and 1mM EDTA (pH 8.0). This solution was incubated for 30min at 37℃ with 10ng/ml of RNase A (Sigma, U.S.A.) and the DNA was purified by Shepadex G-50 (Sigma, U.S.A.).

RAPD

A total of twelve 20-mer random primer (SRILS Uniprimer™; Seolin Science) were

used in the RAPD analysis. Each 30μl PCR mixture contained 200ng DNA, 50mM KCl, 10mM Tris-HCl (pH9.0), 200μ M dNTP, 1.5mM $MgCl_2$, 150ng of random primer, and 2.5U of Taq polymerase (Takara). Amplification was performed in a Cyclogene Thermal Cycler for 35 cycles. The initial cycle was 5min at 94℃. Subsequent cycles were 1min at 94℃, 1min at 55℃, and 2 min at 72℃, followed by 7min at 72℃ for the last cycle. RAPD fragments were separated electrophoretically in 2% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator. In order to optimize the RAPD condition, the PCR mixture contained 50, 100, 200, 300, or 500ng of genomic DNA, 20, 100, 200, 300, or 500 μM dNTP, 0.5, 1, 1.5, 2, or 2.5mM $MgCl_2$, 0.5, 1, 2.5, 3.5, or 5 units of Taq polymerase, and performed at 25, 30, 35, 40, or 50 cycles, 45, 50, 55, 60, or 65℃ of annealing temperature, respectively.

RAPD data analysis

RAPD band were transformed into a binary character matrix ("1" for the presence and "0" for the absence of a band at a particular position). Similarity matrices were computed based on Jaccard's similarity coefficient, using the SPSS 9.0 for window.

PCR-RFLP of the 18S rRNA gene

Each 50μl PCR mixture contained 100ng DNA, 50mM KCl, 10mM Tris-HCl (pH9.0), 200 μM dNTP, 2.5mM $MgCl_2$, 0.25 μM of each primer, and 1.5U of Taq polymerase (Takara). Amplification was performed in a Cyclogene Thermal Cycler. The initial cycle was 3min at 94℃, and 8min at 55℃. Subsequent 30 cycles were 40s at 94℃, and 8min at 55℃, followed by 8min at 55℃ for the last cycle. Each primer had the following sequences : 18S F: 5' CAA CCT GGT TGA TCC TGC CAG T 3' and 18S R: 5' CTG ATC CTT CTG CAG GTT CAC CTA C 3'. The resulting PCR product of 1.8kb in size was purified by treatment with a PCR-Prep kit (Promega). The PCR product was digested with 5 units of restriction enzymes, *Ban* II and *Dde* I (New England Biolab.), respectively at 37℃ for 1.5h, fractionated by a 6% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining under UV.

RESULTS

Initial screening of polymorphic RAPD primers on four ginseng plants

A total of 12 primers were initially screened against four plants, one from China, and the others from Keum-san, Jin-an and Kang-hwa in Korea, respectively. Of the 12, eight primers generated RAPD fragments. Five primers that generated highly repeatable polymorphic RAPD markers were selected.

Optimization of RAPD condition

The PCR condition of RAPD were optimized since it affect to the pattern of PCR products (Munthali *et al.*, 1992). At first, the annealing temperature and number of cycles were optimized (Fig. 1). The other components such as dNTP, $MgCl_2$, Taq polymerase were also tested (data not shown). The optimized conditions were as follows: 200ng of DNA, 200 μM dNTP, 1.5mM $MgCl_2$, 2.5U of Taq polymerase, 35cycles, 55℃ of annealing temperature.

RAPD polymorphism

Four ginseng plants were tested with the five random primers. A total DNA fragments ranging in size from 100 to 2600 bp were scored, corresponding to an

average of 11.4 fragments per primer. All five primers gave distinguishable RAPD amplification patterns in tested plants. Especially, the polymorphic fragments of Chinese ginseng were different from Korean ginseng population. The coefficients of similarity ranged from 0.197 to 0.491 (Table. 1). Each of ginseng plants from Jin_an and Keum_san showed the most close SI, 0.491. The ginseng plants from Jin_an and China, and Keum_san and China showed SI, 0.266 and 0.231, respectively. Kang_hwa and Chinese ginseng plants showed the most low SI, 0.197.

PCR-RFLP analysis on the 18S rRNA gene

Sites of the restriction enzyme for the PCR_RFLP analysis were sought on basis of each sequence of the 18S rRNA gene from the three Korean ginseng. The enzyme *Ban* II recognizes the sequence 5' GGGCTC 3' and *Dde* I, the sequence 5'CTCAG 3'. In the electrophoresis profile digested with the enzyme *Ban* II and *Dde* I, the PCR product of Kang_hwa ginseng showed distinctive fragments from those of Jin_an and Keum_san ginseng plants (Fig. 2).

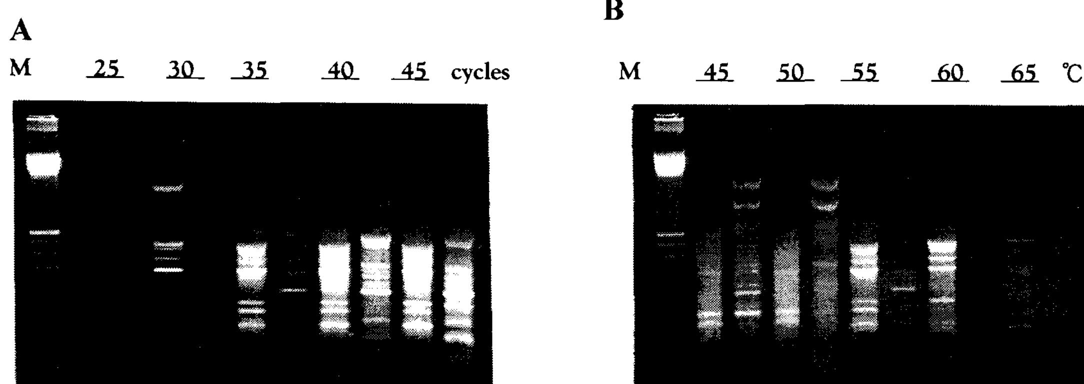

Fig. 1. Optimization of RAPD condition. Using DNA of Jin_an or China and primer 5, each PCR reaction was performed to 25, 30, 35, 40, or 50 cycles (A) and at 45, 50, 55, 60, or 65℃ of annealing temperature (B).

Table 1. Similarity coefficient of four *Panax ginseng* (Jaccard Measure)

	Jin_an	Kang_hwa	Keum_san	China
Jin_an	1	0.345	0.491	0.266
Kang_hwa	0.345	1	0.203	0.197
Keum_san	0.491	0.203	1	0.231
China	0.266	0.197	0.231	1

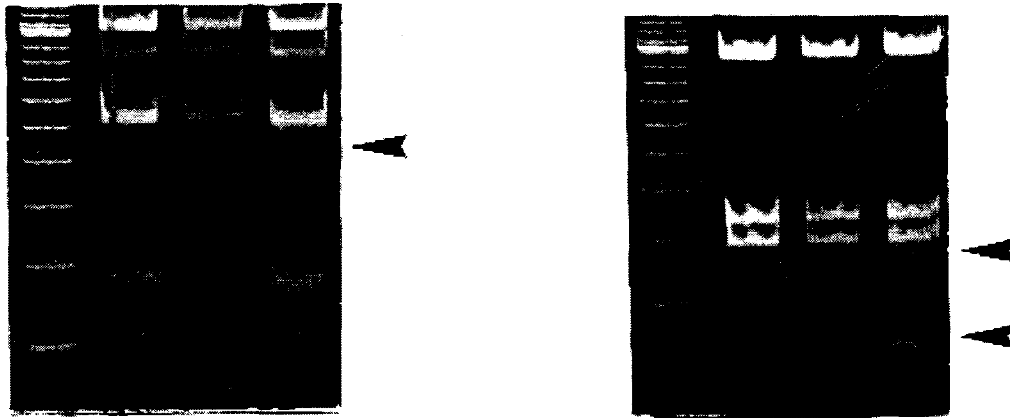

Fig. 2. PCR_RFLP Analysis using Restriction Enzymes *Ban* II (A) and *Dde* I (B).
M represents molecular weight markers. Each lane 1: P. ginseng from Jin_an: lane 2: P *ginseng* from Keum_san : lane 3: P. *ginseng* from Kang_hwa

Examination of reproducibility to randomly selected samples

In order to verify the reproducibility of the RAPD polymorphism in ginseng plants used to extract genomic DNA, ten samples were randomly selected from each of region in Korea. The randomly selected individual samples showed reproducible RAPD patterns (Fig. 3).

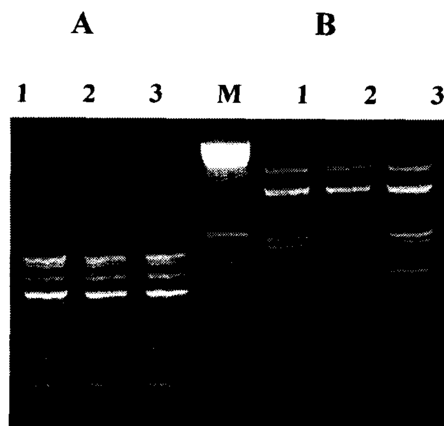

Fig. 3. RAPD profiles of P. *ginseng* from Jin_an (A), P. *ginseng* from China (B) generated by primers 5. M represents molecular weight markers. Each lane: randomly selected individual samples.

DISCUSSION

RAPD (Randomly amplified polymorphic DNA) and PCR_RFLP (PCR_Restriction fragment length polymorphism) analysis were used to authenticate ginseng plants within *Panax* species. A total of 12 primers were screened against four plants. Of the 12, 5 primers generated reproducible and obvious fingerprints. This result is in close agreement with previous report by Fritsch, P. *et al.* which showed only a part of used primers generated obvious fingerprints (Fritsch *et al.*, 1993). These polymorphic fragments were subjected to further data analyses. Simple matching similarity coefficients ranged from 0.491 for the most closely related ginseng, Jin_an and Keum_san to 0.197 for those most distantly related, Kang_hwa and Chinese ginseng . Thus the RAPD analysis was suitable to authenticate among Korean and Chinese ginseng.

And also, The extracted DNA of each ginseng plants collected from three different region in Korea were amplified, and digested with *Ban* II and *Dde* I. Each fragment exhibited unique electrophoretic profiles. Jin-an and Keum-san with high value of SI showed same fingerprints, while Kang-hwa with low SI showed a different unique fingerprint from the others. The band pattern of our study is different from previous report by Fushimi et al. (1997 b). This difference, however, can be explained by genetic diversity that is caused by growth conditions of plants, breeding time etc. even within species.

The authentication of *Panax* species using RAPD analysis have been reported (Shaw et al., 1995; Ngan et al., 1999). However, these reports focused on the authentication not within *Panax* species, but between them. And the random primers they used have low reproducibility due to short length (10 mer), while we used 20-mer random primer with high reproducibility.

In this study, we suggest that RAPD and PCR-RFLP methods are convenient for identifying ginseng plants within *Panax* species. Furthermore, it can be applied to the authentication of the morphologically similar oriental medicines.

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