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Molecular Differentiation of *Panax* Species by RAPD Analysis

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Traditional taxonomic methods used for the identification and differentiation of ginsengs rely primarily on morphological observations or physiochemical methods, which cannot be used efficiently when only powdered forms or shredded material is available. Randomly amplified polymorphic DNA (RAPD) was used to determine the unique DNA profiles that are characteristic not only of the genus *Panax* but also of various *Panax* subgroups collected from five different countries. RAPD results of OP-5A primer showed a specific single band that is characteristic of all ginseng samples. RAPD results of OP-13B primer demonstrated that OP-13B primer could be used as a unique RAPD marker to differentiate *Panax* species. These results support that this approach could be applied to distinguish Korean Ginseng (*Panax ginseng*) from others at the molecular level.

Key words: RAPD, PCR, *Panax* species

INTRODUCTION

The herbal products of various species are imported from several countries including China. Given the increased of demand for Chinese medicine, imitated and adulterated products have been circulated in the market. These illegal practices have become a common problem in recent years. Therefore, an effective standard for the authentication of Chinese herbs is needed to minimize the risk of misusing medicines, and to protect consumers from low quality adulterants, and to develop a healthy herbal industry.

Panax Ginseng cultivated in Korea is highly valued in Chinese medicine (Jeong 2002; Yun *et al.*, 2001; Yun *et al.*, 2001). Korean ginseng (*Panax Ginseng*) has a longer period of development than *Panax Ginseng* cultivated in other countries (Ko *et al.*, 1994; Ko *et al.*, 1996). It has hard and dense internal tissues and preserves its innate flavor longer than other ginsengs. Also, it is known that Korean ginseng contains more vitamins and ginsenosides of high pharmacological activity than Western or other Asian ginsengs (Chung *et al.*, 1995; Ko *et al.*, 1994; Ko *et al.*, 1996). However, the traditional identification and authentication of Korean ginseng depend on morphological and histological examinations. Many commercial ginseng products are traded as powder or as shredded material, making it difficult or impossible to distinguish the source. Various methods have been investigated to differentiate ginseng samples based on their ginsenoside profiles (Fuzzati *et al.*, 1999; Ji *et al.*, 2001; Li *et al.*, 2000; Wang *et al.*, 1999). The application of physico-chemical analysis requires a large quantity of material for proper analysis and ginsenoside profiles are affected by growth condition, storage condition, the freshness of the samples, post-harvest processing etc. (Ngan *et al.*, 1999).

Recently, randomly amplified polymorphic DNA (RAPD) analysis has become a general method for estimating genetic diversity and variation among plant and cultivars (Ha *et al.*, 2001; Oiki *et al.*, 2001; Wang *et al.*, 2001; Shaw and But., 1995). Furthermore, the 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 (Um *et al.*, 2001; Tochika-Komatsu *et al.*, 2001).

Here, we report upon RAPD, a more reproducible form of analysis to authenticate each of 6 *Panax* populations obtained from 5 different countries, and to differentiate Korean ginseng at the molecular level.

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MATERIALS AND METHODS

Plant materials

Panax Ginseng (Korea), Chinese SheoAn Ginseng (China), *Panax notoginseng* (China), *Panax japonica* (Japan), 2 kinds of *Panax quinquefolium* (U.S.A. and Canada) were collected from 6 different areas. The origin of Chinese SheoAn Ginseng was not clear.

Identification of ginseng by High Performance Thin Layer Chromatography (HPTLC)

1.0 g of each sample powder was extracted in 50 mL of methanol for 30 min by reflux-extraction, filtered, and evaporated. Extracts were dissolved in 2 mL of methanol and spotted on silica-gel thin layer plate. Chloroform-methanol-distilled water (13:7:2) was used as the developing solution and bands were visualized by spaying with dilute H_2SO_4 solution at 120°C.

Extraction of ginseng DNA

Total DNA was extracted from ginseng leaves and roots by the cetyltrimethylammonium bromide (CTAB) method with some modification (Tsumura *et al.*, 1995). All samples were frozen in liquid nitrogen, ground into a powder and then filtered. Samples were treated with extraction buffer consisting of 50 mM Tris-HCl (pH 8.0), 0.7 M NaCl, 50 mM EDTA (pH 8.0), 140 mM β-mercaptoethanol for 1 h at 60°C and centrifuged at 3,500 g for 10 min after mixing with phenol-chloroform-isoamylalcohol (25:24:1). The upper lysate was further extracted with 1 vol. of chloroform:isopropanol (24:1) and centrifuged at 3,500 g for 5 min. The upper solution was then mixed with 1 vol. of isopropanol at -20°C for 30 min and the resulting pellet was washed with 70% ethanol. The dried pellet, composed of nucleic acids, was resuspended in TE buffer consisting 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. This solution was then incubated for 30 min at 37°C with 1 mg/mL RNase A and subjected to 0.8% agarose gel electrophoresis.

RAPD

10-mer operon random primers (Operon Technologies, U.S.A) were used (Table I). Each 50 μL PCR mixture contained 0.1 pmol operon random primers and 0.05-0.01 μg/mL template DNA in a PCR-premix kit (Bioneer, Korea), 250 μM dNTP, 10 mM Tris-HCl pH 9.0, 40 mM KCl, 1.5 mM $MgCl_2$ and 2.5 U of *Taq* DNA polymerase. Amplification was performed in an Applied Biosystems 9700 (U.S.A) for 45 cycles. The initial cycle was 5 min at 94°C, this was followed by 45 cycles of 1 min at 94°C for denaturation, 1 min at 37°C for annealing, and 2 min at 72°C for extension, and finally a single cycle of 5 min at 72°C. The PCR product was fractionated by 3.0% agarose (Nusieve 3:agarose 1) (FMC, U.S.A) electrophoresis at 30 volts for 5 h and visualized by ethidium bromide staining under UV.

Table I. Nucleotide sequence of primers used in RAPD

Primer Name	Sequences
OP-5A	5-AGGGGTCTTG-3
OP-8A	5-GTGACGTAGG-3
OP-8B	5-GTCCACACGG-3
OP-13B	5-TTCCCCCGCT-3
OP-14B	5-TCCGCTCTGG-3

RESULTS

The HPTLC results exhibited similar electrophoretic band patterns as shown in Fig. 1, making it difficult to differentiate *Panax Ginseng* (Korea) from the other ginseng strains.

Eighty 10-mer random primers were screened against 6 different ginsengs. The results of the PCR amplification using Operon random primers showed unique electrophoresis band patterns for the different *Panax* strains, and this make it easy to differentiate *Panax* populations. In particular, the OP-5A primer reproducibly produced the most distinct polymorphism, and a 400 bp single fragment was found to be common to the 6 different ginsengs (Fig. 2). In case of OP-8A primer, samples from Korean ginseng and Canadian ginseng showed the similar pattern, and these were distinguished from the others (Fig. 3). The PCR results of OP-8B primer showed that Korean ginseng and Chinese SheoAn ginseng have similar band patterns with two distinctive bands at 250 bp and 450 bp, but Japanese ginseng showed no amplified band (Fig. 4). In Fig. 5, all samples showed amplified products of 100 bp and 800 bp by PCR using OP-13B primer. Also, the 1.1 Kb and 650 bp bands in Korean ginseng, 1.4 Kb and 480 bp in Chinese SheoAn ginseng, 1.4 Kb and 650 bp in Japanese ginseng, 650 bp and 480 bp in Canadian ginseng, and 650 bp in

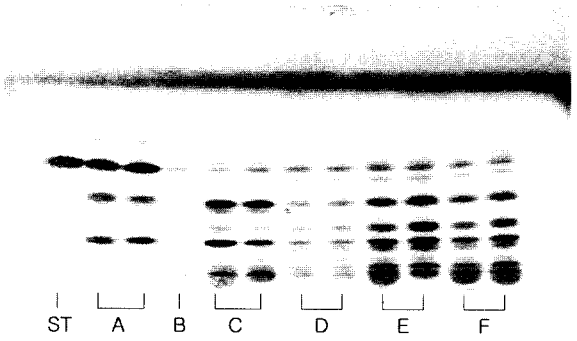

Fig. 1. HPTLC Analysis of samples obtained from 6 different regions. ST; Ginsenoside, A; *Panax Ginseng*, B; Chinese SheoAn ginseng, C; *Panax notoginseng*, D; *Panax japonica*, E; American ginseng, F; Canadian ginseng.

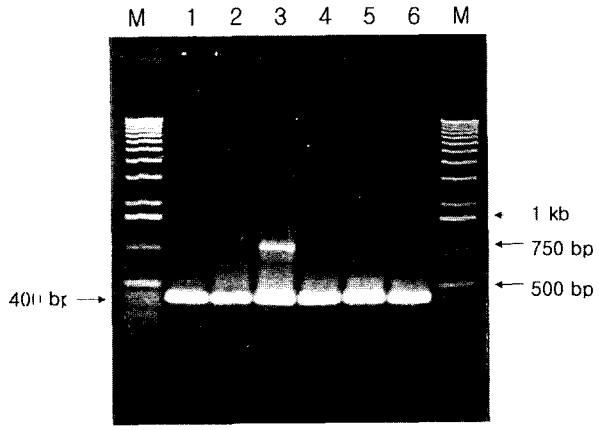

Fig. 2 RAPD Analysis by Operon Primer Type A 5 (OP-5A) primer. M; 1 Kb size marker, 1; *Panax Ginseng*, 2; Chinese SheoAn ginseng, 3; *Panax notoginseng*, 4; *Panax japonica*, 5; American ginseng, 6; Canadian ginseng.

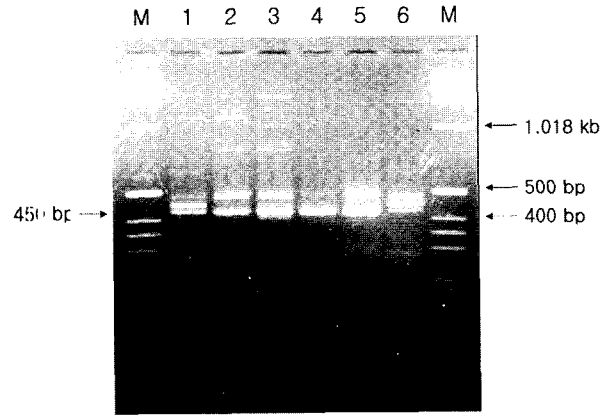

Fig. 3 RAPD Analysis by Operon Primer Type A 8(OP-8A) primer. M; 1 Kb size marker, 1; *Panax Ginseng*, 2; Chinese SheoAn ginseng, 3; *Panax notoginseng*, 4; *Panax japonica*, 5; American ginseng, 6; Canadian ginseng.

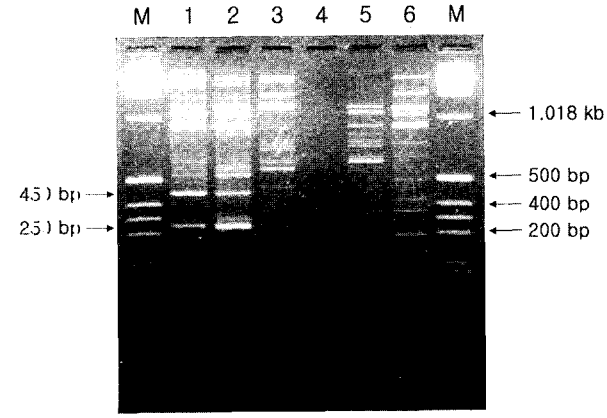

Fig. 4 RAPD Analysis by Operon Primer Type B 8(OP-8B) primer. M; 1 Kb size marker, 1; *Panax Ginseng*, 2; Chinese SheoAn ginseng, 3; *Panax notoginseng*, 4; *Panax japonica*, 5; American ginseng, 6; Canadian ginseng.

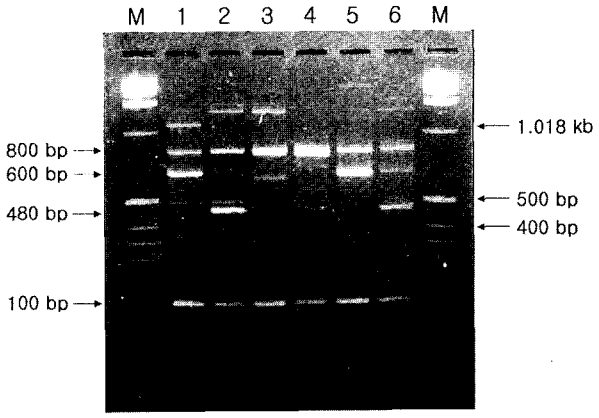

Fig. 5. RAPD Analysis by Operon Primer Type B 13(OP-13B) primer. M; 1 Kb size marker, 1; *Panax Ginseng*, 2; Chinese SheoAn ginseng, 3; *Panax notoginseng*, 4; *Panax japonica*, 5; American ginseng, 6; Canadian ginseng.

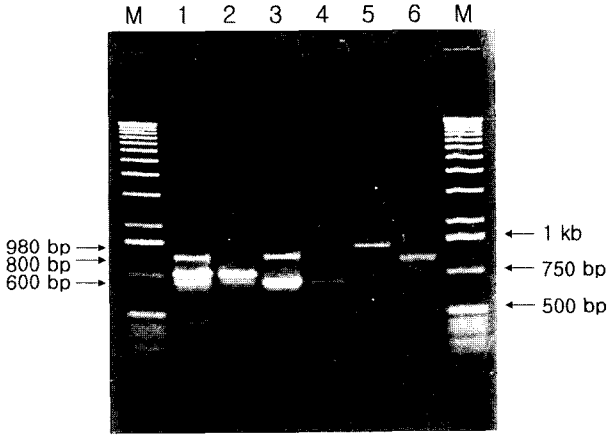

Fig. 6. RAPD Analysis by Operon Primer Type B 14(OP-14B) primer. M; 1 Kb size marker, 1; *Panax Ginseng*, 2; Chinese SheoAn ginseng, 3; *Panax notoginseng*, 4; *Panax japonica*, 5; American ginseng, 6; Canadian ginseng.

American ginseng were unique. Amplification with OP-14B primer produced a distinctive band pattern for Canadian ginseng and American ginseng with apparent bands of 800 bp and 980 bp, respectively, whereas Japanese ginseng had a distinctive apparent band at 600 bp (Fig. 6).

DISCUSSION

RAPD analysis was used to differentiate *Panax* species because physicochemical methods such as HPTLC cannot distinguish between *Panax* populations. Eighty 10-mer random primers were screened to determine whether they displayed polymorphisms. Primers 5, 8, 13, and 14 generated reproducible and obvious amplified products. RAPD analysis using OP-5A primer produced the most distinctive single band that was common to all 6 ginseng types, with high reproducibility. Therefore, it could be used

to identify ginseng from morphologically similar oriental plants. RAPD analysis of the OP-8A, OP-8B, OP-13B, and OP-14B primers exhibited unique epigenetic electrophoretic patterns, caused by variations in the growth conditions of plants and cultivated areas, even within a species. In particular, Chinese SeoAn ginseng from China is believed to have the same origin as Korean ginseng on the basis of the RAPD results obtained using OP-8B primer (Fig. 4).

Generally, American ginseng and Canadian ginseng are placed in the same family, namely, *Panax quinquefolium*. Although they grow in similar geographical and environmental condition and HPTLC shows that they contain similar ginsenosides, RAPD analysis of the OP-14B primer showed obviously different band patterns, supporting the notion that these ginsengs are of different origin. The identification of *Panax* species using RAPD analysis has been reported (Ngan *et al.*, 1999; Um *et al.*, 2001; Tochika-Komatsu *et al.*, 2001). However, these reports focused on differentiation within *Panax* populations collected from 5 countries. The RAPD results obtained exhibited high reproducibility using a short length random primer (10-mer). To enhance the specificity and reproducibility of the method, a combination of RAPD and PCR-RFLP analysis is currently being developed.

In conclusion, we suggest that the RAPD method is convenient and efficient for identifying ginseng plants from similar plants and more importantly for differentiating ginseng plants within the *Panax* species. Furthermore, the method can be applied to authenticate Korean ginseng (*Panax ginseng*) from similar ginsengs and to identify commercial imitations.

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REFERENCES

Chung, Y. Y., Chung, C. M., Ko, S. R., and Choi, K. T., Comparison of Agronomic Characteristics and Chemical Component of *Panax ginseng* C.A. Meyer and *Panax quinquefolium* L. *Korean J. Ginseng Sci.*, 19(2), 160-164 (1995).

Fuzzati, N., Gabetta, B., Jayakar, K., Pace, R., and Peterlongo, F., Liquid Chromatography-Electrospray Mass spectrometric Identification of ginsenosides in *Panax ginseng* roots. *J Chromatogr A.*, 854(1-2), 69-79 (1999).

Ha, W. Y., Yau, F. C., But, P. P., Wang, J., and Shaw, P. C., Direct amplification of Length Polymorphism Analysis differentiates *Panax ginseng* from *P. quinquefolius*. *Planta Med.*, 67(6), 587-589 (2001).

Jeong, C. S., Effect of Butanol Fraction of *Panax ginseng* head on Gastric lesion and Ulcer. *Arch. Pharm. Res.*, 25(1), 61-66 (2002).

Ji, Q. C., Harkey, M. R., Henderson, G. L., Gershwin, M. E., Stern, J. S., and Hackman, R. M., Quantitative Determination of Ginsenosides by High-Performance Liquid Chromatography-tandem mass spectrometry. *Phytochem. Anal.*, 12(5), 320-326 (2001).

Ko, J. G., Kim, Y. S., Kim, H. Y., Na, K. C., Do, J. H., Park, J. D., Park, J. K., Park, H. J., Baek, N. I., Lee, S. J., Lee, J. H., Lee, H. O., and Jeong, K. T., Korean Ginseng. Korea Ginseng & Tobacco Research Institute (1994).

Ko, S.-R., Choi, K.-J., and Han, K.-W., Comparison of Proximate Composition, Mineral Nutrient, Amino Acid and Free Sugar Contents of Several Panax Species. *Korean J. Ginseng Sci.*, 20(1), 36-41 (1996).

Li, W., Gu, C., Zhang, H., Awang, D. V., Fitzloff, J. F., Fong, H. H., and van Breemen, R. B., Use of High-Performance Liquid Chromatography-tandem mass Spectrometry to distinguish *Panax ginseng* C. A. Meyer (Asian ginseng) and *Panax quinquefolius* L. (North American ginseng). *Anal. Chem.*, 72(21), 5417-5422 (2000).

Ngan, F., Shaw, P., But, P., and Wang, J., Molecular Authentication of *Panax* species. *Phytochemistry*, 50, 787-791 (1999).

Oiki, S., Kawahara, T., Inoue, K., Ohara, M., and Maki, M., Random Amplified Polymorphic DNA (RAPD) Variation among Populations of the Insular Endemic Plant *Campanular microdonta* (Campanulceae). *Annals of Botany.*, 87, 661-667 (2001).

Shaw, P. C. and But, P. P., Authentication of *Panax* species and their adulterants by Random-Primed Polymerase Chain Reaction. *Planta Med.*, 61(5), 466-469 (1995).

Tochika-Komatsu, Y., Asaka, I., and Ii, I., A random amplified polymorphic DNA (RAPD) primer to assist the Identification of a selected strain, aizu K-111 of *Panax ginseng* and the Sequence amplified. *Biol. Pharm. Bull.*, 24(10), 1210-1213 (2001).

Tsumura, Y., Yoshimaru, K., Tomaru, N., and Ohba, K., Molecular phylogeny of conifers using RFLP analysis of PCR-amplified specific chloroplast genes. *Theoretical and Applied Genetics*, 91, 1222-1236 (1995).

Um, J. Y., Chung, H. S., Kim, M. S., Na, H. J., Kwon, H. J., Kim, J. J., Lee, K. M., Lee, S. J., Lim, J. P., Do, K. R., Hwang, W. J., Lyu, Y. S., An, N. H., and Kim, H. M. Molecular Authentication of *Panax ginseng* Species by RAPD Analysis and PCR-RFLP. *Biol. Pharm. Bull.*, 24(8), 872-875 (2001).

Wang, J., Ha, W. Y., Ngan, F. N., But, P. P., and Shaw, P. C., Application of Sequence Characterized Amplified Region (SCAR) Analysis to authenticate *Panax* species and their adulterants. *Planta Med.*, 67(8), 781-783 (2001).

Wang, X., Sakuma, T., Asafu-Adjaye, E., and Shiu, G. K., Determination of Ginsenosides in Plant extracts from *Panax ginseng* and *Panax quinquefolius* L. by LC/MS/MS. *Anal. Chem.*, 71(8), 1579-1584 (1999).

Yun, T. K., Lee, Y. S., Lee, Y. H., Kim, S. I., and Yun, H. Y., Anticarcinogenic effect of *Panax ginseng* C.A. Meyer and identification of active compounds. *J. Korean Med. Sci.*, Suppl, S6-18 (2001).

Yun, Y. P., Do, J. H., Ko, S. R., Ryu, S. Y., Kim, J. H., Song, H. C., Park, Y. D., Ahn, K. S., and Kim, S. H., Effects of Korean red ginseng and its mixed prescription on the high molecular weight dextran-induced blood stasis in rats and human platelet aggregation. *Ethnopharmacol.*, 277(2-3), 259-264 (2001).