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Authentication of Korean *Panax ginseng* from Chinease *Panax ginseng* and *Panax quinquefolius* by AFLP analysis

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Abstract

***Panax ginseng* is one of the most important medicinal plants in the world. The international trade of ginseng is increasing yearly. The disguise of Chinese and American ginseng into Korean ginseng became a problem in recent years in abroad and Korea. An effective method to authenticate the Korean *Panax ginseng* from others at a DNA level is necessary for the healthy development of the ginseng market. Amplified fragment length polymorphism (AFLP) analysis was applied to develop a method for the identification of Korean *ginseng* between Chinese *ginseng* and American ginseng. It is very difficult to detect the different polymorphic bands among Korean field cultivated ginseng, and between field and wild-cultivated ginseng. The genetic distance coefficient by AFLP analysis between field- and wild cultivated Korean ginseng was very low, 0.056. Whereas, polymorphic bands between Korean and Chinese wild-cultivated ginseng was significantly different. The genetic distance coefficient between wild-cultivated Korean and Chinese ginseng was 0.149. The genetic distance coefficients between the *P. ginseng* and *P. quinquefolius* were ranging from 0.626 to 0.666. These results support that the AFLP analysis could be applied to authenticate Korean *P. ginseng* from others Chinese *P. ginseng* and American ginseng (*P. quinquefolius*).**

Key words: AFLP, authentication, PCR, *Panax ginseng*, *Panax quinquefolius*

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Introduction

About 12 ginseng species are distributed in the world and each of them has been used as traditional medicines. *Panax. ginseng*, *P. quinquefolius*, and *P. notoginseng* were commercialized for various medicinal products. Among Panax species, Korean P. ginseng is the best-known product with high medicinal values in the world. Due to this fact, disguised Chinese and American ginseng into Korean ginseng became a problem in world and also in Korean market. In Korea, wild-grown and wild-cultivated Korean ginseng is sold with high prices and the disguise of Chinese wild ginseng into Korean ginseng is serious problem in Korea, which may be resulted in the depression of ginseng market.

People consume only root parts of ginseng, and dried roots were imported and exported. It is nearly impossible to differentiate the *Panax* species by visual inspection of dried and manufactured root parts. Especially discrimination of *P. ginseng* produced in different country (Korea, China, Japan and Russia) is more difficult. Therefore, it is very important to authenticate the Korean ginseng from Chinese ginseng and from other different ginseng species.

There are some reports on the morphological and chemical authentication of Korean ginseng between other foreign *P. ginseng* and different ginseng species. There are some differences between *P. ginseng* and *P. quinquefolius* in morphological characters and saponins (Chung et al. 1995, 1998), soluble peptides (Park et al. 1996), phenolic compounds (Wee et al. 1998), and volatile components (Noh and Ko 1997). However, it is very difficult to differentiate the characters of ginseng within same species although they were cultivated in different country.

The molecular markers were accepted as more confident methods as the morphological and chemical analysis. Molecular authentication of different *Panax* species were reported in some extent using RAPD (Shaw et al. 1995; Tochika-Komatsu et al. 2001; Um et al. 2001; Shim et al. 2003; Cui et al. 2003), PCR-RFLP (Ngan et al. 1999; Fushimi et al. 1997), gene sequences (Komatsu et al. 2001; Shu et al. 2003). However, molecular marker to authenticate the Korean *P. ginseng* from other foreign *P. ginseng* is remained to be clear.

Vos et al. (1995) developed an AFLP (amplified fragment length polymorphism) analysis, which has been developed as an excellent method for the detection and study of the genetic polymorphisms in plant species (Lin and Kuo 1995; Mackill et al. 1996). AFLPs have the advantage of detecting length differences as small as 1 bp, and are able to detect more independent loci for polymorphism than other currently available PCR-based techniques (Blears et al. 1998). Authentication of *Panax ginseng* and *Panax quinfolium* by AFLP was reported by Ha et al. (2002). Here, we report a more reproducible approach to authenticate the Korean *P. ginseng* from Chinese *P. ginseng* and *P. quinquefolius* by AFLP analysis

Materials and Methods

Plant materials and extraction of genomic DNA

Fresh roots of *Panax ginseng* collected from Korea and China, and *Panax quinquefolius* collected from Canada and USA were as shown in Table 1. These were used to study the inheritance of AFLP markers.

Total genomic DNA was extracted from ginseng roots with a DNeasy Plant Mini kit (Qiagene, Germany) using the procedure specified by the manufacturer. Two hundred gram of samples were frozen in liquid nitrogen, ground into powder and then followed procedure by the manufacturer. DNA concentration was determined by absorbance at 260 nm.

Genomic DNA digestion and Adapter ligation

The AFLP procedure was used as describe by Vos et al. (1996). To obtain the restriction fragments, 2 μg of genomic DNA was double-digested with 5 units of *Eco*RI (TaKaRa, Japan) and *Mse*I (TaKaRa, Japan) for 12 hours at 37℃.

Table 1. Data of collected samples of various kinds of ginseng roots for AFLP analysis

Samples	Scientific name	Collection site	Putative age
Field cultivated ginseng in Korea			
1	*Panax ginseng*	Jinan, Chonbok	4
2	*Panax ginseng*	Geumsan, Chungnam	4
3	*Panax ginseng*	Ansung, Kyunggi	4
Wild-cultivated ginseng in Korea			
4	*Panax ginseng*	Jinan, Chonbuk	15
5	*Panax ginseng*	Gangneung, Gangwon	17
6	*Panax ginseng*	Namyangju, Kyunggi	17
Wild-Cultivated ginseng in China			
7	*Panax ginseng*	Musong, China	10
8	*Panax ginseng*	Chang-back mountain, China	15
9	*Panax ginseng*	Chang-back mountain, China	15
Wild-Cultivated ginseng in USA			
10	*Panax quinquefolius*	Wisoconsin, USA	30
11	*Panax quinquefolius*	Wisoconsin, USA	40
12	*Panax quinquefolius*	Wisoconsin, USA	40
Field cultivated ginseng in Canada			
13	*Panax quinquefolius*	Ontraio, Canada	4
14	*Panax quinquefolius*	Ontraio, Canada	2
15	*Panax quinquefolius*	Ontraio, Canada	2

Table 2. Oligonucleotide adapters and primers used for AFLP analysis.

Adaptor (5' to 3')	Primer sequences (5' to 3')
	E0 : GAC TGC GTA CCA ATT C
*Eco*RI-adapter	
	E1 : GAC TGC GTA CCA ATT CAT
Forward : CTCGTAGACTGCGTACC	E2 : GAC TGC GTA CCA ATT CAC
Reverse : AATTGGTACGCAGTCTAC	E3 : GAC TGC GTA CCA ATT CTA
	E4 : GAC TGC GTA CCA ATT CTG
	E5 : GAC TGC GTA CCA ATT CACA
*Mse*I-adaptor	
	M0 : GAT GAG TCC TGA GTA A
Forward : GACGATGAGTCCTGAG	
Reverse : TACTCAGGACTCAT	M1 : GAT GAG TCC TGA GTA ACT G
	M2 : GAT GAG TCC TGA GTA AGT T

After addition of ethanol, DNA was precipitated by centrifuge at 14,000 rpm for 30 min. The end of double-digested DNA fragments were ligated with *Eco*RI and *Mse*I adapters for 12 hours at 14℃ (Table 2). After ligation, 10-fold diluted DNA solution was used for pre-amplification.

AFLP analysis

Each 20 ㎕ PCR mixture contained 5 ㎕DNA, 200 μM dNTP, 0.5 ㎛M DNA primers (E0 and M0 of Table 2) and 1 U Taq DNA polymerase (EX Taq, TaKaRa, Japan). Amplification was performed in a DNA thermal cycler (Applied Biosystems 2700, Foster City, Calif.) for 20 cycles. The initial cycle was 5 min at 94℃. Subsequent cycles were 30 second at 94℃, 1 min at 60℃, and 1 min at 72℃, followed by 7 min at 72℃ for the last cycles. Pre-amplification PCR products were diluted 50-fold with water and used for selective amplification. The amplification mixture (20 ㎕, final volume) contained 5 ㎕ pre-amplification mixture, 1 X buffer, 200 μM dNTP, 0.5 μM DNA primer (total 10 kinds of primers (E1-5 and M1-2 of Table 2.), 1 U Taq DNA polymerase (EX Taq, TaKaRa, Japan). After 30 sec at 94℃, 30 sec at 65℃, 1 min at 72℃ for first cycle, followed by a lowering of temperature (1℃) in the next 12 cycle, then at 56℃ for the remaining 23 cycle; extension for 1 min at 72℃. A total of 1.5 ㎕ of the PCR-amplified mixture was added to an equal volume of leading buffer (95% formamide, 10 mM EDTA, pH 8.0, 0.05% bromo phenol blue, 0.05% xylene cyanol FF), denaturated for 5 min at 95℃, load on to a 6% denaturing polyacrylamide gel (5.75% Long Ranger, BMA USA, 7 M urea, 1X TBE) and electrophoresed for 4 h at 15 mA. The gel was fixed in 10% acetic acid and silver stained.

Statistical analysis

AFLP polymorphic fragments were scored as 1 for presence and 0 for absence of a fragment. Genetic distance between eleven ginsengs was calculated using Jaccard' s coefficients of similarity (Jaccard 1908). Neighbor-joining tree (Nei and Li 1979) was constructed with NTSYS software (Rohlf 1998) by the method of unweighted pair-group method using arithmetic averages (UPGMA) (Sneath and Sokai 1973).

Results and Discussion

Genetic distance among Korean *P. ginseng*

Ten AFLP primers as shown in Table 2 were screened for polymorphism among *Panax* species. The number of AFLP bands generated from the Korean *P. ginseng* samples ranged from 65 to 73 in each primer combination. AFLP bands of Korean cultivated ginseng collected from different places (Jinan, Kumsan, and Ansung) showed very little polymorphic bands, and only about 1 to 3 different polymorphic fragments were obtained in each primer combination (Fig. 3). It was generally accepted that there are high genetic variances among *P. ginseng* even in same field because ginseng seeds has been used traditionally maintained by native seeds and not genetically selected or improved. The close similarity of AFLP bands of Korean field-cultivated *P. ginseng* cultivated different places indicates that they are genetically very similar. This might be mainly resulted from the same genetic pool due to geographically non-isolated area in Korea peninsular. On the

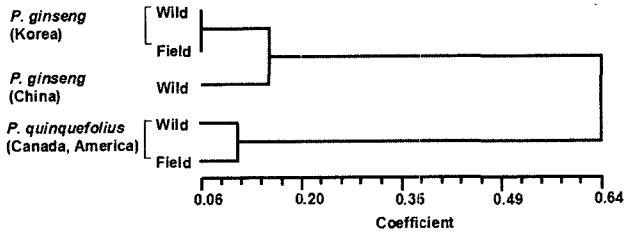

Figure 1. Dendrogram calculated using Jaccard's coefficient of similarity and UPGMA cluster analysis based on 325 AFLP polymorphic fragments from the squared Euclidian distance of AFLP marker frequencies per *P. ginseng* and *P. quinquefolium* population. The numerical codes used to identify each genotype are described in Table 1.

one hand, it is not rule out the possibility that Korean cultivated *P. ginseng* is propagated and spread from some people who invented the cultivation method of *P. ginseng* in ancient time. On the other hand, recently one year-old root after tentative planting in sowing bed were produced massively from the North area of Kyunggi-do and were provided to all local ginseng farmer. The close similarity among Korean *P. ginseng* with different locality was demonstrated by RAPD (Hon et al. 2003). Contrary to Korean ginseng, there are some distinct differences on the bands among different local *P. quinquefolius* in Canada and America (Ha et al. 2002).

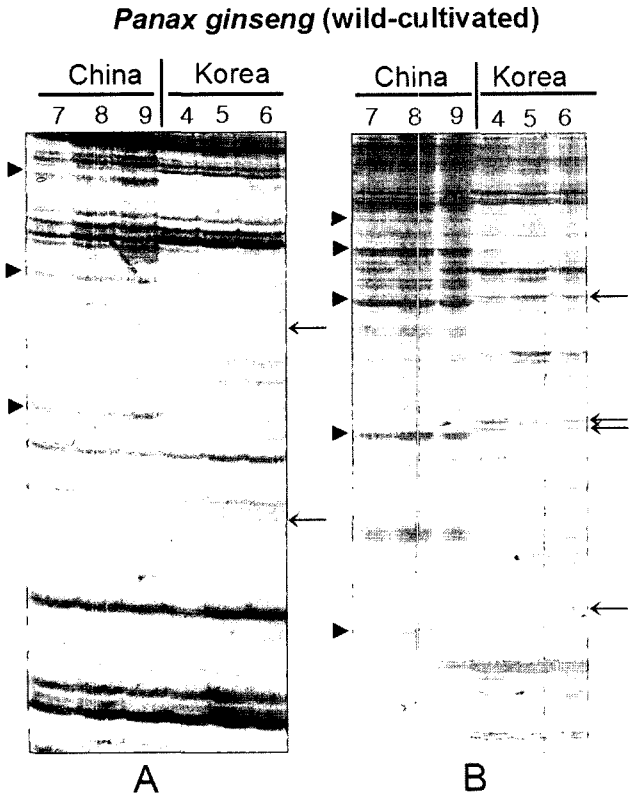

Figure 2. AFLP fingerprints of wild-cultivated *P. ginseng* from Korea (lanes 4-6) and China (lanes 7-9) using primer combination *Eco*RI+TA/Mse I+CTG (A) and *Eco*RI+TG/Mse I+CTG (B). Arrowheads indicate molecular markers observed wild-cultivated ginseng in China. Arrows indicate that molecular markers observed in wild-cultivated ginseng in Korea.

We compared the AFLP bands between wild- and field-cultivated Korean *P. ginseng*. We expect that there are some polymorphic differences between field- and wild cultivated Korean *P. ginseng*. However, it was no conspicuous polymorphic band between wild- and field-cultivated Korean *P. ginseng* and genetic distance coefficient was 0.056. This result indicates that wild- and field-cultivated ginseng were not far from genetically. This is the first attempt to authenticate the field and wild cultivated Korean *P. ginseng* and resulted in the close homogeneity between them.

It is generally accepted that the ginseng seeds for wild-cultivation were originated from wild-grown ginseng. However, it is not rule out the possibility that filed-cultivated ginseng seeds can move to mountain by feces of bird or animals after feeding of ginseng fruits. Therefore, it is very difficult to get true wild-grown ginseng maintained only in wild habitat. It remains to be elucidated for further experiment on the genetic distance between true wild ginseng and cultivated ginseng.

Genetic distance between Korean and Chinese ginseng

There were some significantly different polymorphic bands between Korean and Chinese wild-cultivated *P. ginseng* (Fig. 2). The genetic distance coefficient between Korean and Chinese wild-cultivated ginseng was 0.149. (Fig. 1, Table 3). The level of polymorphism was similar to that by RAPD analysis and that the coefficients of genetic distance ranged from 0.197 to 0.491 among Korean and Chinese ginseng population by Um et al. (2001). Every primer showed distinct polymorphic fragments. Among 10 primers, *Eco*RI+TA/*Mse*I+CTG (Fig. 2A) and *Eco*RI+TG/*Mse* I+CTG (Fig. 2B) showed better result. About 16.8% of bands from totals was polymorphic bands, and the polymorphic AFLP bands was ranging from 5 to 11 per primer-pair between Korean and Chinese wild-cultivated *P. ginseng*. Thereby it confirms the considerable multiplex ration by AFLP technique might be resulted from the geographical isolation between China and Korean peninsular. The obvious polymorphism between Korean and Chinese *P. ginseng* is very important to develop the DNA finger printing methods to discriminate the Korean ginseng from Chinese ginseng. The

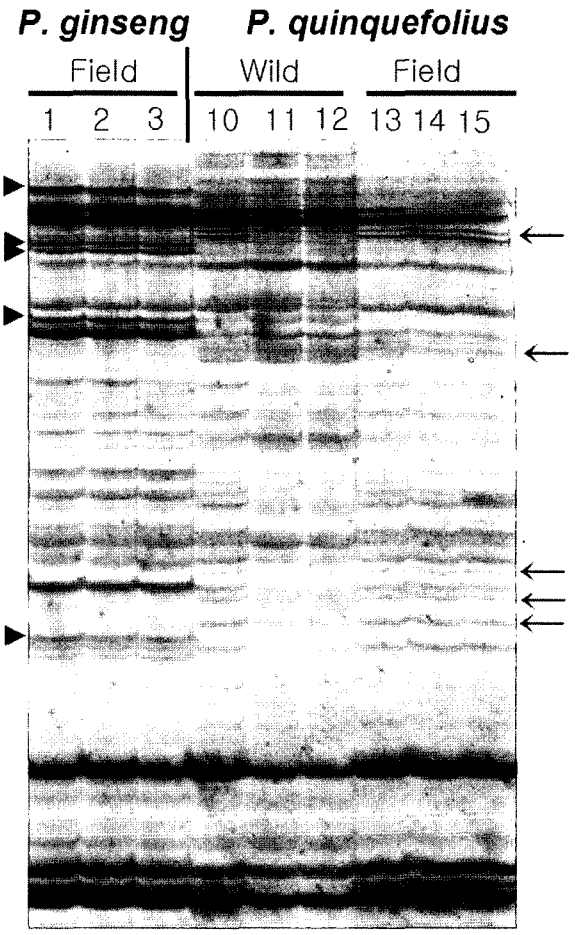

Figure 3. AFLP analysis of the Korean *P. ginseng* (lanes 1-3: field cultivated) and *P. quinquefolius* (lanes 10-12: wild-cultivated in America, lanes 13-15: field cultivated in Canada) using primer combination *Eco*RI+TA/*Mse*I+CTG. Arrowheads indicate molecular markers observed in Korean *P. ginseng*. Arrows indicate that specific bands observed in *P. quinquefolius*.

disguise of Chinese ginseng into Korean ginseng in world market is somewhat problematic and this will be more serious when the trade of agricultural products is open.

Genetic distance between Korean *P. ginseng* and American *P. quinquefolius*

AFLP bands between Korean *P. ginseng* and American wild cultivated- and Canadian field cultivated *P. quinquefolius* were considerably different (Fig. 3). The genetic distance coefficient between the population of *P. ginseng* and *P. quinquefolius* was 0.573 - 0.611 (Table 3). In addition, there were some differences in the bands field- and wild-cultivated local lines among *P. quinquefolius* (Fig. 3). The genetic distance coefficient between wild- and field-cultivated *P. quinquefolius* was 0.112 (Table 3). This result indicates that *P. quinquefolius* grown in North America is highly heterogenous and polymorphic. Ha et al. (2002) reported that samples of *P. quinquefolius* from different localities appear to have heterogenous genetic makeup.

Molecular authentication of different *Panax* species was reported by various techniques such as RAPD (Shaw and But 1995; Boehm et al. 1999), PCR-RFLP (Ngan et al. 1999), polymorphic microsatellite marker (Hon et al. 2003) and internal transcribed spacers (ITS) sequences of ribosomal DNA (Wen and Zimmer 1996).

Conclusively, we found homogeneity between field and wild-cultivated Korean *P. ginseng*, and distinct polymorphic bands of Korean *P. ginseng* from Chinese *P. ginseng*, and American *P. quinquefolius* using AFLP analysis technique. This will be used for molecular marker to authenticate the Korean ginseng among other foreign ginseng.

Table 3. Genetic distance coefficient

Type of ginseng	*P. ginseng* (Wild-cultivated, Korea)	*P. ginseng* (Field cultivated, Korea)	*P. ginseng* (Wild-cultivated, China)	*P.quinquefolius* (Wild-cultivated, USA)	*P. quinquefolius* (Field cultivated, Canada)
P. ginseng (Wild-cultivated, Korea)	0				
P. ginseng (Field cultivated, Korea)	0.056	0			
P. ginseng (Wild-cultivated, China)	0.149	0.164	0		
P. quinquefolius (Wild-cultivated, USA)	0.573	0.626	0.650	0	
P. quinquefolius (Field cultivated, Canada)	0.611	0.666	0.692	0.112	0

Genetic distance was calculated by Nei (1978). Dendrogram was obtained using UPGMA method of NTSYS (V2.0).

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