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Full Length Research Paper

Molecular authentication of *Panax notoginseng* by specific AFLP-derived SCAR marker

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The genus *Panax* contains several medicinal species highly treasured in the Orient. *Panax notoginseng* (Burkill) F. H. Chen (Sanchi) produced in Southern China are used as a hematostatic for hemorrhage. In order to develop species-specific molecular markers for *P. notoginseng*, amplifed fragment length polymorphism (AFLP) analyses were applied among *Panax ginseng, Panax japonicus, Panax quinquefolius* and *P. notoginseng*. A clear species-specific AFLP marker for *P. notoginseng* was generated. After isolation and sequencing of AFLP fragments, a DNA sequence (509 bp) was obtained and named of PNG 8. Two specific primers (PNG 8-1 and PNG 8-2) were designed within the sequence of PNG 8 for amplification of DNA at 402 and 260 bp respectively. PCR analysis revealed a unique amplified band pattern for *P. notoginseng* compared to other *Panax* species (*P. ginseng, P. japonicus,* and *P. quinquefolius*). This sequence characterized amplified regions (SCAR) marker which will be used for rapid authentication of *P. notoginseng* among other related *Panax* species. This is the first report of species-specific SCAR marker development in *P. notoginseng*.

Key words: AFLP, authentication, molecular marker, *Panax notoginseng*, SCAR marker.

INTRODUCTION

The genus Panax belongs to the family Araliaceae and has been regarded as one of the most famous and expensive crude drugs. Ginseng is commonly used to promote quality of life (Ellis and Reddy 2002; Vogler et al., 1999). Immune system modulation, anti-stress activity, anti-cancer and anti-diabetic activities are the most notable features of ginseng in laboratory and clinical trials (Vogler et al. 1999; Shibata 2001). About 12 ginseng species are distributed in the world (Wen and Zimmer 1996; Zhu et al. 2004). The most commonly used *Panax* species are *Panax ginseng, Panax quinquefolius,* and *Panax notoginseng*. Ginsenosides, the steroid saponins, are major biologically active compounds in *Panax* species, and may play critical roles in its diverse physiological actions. Over 30 ginsenosides have been identified to date (Shibata 2001).

P. notoginseng (*Panax pseudoginseng*, Sanqi) is well-known traditional Chinese medicine and is mainly cultivated in Wenshan of Yunnan, China. *P. notoginseng* has therapeutic abilities to stop haemorrhages and to influence blood circulation and to act as an adaptogenic substance (Chang and But, 1986; Ng, 2006). Both *P. ginseng* and *P. quinquefolius* are used as tonics for anti-stress, anti-fatigue (Wang et al., 1983) and anti-aging purposes (Cheng et al., 2005). Like other *Panax* species, *P. notoginseng* contains dammarane-type ginsenosides as the major constitiuents. Total ginsenoside content of *P. notoginseng* is found to be higher than that of *P. ginseng* and *P. quinquefolius* (Dong et al., 2003).

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. Therefore, it is very important to authenticate the *Panax* species among other different species. Analysis of well-characterized compounds, ginsenosides, is the most popular method for identifying the *Panax* species and quality control of ginseng

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Abbreviation: AFLP; Amplifed fragment length polymorphism, **SCAR;** sequence characterized amplified regions.

Table 1. Oligonucleotide adapters and primers used for AFLP analysis.

Adapter (5' to 3')	Primer sequences (5' to 3')
*EcoR*I-adapter	E0 : GAC TGC GTA CCA ATT C
Forward : CTCGTAGACTGCGTACC	E1 : GAC TGC GTA CCA ATT CAT
Reverse : AATTGGTACGCAGTCTAC	E2 : GAC TGC GTA CCA ATT CAC
	E3 : GAC TGC GTA CCA ATT CTA
	E4 : GAC TGC GTA CCA ATT CTG
	E5 : GAC TGC GTA CCA ATT CAC A
*Mse*I-adaptor	M0 : GAT GAG TCC TGA GTA A
Forward : GACGATGAGTCCTGAG	M1 : GAT GAG TCC TGA GTA ACT G
Reverse : TACTCAGGACTCAT	M2 : GAT GAG TCC TGA GTA AGT T
	M3 : GAT GAG TCC TGA GTA ACT A
	M4 : GAT GAG TCC TGA GTA ACA C

products (Chan et al., 2000). The profiles of ginsenosides in roots of *Panax* species are very similar and affected significantly by growth and storage condition, and harvest times. Genetic tools are considered to provide more standardized and reliable methods for authentication of plant materials at the DNA level. Using Panax species, the methods developed previously include randomly amplified polymorphic DNA (RAPD; Bai et al., 1997; Shaw and But, 1995), low-Cot DNA fingerprinting (Ho and Leung, 2002), arbitrarily primed polymerase chain reaction (AP-PCR; Cheung et al., 1994), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP; Ngan et al., 1999; Um et al., 2001), Amplified fragment length polymorphism (AFLP; Kim et al., 2005), and microsatellite markers (Hon et al., 2003) and internal transcribed spacers (ITS) sequences of ribosomal DNA (Wen and Zimmer, 1996), and microchip electrophoresis (Qin et al., 2005).

Paran and Michelmore (1993) developed a technique known as sequence-characterized amplified regions (SCAR). SCAR markers have been derived from RAPD and AFLP markers, and have proven useful in identifying the plants at intra- and/or inter-specific level. SCAR as PCR based genetic markers is a genomic DNA fragment that is identified by PCR amplification using a pair of specific oligonucleotide primers (Paran and Michelmore, 1993). The conversion of such markers into SCARs based on the marker sequence information significantly improves the reproducibility and reliability of PCR assays (Paran and Michelmore, 1993). SCAR markers are highly advantageous for quick and easy assessment (Paran and Michelmore, 1993). Wang et al. (2001) developed the SCAR marker from RAPD fragments to authenticate *P. ginseng* and *P. quinquefolius* species.

The identification of species-specific DNA SCAR markers of *P. notoginseng* would be of great importance to discriminate this species. In this study, a species-specific SCAR marker (named PNG 8-1 and PNG 8-2) for *P. notogiseng* was obtained from AFLP fragments. PCR amplification using specific primers clearly demonstrated the specific band for *P. notoginseng*.

MATERIALS AND METHODS

Plant materials and extraction of genomic DNA

Seeds of P. ginseng were collected from Kangwon-do (Hambaek Mountain), Gyeongsangbuk-do (Punggi), and Chungcheongnam-do (Geumsan) in South Korea, and Jilin Province (Chang Bai Mountain) in China. Seeds of P. quinquefolius were collected from Wisconsin (field-cultivated ginseng) in USA. Seeds of P. japonicus were collected from Tochigi (Nikko national park) in Japan. Seeds of *P. notoginseng* were collected from Yunnan province (four individuals from field-cultivated plants) of southwestern China. Dehisced seeds after moisture-chilling treatment for 6 months were sowed in soil. After two months of culture, roots of plants were used to study the AFLP analysis. Total genomic DNA was extracted from approximately 200 mg of ginseng roots using the MagExtractor (TOYOBO, Japan). DNA concentration was measured with an UV spectrophotometer (Shimazu Co., Japan).

Genomic DNA digestion and adapter ligation

The AFLP analysis was performed according to the procedure described by Vos et al. (1995). In order to identify AFLP markers, 500 ng of genomic DNA were double-digested with 5 units of EcoRI (TaKaRa, Japan) and MseI (BioLabs, New England) for 12 h at 37℃. The ends of double-digested DNA fragments were ligated with EcoRI and MseI adapters for 12 hours at 14℃ (Table 1). After ligation, 10-fold diluted DNA solution was used for pre-amplification.

AFLP analysis

Each 20 µl PCR mixture contained 25 ng DNA, 1× reaction buffer (TaKaRa, Japan), 200 µM of each dNTP, 0.5 µM DNA primers (Table 1), and 1 U Taq DNA polymerase (EX Taq, TaKaRa, Japan). PCR amplification was performed in a DNA thermal cycler (Applied Biosystems 9800, Foster City, Calif.). The amplification profile consisted of one cycle of 5 min at 95℃, followed by 20 cycles of 30 s at 95℃, 1 min at 60℃, and 1 min at 72℃, with a final extension of 10 min at 72℃. Pre-amplification PCR products were diluted 50-fold with distilled water and used as templates for the subsequent

selective PCR amplification. The amplification mixture (20 µl, final volume) contained 5 µl pre-amplification mixture, 1x buffer, 200 µM of each dNTP, 0.5 µM DNA primer (total 10 kinds of primers (E1-5 and M1-4 of Table 1), and 1 U Taq DNA polymerase (Invitrogen, USA). After 30 s at 94°C, 30 s at 65°C, 1 min at 72°C for first cycle, followed by a lowering of temperature (1°C) in the next 12 cycles, then at 56°C for the remaining 23 cycles; extension for 1 min at 72°C. The AFLP amplified products were separated by electrophoresis on a 6% denaturing polyacrylamide gels (5.75% Long Ranger, BMA USA, 7 M urea, 1x TBE) and the fingerprint patterns were visualized by silver staining method (Promega, USA).

Statistical analysis

Each AFLP polymorphic fragments were scored as 1 for presence and 0 for absence. Statistical analysis was performed using the NTSYS-pc software (Rohlf, 1998). The genetic similarity was estimated according to Jaccard's similarity coefficients (Jaccard, 1908). The dendrogram was produced by using similarity matrix of Jaccard's coefficients and UPGMA (unweighted pair group method using arithmetic averages) method (Sneath and Sokal, 1973). The genetic distance was calculated as described by Nei (1978).

AFLP fragment cloning and DNA sequencing

Amplified specific bands were excised from AFLP gel with a razor blade and the DNA was extracted using the HiYield Gel/PCR DNA Extraction kit (Real Biotech, Taipei, Taiwan) according to the manufacturer's instructions. Ligation of the PCR product was carried out with a pGEM-T Easy vector (Promega) according to manufacturer's instructions and then re-amplified alongside the original AFLP reactions to ensure that the correct bands had been cloned. The purified ligation reaction was diluted with 50 µl water, and a 5 µl aliquot was subsequently mixed with 40 µl competent cells and placed on ice for 1 h and then placed into a cuvette and electroporated (GenePulser; BioRad, Hercules, Calif.). 1 ml LB medium was added immediately following electroporation and incubated for 1 h at 37°C with shaking, after which a 200 µl aliquot was plated onto LB agar plates containing ampicillin, IPTG and X-gal and incubated overnight at 37°C. Up to six white colonies from each transformation reaction were streaked onto LB plates to produce single colonies.

Plasmid DNA was extracted from overnight cultures of transformed bacterial cells, and samples diluted 1:50 in sterile water. 10 µl aliquots of the diluted miniprep were mixed with 6 µl sterile water, 10X buffer (Roche), and 2 µl EcoRI restriction enzyme (Roche) and the reactions incubated at 37°C for 1 h. The entire reaction was electrophoresed on a 1% agarose gel and stained with ethidium bromide. Two sequencing reactions were set up using 1 µg plasmid DNA combined with 4 µl of M13 forward and reverse primer, respectively. These reactions were brought up to a total volume of 18 µl with sterile water. DNA sequencing was performed using pUC/M13 primers on an automated sequencer (Applied Biosystems 3700, USA).

SCAR primer design and PCR analysis

The software EnCyclon and Primer3 (Steve and Skaletsky, 1996) were used for sequence analysis and SCAR marker design. Melting temperature, GC contents, and molecular weight were verified using oligo software, then primers were commercially synthesized.

The designed SCAR primer pairs (one forward and one reverse primer) were tested among the four Panax species (*P. ginseng, P. quinquefolius, P. japonicus*, and *P. notoginseng*). Testing was done to ensure amplification of the band with exact molecular weight and determine optimal annealing temperature. Two different annealing temperatures (58 and 62°C) were screened to determine the optimal annealing temperature. The PCR reaction to amplify the SCAR marker consisted of 32 cycles, each one consisting of a 30 s at 95°C, a 30 s at 58°C or 62°C, and a 30 s at 72°C. PCR products were run on a 1.3% (w/v) agarose gel and stained in ethidium bromide as stated above. Presence and absence of the SCAR band was visually scored and compared with samples of each species.

RESULTS

AFLP analysis

Ten AFLP primers as shown in Table 1 were screened for polymorphism among Panax species. The number of AFLP bands generated from Panax samples ranged from 48 - 73 in each primer combination (Figure 1). Every primer showed distinct polymorphic fragments. EcoRI+TG/MseI+CAC primer combination showed several polymorphic AFLP bands (Fig. 1B) in *P. notoginseng* compared to those of *P. ginseng, P. quinquefolius* and *P. japonicus*. Polymorphism showed more striking differences in *P. notoginseng* when compared to P. ginseng, P. quinquefolius and P. japonicus. Thereby it confirms the distinct polymorphic bands by AFLP technique among the different *Panax* species that can use for molecular marker to authenticate the *Panax* species.

Genetic distance among *Panax* species

Polymorphic AFLP fragments were used for the cluster analysis. The dendrogram shows the genetic relationship calculated from an AFLP analysis of *Panax* species (Figure 2). The coefficients of genetic similarity among Panax species ranged from 0.638 - 0.896. The *Panax* species clustered into three groups at a similarity level of 0.67. Among the *Panax* species, the most distant genetic relationship was found between Korean *P. ginseng and P. notoginseng*, and *P. ginseng* was also similar to the *P. quinquefolius* rather than to the P. japonicus or *P. notoginseng* (Figure 2).

The genetic distance among *Panax* species revealed that *P. notoginseng* showed the most distant genetic relationship among *Panax* species as the average value of the genetic distance between *P. notoginseng* and other *Panax* species was 0.555 ± 0.012 (Table 3).

Isolation of AFLP fragment for SCAR marker design

Twenty DNA fragments revealed *P. notoginseng* specific bands were obtained from AFLP gel and cloned and sequenced. After preliminary screening using 20 primers based on sequenced fragments, a 509 bp of DNA fragment named PNG 8 was choose for SCAR marker development (Figure 1B, arrow). Sequences were analyzed for sequence homology in the NCBI GenBank database, and the BLAST results revealed that the sequences have no homology with known plant nucleo-

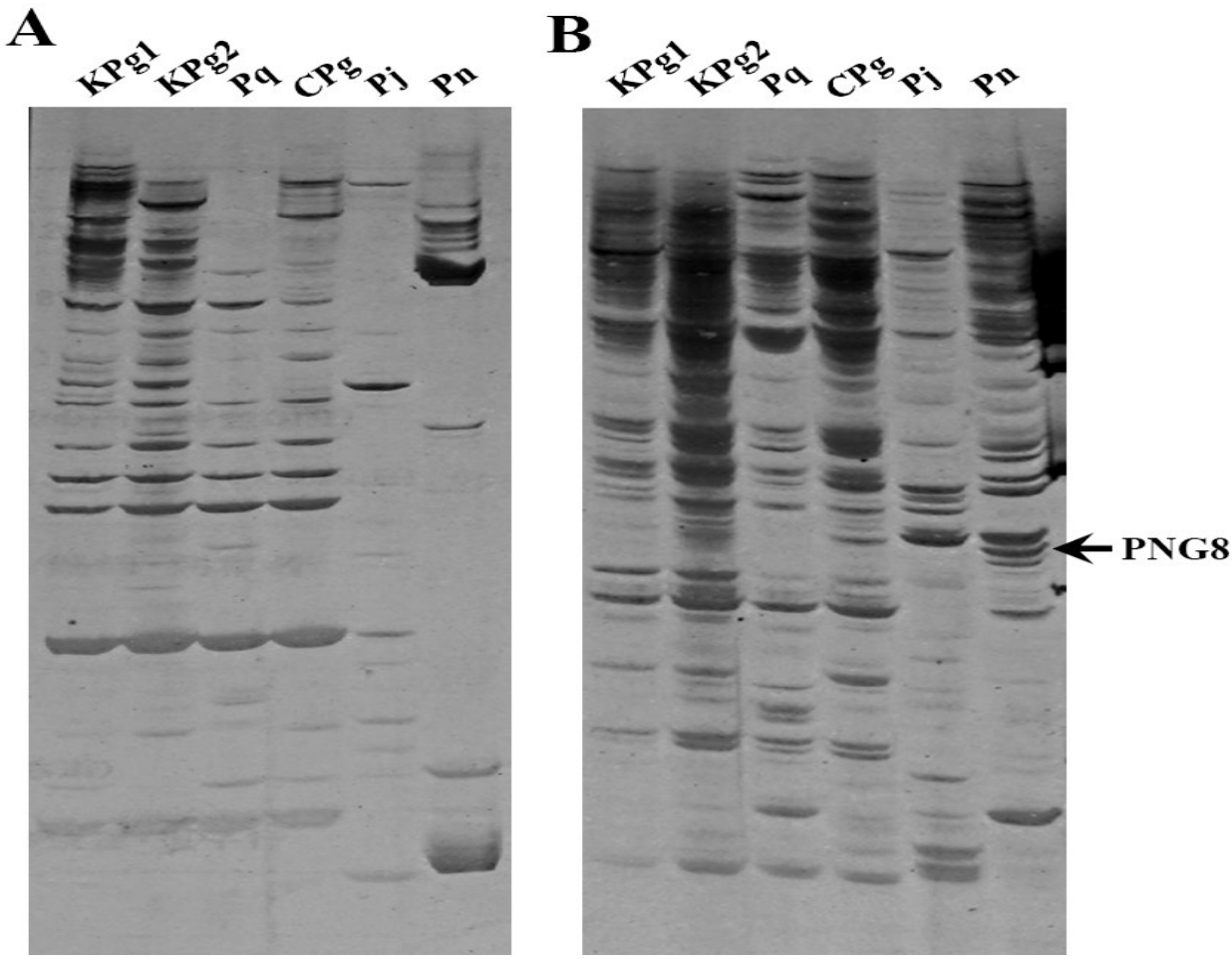

Figure 1. AFLP analysis of *Panax* species using primer combination of *EcoR*I+AT / *Mse*I+CTG (A), and *EcoR*I+TG / *Mse*I+CAC (B). KPg1; Wild-harvested Korean *P. ginseng*, KPg2; Field-cultivated Korean *P. ginseng*, Pq; *P. quinquefolius*, CPg; Chinese *P. ginseng*, Pj; *P. japonicus*, Pn; *P. notoginseng*. The arrow indicate specific band for gene sequencing.

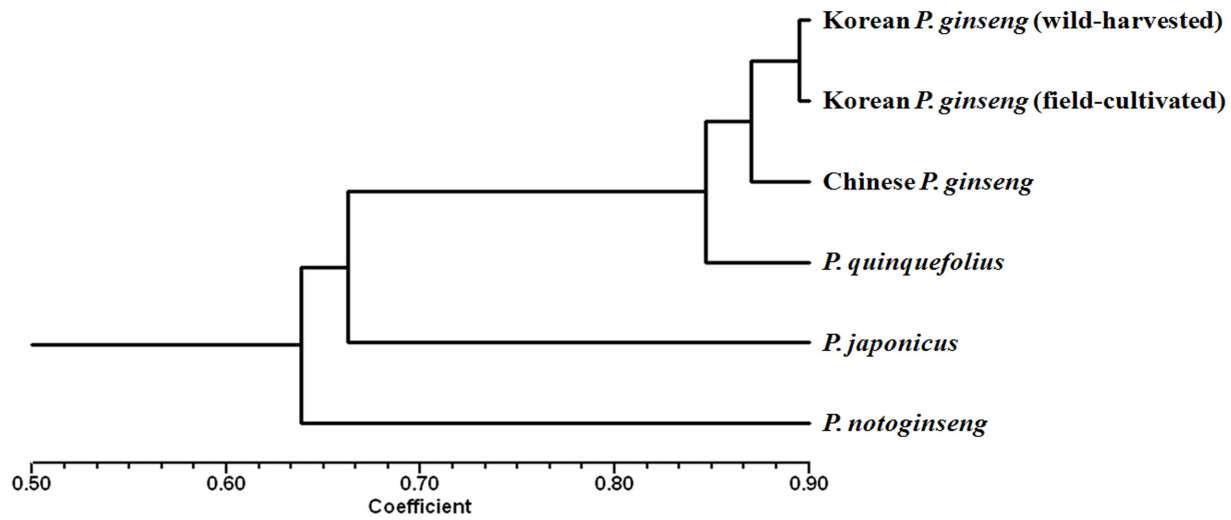

Figure 2. UPGMA dendrograms based on the Jaccard similarity index calculated from an AFLP analysis of *Panax* species.

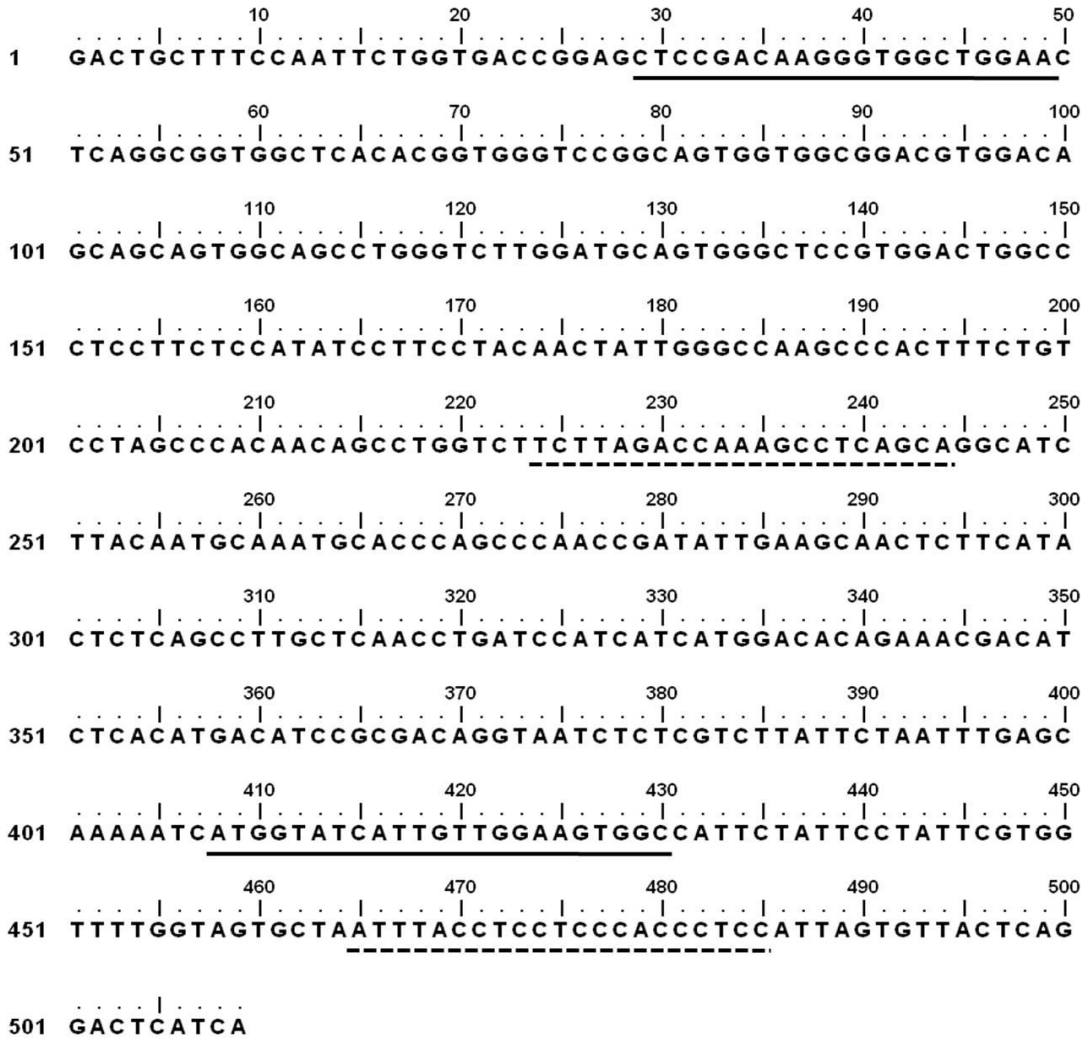

Figure 3. Sequence of the specific DNA fragments of *P. notoginseng*. Total length of the fragment (named PNG 8) is 509 bp. Designed primers for PCR analysis are underlined and named PNG 8-1 (▪▪▪▪▪) and PNG 8-2 (▬▬).

tide sequences at sequence-similarity levels. Two SCAR primers (PNG 8-1 and PNG 8-2) were designed to amplify this AFLP fragment from PNG 8 sequences (Figure 3). The nucleotide sequences of the primer pairs used for amplifying the SCAR fragments are shown in Table 2. The two primers PNG 8-1 and PNG 8-2 were designed for amplication of DNA at 402 and 260 bp fragments, respectively.

Table 2. Sequences of primers used for the SCAR markers.

SCAR marker	Sequence (5' to 3')
PNG 8-1	F : CTCCGACAAGGGTGGCTGGAA
	R : GCCACTTCCAACAATGATACCAT
PNG 8-2	F : CTTAGACCAAAGCCTCAGCA
	R : GAGGGTGGGAGGAGGTAAA

PCR amplification using SCAR primers

PCR amplication was performed using two SCAR marker primers (PNG 8-1 and PNG 8-2) and DNA samples of *P. ginseng* (wild-harvested and field-cultivated Korean *P. ginseng*, and wild-cultivated Chinese *P. ginseng*), *P. quinquefolius*, P. japonicus, and *P. notoginseng*. In order to determine the precise condition for PCR amplification, PCR analysis was performed using different DNA polymerases and annealing temperatures. Using the two commercially available DNA polymerases; Platinum Taq DNA polymerase (Invitrogen) and DNA master mix (Promega), PCR amplification did not show a significant difference between the two different DNA polymerase (Figure 4).

Two different annealing temperatures (58 and 62℃)

Table 3. The genetic distance among *Panax* species.

	Korean *P. ginseng* (Wild-harvested)	Korean *P. ginseng* (Field-cultivated)	Chinese *P. ginseng*	*P. quinquefolius*	*P. japonicus*	*P. notoginseng*
Korean *P. ginseng* (Wild-harvested)	0.000					
Korean *P. ginseng* (Field-cultivated)	0.134	0.000				
Chinese *P. ginseng*	0.164	0.171	0.000			
P. quinquefolius	0.190	0.214	0.190	0.000		
P. japonicus	0.443	0.482	0.457	0.522	0.000	
P. notoginseng	0.552	0.552	0.568	0.532	0.573	0.000

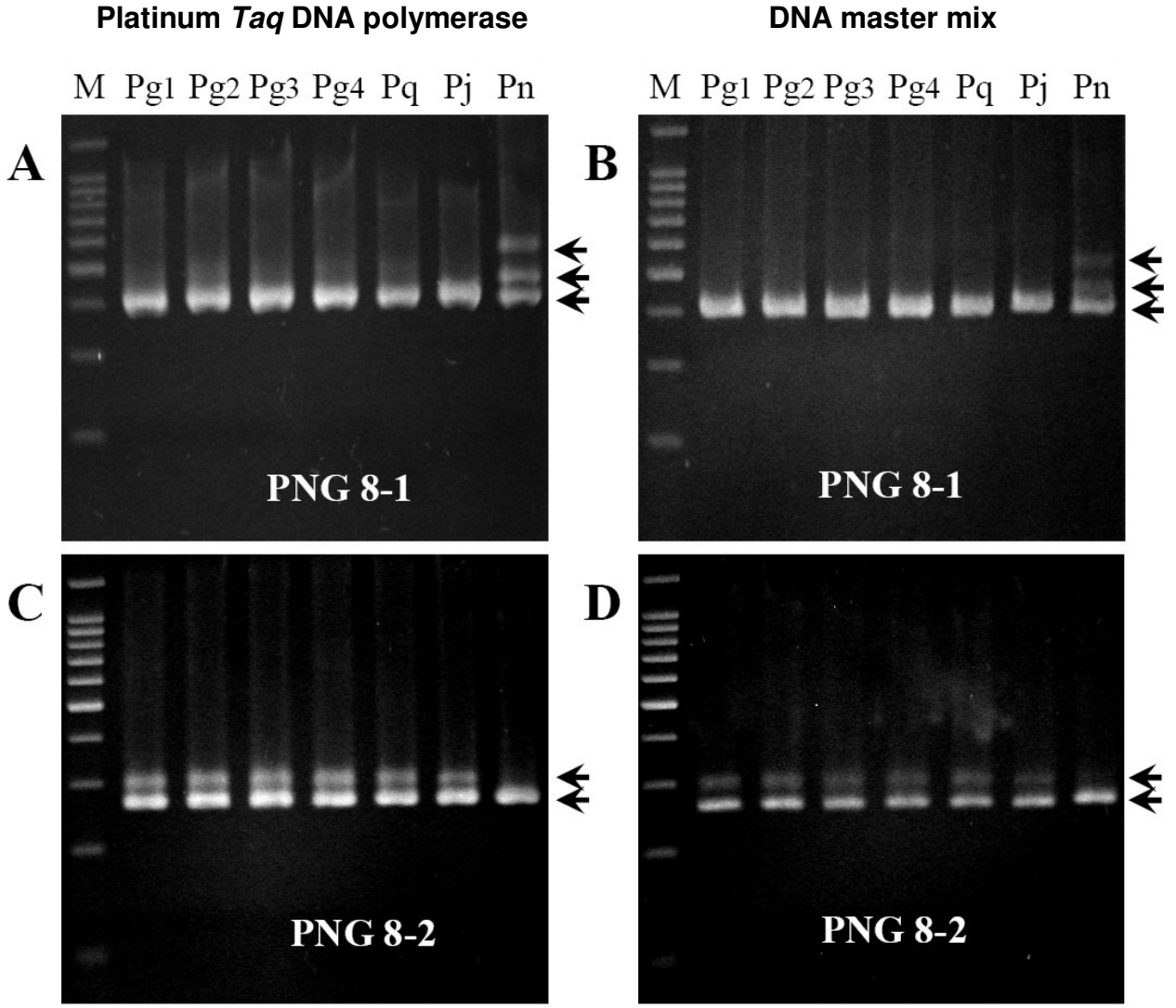

Figure 4. The optimal PCR amplification using different commercially-available DNA polymerases; Platinum *Taq* DNA polymerase (Invitrogen, A, C), and DNA master mix (Promega, B, D). SCAR fragments of *P. notoginseng* were amplified by PNG 8-1 (A-B) and PNG 8-2 (C-D) primer at 62°C annealing temperature. Lanes M; 100-bp marker, Pg1; wild-harvested Korean *P. ginseng* (Mt. Hambaek in Korea), Pg2; field-cultivated Korean *P. ginseng* (Geumsan in Korea), Pg3; field-cultivated Korean *P. ginseng* (Punggi in Korea), Pg4; Chinese *P. ginseng* (Jilin Province in China), Pq; *P. quinquefolius* (Wisconsin in USA), Pj; *P. japonicus* (Tochigi in Japan), Pn; *P. notoginseng* (Yunnan province in China). Arrows indicates PCR products.

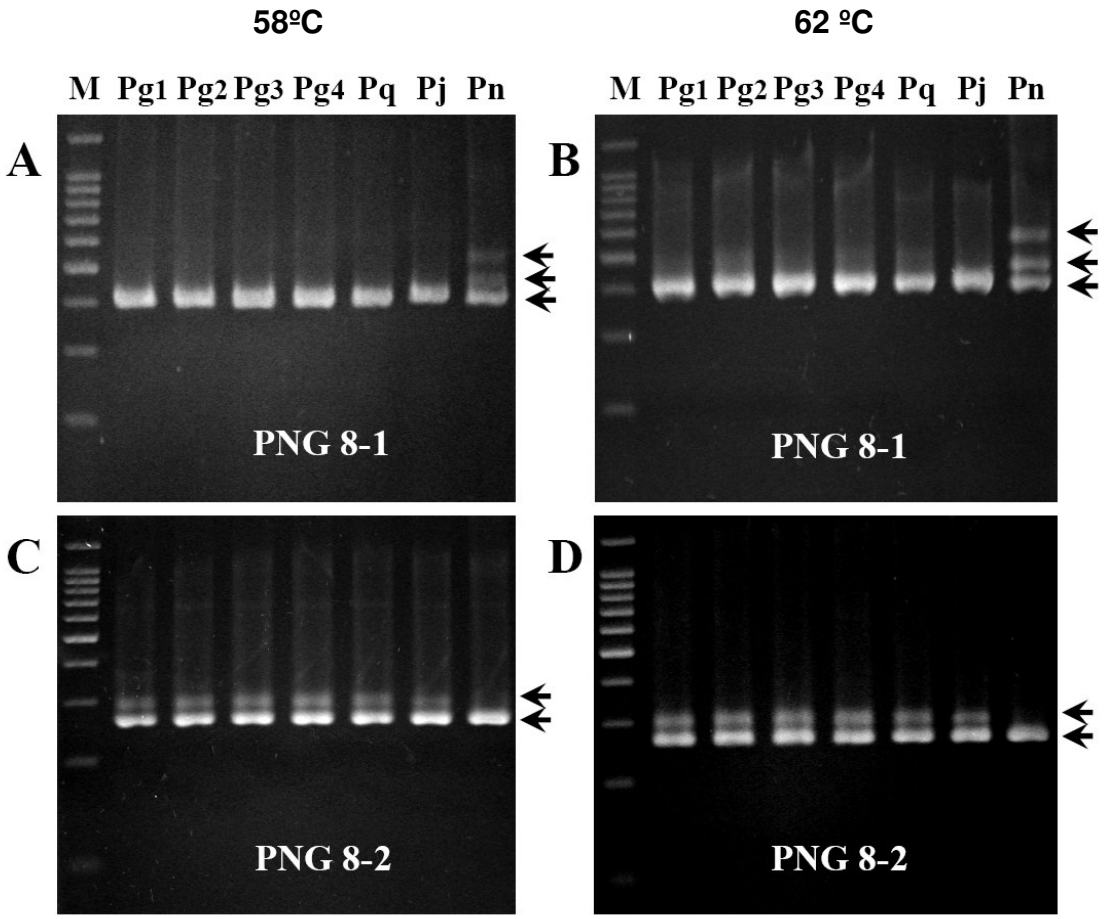

Figure 5. PCR patterns of SCAR markers, PNG 8-1 (A-B) and PNG 8-2 (C-D). PCR amplification was optimalized by different annealing temperatures (58°C; A, C and 62°C; B, D) and was performed with Platinum *Taq* DNA polymerase (Invitrogen). Lane M; 100-bp marker, Pg1; wild-harvested Korean *P. ginseng* (Mt. Hambaek in Korea), Pg2; field-cultivated Korean *P. ginseng* (Geumsan in Korea), Pg3; field-cultivated Korean *P. ginseng* (Punggi in Korea), Pg4; Chinese *P. ginseng* (Jilin Province in China), Pq; *P. quinquefolius* (Wisconsin in USA), Pj; *P. japonicus* (Tochigi in Japan), Pn; *P. notoginseng* (Yunnan province in China). Arrows indicates PCR products.

were also screened to determine the optimal annealing temperature (Figure 5). Annealing temperature at 62℃ was superior to authenticate the *P. notoginseng* among other species compared to 58℃ (Figure 5B and 5D). The optimized conditions to amplify the SCAR markers were as follows: an initial denaturation of 5 min at 95℃; 32 cycles of 30 s at 95℃, 30 s step at 62℃, and 30 s at 72℃; a final extension of 10 min at 72℃.

Using PNG 8-1 SCAR marker, single fragment at 402 bp was amplified in all the tested *Panax* species except for *P. notoginseng* (Figure 5A-B). However, three fragments (402 bp, about 470 bp, and 600 bp) were observed in *P. notoginseng*. Using PNG 8-2 SCAR marker, PCR product revealed single fragment of 260 bp for *P. notoginseng*, and two fragments at 260 and 290 bp for other *Panax* species (Figure 5C-D). When the annealing temperature was decreased to 58℃ (Figure 5A and 5C), PCR products of two SCAR markers There was no significant differences of PCR products between 58 and 62℃ in *P. notoginseng* although PCR products showed sharper band at 62℃ than those at 58℃ (Figure 5).

Using four independent individuals of *P. notoginseng* collected from Yunnan province of southwestern China, PCR amplication revealed the same PCR products in all samples (Figure 6).

DISCUSSION

AFLP is based on selective PCR amplification of restriction fragments from a digest of total genomic DNA using PCR. The procedure of the AFLP analysis is more time-consuming than RAPD. However, a major advantage of AFLP markers is the capacity to reveal many polymorphic bands in one lane compared to RAPD markers (Barker et al., 1999; Ha et al., 2002). Because of the higher sensiti-

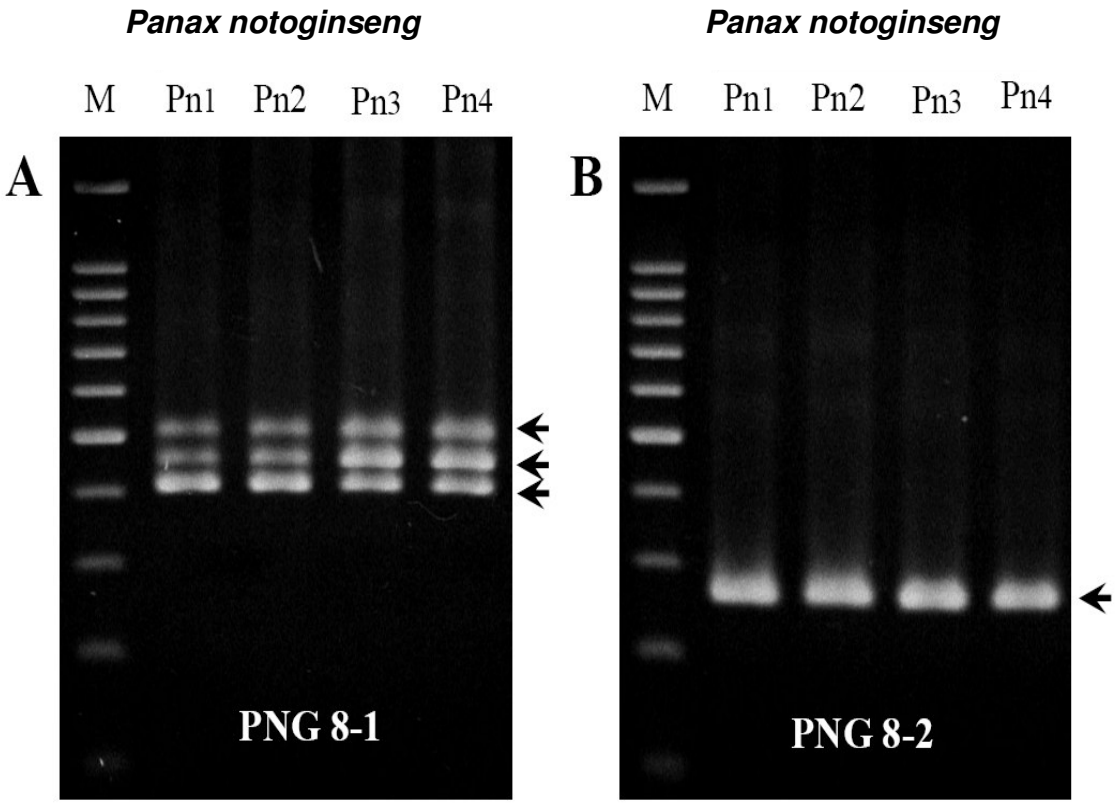

Figure 6. PCR amplification of SCAR fragments in different samples of *P. notoginseng.* Different individuals (Pn1, Pn2, Pn3, and Pn4) of *P. notoginseng* were collected from Yunnan province of southwestern China. PCR analysis of specific fragments amplified with PNG 8-1 (A) and PNG 8-2 (B). PCR reaction was performed with the Platinum *Taq* DNA polymerases and 62°C annealing temperatures. M is the 100-bp marker. Arrows indicates PCR products.

vity of AFLP than that of RAPD, the weak divergence can be efficiently detected by AFLP. Also, AFLP technique is a reliable, stable, and rapid assay for use in molecular marker screening (Jia et al., 2001). Thus the AFLP technique has been increasingly used to assess genetic diversity in a variety of organisms (Winfield et al., 1998; Das et al., 1999; Cresswell et al., 2001).

In this study, we attempted to use the AFLP fingerprints in phylogenetic analysis of the *Panax* species. Three kinds of *P. ginseng* (wild-harvested and field-cultivated Korean *P. ginseng*, and Chinese *P. ginseng*) were closely clustered together (Figure 2). Relatively higher genetic diversity was shown in *P. notoginseng*. The average value of the genetic distance between *P. notoginseng* and other *Panax* species was 0.555 ± 0.012.

Compared with AFLP markers, SCAR markers are stable, repeatable, clear, and convenient in practical application. Thus, conversion of AFLP marker to SCAR marker by sequencing of amplified bands can provide effective methods for the identification and classification in a various species (Radisek et al., 2004; Sun et al., 2005; He et al., 2008). In this study, 20 DNA fragments from AFLP gel were cloned and sequenced. From preliminary screening of 20 AFLP derived SCAR markers, one sequence, named PNG8, utilized for *P. notoginseng* species-specific SCAR marker.

There are many reports for authentication of ginseng species using various techniques such as PCR-RFLP (Ngan et al., 1999), AFLP (Ha et al., 2002; Choi et al., 2008), RAPD (Ho and Leung, 2002), DALP (Ha et al., 2001), low-Cot DNA fingerprinting (Bai et al., 1997), AP-PCR (Cheung et al., 1994), microsatellite markers (Hon et al., 2003; Kim et al., 2007; Ma et al., 2007), internal transcribed spacers (ITS) sequences of ribosomal DNA (Wen and Zimmer, 1996; Zhu et al., 2004), and microchip electrophoresis (Qin et al., 2005). For molecular authentication of *P. notoginseng*, RAPD (Cui et al., 2003) or sequencing of rRNA and chloroplast matK gene (Cao et al., 2001), and sequencing of 5S-rRNA spacer domain (Cui et al., 2003) were reported. Compared to RAPD and sequencing of DNA, development of SCAR markers in *P. notoginseng* is highly advantageous for quick and easy assessment. Our present result is the first attempt to authenticate the *P. notoginseng* among other Panax species using SCAR marker.

In conclusion, two species-specific SCAR markers for *P. notoginseng* were obtained from AFLP-derived DNA fragments. PCR amplication of PNG 8 specific primers

clearly demonstrated the different band pattern for *P. notoginseng* among other P. ginseng, P. quinquefolius and P. japonicus. Two SCAR markers for *P. notoginseng* will be used for rapid authentication of this species among other Panax species.

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