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Authentication of *Panax ginseng* and *Panax quinquefolius* Using Amplified Fragment Length Polymorphism (AFLP) and Directed Amplification of Minisatellite Region DNA (DAMD)

W. Y. HA,[†] P. C. SHAW,[†,‡] J. LIU,[†] FORREST C. F. YAU,[‡] AND J. WANG*,[†,‡]

Department of Biochemistry and Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong, China

AFLP profiles characteristic to *Panax ginseng* and *Panax quinquefolius* were generated using primers E-AGG/M-CAA. *P. ginseng* samples from different farms in China and Korea are homogeneous genetically [similarity index (SI) = 0.88−0.99], whereas samples of *P. quinquefolius* from different sources are much more heterogeneous (SI = 0.64−0.96). Detailed analysis of one of the polymorphic bands in *P. ginseng* led to the identification of a minisatellite Pg2, which contains eight repeats of 5′-AGGACTCATCACATTGTTACTC. The minisatellite DNA was consequently used in directed amplification minisatellite region DNA analysis to authenticate the two ginsengs.

KEYWORDS: ***Panax ginseng*; *Panax quinquefolius*; AFLP; DAMD**

INTRODUCTION

Panax ginseng (ginseng) and *Panax quinquefolius* (American ginseng) belong to the family Araliaceae and are highly recognized and valued as Chinese herbal medicines. *P. ginseng*, which was used for medicinal treatment and as a tonic as early as 202 B.C. (*1*), is now cultivated in northeastern China, Japan, and Korea. *P. quinquefolius* was used by Native American tribes as a health food long before it was recorded in the early 1700s and was exported to China in the late 18th century and is now farmed in the United States (Wisconsin), Canada (British Columbia), and other parts of the world. Ginsenosides (ginseng saponins) were demonstrated to be the active constituents in both species. The two ginsengs contain chemically related but pharmacologically distinctive ginsenosides and are used for the treatment of different diseases in Chinese medicine. Hong Kong is a major trade center for both *P. ginseng* and *P. quinquefolius*. As the price of cultivated *P. quinquefolius* is usually 5−10 times greater than that of cultivated *P. ginseng*, the latter is frequently misrepresented as the former in the market. Therefore, it is essential to develop effective authentication methods to differentiate the two ginsengs to safeguard public health and to protect consumers' rights.

We have previously applied AP-PCR, RAPD, PCR-RFLP, and DALP to differentiate *Panax* and various species of Chinese medicinal materials (*2*−*7*). In this paper, we explore the usage of AFLP and DAMD technology to generate DNA fingerprints for distinguishing *P. ginseng* and *P. quinquefolius by* measuring the relatedness between the two ginsengs and among the ginseng samples from different localities. We also report, for the first time, the identification of a minisatellite from AFLP fingerprints.

* Corresponding author [telephone (852) 2609-6122; fax (852) 2603-7246; e-mail wangjun@cuhk.edu.hk].
[†] Department of Biochemistry.
[‡] Institute of Chinese Medicine.

MATERIALS AND METHODS

Medicinal Materials. Dried or fresh roots of *Panax* species were obtained from the following sources: *P. ginseng* C.A. Meyer samples were from China (Ji'an County of Jilin Province and Xingbing County of Liaonin Province) and Korea (Kangwon, Pochon, Kanghwa, Kumsan, and Kimpo); *P. quinquefolius* L. samples were from farms in Ontario and British Columbia, Canada, and Wisconsin, USA. Samples were identified and deposited in the Institute of Chinese Medicine, The Chinese University of Hong Kong. Genomic DNA was extracted using a modified KOAc/SDS method (*8*).

AFLP Assay. The AFLP assay was done using an AFLP Analysis System II (GIBCO BRL) according to the manufacturer's instructions. Briefly, 125 ng of genomic DNA was digested in 12.5 μL of 1× reaction buffer with 15 units of *Eco*RI and *Mse*I. The reaction mixture was incubated at 37 °C for 2 h and inactivated at 70 °C for 15 min. The mixture was then ligated with 12 μL of adapter/ligation solution containing *Eco*RI and *Mse*I adapters in a final volume of 25 μL. After incubation at 20 °C for 2 h, it was diluted 10-fold with TE buffer. Preamplification reaction was carried out in a volume of 25 μL containing 5 μL of the 10-fold diluted ligation mixture, 1× PCR buffer containing 1.5 mM $MgCl_2$, 20 μL of preamplification primer mix, and 0.5 unit of *Taq* DNA polymerase. PCR amplification was performed using the following cycling profile: 20 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min. The amplified solution was diluted 20-fold. In subsequent selective amplification, primers were first labeled in a final volume of 10 μL containing 1× T4 polynucleotide kinase buffer, 3.6 μL of *Eco*RI primer, 2 μL of [γ-^{33}P]ATP (3 μCi/mmol, 10 mCi/mL), and 10 units of T4 polynucleotide kinase and incubated at 37 °C for 1 h. It was mixed with 90 μL of *Mse*I primer containing dNTPs to produce a primers/dNTP mix. This primer/dNTPs mix was mixed with 2.5 μL of diluted template DNA, 1× PCR buffer with 1.5 mM $MgCl_2$, and 0.25 unit of *Taq* DNA polymerase to a total volume of 10 μL. The amplification was performed using the following "touchdown" cycling profile: first cycle of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min; a subsequent 12 cycles of lowering the annealing temperature (65 °C) by 0.7 °C per cycle; and a final 23 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min.

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Table 1. SI of *P. ginseng* Compiled from E-AGG/M-CAA AFLP Profile[a]

		Jilin		Liaonin		Kangwon			Pochon			Kanghwa			Kumsan				Kimpo	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Jilin	2	0.93																		
Liaolin	3	0.91	0.97																	
	4	0.93	0.92	0.93																
	5	0.96	0.93	0.92	0.93															
Kangwon	6	0.95	0.94	0.89	0.94	0.95														
	7	0.95	0.93	0.90	0.95	0.95	0.92													
	8	0.96	0.92	0.91	0.93	0.96	0.96	0.95												
Pochon	9	0.95	0.92	0.90	0.89	0.93	0.93	0.89	0.95											
	10	0.93	0.92	0.93	0.95	0.94	0.88	0.91	0.93	0.95										
	11	0.95	0.92	0.90	0.92	0.96	0.96	0.95	0.98	0.96	0.97									
Kangwha	12	0.95	0.92	0.90	0.92	0.96	0.96	0.95	0.98	0.96	0.95	0.99								
	13	0.94	0.94	0.93	0.97	0.98	0.90	0.90	0.95	0.95	0.96	0.98	0.98							
	14	0.96	0.93	0.91	0.94	0.96	0.96	0.95	0.97	0.94	0.97	0.96	0.96	0.99						
	15	0.93	0.92	0.92	0.93	0.95	0.93	0.92	0.95	0.93	0.95	0.96	0.96	0.95	0.96					
Kumsan	16	0.95	0.93	0.95	0.95	0.96	0.91	0.91	0.95	0.94	0.95	0.92	0.94	0.96	0.95	0.93				
	17	0.95	0.92	0.94	0.95	0.97	0.92	0.90	0.95	0.92	0.95	0.95	0.95	0.96	0.97	0.94	0.96			
	18	0.96	0.92	0.91	0.94	0.96	0.95	0.95	0.97	0.94	0.89	0.96	0.96	0.95	0.96	0.95	0.98	0.95		
Kimpo	19	0.96	0.93	0.91	0.94	0.97	0.95	0.94	0.96	0.93	0.89	0.96	0.96	0.95	0.96	0.96	0.97	0.96	0.96	
	20	0.95	0.93	0.95	0.94	0.96	0.94	0.95	0.96	0.92	0.9	0.95	0.95	0.96	0.94	0.95	0.95	0.95	0.97	0.96

[a] Two to four individuals from each locality are examined. Lanes 1 and 2 are samples from Ji'an County, Jilin Province; lanes 3 and 4, from Xingbing County, Liaonin Province; lanes 5–7, from Kangwon; lanes 8–10, from Pochon; lanes 11–13, from Kanghwa; lanes 14–17, from Kumsan; and lanes 18–20, from Kimpo.

The PCR solution was mixed with an equal volume (10 μL) of formamide dye (98% formamide, 10 mM EDTA, bromophenol blue, and xylene cyanol) and electrophoresed on 6% denaturing polyacrylamide gel [50 cm long, 0.4 mm thick, acrylamide/bis(acrylamide) 19:1, and in 7 M urea]. A sequencing reaction of PUC18 DNA was run in parallel with the samples as a size marker. All samples were simultaneously run at a constant temperature of 55 °C until xylene cyanol reached two-thirds the length of the gel. After electrophoresis, the gel was dried and autoradiographed with a Biomax MR film for two to three days.

SI Analysis. AFLP bands were scored as 0 (absent) or 1 (present). The relatedness of the two *Panax* species from different localities was established on the basis of SI, the fraction of shared fragments between two samples, using the formula $SI = 2N_{xy}/N_x + N_y$, where N_{xy} is the number of bands shared between two samples, N_x is the number of bands in sample X, and N_y is the number of bands in sample Y. The value of SI ranges from 0 to 1 (*9*). Only clear and distinctive bands were scored; diffuse and low-intensity bands were excluded from the scoring.

Purification and Amplification of AFLP Polymorphic Band. The *P. ginseng*-specific AFLP band was excised from the polyacrylamide gel and eluted by soaking it in 100 μL of TE for 3 h at 65 °C. With 1 μL of the DNA eluant as the template, PCR amplification was performed in a volume of 20 μL using the same cycle profile and conditions as that of the selective AFLP amplification, except that 75 ng of unlabeled AFLP primers was used. The reamplified product was electrophoresed in a 2% TAE agarose gel, excised, and purified using a BIO 101 GENECLEAN III kit. The band was cloned in pUC18 and sequenced using a T7 sequencing kit (Pharmacia). The 22 bp minisatellite Pg2, AGGACTCATCACATTGTTACTC, was used as the primer for direct amplification of the minisatellite region. The direct amplification of the minisatellite region DNA (DAMD) was done in a 20 μL volume containing 40 ng of plant DNA, 1× PCR buffer, 0.125 mM dNTPs, 2.5 mM $MgCl_2$, and 40 pmol of primer using the following cycling profile: 95 °C for 3 min, and, after the addition of 1 unit of *Taq* DNA polymerase, 94 °C for 4 min, 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 30 s. After the amplification, the products were resolved on 2% agarose TBE gel, stained with ethidium bromide, and visualized and photographed under UV illumination.

RESULTS

AFLP Analysis of *P. ginseng* and *P. quinquefolius*. Ten AFLP primers in various combinations were screened for polymorphism between the two *Panax* species. Primer pair E-AGG/M-CAA was found to be able to detect polymorphic bands among the samples of different localities of the same species (**Figure 1**). The numbers of AFLP bands generated from the *P. ginseng* samples ranged from 66 to 77, whereas that from *P. quinquefolius* ranged from 57 to 63. Samples of *P. ginseng* of different localities show high values of similarity index (SI = 0.88−0.99) (**Table 1**), whereas samples of *P. quinquefolius* of different localities appear to have a more heterogeneous genetic makeup with SI varying between 0.64 and 0.96 (**Table 2**). The primer has also revealed DNA polymorphism between the two closely related species. Many of the AFLP bands are shared by the two ginsengs; some of them are indicated in Figure 1 by open arrows. Other bands, indicated as solid arrows in Figure 1, are polymorphic between the two species. These polymorphic bands are further analyzed.

Identification of Minisatellite Pg2 and Its Usage in Authentication. Determination of the sequences of the polymorphic bands discloses that one of the polymorphic bands from *P. ginseng* (Kanghwa), indicated by an asterisk in **Figure 1A**, contains a minisatellite, designated Pg2, of eight repeats of 22 nucleotides (AGG ACT CAT CAC ATT GTT ACT C) (**Figure 2**). The sequence does not match any entries in GenBank. Using the 22 bp minisatellite sequence as a primer, a fingerprinting profile discriminatory for the two *Panax* species was established (**Figure 3**). In the profile, four common bands were shared by all nine samples of both *Panax* species, but the other three bands of 990, 1490, and 1640 bp in size, indicated by arrows, are amplified only from each of the five *P. quinquefolius* samples but from none of the four *P. ginseng* samples (**Figure 3**).

DISCUSSION

The AFLP technique has two characteristics. First, the template DNAs are restriction fragments. Second, the primers contain variable selective nucleotides at the 3′ end. Consequently, by addition or removal of nucleotides at the 3′ end of the AFLP primer, one can selectively amplify different sets of restriction fragments of genomic DNA. The number of selective nucleotides added to the 3′ end of the primers dictates the level of complexity for the AFLP profile. For example, primers with no or one selective nucleotide were used for molecular typing

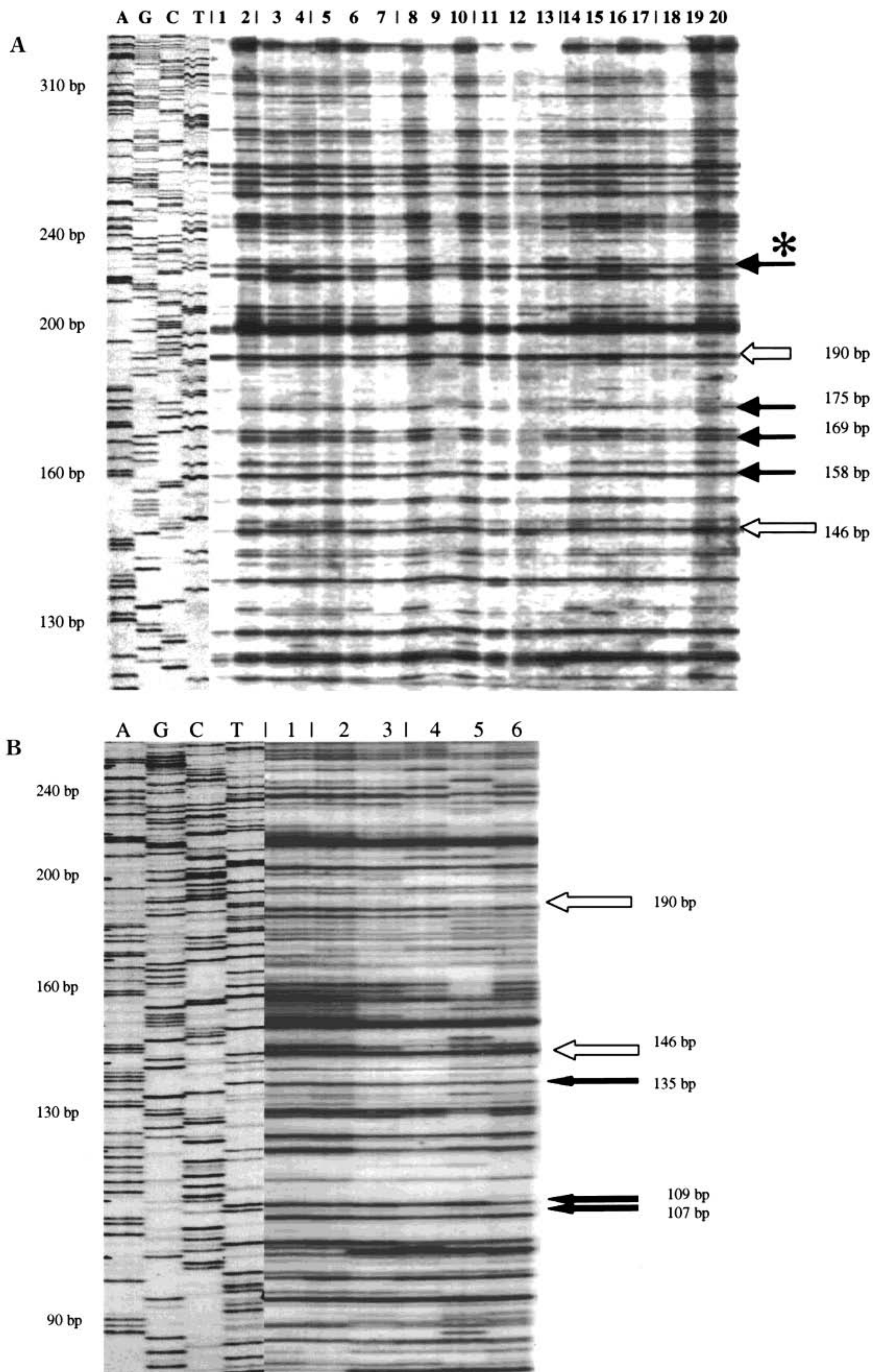

Figure 1. (A) AFLP profile of *P. ginseng* generated by primer pair E-AGG/M-CAA. Two to four individuals from each locality are examined: (lanes 1, 2) samples from Ji'an County, Jilin Province; (lanes 3, 4) from Xingbing County, Liaonin Province; (lanes 5–7) from Kangwon; (lanes 8–10) from Pochon; (lanes 11–13) from Kanghwa; (lanes 14–17) from Kumsan; (lanes 17–20) from Kimpo. Asterisk indicates sequence containing minisatellite repeat Pg2. (B) AFLP profile of *P. quinquefolius* generated by primer pair E-AGG/M-CAA: (lane 1) from Ontario; (lanes 2, 3) from two farms in British Columbia; (lane 4) from a farm in Canada; (lane 5) from a farm in America; (lane 6) from a farm in Wisconsin. Solid arrows indicate bands unique to *P. ginseng* (A) or *P. quinquefolius* (B). Open arrows represent bands common to both *Panax* species. Lanes A, G, C, and T are the sequence of pUC 18, used as a size marker.

Table 2. SI of *P. quinquefolius* Compiled from E-AGG/M-CAA AFLP Profile[a]

		Ontario	British Columbia		Canada	America
		1	2	3	4	5
British Columbia	2	0.96				
	3	0.89	0.88			
Canada	4	0.76	0.95	0.75		
America	5	0.72	0.75	0.72	0.72	
Wisconsin	6	0.87	0.85	0.70	0.82	0.64

[a] Lane 1 is from Ontario; lanes 2 and 3, from two farms in British Columbia; lane 4, from a farm in Canada; lane 5, from a farm in America; and lane 6, from a farm in Wisconsin.

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        10          20          30          40          50
CTGCGTACCA ATTCAGGTCA AGATACTTAC ATAGACATCA AGACGCGTTG
        60          70          80          90         100
TTACTC AGGACTCATCACATTGTTACTC AGGACTCATCACATTGTTACTC
       110         120         130         140         150
AGGACTCATCACATTGTTACTC AGGACTCATCACATTGTTACTC AGGAC
       160         170         180         190         200
TCATCACATTGTTACTC AGGACTCATCACATTGTTACTC AGGACTCATCA
       210         220         233
CATTGTTACTC AGGACTCATCACATTGTTACTC
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Figure 2. Sequence of minisatellite Pg2, with the repeating sequences (AGGACTCATCACATTGTTACTC) in bold.

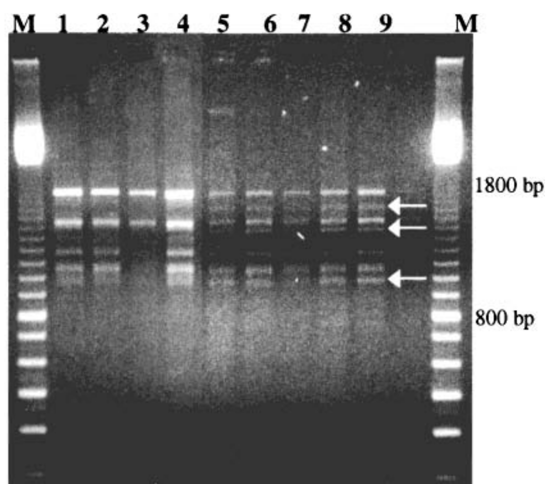

Figure 3. Directed amplification minisatellite region DNA (DAMD) analysis of *P. ginseng* and *P. quinquefolius*: (lanes 1–4) *P. ginseng* from Ji'an County, Jilin Province; Xingbing County, Liaonin Province; and Kanghwa and Kimpo of Korea, respectively; (lanes 5–9) *P. quinquefolius* from British Columbia, Ontario, Wisconsin, and a farm in America, respectively. Arrows indicate the polymorphic bands. M is the 100 base pair marker (Pharmacia).

of bacteria (*10*), and primers having seven to eight selective nucleotides were used for the study of organisms of a larger genome (*11*). In practice, primer selectivity can be optimized using touchdown PCR, whereby the annealing temperature is lowered gradually to a temperature at which efficient primer binding occurs and is maintained for the rest of the PCR cycles. AFLP polymorphisms may be the consequence of single nucleotide changes either at or adjacent to the restriction site(s). Deletions, insertions, and rearrangements that affect the restriction fragment will also lead to polymorphisms (*11*). This technique is commonly used in biodiversity studies, analysis of germplasm collections, and genetic relationship studies.

Using primer pair E-AGG/M-CAA, profiles containing 57−77 PCR fragments and discriminatory between the two ginsengs were generated (**Figure 1**). Bands with different intensities were observed. Faint bands are caused by low efficiency of amplification and may be due to the existence of a secondary structure in the DNA template that interferes with the PCR reaction or due to imperfect match of primers to their binding sites. Apart from the existence of polymorphism between the samples of the two ginsengs, polymorphism was also identified among samples from different localities. Samples of *P. quinquefolius* from different localities appear to have heterogeneous genetic makeup with SI values varying between 0.64 and 0.96 (**Table 2**), whereas samples of *P. ginseng* are much more homogeneous with SI = 0.88−0.99 (**Table 1**).

Compared with other methods that detect genome-wise polymorphism simultaneously, such as RAPD and AP-PCR (*2*, *5*, *7*), AFLP is more robust and reliable. A reproducibility testing of AFLPs by a network of European laboratories has demonstrated that the AFLP profiles produced were identical and highly reproducible among the seven European laboratories (*12*). Nevertheless, AFLP is a delicate technique, as (1) meaningful fingerprints can be obtained only from complete digestion of genomic DNA, (2) AFLP fingerprints will be too diversified when sequence homology is <90% (*13*), and (3) high-quality DNA, especially high molecular weight DNA, is required.

To develop a rapid, simple, and reliable method for authentication between the two ginsengs, several species-specific bands, identified after comparison with AFLP profiles of the samples of the two ginsengs, were sequenced, and primers designed accordingly were screened for species-specific PCR fragments. A 22 bp minisatellite repeat of the polymorphic band Pg2 has been found to be present in all of the *P. ginseng* samples but not in *P. quinquefolius* (**Figure 3**).

Minisatellites are highly polymorphic tandem DNA repeats found in diverse species of plants and animals (*14*). They are ∼10−60 bp long, concentrated in the telomeric regions of chromosomes. In comparison, microsatellites are made up of simple repeats of mononucleotide to pentanucleotides, with variable size of a few tens to a few hundred bps. A microsatellite usually has more alleles than a minisatellite and therefore is particularly suitable for individual identification within the same species.

Possible mechanisms for the origin of minisatellites include replication slippage, transposition, and unequal crossover between sister chromatids at mitosis or meiosis or between homologous recombination at meiosis and gene conversion (*15*). If the orientation of minisatellites happens to enclose a single-copy DNA with amplifiable length, then it can be used as a primer to amplify the DNA fragment.

Recently, DAMD−PCR has been successfully applied for genotyping of wheat cultivars and rice species (*16*). As reported (*17*), PCR fragments amplified by DAMD, using the sequence of the minisatellite as a single primer, showed minor variation within a single species but large differences among three species of salmonids. Similarly, in our DAMD profile of the ginsengs using the Pg2 primer, in addition to the common bands shared by both of the species, three bands of 990, 1490, and 1640 bp in size are uniquely amplified from all of the *P. quinquefolius* samples but not from any of the *P. ginseng* samples tested (**Figure 3**). Our study demonstrates that by using the primer

flanking the 22 bp minisatellite repeat, the simple method of DAMD is capable of differentiating *P. ginseng* and *P. quinquefolius*.

ABBREVIATIONS USED

AFLP, amplified fragment length polymorphism; AP-PCR, arbitrarily primed Polymerase Chain Reaction; DALP, direct amplification of length polymorphisms; DAMD, directed amplification of minisatellite region DNA; PCR-RFLP, Polymerase Chain Reaction−restriction fragment length polymorphism; RAPD, random amplified polymorphic DNA; SI, similarity index.

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