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Molecular authentication of *Panax ginseng* and ginseng products using robust SNP markers in ribosomal external transcribed spacer region

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ABSTRACT

Panax ginseng and *Panax quinquefolius* are the most widely used *Panax* species, but they are known to have different properties and medicinal values. The aim of this study is to develop a robust and accurate DNA marker for identifying *P. ginseng* and the origins of ginseng products. Two single nucleotide polymorphism (SNP) sites specific to *P. ginseng* were exploited from nuclear ribosomal external transcribed spacer (ETS) region. Based on the SNP sites, two specific primers were designed for *P. ginseng* and *P. quinquefolius* respectively. *P. ginseng* can be easily discriminated from *P. quinquefolius* by amplifying the two specific alleles using multiplex allele-specific PCR. Favorable results can also be obtained from commercial ginseng products. The established method is highly sensitive and can detect 1% of intentional adulteration of *P. quinquefolius* into *P. ginseng* down to the 0.1 ng level of total DNA. Therefore this study provides a reliable and simple DNA method for authentication of the origins and purities of ginseng products.

1. Introduction

Panax ginseng (Korean ginseng) and *Panax quinquefolius* (American ginseng) are the most widely used *Panax* species, and the products of these two ginsengs have attracted worldwide consumption. Ginsenosides are the major bioactive constituents in both species, but they are known to have different properties and medicinal values [1,2]. *P. ginseng* is considered to be "warm" and used in "yang-deficient" conditions, whereas *P. quinquefolius* is "cool" and is mainly used in "yin-deficient" conditions [3]. However, American ginseng is sometimes adulterated in commercial Korean ginseng products as material by some dishonest merchants to reduce production costs, as American ginseng has much higher total ginsenosides content [4]. Therefore, it is essential to develop effective methods to authenticate the origin of commercial ginseng products to safeguard public health as well as consumers' rights.

Although several efforts have been made for discrimination of Korean ginseng from American ginseng, authentication of the origin of *P. ginseng* products is not an easy task. First, the sterilization and extraction treatments in processing red ginseng extracts will result in a high degree of DNA degradation, therefore DNA molecular markers such as RAPD (random amplified polymorphic DNA), ISSR (inter-simple sequence repeat), SSR (simple sequence repeat), and AFLP (amplified fragment length polymorphisms) may get unfavorable identification results. Second, with the development of new *P. ginseng* cultivars [5], the current DNA molecular markers cannot identify the ginseng origins accurately. The chemical analysis method [6,7] encounters the same problem because ginsenoside profile used for authentication markers may be affected by commercial processing. In the present study, we describe a robust and reliable method for authentication of the origin of *P. ginseng* products, by using two SNP markers exploited from the external transcribed spacer (ETS) region of ribosomal DNA.

2. Materials and methods

2.1. Materials and DNA isolation

Samples of ginseng plants list in Table 1 were provided by *Korean Ginseng Center for Most Valuable Products & Ginseng Genetic Resource Bank*. The plant roots were frozen in liquid nitrogen and ground into fine powders. Genomic DNA was isolated using a plant DNA isolation kit (Exgene Plant SV mini, GeneAll), according to manufacturer's instructions. Commercial products of Korean ginseng and American ginseng were purchased from local market and USA, respectively. Ginseng commercial samples were used directly in DNA isolation procedure.

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Table 1
Plant samples used in this study.

Ginseng samples	Voucher	Localities
Chunpoong[a]	GB001	Kochang, Korea
Yunpoong[a]	GB002	Kochang, Korea
Gopoong[a]	GB003	Kochang, Korea
Sunpoong[a]	GB004	Kochang, Korea
Gumpoong[a]	GB005	Kochang, Korea
Sunwon[a]	GBD048	Daejeon, Korea
Chungsun[a]	GBD073	Daejeon, Korea
Sunweon[a]	GBD043	Daejeon, Korea
Sunhyang[a]	GBD058	Daejeon, Korea
Damaya[b]	GB090	Jilin, China
Ermaya[b]	GB091	Jilin, China
Biantiao[b]	GB092	Jilin, China
P. quinquefolius	GB099	Wisconsin, USA
P. quinquefolius	GB100	Minnesota, USA
P. quinquefolius	GB101	Iowa, USA
P. quinquefolius	GB102	Shandong, China
P. quinquefolius	GB103	Daejeon, Korea
P. notoginseng	GB031	Yunnan, China
P. notoginseng	GB032	Yunnan, China
P. notoginseng	GB033	Yunnan, China
P. notoginseng	GB034	Yunnan, China

[a] Cultivars of Korean *P. ginseng*.
[b] Landraces of Chinese *P. ginseng*.

2.2. PCR amplification of ETS region

PCR amplification of ribosomal ETS region was performed on ginseng plant DNA samples. Oligonucleotide primers ETSF (5′-TTTGCAAGTCGTGTGAGTTG-3′) and ETSR (5′-AGACAAGCATATGACTACTGGCAGG-3′) specific for the ribosomal ETS region were designed according to the 26S–18S ribosomal DNA intergenic spacer region of *P. ginseng* (GenBank accession EU232126). The 20 μl PCR reaction mixture consist of 10 ng of template DNA, 0.5 μM of each primer, and 10 μl of 2X Premix DNA polymerase (Genotech). PCR amplification was performed using 1 predenaturation cycle of 4 min at 94 °C, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were analyzed on a 1.0% agarose gel stained with ethidium bromide.

Table 2
Oligonucleotide sequences of primers used in this study.

Primer name	Nucleotide sequence (5′ → 3′)
ETSF	TTTGCAAGTCGTGTGAGTTG
ETSR	AGACAAGCATATGACTACTGGCAGG
AgF	GTGTTGGCATAGTGTACGT**T**A (A → T)
PgF	AGAGCAGTAAGCCTTGGAA**A**AT (C → A)

2.3. DNA sequencing and analysis

The PCR products were purified with a PCR DNA Purification Kit (GeneAll), as described in the manufacturer's instructions. DNA was sequenced in both directions on an automatic DNA sequencer (ABI PRISM 3700, USA), by using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). DNA sequences were assembled using SeqMan software, Multiple sequence alignments were conducted using the Clustal W 2.0 program [8].

2.4. Design of allele-specific primers

Two primers AgF and PgF, specific to American ginseng and Korean ginseng respectively, were designed based on the SNP sites detected in ETS region. The substitutions of T for A in primer AgF and of A for C in primer PgF were additional mismatches introduced deliberately (Table 2), to ensure required allelic specificity [9,10]. Primer ETSR was used as the corresponding reverse primer of AgF and PgF. The orientations of these three primers are shown in Fig. 1.

2.5. Multiplex allele-specific PCR

Multiplex allele-specific PCR was used for authentication of *P. ginseng* and *ginseng* products. For authentication of ginseng plants, the 20 μl PCR reaction mixture consist of 10 ng of template DNA, 0.5 μM of ETSR, 0.5 μM of PgF, 0.15 μM of AgF, and 10 μl of 2X Premix DNA polymerase (Genotech). PCR amplification was performed using 1 predenaturation cycle of 4 min at 94 °C, 35 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 for 30 s, and a final extension at 72 °C for 5 min. The annealing temperature range from 65 °C to 68 °C was tested in the experiment to check the robustness of this method.

For authentication of the mixture of *P. ginseng and P. quinquefolius*, the total amount of mixed template DNA in the 20 μl PCR reaction mixture was set to 0.1 ng. Seven mixed DNA samples con-

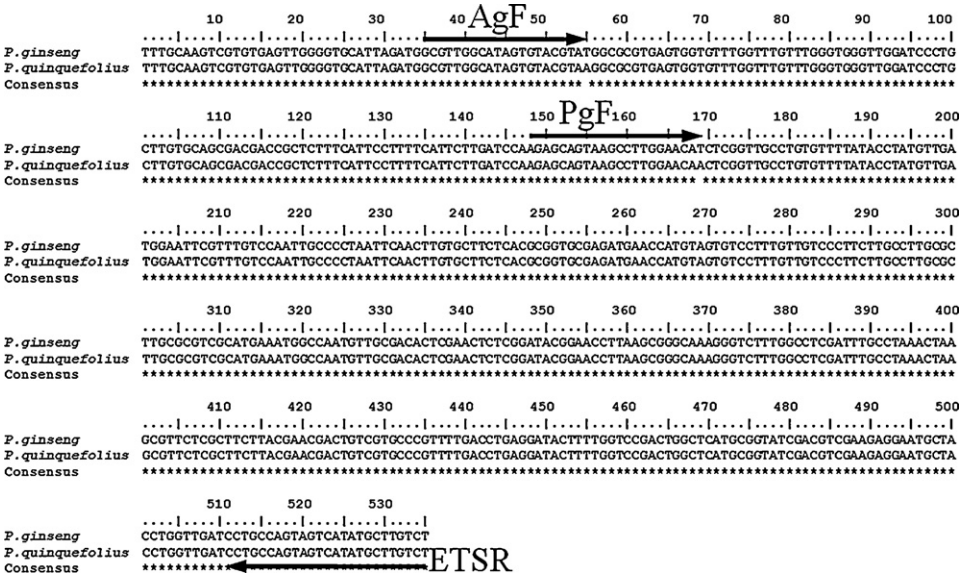

Fig. 1. Comparison of the ETS sequences of *P. ginseng* and *P. quinquefolius*.

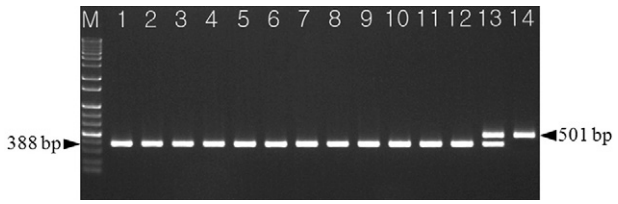

Fig. 2. Multiplex allele-specific PCR products using DNA from ginseng plants. Lane M: 1 kb DNA ladder; Lane 1: Yunpoong; Lane 2: Gopoong; Lane 3: Sunpoong; Lane 4: Gumpoong; Lane 5: Chunpoong; Lane 6: Sunwon; Lane 7: Sunweon; Lane 8: Sunhyang; Lane 9: Chungsun; Lane 10: Damaya; Lane 11: Ermaya; Lane 12: Biantiao; Lane 13: mixture of *P. ginseng* and *P. quinquefolius*; Lane 14: *P. quinquefolius*.

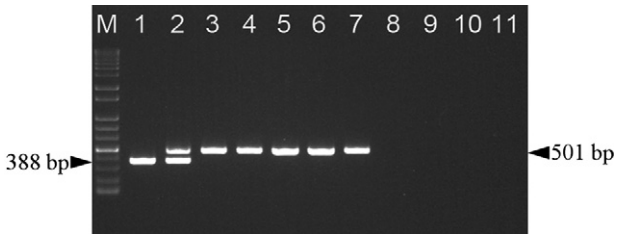

Fig. 3. Multiplex allele-specific PCR products using DNA from different ginseng species. Lane M: 1 kb DNA ladder; Lane 1: *P. ginseng*; Lane 2: mixture of *P. ginseng* and *P. quinquefolius*; Lanes 3–7: *P. quinquefolius* from different localities; Lanes 8–11: *P. notoginseng*.

tain 1%, 2%, 5%, 10%, 20%, 50%, 80% amount of *P. quinquefolius* were respectively used. The reaction mixture and PCR profile for authentication of ginseng mixture and commercial products were the same as described above, except that the cycle number was changed to 40.

3. Results

3.1. DNA sequences and alignments of ETS region

The ribosomal ETS regions amplified from 12 *P. ginseng* and five *P. quinquefolius* samples were determined to be 535 bp. The compiled DNA sequences of *P. ginseng* and *P. quinquefolius* were registered in GenBank with accessions of HQ650811 and HQ650812. As had been expected, DNA sequences of nine Korean ginseng cultivars and three Chinese *P. ginseng* landraces exhibit complete homology. However, two SNP sites specific to *P. ginseng* were detected when compared with *P. quinquefolius*. The 55th and 169th nucleotides of the ETS region in *P. ginseng* are both T, but they are replaced with A at the same positions in *P. quinquefolius* (Fig. 1). Based on these two mutations, two primers AgF and PgF, were designed respectively for specific authentication of *P. quinquefolius* and *P. ginseng*.

3.2. Molecular authentication of P. ginseng and ginseng products

Molecular authentication of *P. ginseng* and the origin of ginseng products were performed using multiplex allele-specific PCR. The combination of three primers AgF, PgF, and ETSR generated different fragment patterns for different *Panax* species. As shown in Figs. 2 and 3, all the Korean ginseng cultivars and Chinese *P. ginseng* landraces yielded the 388 bp specific bands representing the T allele, whereas *P. quinquefolius* samples from different localities generated the specific amplicon of 501 bp representing the A allele. No band was detected when DNA of *Panax notoginseng* was used. For the DNA mixture of *P. ginseng* and *P. quinquefolius*, the two specific bands were both amplified. Therefore, *P. ginseng* can be easily discriminated from *P. quinquefolius* and *P. notoginseng* by amplifying its two specific alleles in ETS region.

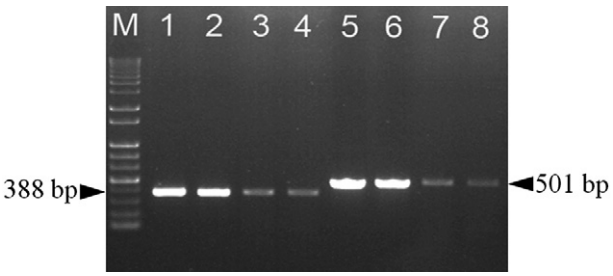

Fig. 4. Multiplex allele-specific PCR products using DNA from ginseng products. Lane M: 1 kb DNA ladder; Lanes 1–4: powder, capsule, tea, red ginseng extract of Korean ginseng, respectively; Lanes 5–8: powder, capsule, tea, red ginseng extract of American ginseng, respectively.

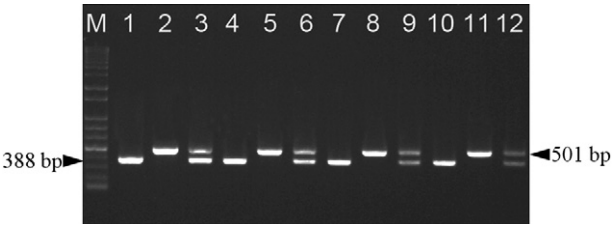

Fig. 5. Multiplex allele-specific PCR products under different annealing temperatures. Lanes 1–3: 65 °C; Lanes 4–6: 66 °C; Lanes 7–9: 67 °C; Lanes 10–12: 68 °C. Lane M: 1 kb DNA ladder; Lane 1, 4, 7, 10: *P. ginseng*; Lane 2, 5, 8, 11: *P. quinquefolius*; Lane 3, 6, 9, 12: Mixture of *P. ginseng* and *P. quinquefolius*.

In order to determine the feasibility of this method on species authentication of ginseng products, commercial ginseng products in the forms of ginseng powder, capsule, tea, and red ginseng extract of *P. ginseng* and *P. quinquefolius* were analyzed. The authentication result is same with that of the ginseng plants, except for the amount of PCR products of ginseng tea and red ginseng extracts is decreased (Fig. 4). This is mainly due to the serious DNA degradation during the manufacturing process of red ginseng extracts.

3.3. Robustness test of multiplex allele-specific PCR

Allele-specific PCR is sensitive to annealing temperature. In order to check the robustness of this method, an annealing temperature range from 65 °C to 68 °C was tested. As shown in Fig. 5, *P. ginseng*, *P. quinquefolius*, and the mixture of the two species generated their specific fragments effectively under different annealing temperatures.

3.4. Molecular authentication of mixture of P. quinquefolius and P. ginseng

To check the adulteration of *P. quinquefolius* into *P. ginseng*, the total amount of mixed template DNA was set to 0.1 ng, by taking into consideration of DNA degradation in ginseng products. Seven DNA mixture samples contain 1%, 2%, 5%, 10%, 20%, 50%, 80% amount of *P. quinquefolius* were respectively used for PCR. As shown in Fig. 6, the established method can detect 1% of intentional adulteration of *P. quinquefolius* into *P. ginseng* at 0.1 ng level of total DNA.

4. Discussion

In recent years, several technologies have been developed for authentication of *P. ginseng* and ginseng products, including chemical analysis and DNA molecular markers. Ginsenosides are the most widely used marker compounds for chemical authentication. Ginsenoside Rf is found only in *P. ginseng* while 24(*R*) – pseudoginsenoside F_{11} is *P. quinquefolius* specific. Chan et al. [6] differentiated

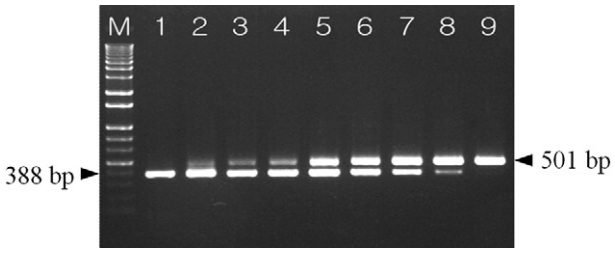

Fig. 6. Multiplex allele-specific PCR products using DNA mixture of *P. ginseng* and *P. quinquefolius*. Lane M: 1 kb DNA ladder; Lane 1: *P. ginseng*; Lanes 2–8: DNA mixture contain 1%, 2%, 5%, 10%, 20%, 50%, 80% amount of *P. quinquefolius*, respectively; Lane 9: *P. quinquefolius*.

P. ginseng from *P. quinquefolius* by distinguishing ginsenoside Rf and 24(*R*) – pseudoginsenoside F_{11} by using the HPLC/MS method. Besides the indicative ginsenoside Rf and F_{11}, the ginsenoside ratios of Rg_1/Rf and Rb_2/Rc were found specific for these two species of ginseng [11]. However, the contents of ginsenosides may be affected by external environment and commercial processing conditions, thus quantitative variations in ginsenosides between the two species do not give us complete confidence in the confirmation of the identity of ginseng [12].

Benefiting from the advances in molecular biotechnologies in the past few decades, DNA molecular markers have become popular means for authentication of ginseng. The molecular markers developed for *P. ginseng* include RAPD [13–16], ISSR [14,17], RFLP [16,3,18], SCAR [19,20], SSR [12,21], AFLP [22], and ARMS [23]. The main drawback of RAPD and ISSR is their lack of reliability and reproducibility. RFLP and AFLP need stringent reaction conditions and tedious operation, and they are not suitable for identification of processed samples due to the degradation of genomic DNA. SSR is a reliable and reproducible molecular marker for ginseng identification. However, the size differences between the PCR products amplified from each allele are too small to differentiate on standard agarose gel electrophoresis. Therefore silver stained PAGE is usually needed, which is time consuming and limits the use of SSR.

Allele-specific PCR is also known as ARMS [9]. SNP genotyping with allele-specific PCR has been used for identification of *P. ginseng* by amplifying its specific allele in nuclear 5.8S rRNA [23], 5S rRNA non-transcribed spacer [24], 18S rRNA and chloroplast *trn*K gene [25]. The 'Gumpoong' and 'Gopoong' cultivars of Korean ginseng, however, share the same sequences with *P. quinquefolius* in 5.8S rDNA [18], so the ginseng products made from 'Gumpoong' and 'Gopoong' may be wrongly identified as American ginseng using 5.8S rRNA. The ETS region of the 18S–26S rDNA repeat evolve at least as rapidly as the ITS at the nucleotide sequence level [26], and it has shown potential for phylogenetic studies at a low taxonomic level. In this study, we exploited two SNP sites specific for all the Korean ginseng cultivars and three main Chinese *P. ginseng* landraces in ribosomal ETS region. By simultaneous amplifying the two specific alleles using multiplex allele-specific PCR, *P. ginseng* can be easily differentiated from *P. quinquefolius* and *P. notoginseng*. Our result showed that favorable PCR products could be obtained even from ginseng tea and red ginseng extracts in which DNA has been extremely degraded.

Compared with previously developed SNP markers, the SNP marker developed in ETS region is common to all *P. ginseng* cultivars and accessions, thus it can be used for authentication of *P. ginseng* and ginseng products accurately. Moreover, the ribosomal ETS region appears in multiple copies in the cell, PCR amplification of partial ETS region is therefore not appreciably affected by DNA degradation and therefore suitable for authentication of ginseng products. Finally, this SNP marker is highly sensitive and can detect trace amount of intentional adulteration of *P. quinquefolius* into *P. ginseng*.

5. Conclusion

Several chemical analysis methods and DNA molecular markers have been employed for authentication of ginseng. By simultaneous amplifying the specific alleles of *P. ginseng* and *P. quinquefolius* in ribosomal ETS region, we have developed a more reliable and reproducible method for *P. ginseng* and ginseng products. The established method is highly sensitive and can detect 1% of intentional adulteration of *P. quinquefolius* into *P. ginseng* down to the 0.1 ng level of total DNA. Therefore this study provides a robust DNA method for authentication of the origins and purities of ginseng products. We hope the described method should serve as a powerful tool in the quality control and validation of commercial *P. ginseng* products.

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