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DNA 바코딩과 고해상 융해곡선분석에 기반한 인삼속 식물의 종 판별

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Internal Transcribed Spacer Barcoding DNA Region Coupled with High Resolution Melting Analysis for Authentication of *Panax* Species

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ABSTRACT

Background : Correct identification of *Panax* species is important to ensure food quality, safety, authenticity and health for consumers. This paper describes a high resolution melting (HRM) analysis based method using internal transcribed spacer (ITS) and 5.8S ribosomal DNA barcoding regions as target (Bar-HRM) to obtain barcoding information for the major *Panax* species and to identify the origin of ginseng plant.
Methods and Results : A PCR-based approach, Bar-HRM was developed to discriminate among *Panax* species. In this study, the ITS1, ITS2, and 5.8S rDNA genes were targeted for testing, since these have been identified as suitable genes for use in the identification of *Panax* species. The HRM analysis generated cluster patterns that were specific and sensitive enough to detect small sequence differences among the tested *Panax* species.
Conclusion : The results of this study show that the HRM curve analysis of the ITS regions and 5.8S rDNA sequences is a simple, quick, and reproducible method. It can simultaneously identify three *Panax* species and screen for variants. Thus, ITS1HRM and 5.8SHRM primer sets can be used to distinguish among *Panax* species.

Key Words **:** *Panax* Species, High Resolution Melting, ITS Barcoding, Real Time PCR

INTRODUCTION

Medicinal plants of the *Panax* genus belonging to the Araliaceae family are well-known rare plants used as tonics in traditional Oriental medicine, and have been described in the Korean Pharmacopoeia. The most commonly used *Panax* species are *Panax ginseng* C. A. Meyer (Korean or Asian ginseng), *P. quinquefolius* L. (American ginseng) and *P. notoginseng* (Burkill) F. H. Chen (Chinese ginseng). The common name ginseng is somewhat misleading nowadays because several closely related ginseng species exist and are known to have varying phytochemical compositions, and different pharmacological properties (Shaw and But, 1995). Each ginseng species exhibits different pharmacological actions and different clinical indications. Therefore, correct identification of the starting material is an essential part to ensure the quality, safety, and efficacy of herbal products.

Traditionally, subjective methods based on morphological features such as leaf shape, berry skin color, and stem

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length, were used to discriminate among *Panax* species (Jo *et al*., 2014). However, this approach is impractical in many situations because of time constraints, the need for specialized taxonomic knowledge, and the difficulty in species identification in cases where only partial or trace samples are available. Different methods have been used to discriminate among *Panax* species, including liquid chromatography-tandem mass spectrometry (LC-MS-MS), gas chromatographic-mass spectrometry (GC-MS), NMR spectroscopy, and Fourier transform infrared (FT-NIR) spectroscopy (Li and Fitzloff, 2001; Chan *et al*., 2000; Cui *et al*., 1999; Kang *et al*., 2008; Mao *et al*., 2014).

Recently, the classification of *Panax* species using metabolic profiling has been greatly facilitated by the analysis of certain secondary metabolites such as ginseosides, which are considered chemotaxonomic markers of the genus (Yang *et al*., 2013). However, there are cases where the plant metabolite profile can change because of external factors such as light, temperature, microbial infections, and storage conditions (Yang *et al*., 2014).

In traditional Oriental medicine, crude plants are usually dried and thus lose their diagnostic features; therefore, the molecular marker is extremely useful for authenticating the composition of herbal medicines. Molecular marker analysis of *Panax* species has been carried out by comparing the DNA sequence variations within 5.8S ribosomal DNA (rDNA) and internal transcribed spacer (ITS) regions (Choi and Wen, 2000; Wang *et al*., 2011; Lee *et al*., 2012). However, this technique requires an agarose gel electrophoresis confirmation step, which is laborious and tends to feature carry-over contamination. Thus, this technique is difficult to utilize for the practical and systematic authentication of material purporting to be comprised of various *Panax* species.

Recently, high resolution melting (HRM) assays using real-time PCR have been introduced as a powerful tool not only for genome-wide SNP discovery but also for the diagnostic analysis of mutated genes causing human diseases (Stephens *et al*., 2008; Wittwer *et al*., 2003; Zhou *et al*., 2004). This technique is particularly useful for plant cultivar identification, genetic mapping, QTL analysis, the identification of pathogenic species, and gene discovery. Very recently, Jaakola *et al*. (2010) developed an HRM analysis method targeting DNA barcoding regions, which was intended for use in the verification of the authenticity of berry species, in particular for distinguishing bilberry (*Vaccinium myrtillus* L.) from other berry species.

The objectives of this study were as follows; (a) to analyze the sequence variations of the ITS1, 5.8S rDNA, and ITS2 regions and test their usefulness for the identification of *Panax* species; (b) to develop a rapid, simple, and stable barcode DNA high resolution melting (Bar-HRM) assay targeting the ITS region for the identification of *Panax* species, as an alternative and efficient approach that can be used in molecular taxonomy studies of closely related ginseng species; and (c) to trace the origins of ginseng via Bar-HRM analysis, in particular the ginseng species of high commercial value, that is, *P. ginseng* and *P. quinquefolius*.

MATERIALS AND METHODS

1. Plant materials and DNA isolation

P. ginseng and other *Panax* species (*P. quinquefolius* and *P. notoginseng*) were preserved and cultivated at the experimental field of the NIHHS, RDA, Chungbuk Province, Korea. These samples were deposited at Korean medicinal herbarium in NIHHS (Table 1). Fresh leaves of 4-years-old plants from *P. ginseng* and two *Panax* species were quickly cut the tissue into small pieces with a sterile razor blade, freeze in liquid nitrogen and grind well using mortar and pestle. Total genomic DNAs were extracted from five leaves of five plants per each specie (*P. ginseng, P. quinquefolius* and *P. notoginseng*) using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. The quantity and quality of DNA samples were measured using a NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA) and the final DNA concentration was adjusted to 10 ng/$\mu\ell$.

Table 1. Details of plant materials used in the high resolution meting (HRM) analysis.

Name	Voucher No.	Classification
P. ginseng C. A. Meyer	MPS002502	Korean ginseng
P. quinquefolius L.	MPS003116	American ginseng
P. notoginseng (Burkill) F. H. Chen	MPS004006	Chinese ginseng

Table 2. Information of primer sequence used in Bar-HRM analysis.

Primer name	Locus	5' Primer sequence	3' Primer sequence	Product size
ITS1HRM	ITS1	CGTTACAATACCGGGTGAGG	GACGCGTGCAGTTCAGTTT	148
5.8SHRM	5.8S rDNA	CGCCAAGGAAATCAAACTGA	ATCGCATTTCGCTACGTTCT	138
ITS2HRM	ITS2	TCGAGTCTTTGAACGCAAGTT	CCAAGGACTCGCATTTGG	175

2. PCR amplification and gel electrophoresis

To amplify fragments of ITS1, 5.8S rRNA, and ITS2 *P. ginseng* cultivar and two *Panax* species, we used *Taq* DNA polymerase (Inclone, Jeonju, Korea) and the oligonucleotide was synthesized by Bioneer (Daejeon, Korea). The primer set consisted of ITS forward (5'-GTCCACTGAACCTTATCATT-3') and ITS reverse (5'-TCCTCCGCTTATTGATATG-3'). PCR amplification was performed using the following mixture; 10 ng of genomic DNA, 0.2 mM of each primer, 0.2 mM dNTPs, 2.5 U DNA polymerase (5 U/$\mu\ell$), 1 $\mu\ell$ reaction buffer; giving a 20 $\mu\ell$ reaction mixture according to the manufacturer's protocol. Amplification reactions were carried out on a Bio-Rad CFX96 RealTime PCR machine (Bio-Rad, Hercules, CA, USA); the procedure used was an initial 5 min at 95℃ followed by 35 cycles of 1 min at 95℃, 1 min at 55℃, 1 min at 72℃, and a final 10 min at 72℃. Amplification products were analyzed by electrophoresis on 1.2% agarose gel in TBE buffer (45 mM Tris-HCl, pH 8.0, 45 mM boric acid, 1 mM EDTA).

3. DNA sequencing and ITS sequence comparison

The PCR products from three *Panax* species (*P. ginseng*, *P. quinquefolius* and *P. notoginseng*) were PCR-amplified and purified with a PCR purification kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Purified PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into competent DH5*a Escherichia coli* cells. Plasmid DNA was prepared from several transformants and sequenced using an Automatic Genetic Analyzer 3100 (Applied Biosystems, Foster City, CA, USA). The DNA sequences obtained via the sequencing experiments were then used to conduct a comparison of the ITS regions. The entire sequence of the ITS1-5.8S-ITS2 was compiled using the SeqEd software package (Applied Biosystems, Foster City, CA, USA).

4. Primer design and high resolution melting analysis

According to the sequence information from the barcoding analyses, three primer pairs were designed from the rDNA ITS region (Table 2). Primers were designed using the following criteria: (1) a minimum primer length of 18 bp, (2) melting temperature between 58℃ and 62℃ with a maximum discrepancy of 2℃ among primers, and (3) PCR product size ranging from 100 to 200 bp. The HRM analysis PCR amplification was performed in 10 $\mu\ell$ on a Bio-Rad CFX96 RealTime PCR System (Bio-Rad, Hercules, CA, USA). A standard PCR was performed in 10 $\mu\ell$ reaction volumes with 50 ng of genomic DNA as template (five samples per species), 10 pmol of reverse and forward primers, and 5 $\mu\ell$ 2X Precision Melt Supermix (Bio-Rad, Hercules, CA, USA). HRM was performed using a CFX96 real-time PCR detection system and the cycling conditions for HRM followed the manual of Precision Melt Supermix, 95℃ for 2 min, 40 cycles of denaturation at 95℃ for 10 s and annealing/extension at 60℃ for 30 s. To assess product specificity, amplicons were systematically checked by the melting curve analysis. Melting curves were generated from 65℃ to 95℃ with increments of 0.2℃/cycle. Melting profiles were analyzed with the Bio-Rad Precision Melt Software version 1.0, as described in the following paragraphs.

RESULTS

1. DNA sequencing and ITS sequence comparison

The DNA sequences in the ITS1-5.8S-ITS2 of rDNA were PCR-amplified from the leaves of three *Panax* species (Fig. 1); *P. ginseng*, *P. quinquefolius* and *P. notoginseng*. We obtained PCR products approximately 750 bp long from the *Panax* species tested. The length of the amplified products was determined to be 746 bp via sequencing, and the pure ITS1-5.8S-ITS2 region was determined to be 682 bp long, via a comparison with sequence data in the NCBI GenBank nucleotide databases

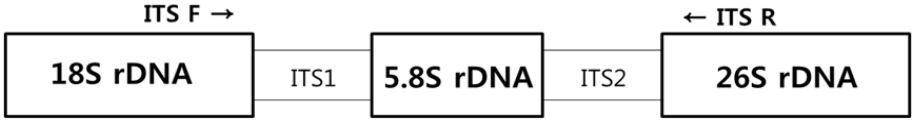

Fig. 1. A diagram of the nuclear ITS (Internal Transcribed Spacer) region and the positions of the primers used for PCR.

Fig. 2. DNA sequences in the ITS1-5.8S-ITS2 region for three *Panax* species. Arrows indicate the ranges of ITS1, 5.8S rRNA, and ITS2. Primer binding regions and directions were indicated with dot arrows. The areas enclosed by the boxes indicates SNP variations.

(AY548192, FJ606755, and U41685). The amplified DNA of the three plant samples was sequenced as described in Figure 1. As expected, the sequences of the species showed a very high degree of homology (97.9 - 99.4%) in the ITS1-5.8S-ITS2 region (Fig. 2). The ITS and 5.8s rDNA sequences of the three *Panax* species had a G + C content of 59.5 - 59.7%. SNPs were observed at the 27 bp, 48 bp, 70 bp, 81 bp, 83 bp, 117 bp, 129 bp, 130 bp, and 248 bp locations of ITS1; at 328 bp and 423 bp of the 5.8s rDNA gene; and at 427 bp, 429 bp, 438 bp, 441 bp, 535 bp, 602 bp, and 613 bp of the ITS2 region (Fig. 2 and Table 2).

2. HRM curves analysis

Three *Panax* species (*P. ginseng*, *P. quinquefolius* and *P. notoginseng*) were tested with real-time PCR using three primer sets: ITS1HRM, 5.8SHRM, and ITS2HRM (Table 3). The resulting melting curves showed that the three *Panax* species could be clearly discriminated between by using the ITS1, ITS2, and 5.8S rDNA barcoding region in combination with HRM analysis. Distinct HRM peaks were observed for each SNP genotype of the *Panax* species, each of which is represented by a different color (Fig. 3). In all the samples evaluated, the five replicates always had similar curves and peaks, and were assigned

Table 3. Nucleotide variations in the ITS region among *Panax* species tested.

Target region	Nucleotide position (bp)	Nucleotide variations		
		Panax ginseng	*Panax quinquefolius*	*Panax notoginseng*
ITS1	27	T	T	C
	48	C	C	A
	70	C	C	T
	81	C	C	T
	83	A	A	G
	117	A	A	G
	129	A	C	C
	130	T	T	C
	248	C	C	T
5.8S rDNA	328	A	G	G
	423	C	C	T
ITS2	427	C	T	C
	429	T	T	C
	438	T	C	C
	441	G	G	T

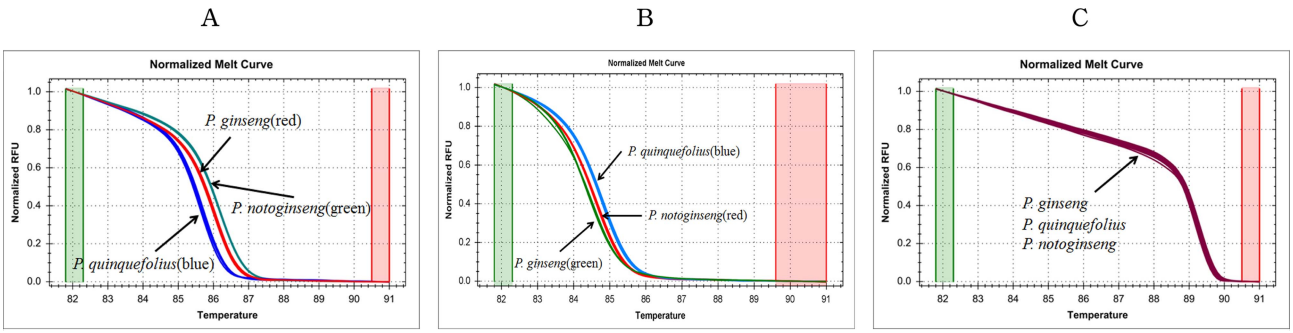

Fig. 3. Normalized high resolution melting (HRM) curve profiles of PCR amplicons using the primers. A; ITS1HRM, B; 5.8SHRM, and C; ITS2HRM.

to the same group, demonstrating the consistency and reproducibility of the HRM assay.

The melting characteristics of the ITS1 amplicons of *P. ginseng*, *P. quinquefolius* and *P. notoginseng* were assessed by plotting three different curves (Fig. 3A). The melting curves were characterized by peaks of 85.60℃ in profile 1 (*P. quinquefolius*), 86.00℃ in profile 2 (*P. ginseng*), and 86.20℃ in profile 3 (*P. notoginseng*). The three *Panax* species tested generated distinctive HRM profiles and normalized HRM profiles, allowing for the discrimination of each species. They were easily discriminated because they produced different HRM patterns because of the differences in the nucleotide sequences of the ITS1 fragments amplified in this assay.

The analysis of the normalized HRM curves using the barcode marker 5.8SHRM is shown in Fig. 3B. It shows that each genotype was represented by three peaks. The first peak (profile 1, *P. ginseng*) ranged from 84.20 to 84.60℃, the second peak (profile 2, *P. quinquefolius*) from 84.60 to 85.00℃, and the third peak (profile 3, *P. notoginseng*) was at 84.60℃ (Fig. 3B). Analysis of the normalized HRM curves produced using the 5.8SHRM marker revealed that *Panax* species can easily be distinguished from each other. The primer ITS2HRM produced a single melt peak, and the melt temperature for each sample at 89.20℃ (Fig. 3C). Sequence variations in the ITS1 region and 5.8s rDNA genes allowed for very clear and reproducible HRM curve analysis differentiation among the *Panax* species analyzed in this study.

DISCUSSION

The Bar-HRM method allows the fast analysis of genetic variation in various plans using universal barcoding regions (ITS or chloroplast) while at the same time is a close tube post PCR method reducing the risk of contamination.

The ITS nuclear ribosomal DNA is considered one of the best barcoding regions available (Bladwin, 1992). This region has a number of valuable characteristics, such as conserved regions that can be used for designing a universal primer, the ease with which it can be amplified, and sufficient variability to distinguish between even closely related species (Yao *et al*., 2010).

Thus, several researchers have tried to detect variations in the ITS regions in order to carry out phylogenetic studies of the genus *Panax* (Wen and Zimmer, 1996). Studies for distinguishing among *Panax* species were performed using the cleaved amplified polymorphic sequences (CAPS) marker system (Kim *et al*., 2007), and species-specific PCR based on SNPs detected in the ITS regions (Park *et al*., 2006; Bang *et al*., 2012). However, this technique requires the inclusion of a confirmation step using agarose gel electrophoresis, which is laborious and tends to feature carry-over contamination. Thus, they are difficult to utilize for the practical and systematic authentication of *Panax* species.

Recently, a new methodology known as plant DNA barcoding with high resolution melting (plant Bar-HRM) analysis has been developed (Kalivas *et al*., 2014). Plant Bar-HRM has been used for species identification, evolution studies, and forensics as it targets small conserved DNA regions such as the chloroplast, mitochondria, and ITS (Ganopoulos *et al*., 2013; Madesis *et al*., 2012; Bosmali *et al*., 2012). It is a closed tube assay that does not employ additional fluorescent probes and simply utilizes a DNA melting assay and computerized analysis of the results to produce a graphic output, thus decreasing the risk of sample contamination.

According to Ganopoulos *et al*. (2012) the use of DNA markers to authenticate legume species requires polymorphic and high-copy analytical targets such as the universal nuclear plant DNA barcoding region ITS, which has been used to discriminate among several plant species. Similarly, we used the PCR-amplified universal barcoding region as an analytical target for the HRM curve assay in order to discriminate among *Panax* species (Fig. 3). A melting curve analysis using ITS barcoding regions was sufficient to distinguish among three *Panax* species (Table 1). The authentication of *Panax* species is of great importance, especially when seeds are mixed with species that can be a source of seed purity management and quality control problems in the manufacture of ginseng products.

In conclusion, we have successfully developed a Bar-HRM method coupled with real-time PCR, which can be used for the rapid identification of three *Panax* species. The Bar-HRM method has been proven to be a universal method, as it allowed for discrimination among three species using two barcoding regions, that is, ITS1 and 5.8S rDNA (Fig 1). The ITS1HRM and 5.8SHRM primers could be used to differentiate among three *Panax* species in conjunction with HRM curve analysis.

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