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Research Article

Molecular markers based on chloroplast and nuclear ribosomal DNA regions which distinguish Korean-specific ecotypes of the medicinal plant *Cudrania tricuspidata* Bureau

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Abstract *Cudrania tricuspidata* Bureau is a widely-used, medicinal, perennial and woody plant. Obtaining information about the genetic diversity of plant populations is highly important with regard toconservation and germplasm utilization. Although *C. tricuspidata* is an important medicinal plant species registered in South Korea, no molecular markers are currently available to distinguish Korean-specific ecotypes from other ecotypes from different countries.

In this study, we developed single nucleotide polymorphism (SNP) markers derived from the chloroplast and nuclear genomic sequences, which serve to to identify distinct Korean-specific ecotypes of *C. tricuspidata* via amplification refractory mutation system (ARMS)-PCR and high resolution melting (HRM) curve analyses. We performed molecular authentication of twelve *C. tricuspidata* ecotypes from different regions using DNA sequences in the maturaseK (*MatK*) chloroplast intergenic region and nuclear ribosomal DNA internal transcribed spacer (ITS) regions. The SNP markers developed in this study are useful for rapidly identifying specific *C. tricuspidata* ecotypes from different regions.

Keywords ARMS-PCR, chloroplast genome, HRM curve analysis, single nucleotide polymorphisms, nuclear ribosomal DNA internal transcribed spacers

Introduction

Cudrania tricuspidata Bureau is a deciduous tree found in Korea, China, Japan, and Eastern Russia. The root bark and cortex of *C. tricuspidata* have long been used as crude drug materials, yielding one of the most ubiquitous traditional herbal medicines in East Asia (Hano et al. 1990). The beneficial effects of these plants primarily include their anti-tumor (Zou et al. 2004), anti-inflammatory (Park et al. 2006), and cytotoxic activities (Park 2005). Understanding the genetic variation, structure, and phylogenetic characteristics of this useful plant species is important for its conservation and sustainable use, but molecular markers to classify the genetic diversity in *C. tricuspidata* have not yet been reported.

DNA markers based on the chloroplast genome can be used to quickly and reliably classify specific plant species, cultivars, or ecotypes due to their unique features. Chloroplasts are maternally inherited intracellular plant organelles with specific functions that contain their own genomes (Reboud 1994). A plant cell can contain up to 1,000 copies of the chloroplast genome, which is over 100-times greater than the number of copies of the nuclear genome found in plant cells (Pyke 1999). Therefore, a target region in the chloroplast genome can be amplified by PCR more easily than a target region in the nuclear genome using trace amounts of genomic DNA. Most gene sequences are also highly conserved in various plant species, but considerable amounts of nucleotide variation have been identified in chloroplast intergenic spacer regions at the interspecies level and (rarely) at the intraspecies level (Wolfe et al. 2004). In addition, nuclear ribosomal DNA internal transcribed spacer (ITS) sequences have recently been used to develop molecular markers to identify various medicinal plant species originating from Korea and China (Yang et al. 2012; Han et al. 2016). Hybrids may also be produced via cross-fertilization when similar species or ecotypes are cultivated in the same field. Genetic markers based only on chloroplast intergenic sequences are likely to be insufficient for identifying specific species among hybrid

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plants, since chloroplast genomes are inherited maternally, whereas nuclear genomes are inherited by hybridization; thus ITSs are at the forefront of DNA barcoding research. Sequence-based DNA markers have practical advantages for authenticating plant species, as they can be used to differentiate similar medicinal plants in a time- and cost-effective manner (Jung et al. 2014). Various DNA markers based on random polymorphic sequences have been used to classify similar medicinal plant species, including single nucleotide polymorphisms (SNPs) (Kim et al. 2013; Han et al. 2016). While the use of highly variable sequences from the plastid and nuclear genomes is important for barcoding, molecular markers to classify genetic diversity in *C. tricuspidata* have not yet been reported. Sequence analysis of PCR products via amplification refractory mutation system (ARMS) is a simple, timesaving, effective method for SNP genotyping. The main advantage of ARMS is that the amplification and authentication steps are combined, such that the presence of an amplified product indicates the presence of a particular allele. High resolution melting (HRM) curve analysis was recently developed to detect SNPs located on amplicons (Ririe et al. 1997; Gundry et al. 2003; Liu et al. 2012). In this method, amplified DNA strands are melted apart via a gradual increase in temperature, and different melting patterns are detected based on subtle changes in fluorescence generated by double-stranded DNA-binding dyes. The rapid, inexpensive detection of a broad range of SNPs via HRM analysis makes this technique suitable for genotype discrimination (Lehmensiek et al. 2008) and genetic mapping (Chagne et al. 2008); various cultivars have been identified using SNPs via these technique (Mackay et al. 2008).

Therefore, in this study, we developed an effective method for identifying Korean-specific *C. tricuspidata* ecotypes using markers derived from plastid and nuclear DNA sequences and demonstrated that marker polymorphisms can be efficiently detected by ARMS-PCR and HRM analysis. This is the first report of the development and characterization of Korean-specific *C. tricuspidata* ecotypes using SNP markers derived from plastid and nuclear DNA sequences. Our data from DNA barcoding analysis using chloroplast and nuclear genomic sequence regions (*MatK* and *ITS*) reveal inter- and intraspecific variation among *C. tricuspidata* ecotypes.

Materials and Methods

Plant materials

Twelve *Cudrania tricuspidata* Bureau ecotypes from different regions were used in this study (Table 1 and Fig. 1). Sample identities were confirmed by comparing sequences from the chloroplast *MatK* and nuclear *ITS* regions in these samples with those in NCBI GenBank: *MatK* (accession number JF317421.1) and *ITS* (accession number JF980330.1). All plant materials were assigned identification numbers and preserved at the Gyeongnam National University of Science and Technology (Jinju, Korea)(Table 1).

Genomic DNA extraction

Genomic DNA was isolated using a Plant DNA Extraction kit (GeneAll Co. Exgene^{TM}, Seoul, Korea) from plant samples that had been snap frozen in liquid nitrogen and ground into a powder. The concentration and purity of the DNA samples were measured using a micro-spectrophotometer (Bio-Prince, SD-2000, Gangwon, South Korea). All samples had

Table 1 List of plant materials used in this study

Identification code	Scientific name	Cultivated regions (sources)	Identified origin	Material used
2014-30	*Cudrania tricuspidata* Bureau	Haenam, Jeonam, Korea	South Korea	Leaves
2014-31	*Cudrania tricuspidata* Bureau	Haenam, Jeonam, Korea	South Korea	Leaves/stems
2014-33	*Cudrania tricuspidata* Bureau	Sancheong, Gyeongnam, Korea	South Korea	Leaves
2014-34	*Cudrania tricuspidata* Bureau	Sacheon, Gyeongnam, Korea	South Korea	Leaves
2014-36	*Cudrania tricuspidata* Bureau	Jinju, Gyeongnam, Korea	South Korea	Leaves
2014-37	*Cudrania tricuspidata* Bureau	Uiryeong, Gyeongnam, Korea	South Korea	Leaves
2014-38	*Cudrania tricuspidata* Bureau	Sancheong, Gyeongnam, Korea	South Korea	Leaves
2014-39	*Cudrania tricuspidata* Bureau	Jinju, Gyeongnam, Korea	South Korea	Stems
2014-41	*Cudrania tricuspidata* Bureau	Sancheong, Gyeongnam, Korea	China	Leaves
2014-42	*Cudrania tricuspidata* Bureau	Sancheong, Gyeongnam, Korea	South Korea	Leaves
2016-10	*Cudrania tricuspidata* Bureau	Miryang, Gyeongnam, Korea	China	Leaves/stems
2016-47	*Cudrania tricuspidata* Bureau	Commercial herbs	China	Dried stems

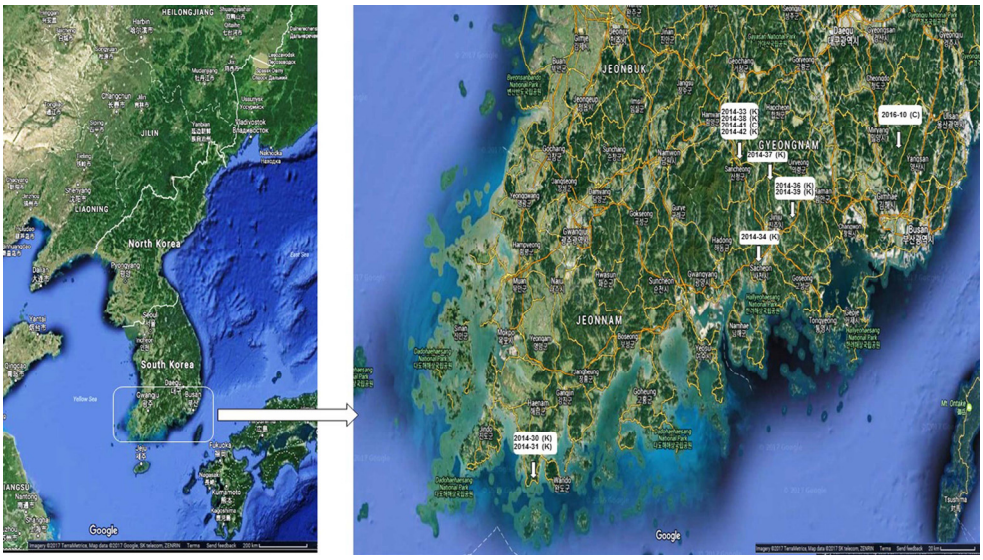

Fig. 1 Sampling locations of 12 *Cudrania tricuspidata* Bureau ecotypes (Google Maps)

Table 2 Primer sequences used in this study

A. SNP analysis

Gene	Primers	Sequences (5'–3')	Tm (°C)	Size (bp)
MatK	MatK forward	ATTGCGGTTTTTTCTTCACGACT	57.8	988
	MatK reverse	ATGATTGACCAGATCGTTGATGC	57.4	
ITS	ITS forward	TCCGTAGGTGAACCTGCGG	58.0	762
	ITS reverse	GCCGTTACTAGGGGAATCCTTG	57.6	

B. ARMS-PCR analysis

Origin	Primers	Sequences (5'–3')	Tm (°C)	Size (bp)
South Korea	Matk-specific forward	ACGATTAACATCTTCTGGTGA	55.5	537
	Matk-specific reverse	GATTTCTGCATATACACGCATAG	59.3	
	ITS-specific forward	GCCAAGTGCGTGCCGCTCATC	68.7	458
	ITS-specific reverse	CGACAACCACCTTTTGCCTCA	60.2	
China	Matk-specific forward	ACGATTAACATCTTCTGGAGG	57.4	537
	Matk-specific reverse	GATTTCTGCATATACACGCAGAT	59.3	
	ITS-specific forward	GCCAAGTGCGTGCCGCTCTGT	66.2	458
	ITS-specific reverse	CGACAACCACCTTTTGTCACG	57.5	

C. HRM analysis

Gene	Primers	Sequences (5'–3')	Tm (°C)	Size (bp)
MatK	MatK forward	GTGTGGTCTCAACCAGGAAG	57.2	197
	MatK reverse	GCCAACGATCCAATCAGAGG	57.7	
ITS	ITS forward	TCCCGTGAACCATCGAGTC	58.2	205
	ITS reverse	GCACGTGACAAGGGACTTG	58.1	

A260/A280 absorbance ratios >1.8 and A234/A260 ratios of 0.5 ~ 0.8.

PCR amplification and nucleotide sequence analyses

Primers were designed based on sequences in the NCBI database to specifically amplify sequences from the *MatK* and *ITS* regions of the plastid and nuclear genomes, respectively (Table 2A). The sequences of each primer pair are provided in Table 2A. PCR amplification was performed using i-pfu DNA polymerase from iNtRON Co. (Seoul, Korea), which minimizes the introduction of mutations during

the amplification reaction. The amplicons were sent to Solgent Co., Seoul, Korea for sequencing analysis without cloning the amplified fragments to avoid introducing any mutations. Each experiment was repeated at least three times, and all amplified fragments were sequenced in both directions.

Construction of a dendrogram and genetic distance analysis

A dendrogram describing the genetic distances between the ecotypes based on their *MatK* and *ITS* sequences was constructed using the Mega 6.0 statistical program. Pairwise comparisons between species were performed by measuring genetic distances using the Tamura-Nei distance method. A genetic distance matrix was used for cluster analysis via the neighbor-joining method (Tamura et al. 2013).

ARMS-PCR

An ARMS-PCR assay was developed to investigate samples collected from different locations (Table 1). Plastid DNA-specific primer sets for each ecotype were designed based on the intergenic sequence of the *MatK* region (Table 2B). The relative positions and sizes of the targeted species-specific amplicons are shown in Fig. 3A. Ecotype-specific amplification of the *ITS* region was performed using a forward primer based on a sequence in *ITS I* and a reverse primer based on a sequence in *ITS II* (Table 2B and Fig. 4A). ARMS primers were designed essentially as described by Newton et al. (1989), and PCR analysis was carried out using Exprime Taq Premix (Genet Bio, Seoul, Korea).

HRM curve analysis

A primer set was designed based on the intergenic sequences of the *MatK* and *ITS* regions to develop a plastid sequence HRM assay for identifying specific plant ecotypes. HRM analysis was performed to detect polymorphisms in this marker sequence. Since short amplicons usually result in better resolution in HRM analysis, primers were designed to amplify a short region of the intergenic sequence. Specific primer sets were designed for HRM analysis to discriminate between each of the three plant species based on specific SNPs (Table 2C). HRM analysis was conducted using the Mx3005P QPCR System (Agilent Technologies, CA, USA). Briefly, 10 ng of purified DNA, 5 pmoles of each primer, and 10 μl of SsoFast™ EvaGreen® Supermix 172-5200 premixture (Bio-Rad, CA, USA) and reaction buffer (provided by the manufacturer) were combined in a total volume of 20 μl and subjected to the following cycling conditions: denaturation for 2 min at 98°C, followed by 30 cycles of 5 sec at 98°C and 20 sec at 57°C for double-strand annealing and extension. At the end of the final cycle, the temperature was reduced to 40°C, followed by an increase to 95°C, and fluorescence signals were plotted in real time against temperature to produce melting curves. Data were normalized to obtain values between 0% and 100%.

Results and Discussion

Alignment of DNA sequences from the chloroplast MatK and nuclear ITS regions

PCR products amplified from the *MatK* region of the chloroplast genome and the *ITS* region of the nuclear genome were 988 and 762 bp long, respectively. Alignment of sequences from each *C. tricuspidata* ecotype originating from the same country, such as Korean and China, revealed a very high degree of sequence homology. Phylogenetic analysis using the *MatK* and *ITS* sequence regions demonstrated more similarity among ecotypes originating from Korea than among those from China, with 100% sequence homology detected between each Korean ecotype, such as 2014-30, 31, 33, 34, 36, 37, 38, 39, and 42 (Fig. 2). Among Chinese

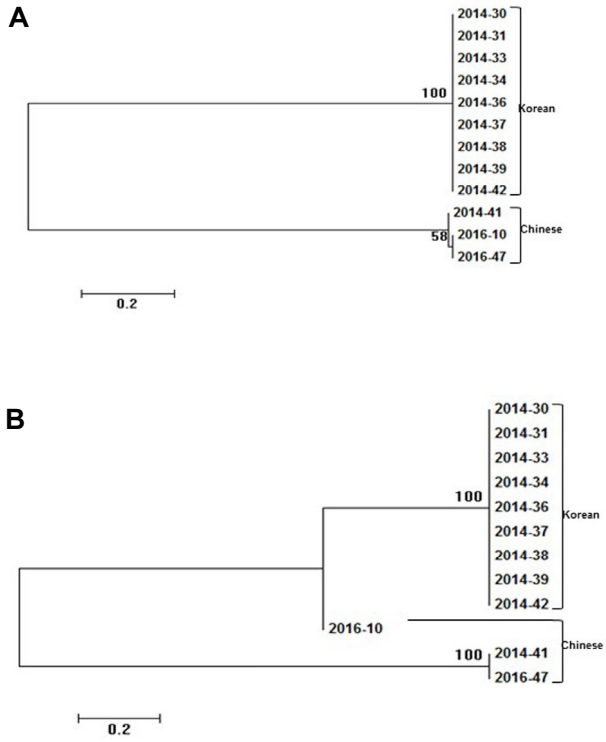

Fig. 2 Phylogenetic tree showing the genetic diversity of 12 ecotypes of Cudrania tricuspidata Bureau. The tree was produced using the neighbor-joining method based on intergenic sequences of MatK (A) and ITS (B)

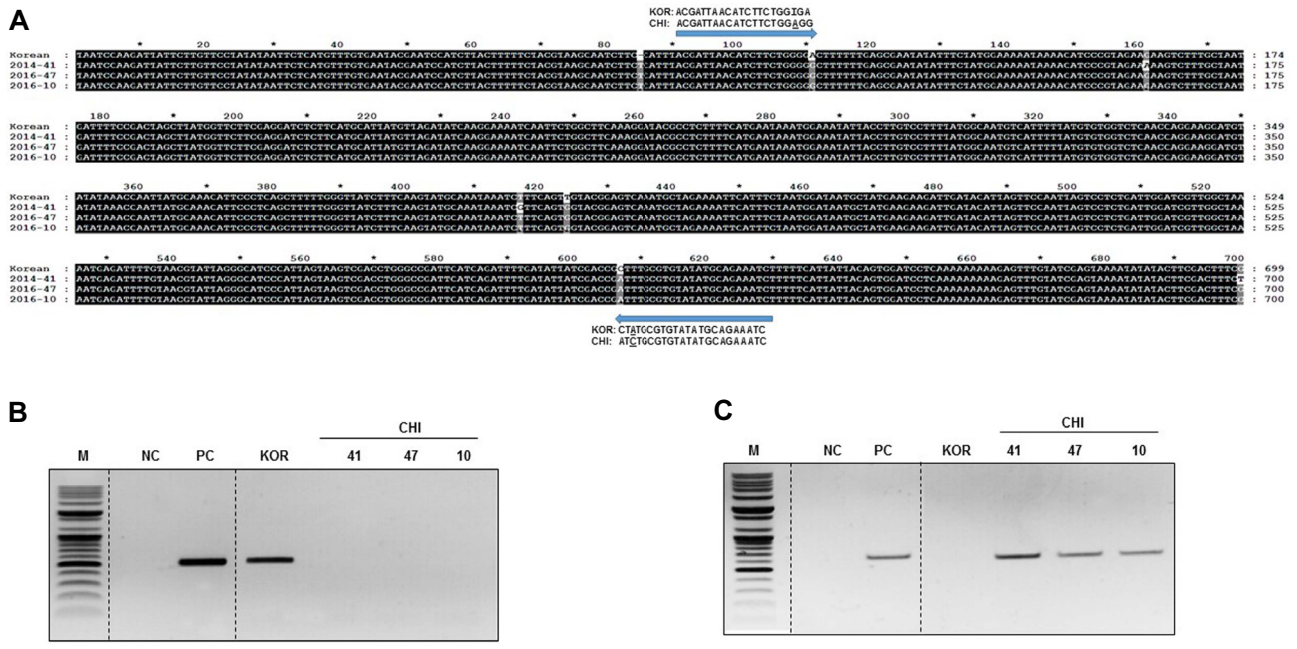

Fig. 3 Sequence alignment and products of ARMS-PCR using the *MatK* (GenBank accession number JF317421.1) chloroplast intergenic regions in various ecotypes of *Cudrania tricuspidata* Bureau. (A) Sequence alignment of the *MatK* chloroplast intergenic region in each ecotype. The gray box indicates the same sequences in two or three ecotypes, while the black box indicates the same sequence in all ecotypes. ▲ indicates polymorphisms. Arrows indicate the positions of the *MatK* primers developed in this study. (B) PCR results using Korean ecotype-specific primers; (C) PCR results using Chinese ecotype-specific primers. M, marker; NC, negative control; PC, positive control; KOR, ecotype originating from Korea; CHI, ecotype originating from China

C. tricuspidata ecotypes, phylogenetic analysis using *MatK* regions demonstrated more similarity between 2016-10 and 2016-47 versus 2014-41 (Fig. 2A), whereas analysis of *ITS* regions suggested that 2014-41 and 2016-47 are more closely related than 2016-10 (Fig. 2B).

ARMS-PCR analysis using ecotype-specific MatK region primers

We performed molecular authentication of Korean and Chinese *C. tricuspidata* ecotypes via ARMS-PCR using specific forward and reverse primers (Fig. 3A). The combination of specific primers yielded a single band of the correct size for each sample examined. We amplified PCR products from only the target species using specific primers. Analysis of many samples from each ecotype confirmed the accuracy of this assay. As shown in Fig. 3B, the use of mismatched *MatK* primer pairs yielded 537 bp amplicons only from Korean *C. tricuspidata*, whereas, for Chinese *C. tricuspidata*, no band was detected using a combination of mismatched SNP forward primer and the reverse *MatK*-specific primer. Chinese *C. tricuspidata* ecotypes produced specific bands only when using mismatched Chinese ecotype-specific *MatK* primer pairs (Fig. 3C). Thus, Korean *C. tricuspidata* could clearly be identified from among different *C. tricuspidata* ecotypes.

Development of ecotype identification markers using nuclear DNA sequences

We designed Korean and Chinese *C. tricuspidata*-specific primer sets based on the intergenic sequences flanking the *5.8S rDNA* gene, *ITS 1* and *ITS II*, to develop markers derived from nuclear sequences that can be used to identify each *C. tricuspidata* ecotype by ARMS-PCR (Table 2B). For ARMS-PCR analysis, we used specific forward and reverse primers in the *ITS I* and *ITS II* regions, respectively. The forward and reverse primers contained G → T and T → A substitutions in the Korean *C. tricuspidata* sequence relative to that of Chinese *C. tricuspidata,* whereas no substitutions were present in the forward and reverse primers for Chinese *C. tricuspidata*. As expected, a specific PCR product was amplified from each ecotype using the ecotype-specific primers (Fig. 4B and 4C). This result indicates that ARMS-PCR analysis of nuclear ribosomal DNA using highly specific primers can be used to identify hybrid *C. tricuspidata* ecotypes.

Development of a chloroplast and nuclear DNA-based HRM assay

Based on the results of nucleotide sequence alignment of

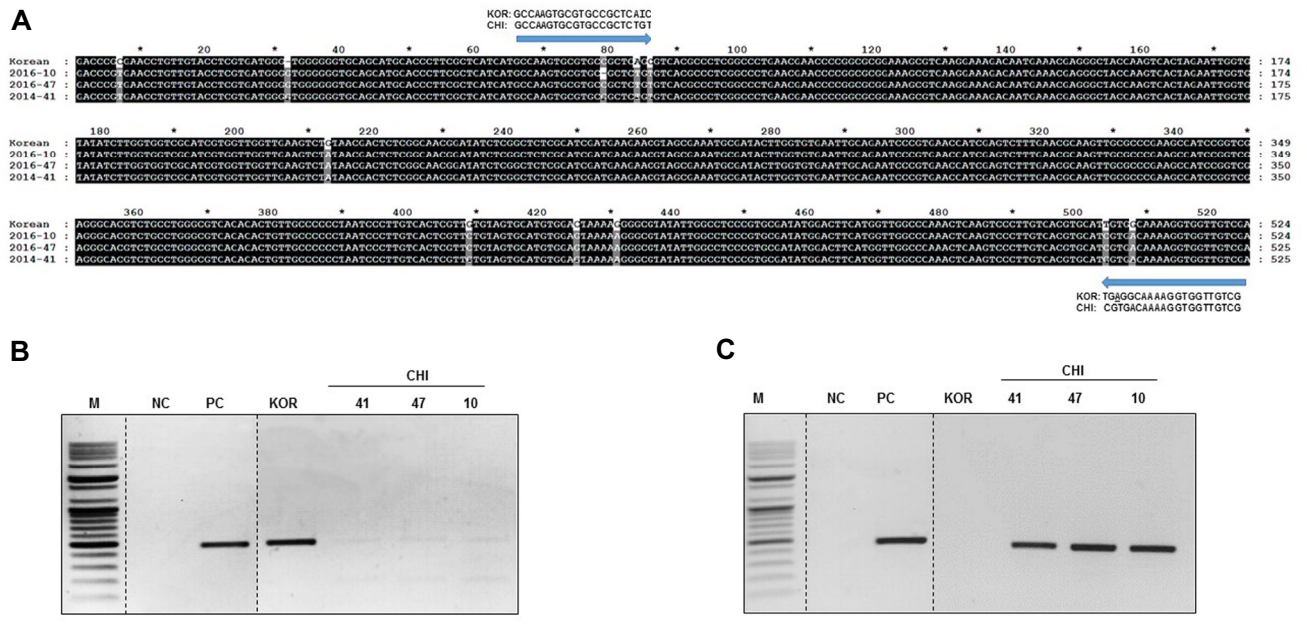

Fig. 4 Sequence alignment and products of ARMS-PCR using the *ITS* (GenBank accession number JF980330.1) nuclear intergenic regions in various *Cudrania tricuspidata* Bureau ecotypes. (A) Sequence alignment of the *ITS* nuclear intergenic regions in each ecotype. Gray box indicates the same sequences in two or three ecotypes, while the black box indicates the same sequence in all ecotypes. ▲ indicates polymorphisms. Arrows indicate the positions of the *ITS* primers developed in this study. (B) PCR results using Korean ecotype-specific primers; (C) PCR results using Chinese ecotype-specific primers. M, marker; NC, negative control; PC, positive control; KOR, ecotype originating from Korea; CHI, ecotype originating from China

each *C. tricuspidata* ecotype, we designed a primer set to amplify a short fragment of each sequence (*MatK*, 197 bp and *ITS*, 205 bp) and to locate the SNP site in the middle of each amplicon for HRM curve analysis (Fig. 5A). When we performed HRM analysis of the *MatK* plastid sequences and the *ITS* nuclear sequences using DNA from each plant sample, two different melting curve patterns were detected (Fig. 5B): the melting curve patterns exactly corresponded with the classification of each ecotype of Korean and Chinese *C. tricuspidata.* When we subjected *C. tricuspidata* plants to HRM curve analysis of the intergenic regions of plastid *MatK* and nuclear *ITS* using the appropriate primer set, a typical melting curve pattern was generated for each ecotype, allowing the various Korean and Chinese *C. tricuspidata* ecotypes to be readily differentiated.

Sequence analysis of highly variable DNA is commonly used for species identification and phylogenetic analysis. In this study, we demonstrated that ARMS-PCR and HRM analysis could be used to detect polymorphic SNPs and to discriminate among ecotypes of *C. tricuspidata* collected from different locations in South Korea. The main advantage of ARMS is that the amplification and authentication steps are combined, in that the presence of an amplified product indicates the presence of a particular allele and vice versa. HRM analysis has several advantages over direct sequencing for both marker development and polymorphism detection

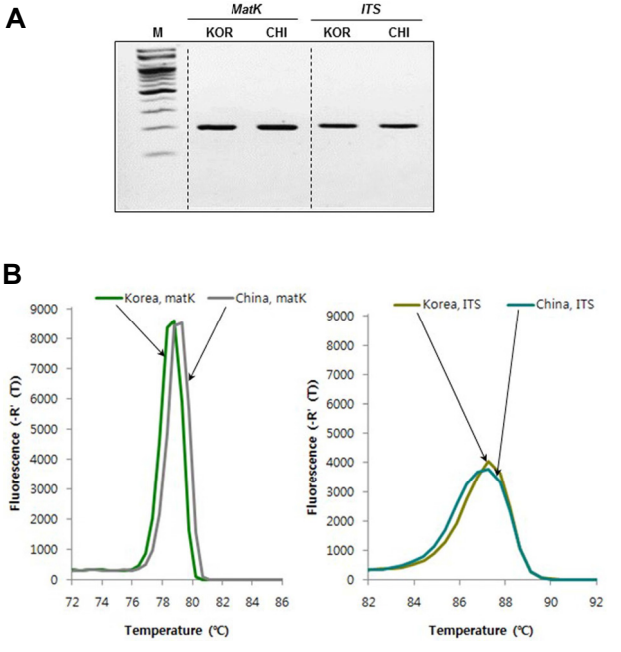

Fig. 5 PCR products and HRM curve analysis using the *MatK* (GenBank accession number JF317421.1) chloroplast intergenic regions and the *ITS* (GenBank accession number JF980330.1) nuclear intergenic regions in *Cudrania tricuspidata* Bureau ecotypes originating from Korea and China. (A) PCR results using ecotype-specific primers. M, marker; KOR, ecotype originating from Korea; CHI, ecotype originating from China. (B) Melting curves of *MatK* and *ITS* from various samples using ecotype-specific primers

(Mackay et al. 2008). HRM analysis is also a highly sensitive method for detecting SNPs, allowing sequence differences between species to be readily detected without the need for direct sequencing. ARMS-PCR and HRM analyses were recently used to discriminate among various medicinal plants, such as members of the diverse *Panax* genus and similar plant species *Cynanchum wilfordii, C. auriculatum*, and *Polygonum multiflorum* (Kim et al. 2013; Han et al. 2016). In the current study, we showed that SNPs in the *MatK* regions of plastid sequences and the *ITS* regions of nuclear sequences are effective, reliable tools for discriminating among *C. tricuspidata* ecotypes originating from the same country, such as Korea and China (Fig. 3 and 4). HRM curve analysis of specific markers in the plastid *MatK* region and the nuclear *ITS* region using DNA samples from *C. tricuspidata* ecotypes originating from Korea and China resulted in melting curve patterns consistent with the nucleotide differences determined by sequence analysis (Fig. 5). As expected, the melting curve patterns differed between Korean- and Chinese-specific *C. tricuspidata* ecotypes based on *MatK* and *ITS* markers. Therefore, the results demonstrate that, even though the ecotypes investigated in this study are related and share morphological characteristics, ARMS-PCR and HRM curve analysis using the plastid marker *MatK* and nuclear marker *ITS* are sufficient for providing an ecotype-specific DNA barcode to distinguish between ecotypes with different countries of origin.

In conclusion, we performed molecular genetic identification of *C. tricuspidata* ecotypes collected from different locations using SNP-based ARMS-PCR and HRM analysis with specific primers. The results suggest that it is possible to identify plant materials using assays based on the chloroplast *MatK* and nuclear *ITS* region. Compared with other methods involving the use of molecular markers, our method is reliable, efficient, and scalable for testing large numbers of ecotypes collected from different locations. The results demonstrate that the plastid DNA region *MatK* and the nuclear DNA *ITS* can be used for intraspecific polymorphism studies and that they represent useful tools for marker-assisted identification and selection of specific *C. tricuspidata* ecotypes or cultivars.

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