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 to conservation 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 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
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™, 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

| Identification code | Scientific name         | Cultivated regions (sources) | Identified origin   | Material used  |
|---------------------|-------------------------|-----------------------------|---------------------|---------------|
| 2014-30             | Cudrania tricuspidata    | Haenam, Jeonam, Korea       | South Korea         | Leaves        |
| 2014-31             | Cudrania tricuspidata    | Haenam, Jeonam, Korea       | South Korea         | Leaves/stems  |
| 2014-33             | Cudrania tricuspidata    | Sancheong, Gyeongnam, Korea | South Korea         | Leaves        |
| 2014-34             | Cudrania tricuspidata    | Sancheong, Gyeongnam, Korea | South Korea         | Leaves        |
| 2014-36             | Cudrania tricuspidata    | Jinju, Gyeongnam, Korea     | South Korea         | Leaves        |
| 2014-37             | Cudrania tricuspidata    | Uiryeong, Gyeongnam, Korea  | South Korea         | Leaves        |
| 2014-38             | Cudrania tricuspidata    | Sancheong, Gyeongnam, Korea | South Korea         | Leaves        |
| 2014-39             | Cudrania tricuspidata    | Jinju, Gyeongnam, Korea     | South Korea         | Stems         |
| 2014-41             | Cudrania tricuspidata    | Sancheong, Gyeongnam, Korea | China              | Leaves        |
| 2014-42             | Cudrania tricuspidata    | Sancheong, Gyeongnam, Korea | South Korea         | Leaves        |
| 2016-10             | Cudrania tricuspidata    | Miryang, Gyeongnam, Korea   | China               | Leaves/stems  |
| 2016-47             | Cudrania tricuspidata    | 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                 | ATGATTGACCAGATCGTGTGATGC           | 57.4    |           |
| ITS  | ITS forward                  | TCCGTAGGTAACCTCGG                 | 58.0    | 762       |
|      | ITS reverse                  | GCCGTTACTAGGGGAATCTTG             | 57.6    |           |

**B. ARMS-PCR analysis**

| Origin | Primers                      | Sequences (5'-3')                  | Tm (°C) | Size (bp) |
|--------|-----------------------------|-----------------------------------|---------|-----------|
| South Korea | MatK-specific forward    | ACGATTAACATCTTCTGTTGA             | 55.5    | 537       |
|        | MatK-specific reverse       | GATTCTCAGTATAACACGCTAG            | 59.3    |           |
|        | ITS-specific forward        | GCCAAGTGCGTGCGCTCATC              | 68.7    | 458       |
|        | ITS-specific reverse        | CGCAACCACCTTTTGCTCA              | 60.2    |           |
| China  | MatK-specific forward       | ACGATTAACATCTTCTGAGG             | 57.4    | 537       |
|        | MatK-specific reverse       | GATTCTCAGTATAACACGCGAT            | 59.3    |           |
|        | ITS-specific forward        | GCCAAGTGCGTGCGGCTGTC             | 66.2    | 458       |
|        | ITS-specific reverse        | CGCAACCACCTTTTGTCAG              | 57.5    |           |

**C. HRM analysis**

| Gene | Primers                      | Sequences (5'-3')                  | Tm (°C) | Size (bp) |
|------|-----------------------------|-----------------------------------|---------|-----------|
| MatK | MatK forward                 | GTGTGGTCTCAACCAGGAAG              | 57.2    | 197       |
|      | MatK reverse                 | GCAAACGATACCAATCAGAGG             | 57.7    |           |
| ITS  | ITS forward                  | TCCCCGTAAACCACCGCTG              | 58.2    | 205       |
|      | ITS reverse                  | GCACGTTCAAACCGGCTG              | 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. 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.

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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References

Chagne D, Gasic K, Crowhurst RN, Han Y, Bassett HC, Bowatte DR, Lawrence TJ, Rikkerink EH, Gardiner SE, Korban SS (2008) Development of a set of SNP markers present in expressed genes of the apple. Genomics 92:353-358

Gundry CN, Vandersteen JG, Reed GH, Pryor RJ, Chen J, Wittwer CT (2003) Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. Clin Chem 49:396-406

Han EH, Cho KM, Goo YM, Kim MB, Shin YW, Kim YH, Lee SW (2016) Development of molecular markers, based on chloroplast and ribosomal DNA regions, to discriminate three popular medicinal plant species, Cynanchum wilfordii, Cynanchum auriculatum, and Polygonum multiflorum. Mol Biol Rep 43: 323-332

Hano Y, Matsumoto Y, Sun JY, Nomura T (1990) Structures of three new isoprenylated xanthones, cudraxanthones E, F and G. Planta Med 56:478-481

Jung J, Kim KH, Yang K, Bang KH, Yang TJ (2014) Practical application of DNA markers for high-throughput authentication of Panax ginseng and Panax quinquefolius from commercial ginseng products. J Ginseng Res 38:123-129

Kim MK, Wang H, Kim YJ, Sathiyanamoththy S, Kwon WS, Yang DC (2013) Molecular authentication by multiplex-PCR of three similar medicinal plant species: Cynanchum wilfordii, Cynanchum auriculatum and Polygonum multiflorum (Fallopia multiflorum). J Med Plant Res 35:2854-2589

Lehmensiek A, Sutherland MW, McNamara RB (2008) The use of high resolution melting (HRM) to map single nucleotide polymorphism markers linked to a covered smut resistance gene in barley. Theor Appl Genet 117:721-728

Liu J, Huang S, Sun M, Liu S, Liu Y, Wang W, Zhang X, Wang H, Hua W (2012) An improved allele-specific PCR primer design method for SNP marker analysis and its application. Plant Meth 8:34

Mackay JF, Wright CD, Bonfiglioli RG (2008) A new approach to varietal identification in plants by microsatellite high resolution melting analysis: application to the verification of grapevine and olive cultivars. Plant Meth 4:8

Newton CR, Graham A, Heptinstall LE (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucl Acids Res 17:2503-2516

Park KH (2005) Antioxidant and cytotoxic activities of xanthones from Cudrania tricuspidata. Bioorg Med Chem Lett 15: 5548-5552

Park KH, Park YD, Han JM, Im KR, Lee BW, Jeong IY (2006) Antithrombotic and anti-inflammatory activities of catecholic xanthones and flavonoids isolated from Cudrania tricuspidata. Bioorg Med Chem Lett 16:5580-5583

Pyke KA (1999) Plastid division and development. Plant Cell 11: 549-556

Reboud X, Zeyl C (1994) Organelle inheritance in plants. Heredity 72:132-140
Ririe KM, Rasmussen RP, Wittwer CT (1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal Biochem 245:154-160

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725-2729

Wolfe AD, Randle CP (2004) Recombination, heteroplasy, haplotype polymorphism, and paralogy in plastid genes: implications for plant molecular systematics. Syst Bot 29:1011-1120

Yang JY, Jang SY, Kim HK, Park SJ (2012) Development of a molecular marker to discriminate Korean Rubus species medicinal plants based on the nuclear ribosomal DNA internal transcribed spacer and chloroplast trnL-F intergenic region sequences. J Kor Soc Appl Biol Chem 55:281-289

Zou YS, Hou AJ, Zhu GF, Chen YF, Sun HD, Zhao QS (2004) Cytotoxic isoprenylated xanthones from Cudrania tricuspidata. Bioorg Med Chem 12:1947-1953