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RESEARCH REPORT

Genetics and Breeding

Molecular identification of *Allium ochotense* and *Allium microdictyon* using multiplex-PCR based on single nucleotide polymorphisms

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Abstract

Allium ochotense and *Allium microdictyon* are commonly known as 'Mountain garlic' and are popular, economically important species in many countries such as Korea, China, and Mongolia. Their leaves are used as culinary side dishes and in traditional medicines. In Korea, these two species are at risk of extinction due to damage to their natural habitat and thus, conservation and breeding programs are needed. However, their identification relies mostly on morphological data, which is limited and until recently, led to classifying these two species under *A. victorialis*. In the present study, a simple and reliable method of molecular identification was developed to distinguish *A. ochotense* from *A. microdictyon* that targets four barcoding regions: the internal transcribed spacer (*ITS*), the maturase K gene (*matK*), the chloroplast *psbA-trnH* intergenic region, and the ribulose-bisphosphate carboxylase large subunit gene (*rbcL*). Single nucleotide polymorphisms (SNPs) were found in *ITS* and *matK* regions, and species-specific primers were designed based solely on the SNP at position 680 of the *ITS* region that could differentiate *A. ochotense* from *A. microdictyon*. Using these primers in amplification refractory mutation system (ARMS)-PCR, *A. ochotense,* and *A. microdictyon* could be simultaneously and efficiently distinguished. This study is the first to report a simple, rapid, and efficient method for discriminating *A. ochotense* and *A. microdictyon*, indicating the utility of species-specific markers in the development of conservation and breeding programs.

Keywords *ITS* · *matK* · *psbA-trnH* · *rbcL* · Amplification refractory mutation system (ARMS)-PCR

1 Introduction

Allium is probably the largest and most diverse monocotyledonous genera, comprised of over 920 species that are mainly distributed in seasonally dry regions of the Northern Hemisphere (Friesen et al. 2006; Fritsch et al. 2010; Herden et al. 2016; Li et al. 2010), with its main center of diversity located in southwest and central Asia (Choi and Oh 2011; Nguyen et al. 2008; Sarker et al. 1997). This genus consists of perennial herbs characterized by tunicate bulbs, narrow-based leaves, and head-like inflorescences with superior ovaries. Most *Allium* species produce cysteine sulfoxide, which causes their characteristic odor and taste (Fritsch and Keusgen 2006). Various *Allium* species are cultivated and constitute valuable crops or garden vegetables with health benefits and medicinal properties. In Korea, 26 taxa are recognized (Choi and Oh 2011) and include 24 species, with *A. ochotense* and *A. microdictyon* some of the most important

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species. They are commonly referred to as 'sanmaneul' in Korean or 'mountain garlic'; their leaves and scaly bulbs are often served as side dishes or garnishing ingredients in many traditional Korean foods, and also used in many traditional Korean medicines and as a detoxing agent.

Allium microdictyon is a diploid species with 16 chromosomes (2n = 2x = 16) (Yoo et al. 1998), and mainly distributed from central Asia to central Korea, but is also found Russia, Mongolia, and China (Friesen 1995). In Korea, *A. microdictyon* grows in shaded and humid forests on mountain slopes 1300 m above sea level (Choi and Oh 2011). *A. ochotense* is a forest species distributed in North America, Russia, northern China, Korea, and Japan (Jung et al. 2013); although the population from the Ulleungdo island far off the Korean peninsula is diploid (2n = 2x = 16), other Asiatic populations are tetraploid (2n = 4x = 32) (Yoo et al. 1998). These two *Allium* species are quite rare in Korea, and the *A. ochotense* population from Ulleungdo in particular needs to be conserved. In addition, the Korean Ministry has listed *A. microdictyon* among the country's most endangered species that are at risk of extinction due to damage to their natural habitats (Choi and Oh 2011).

Initially, *A. ochotense* and *A. microdictyon* were classified under *A. victorialis* (Choi et al. 2004; Kim et al. 2000); however, studies have reported the identification of peculiar differences in their external morphology, especially in their leaves: *A. microdictyon* has thin and narrow leaves, while the leaves of *A. ochotense* are broad and oval. Considerable differences can also be observed in the size and color of their respective reproductive organs (i.e. tepal, stamen, pistil, capsule), that have either a yellow (*A. microdictyon*) or white (*A. ochotense*) perianth. Thus, both species are now considered separate from rather than a synonym of *A. victorialis* (Choi et al. 2004; Choi and Oh 2011).

Identifying species based solely on morphological characters has limitations, in particular for herbs with economic and medicinal importance. The limited availability of trained professionals that can participate in the taxonomic examination of *Allium* spp. limits accurate identification in systematic studies, which is sometimes difficult using only dried specimens. Therefore, genomic fingerprinting using molecular techniques has emerged as a powerful and reliable tool for taxonomic identification and quality control of herbal drugs (El Beyrouthy and Alain AbiRizk 2013; Ganopoulos et al. 2013; Mishra et al. 2016; Whatmore et al. 2005; Yip et al. 2007). Moreover, DNA-based molecular markers have played a pivotal role in identifying or authenticating the species (Arif et al. 2011; Ganie et al. 2015). Molecular markers are DNA fragments with features that are determined by allelic forms of genes that can be transmitted from one generation to the next. They can be used to detect species-specific variation (polymorphism) in nucleotide sequences, resulting in characteristic genomic fingerprints (Semagn et al. 2006). Many PCR-based methods have been applied to develop markers for plant authentication and identification (Ganie et al. 2015; Han et al. 2016), and during the last decades, several candidate genomic regions (i.e. DNA barcodes) have been proposed for identifying species or taxa. These regions generate useful information to resolve phylogenetic relationships at several taxonomic levels, as they show a high level of discriminatory power and many copies per cell.

Several genomic regions or loci from nuclear and cytoplasmic DNA, particularly the internal transcribed spacer (*ITS*) (Gao et al. 2010; Han et al. 2013), the maturase K gene (*matK*) (Lahaye et al. 2008; Newmaster et al. 2008),

Table 1 Codes and collection sites of the *Allium* species used in this study

Sample code	Collection site	Species
TB	Taebaek, South Korea	*A. ochotense*
UL	Ulleungdo, South Korea	
OD	Odae, South Korea	*A. microdictyon*
BH	Baekdu, North Korea	
CH	China	
MO	Mongolia	

Table 2 Primer sets used to amplify each barcoding region and their sequences

Marker	Genomic Source	Type	Primer name	Primer sequence (5′–3′)	Reference
ITS	Nuclear	Transcribed spacers and 5.8S gene	AB 101	ACGAATTCATGGTCCGGTGAAGTGTTCG	Sun et al. (1994)
			AB 102	TAGAATTCCCCGGTTCGCTCGCCGTTAC	
matK	Plastid	Protein coding	matK-390F	CGATCTATTCATTCAATATTTC	Cuenoud et al. (2002)
			matK-1326R	TCTAGCACACGAAAGTCGAAGT	
rbcl	Plastid	Protein coding	rbcLaF	ATGTCACCACAAACAGAGACTAAAGC	Kress et al. (2009)
			rbcLaR	GTAAAATCAAGTCCACCRCG	
trnH-psBA	Plastid	Intergenic spacer	psbA	CGAAGCTCCATCTACAAATGG	Tate and Simpson (2003)
			trnHf	CGCGCATGGTGGATTCACAATCC	

ITS nuclear internal transcribed spacer, *matK* maturase K, *rbcL* ribulose-bisphosphate carboxylase, *trnH-psbA* plastid intergenic spacer

Table 3 Primers sets used for differentiating *Allium ochotense* and *Allium microdictyon* with their sequence information

Primer	Sequence	Orientation	Start position	Tm (°C)	Specificity
ITS-OF	5′-GTCGATGAAGAACGTAGCGAAATG-3′	Forward	429	61	Common
ITS-OR	5′-TGATATGCTTAAACTCAGCGGGTG-3′	Reverse	843	62	Common
ITS-SNP1	5′-AGACGGTCGTCGTTAGGATTGAAC-3′	Forward	657	62	*A. ochotense* specific
ITS-SNP2	5′-AACTCGATCAACATTCGCCTCA-3′	Reverse	703	61	*A. microdictyon* specific

Tm annealing temperature

the chloroplast *psbA-trnH* intergenic region (Ma et al. 2010; Yao et al. 2009), and the large subunit of the ribulose-bisphosphate carboxylase gene (*rbcL*) (Kress and Erickson 2007) have been used in plant species identification. A single nucleotide polymorphism (SNP) is frequently observed in barcoding regions when compared among species or individuals within a species, which can be especially important in the study of medicinal plants (Choi et al. 2017; Wang et al. 2012; Yang et al. 2012). Such variations have been effectively explored to develop species-specific SNP markers in many plants (In et al. 2010; Jigden et al. 2010; Kim et al. 2012, 2017; Park et al. 2006). The tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) is a simple and economic method to genotype organisms using single nucleotide polymorphisms (SNPs) (Ye et al. 2001). It utilizes an allele-specific primer with a mismatch at 3′ terminus, which makes it unique to only one allele of the SNP and distinguishable from the other allele. Consequently, the primer will only amplify the template that is perfectly complementary to its 3′ end, generating a single PCR amplicon. By determining whether an amplicon is produced or not, the target DNA can be genotyped (Kwok 2001).

In the present study, the nucleotide sequences of the barcoding regions *ITS, matK, rbcL*, and *trnH-psbA* in *A. microdictyon* and *A. ochotense* were analyzed and variation between the two species was evaluated. Based on the variation of the SNPs within the *ITS* region, species-specific primers were designed and used in the ARMS-PCR technique to establish a reproducible and robust approach for the molecular identification of *A. microdictyon* and *A. ochotense*.

2 Materials and methods

2.1 Plant material and DNA extraction

Six leaf tissues in total *A. ochotense* (two samples) and *A. microdictyon* (four samples) were collected from different geographical regions (Ulleungdo Island, Mount Taebaek, Mount Odae, and Mount Baekdu in Korea, China, and Mongolia). A code was assigned to each sample (Table 1), and the leaf tissues from each sample were frozen in liquid nitrogen and ground into a fine powder. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Table 4 Summary of the sequence analysis of the four gene regions analyzed for *Allium ochotense* and *Allium microdictyon*

Region	Length (bp)	Single nucleotide polymorphisms (number)	Transitions (number)	Transversions (number)
ITS	908	13	7	6
*mat*K	901	8	3	5
trnH-psbA	592	0	0	0
*rbcL*A	551	0	0	0

2.2 PCR amplification and gel electrophoresis

The *ITS, matK, rbcL,* and *trnH-psbA* regions were amplified using specific primers (Cuenoud et al. 2002; Kress et al. 2009; Sun et al. 1994; Tate and Simpson 2003) (Table 2). The PCR reaction mixtures (20 µl total volume) contained 20 ng template DNA, 2 µl 10 × PCR buffer, 0.20 mM dNTPs, 0.5 µM each forward and reverse primer, and 0.025 U i-star max DNA polymerase (Intron Biotechnology, Korea). The amplification was conducted as follows: pre-denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 35 s, and extension at 72 °C for 45 s; final extension at 72 °C for 5 min to ensure the full extension of the products. The PCR products were analyzed in a 1.0% agarose gel electrophoresis and visualized by ethidium bromide staining under UV.

2.3 DNA sequencing and analysis

Successfully amplified PCR products were purified using a PCR DNA purification kit (Gene All, South Korea) following the manufacturer's instructions, and then cloned into a pGEM-T vector (Promega, Madison USA). Three clones of

each PCR product were sequenced in an ABI4000 machine (ABI Inc., USA). Sequencing was performed in both directions to avoid sequencing errors and verify sequence variants. Consensus sequences were generated for each sample using the online multiple sequence alignment tool MAFFT version 7 (http://www.ebi.ac.uk/Tools/msa/mafft/) with default parameters. To identify and compare SNPs between *A. ochotense* and *A. microdictyon*, a neighbor-joining phylogenetic tree was constructed for *matK* and *ITS* regions using MEGA version 5 package (Hall 2013), using representative sequences.

2.4 ARMS-PCR

Specific *A. microdictyon* and *A. ochotense* SNP alleles were detected in the *ITS* region at position 680 bp (Supplementary Fig. 1) and used to design two species-specific primers in Batch Primer version 3 (You et al. 2008). The forward primer ITS-SNP1 was designed to amplify *A. ochotense* and did not work with *A. microdictyon*. Hence, the reverse primer ITS-SNP2 was specifically designed for *A. microdictyon* (Table 3). The underlined bases in the primers ITS-SNP1 and ITS-SNP2 were deliberately destabilized via substitution of A for C and G for T, respectively, following protocol explained by Liu et al. (2012). These species-specific primers with specific mismatches at the 3′ end allowed for the preferential amplification of one allele over another and were designated 'inner primers.' Two universal primers, ITS-OF and ITS-OR were common to both *Allium* species and designated 'outer primers' in the ARMS technique (Table 3). The ARMS-PCR was carried out using the following thermal profile: pre-denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 67 °C for 30 s, and extension at 72 °C for 30 s; final 5-min extension a 72 °C. Reaction mixtures were identical to those described above, except that 0.5 µM primers of both outer and inner primer sets were used in these reactions.

2.5 Allele-specific PCRs

To further validate the efficiency of ARMS-PCR, an allele-specific PCR was performed separately for *A. ochotense* and *A. microdictyon* using their specific primers and the reaction mixtures and PCR conditions similar to ARMS-PCR.

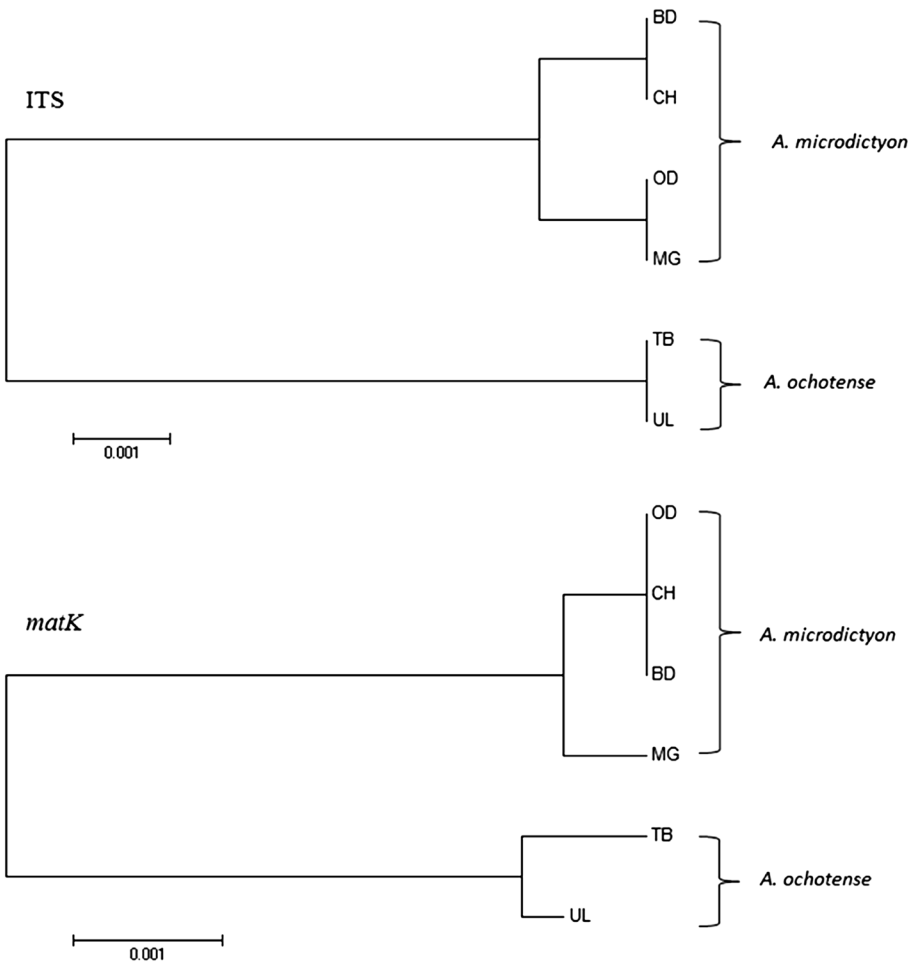

Fig. 1 Construction of neighbor-joining phylogenetic trees based on the internal transcribed spacer (*ITS*) and maturase K (*matK*) gene sequence variations in *Allium ochotense* (TB, Taebaek; UL, Ulleungdo) and *Allium microdictyon* (OD, Odae; BH, Baekdu; CH, China; MO, Mongolia). The scale bar indicates number of substitution per site

2.6 Validation of genotypes by multiplex ARMS-PCR

The developed multiplex PCR was further validated on a set of *Allium* species collected from different geographical areas (Supplementary Table 1). The samples were kindly provided by Prof. Soon-Kwon Hong (Kangwon National University). The reaction mixture and PCR cycle were identical to that of the ARMS-PCR described above.

3 Results

3.1 *ITS*, *matK*, *trnH-psbA*, and *rbcL* sequence comparisons between Allium species

The genomic regions *ITS, matK, trnH-psbA, and rbcL* were amplified from six samples of *Allium* species (Table 1) using gene-specific primer sets (Table 2).

3.1.1 *ITS*

The *ITS1–5.8S–ITS2* rDNA region was amplified from leaf tissues using the AB 101 and AB 102 universal primer set, and a 908 bp product that corresponded to 257 bp for *ITS1*, 162 bp for *5.8S*, and 292 bp for *ITS2*. Based on the multiple sequence alignment of all samples, 13 SNPs were identified, including seven transitions and six transversions (Table 4). Eleven SNPs were observed between *A. ochotense* and *A. microdictyon* species, with only two exclusive to *A. microdictyon*. The two *A. ochotense* samples had 100% sequence homology. The phylogenetic tree produced for the *ITS* region indicated two distinct groups, each corresponding to a species. Group I corresponded to *A. microdictyon* and was further divided into two clusters: one containing samples from Baekdu (BD) and China (CH) and the other containing samples from Odae (OD) and Mongolia (MG) (Fig. 1).

3.1.2 *matK*

Amplification of *matK* yielded 901 bp sequences and multiple alignments indicated eight SNPs: three were transitions, and five were transversions (Table 4). All these SNPs were found between *A. ochotense* and *A. microdictyon*. Similar to the *ITS* tree, phylogenetic analysis using full-length *matK* sequences also grouped the sequences into two clusters, with each corresponding to a different species. However, unlike

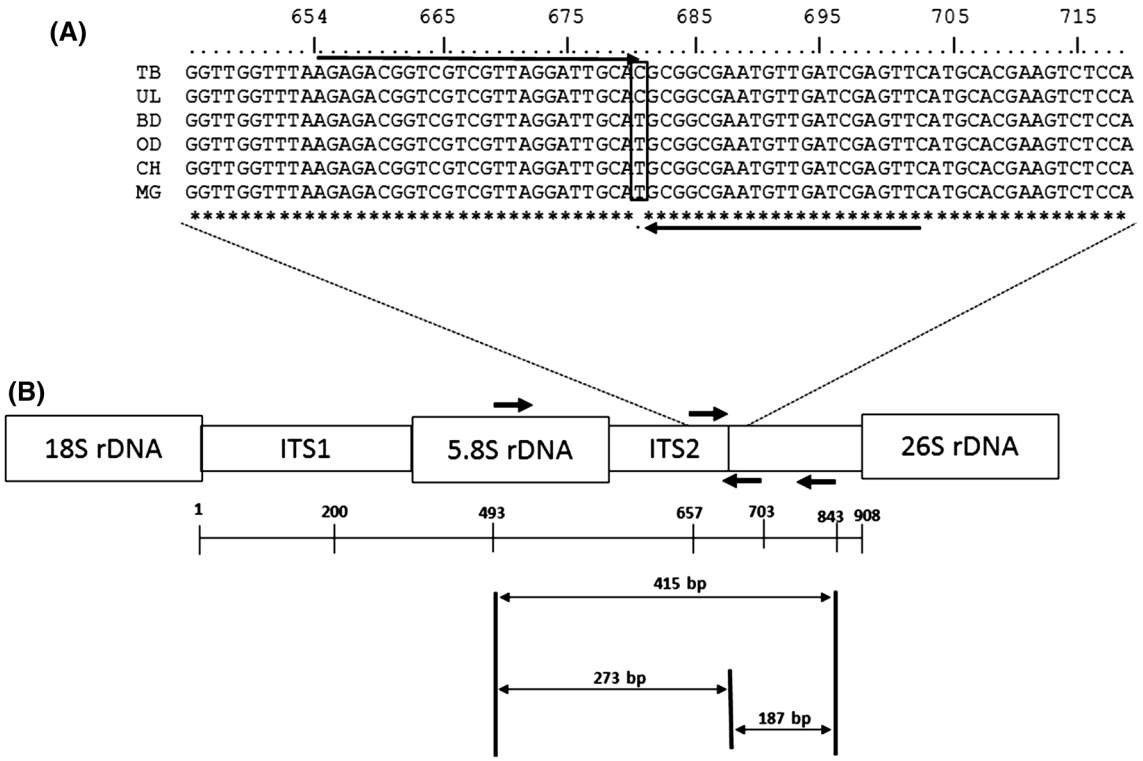

Fig. 2 Graphical overview of **a** the internal transcribed spacer (*ITS*) sequences obtained from *Allium* species that displayed single nucleotide polymorphisms (SNPs), and **b** the *ITS* region including the positions and sequences of the primers used in the multiplex PCR. The arrow indicates the direction of the outer and inner primers used in the amplification refractory mutation system (ARMS)-PCR technique

ITS, a low discrimination power was observed between *A. microdictyon* samples (Group I, Fig. 1).

3.1.3 *trnH-psbA* and *rbcL*

Sequences obtained for these regions were 592 bp and 551 bp in length, respectively. Because the two species exhibited complete homology for both *trnH-pbsA* and rbcL, these regions were not analyzed further. Detailed sequence alignment information is shown in Supplementary Fig. 1, and detailed information on SNPs and their location are presented in Table 4.

3.2 Multiplex ARMS-PCR

The SNPs identified in the *matK* region were not suitable for designing ARMS primers, as they were expressed in both species. Thus, the *ITS* region was used for molecular identification of *Allium* species using species-specific ARMS-PCR. The SNP position located at 680 bp of ITS region was selected for this analysis, and an outer (common) and an inner (species-specific) primer set was designed such that their combined use generated two differently sized amplicons (Fig. 2). The relative position of the four primers is presented in Table 4. The application of ARMS-PCR in the two samples of *A. ochotense* yielded the expected two amplicons that were 415 and 187 bp, respectively, and its application to the four samples of *A. microdictyon* yielded two amplicons that were 415 and 237 bp (Fig. 3). All experiments were repeated multiple times to confirm the reproducibility of the data.

The allele-specific PCRs using the same primer sets produced only one type of amplicon: the combination of *ITS-OF* and *ITS-SNP2* only amplified samples from *A. microdictyon*, whereas the combination *ITS-OR* and *ITS-SNP1* only amplified samples from *A. ochotense* (Fig. 3b, c). Thus, the ARMS-PCR technique could specifically detect *A. ochotense* and *A. microdictyon*. We compared the *ITS* sequence regions from different *Allium* species available in the NCBI database. Variation between species was observed at the SNP position (680 bp) depending on the ARMS primer that was designed, which exemplified the utility of this method for identifying *Allium* species (Supplementary Table 2). Further, to validate the molecular markers we developed, *Allium* species collected from different areas were used in multiplex ARMS-PCR. As expected, ARMS-PCR generated two different sized amplicons that differentiated *A. ochotense* from *A. microdictyon*

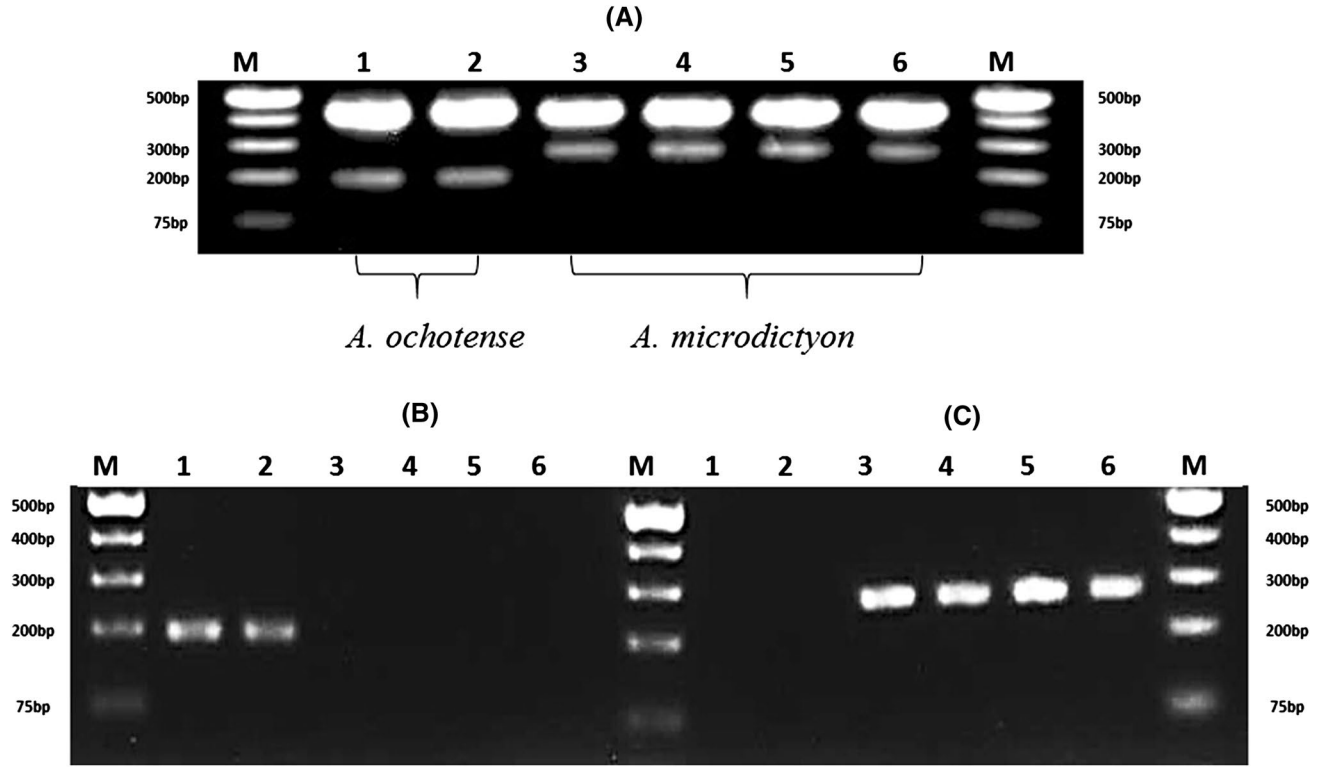

Fig. 3 PCR products obtained in the **a** multiplex PCR using primers ITS-OF, ITS-OR, ITS-SNP1, and ITS-SNP2; **b** allele-specific PCR using primers ITS-OF and ITS-SNP2 that are specific to *Allium ochotense*; and **c** allele-specific PCR using primers ITS-OR and ITS-SNP1 specific to *A. microdictyon*. Lanes: M, 1000 bp DNA ladder; 1 and 2, *A. ochotense*; 3–6, *A. microdictyon*. Sampling sites: TB, Taebaek; UL, Ulleungdo; OD, Odae; BH, Baekdu; CH, China; MO, Mongolia

(Fig. 4). We were, therefore, able to conclue that the high specificity ARMS primers designed in our study can specifically discriminate between *A. ochotense* and *A. microdictyon*.

4 Discussion

In the present study, the frequently used loci in plant molecular studies, *ITS, matK, trnH-psbA*, and *rbcL*, were targeted for the molecular analysis of two culturally and economically important *Allium* species found in Korea. Although the *ITS* region showed more variable sites between *A. ochotense* and *A. microdictyon* than the *matK* region, both regions had species-specific variations that were supported by the two groups, as evidenced in the phylogenetic trees comprosed of sequences from only one of the species. The *trnH-psbA* and *rbcL* sequences were completely homologous and, therefore, were not further analyzed in this study. The SNP site of the *ITS* region and the successfully employed ARMS-PCR technique was about to distinguish between *A. ochotense* and *A. microdictyon*, and was validated using allele-specific PCR amplification. These procedures are part of a standard protocol that includes species-specific primers that can be used in species identification processes.

The taxonomy of *Allium* spp. is complicated and might be prone to errors as shown by the significant number of synonyms and intergenic groupings within the genus (Choi et al. 2004; Choi and Oh 2011). Moreover, until recently, *A. ochotense* and *A. microdictyon* were treated as synonyms of *A. victorialis* (Kim et al. 2000). *Allium ochotense* and *A. microdictyon* are indigenous in Korea and are at risk of extinction due to the destruction of their natural habitats (Choi and Oh 2011); thus, there is a need to preserve and breed both species. However, their identification and distinction from one another relies mainly on morphological diagnosis, which has significant limitations

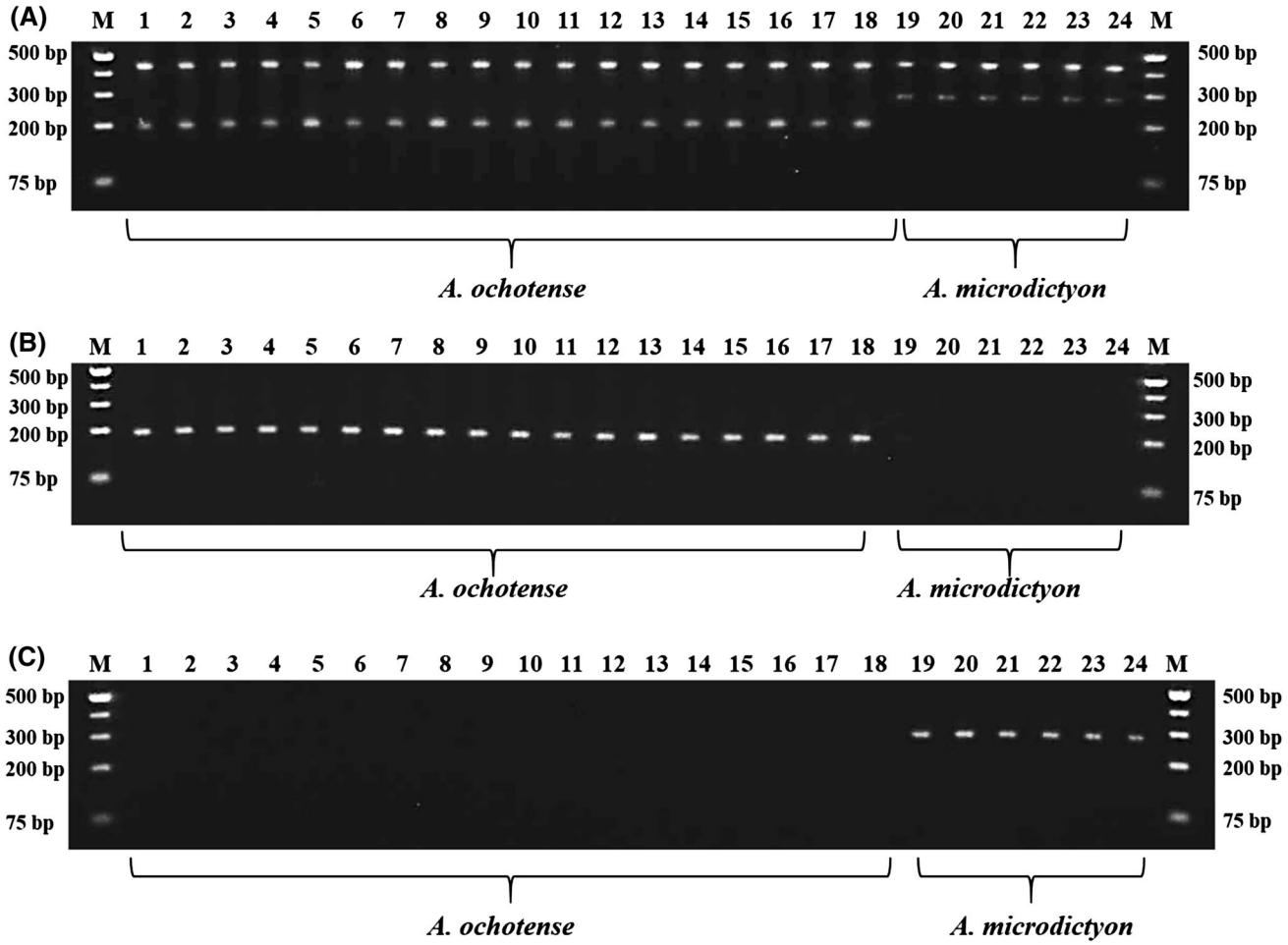

Fig. 4 Validation of the ability for the multiplex ARMS-PCR to differentiate between *A. ochotense* and *A. microdictyon*. **a** PCR products obtained using primers ITS-OF, ITS-OR, ITS-SNP1, and ITS-SNP2; **b** allele-specific PCR using primers ITS-OF and ITS-SNP2 specific to *Allium ochotense*; and **c** allele-specific PCR using primers ITS-OR and ITS-SNP1 specific to *Allium microdictyon*. Lanes: M, 1000 bp DNA ladder; 1–18, *A. ochotense*; 19–24, *A. microdictyon*

(Friesen et al. 2000; Hebert et al. 2003) and genomic approaches based on sequence variation have gained broad acceptance for *Allium* identification at the species level (Mishra et al. 2016; Techen et al. 2014). Moreover, efforts to structure the genus using molecular tools have been undertaken (Friesen et al. 2006; Fritsch et al. 2010; Herden et al. 2016; Li et al. 2010). The *ITS* sequences from *A. ochotense* and *A. microdictyon* indicated that these two congeners were genetically distinct from each other (Choi and Oh 2011). However, a quick and simple method for their identification is needed and the present method, employing species-specific primers, seems to be adequate, as it requires a short analysis time, unsophisticated equipment, and can be applied to immature plants or seeds. The ARMS-PCR technique used in the present study was already proven useful in the identification of many medicinal plants including *Panax ginseng* (In et al. 2010; Wang et al. 2010), *Anemarrhena asphodeloides* sp (Jigden et al. 2010), and *Schisandra chinensis* (Kim et al. 2012). A few SNPs were observed between samples of different origins, for example in *A. microdictyon* from Korean and Chinese origin, indicating that this method may also be applied to designing region-specific SNP primers, which would be very useful for breeding programs.

In conclusion, the authentication of the Korean *Allium* species *A. ochotense* and *A. microdictyon* has relied mainly on time consuming and difficult morphological inspections, and would greatly benefit from a high-throughput and accurate method of gene analysis for species identification. The present study demonstrated a reproducible and reliable approach to distinguish *A. ochotense* from *A. microdictyon* based on *ITS* sequence variation.

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