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Development of a Plastid DNA-Based Maker for the Identification of Five *Medicago* Plants in South Korea

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ABSTRACT

DNA markers have been studied and used intensively to identify plant species based on molecular approaches. The genus *Medicago* belongs to the family Fabaceae and contains 87 species distributed from the Mediterranean to central Asia. Five species of *Medicago* are known to be distributed in South Korea; however, their morphological characteristics alone cannot distinguish the species. In this study, we analyzed the phylogenetic relationships using collected five species of *Medicago* from South Korea and 44 taxa nucleotide information from NCBI. The constructed phylogenetic tree using gibberellin 3-oxidase 1 and $tRNA^{Lys}$ (UUU) to maturase K gene sequences showed the monophyly of the genus *Medicago*, with five species each forming a single clade. These results suggest that there are five species of *Medicago* distributed in South Korea. In addition, we designed polymerase chain reaction primers for species-specific detection of *Medicago* by comparing the plastid sequences. The accuracy of the designed primer pairs was confirmed for each *Medicago* species. The findings of this study provide efficient and novel species identification methods for *Medicago*, which will assist in the identification of wild plants for the management of alien species and living modified organisms.

Keywords: DNA markers, *Medicago*, Phylogenetic tree, Plastid genome, Species specificity

Introduction

The genus *Medicago* L. belongs to the family Fabaceae and consists of approximately 87 species that are predominately distributed from the Mediterranean to Central Asia (Choi *et al.,* 2022; Steele *et al.,* 2010). The genus comprises annual or perennial herbs and a few shrubs, including alfalfa (*Medicago sativa*) as a widely cultivated forage crop and *Medicago truncatula* as a legume model plant (Echeverria *et al.,* 2021; Thanopoulos, 2007). Based on studies of flora and distribution, five taxa, including *M. sativa, Medicago ruthenica, Medicago polymorpha, Medicago minima,* and *Medicago lupulina* are speculated to be distributed in South Korea (Kil *et al.,* 2004; Kim, 2005; Lee, 1976; Lee *et al.,* 2018; Van Berkum *et al.,* 1998). However, the exact number of taxa present in South Korea remains unknown. These *Medicago* plants have been considered alien species introduced from Europe, the Mediterranean, and Mongolia to South Korea (Kim, 2005; Lee *et al.,* 2018; Van Berkum *et al.,* 1998). They are usually distributed in open fields, road verges, pastures, parks, and banks (Kil *et al.,* 2004; Kim, 2005; Song & Park, 2019; You, 2018). *M. sativa* is used as livestock feed, and a living modified (LM) version has been developed recently (Choi *et al.,* 2020; Guertler *et al.,* 2019). This LM organism (LMO) has been approved for use in many countries, including South Korea (Choi *et al.,* 2020).

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However, concerns regarding potential adverse effects on biodiversity related to alien species and LMOs have been increasing (Kim *et al.,* 2020; Terrones *et al.,* 2021). For the management of alien species or LMOs, rapid and accurate identification should be a priority to prevent adverse effects on biodiversity (Kim *et al.,* 2020; Terrones *et al.,* 2021).

Morphological characteristics such as plant type (herb/shrub), legume type (spiral/not spiral), leaflet or petiole shape, flower color, and number of flowers in each inflorescence are generally evaluated when identifying *Medicago* species (Chen *et al.,* 2021; Wu *et al.,* 2010). The five taxa of *Medicago* in South Korea are also identified based on morphological characteristics. *M. sativa* is a shrub that can be easily distinguished from other species (Wu *et al.,* 2010). *M. ruthenica, M. polymorpha, M. minima*, and *M. lupulina* are herbs and they are distinguished based on morphological characteristics such as leaflet or legume shape, and flower color (Wu *et al.,* 2010). However, it is difficult to accurately distinguish the five taxa based on these morphological characteristics outside of their flowering and ripening periods.

Various DNA markers such as random amplified polymorphic DNA, amplified fragment length polymorphisms, single nucleotide polymorphisms, microsatellites, and short sequence repeats are mainly used for studying plant ecology, taxonomy, phylogeny, and genetics (Azizi *et al.,* 2021; Dar *et al.,* 2019; Marakli, 2018). The use of DNA-based markers is advantageous for identifying plant species with similar morphologies as these methods use small amounts of sample material in a time-saving and cost-effective manner (An *et al.,* 2019). Recently, highly variable sequences in plastid genomes have been identified that can facilitate specific plant species identification (Dong *et al.,* 2012; Gao *et al.,* 2011). Analyzing nucleotide sequence data of plastids represents an effective strategy for species identification in plants (Fazekas *et al.,* 2008; Kress & Erickson, 2007).

The target region of the chloroplast genome can be amplified more easily than nuclear DNA when using polymerase chain reaction (PCR) because a plant leaf cell contains up to 10,000 copies of the chloroplast genome (Koya *et al.,* 2005; Lee *et al.,* 2017; Morley & Nielsen, 2016). Although most nucleotide sequences of chloroplast genes are conserved, significant nucleotide variation has been confirmed in chloroplast intergenic spacer regions which allow interspecies comparison (McCauley, 1995). In addition, plastid sequences are generally used to establish molecular markers to identify plants in various genera in ecological and agricultural studies (Abid *et al.,* 2019; Moon *et al.,* 2016; Nguyen *et al.,* 2020; Zhang *et al.,* 2017). However, although species identification is potentially essential for studies on plants, no investigations on species-specific DNA markers in the five aforementioned *Medicago* plant species have been reported yet.

Taxonomic studies using molecular markers can improve our understanding of the relationship among plant species and can improve management of potentially damaging plant management. In this study, we applied molecular phylogeny based on the nuclear gene gibberellin 3-oxidase 1 (*GA3ox1*) and chloroplast region $tRNA^{Lys}$ (UUU) to maturase K (*trnK-matK*) to five *Medicago* taxa collected from South Korea. Additionally, we developed a PCR-based species-specific detection method based on plastid DNA sequences to distinguish species with similar morphology.

Materials and Methods

Plant material

Leaves and mature seeds were collected for DNA extraction and PCR analysis. *M. polymorpha* and *M. ruthenica* samples were collected from Jindo-gun, Jeollanam-do and from Jeju-si, Jeju-do, respectively. The leaf samples were dried using silica gel and stored at 4°C until DNA extraction. Seeds of *M. sativa* (NIBRVP0000540343), *M. minima* (NIBRVP0000499194), and *M. lupulina* (NIBRVP0000600808) were obtained from the National Institute of Biological Resources (NIBR, Incheon, Korea). Thirty-eight species of *Medicago*, *Melilotus indicus, Melilotus albus, Trigonella elliptica,* and *Trigonella anguina* of Fabaceae were included as outgroups, based on a previous study using *trnK-matK* and *GA3ox1* nucleotide sequence data (Steele *et al.,* 2010). GenBank accession numbers of species used in this study are listed in Appendix 1.

DNA extraction and PCR amplification

Genomic DNA was isolated from fresh tissues using the Libex NP968 system (Tianlong, Xi'an, China) according to the manufacturer's instructions. PCR amplification was performed using the ProFlex PCR System (Thermo Fisher Scientific Inc., Waltham, MA, USA). PCR reactions were conducted using 2×LAMP Taq PCR Pre-Mix (Biofact, Daejeon, Korea) in 30 μL volumes containing 100 ng of each template DNA and 1 μL of each primer (10 pmoL/μL). For the phylogenetic analysis, PCR conditions and primers for two regions of *GA3ox1* and *trnK-matK* were obtained from a previous study (Steele *et al.,* 2010). PCR condition for species-specific detection method was determined with pre-denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 1 minutes 30 seconds, with a final extension at 72°C for 7 minutes. PCR products were separated via electrophoresis on 2.0% agarose gels, run in a 1×TAE buffer, and identified using the ChemiDoc XRS^{+} Imaging System (Bio-Rad, Hercules, CA, USA). The PCR products were sequenced using the amplification primers on an ABI 3730XL system (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

The DNA sequences obtained from the National Center for Biotechnology Information (NCBI) and PCR analysis were aligned using ClustalW and BioEdit version 7.2.5 (Hall, 1999). Phylogenetic tree construction was based on two nucleotide sequences as separated data, the maximum parsimony (MP) method was performed using MEGA 11 (Tamura *et al.*, 2021). The consistency index (CI), retention index (RI), and tree length were calculated using MEGA 11. Five hundred bootstrap replications estimated the internal branch strength of a strict consensus tree to support individual clades (Felsenstein, 1985).

Primer design for detection

A species-specific detection method for the five taxa of *Medicago* was developed using plastid sequence data obtained from the NCBI (accession numbers: MK460494, MK460497, MK460498, MK460499, and NC053371).

Table 1. Tree statistics of the *GA3ox1* and *TrnK-matK* region based on maximum parsimony analysis

Characteristics	*GA3ox1*	*trnK-matK*
Number of OTUs (ingroup/outgroup)	53 (49/4)	53 (49/4)
Aligned length (bp)	1,270	2,526
Variable characters (%)	436 (34.3)	538 (21.3)
Parsimony informative characters (%)	256 (20.2)	256 (10.1)
Length of maximum parsimonious trees	921	770
Consistency index	0.61	0.80
Retention index	0.71	0.85

OTU, operational taxonomic unit.

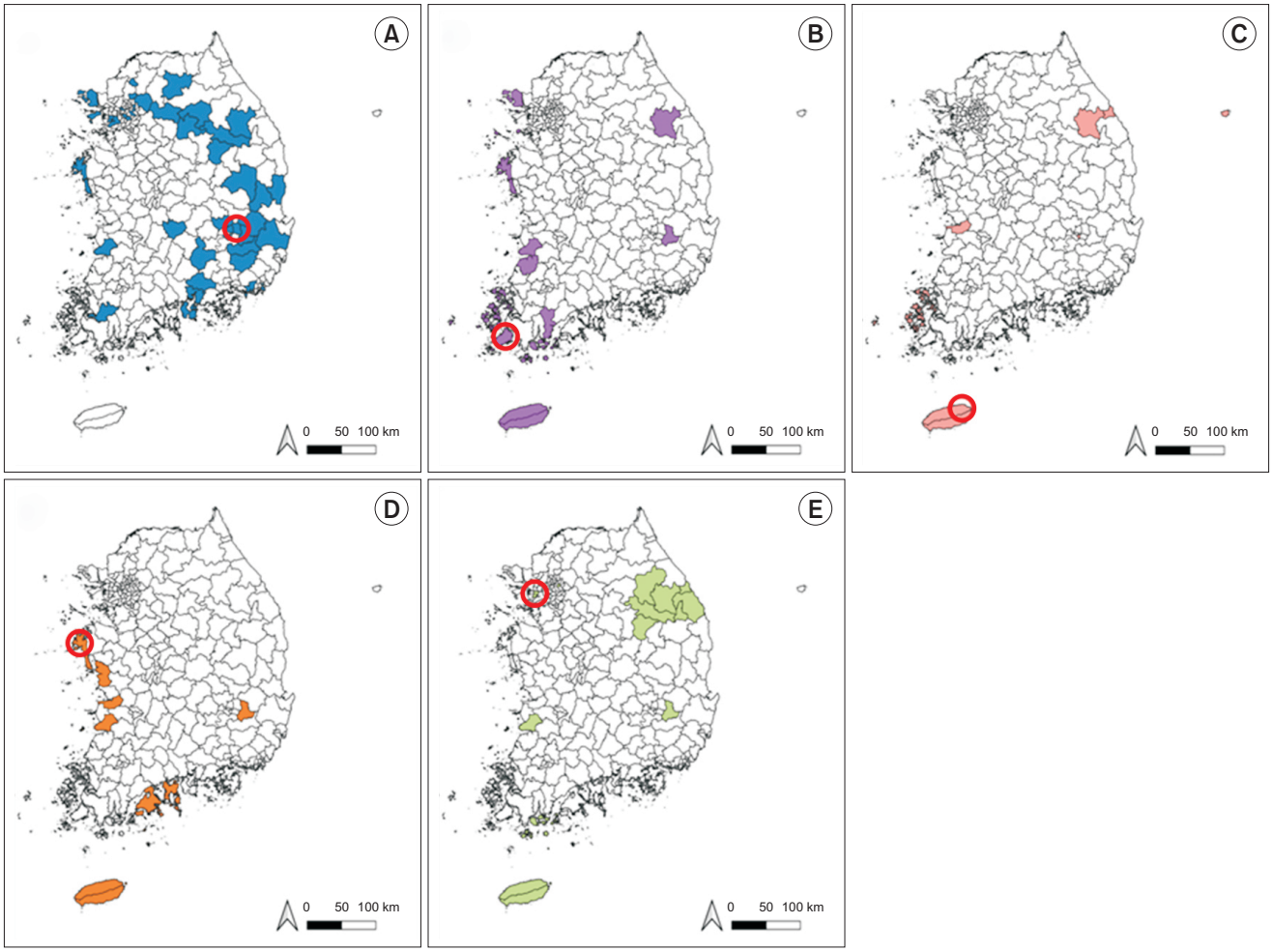

Fig. 1. Maps of *Medicago* distribution in South Korea. Each map shows the distribution areas (colored patches) and sample collection sites (red circles) of (A) *Medicago sativa*, (B) *Medicago polymorpha*, (C) *Medicago ruthenica*, (D) *Medicago minima*, and (E) *Medicago lupulina*.

Plastid sequences were aligned using the ClustalW multiple alignment program in BioEdit version 7.2.5. The primers were designed using regions showing differences in nucleotide sequence between each species (Table 1).

Results

Distribution and sample information of *Medicago*

Five species of *Medicago* distributed in South Korea

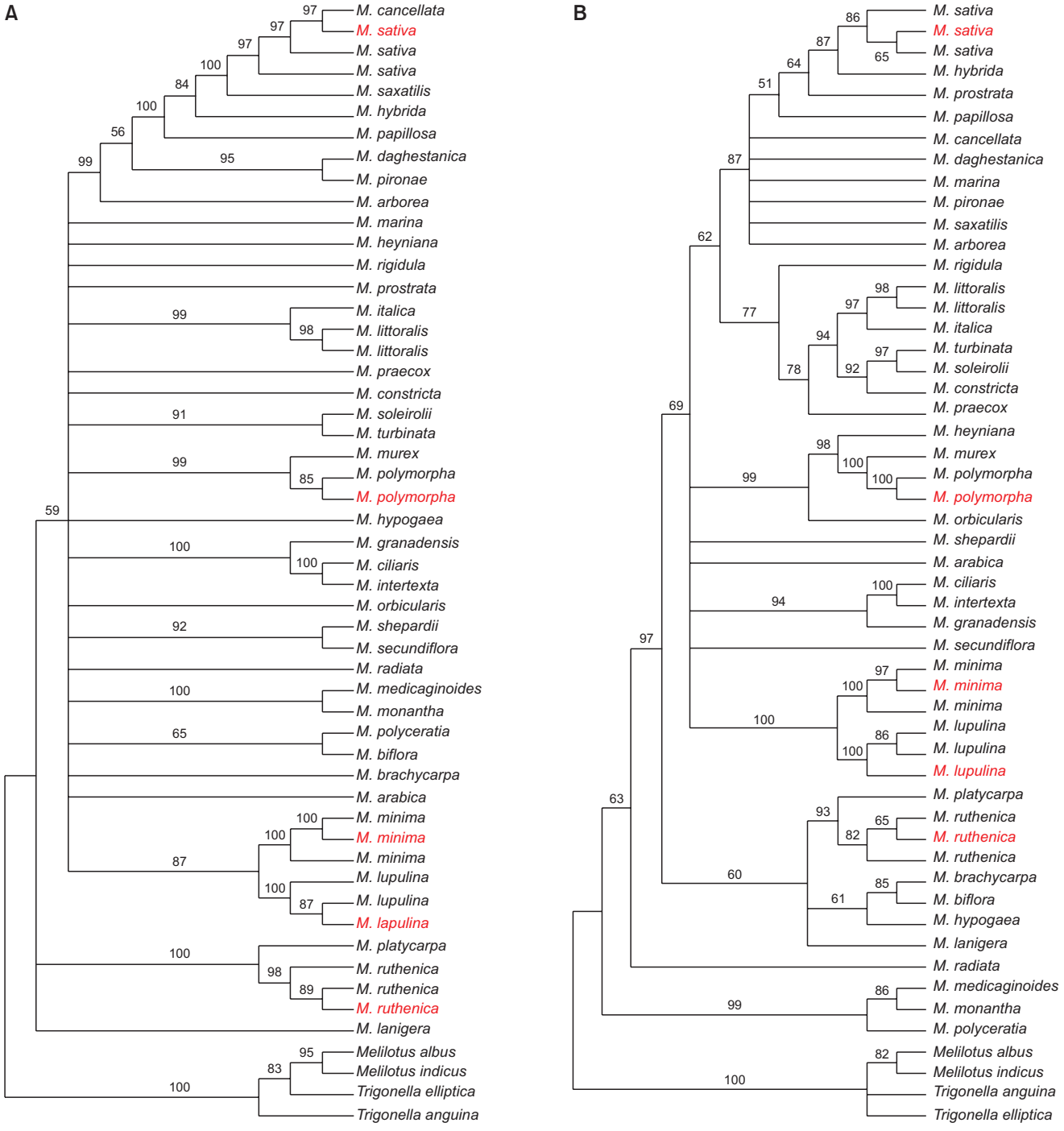

Fig. 2. Molecular phylogenetic tree of *Medicago*. (A) Maximum parsimony strict consensus tree (tree length=921, CI=0.61, RI=0.71) inferred from *GA3ox1* sequence data. (B) Maximum parsimony strict consensus tree (tree length=770, CI=0.80, RI=0.85) inferred from *trnK-matK* sequence data. Numbers in the branches indicate support values. Red-labelled text indicates samples collected in this study. CI, consistency index; RI, retention index.

have been studied previously, however, taxonomic and detailed information remain unavailable (Kil *et al.,* 2004; Kim, 2005; Lee, 1976; Lee *et al.,* 2018; Van Berkum *et al.,* 1998). For performing the taxonomic study of *Medicago*, distribution-related information was collected as specimen information from the website of Korea National Arboretum and previous studies (Choi *et al.,* 2015; Hwang *et al.,* 2013) (Fig. 1). *M. sativa* is widely distributed in South Korea, and other species have limited distribution (Fig. 1). In the present study, we obtained three samples of *M. sativa, M. minima,* and *M. lupulina* from NIBR (Fig. 1A, D, E). Wild samples of *M. polymorpha* and *M. ruthenica* were collected from Jindo-gun, Jeollanam-do and from Jeju-si, Jeju-do, respectively (Fig. 1B, C).

Phylogenetic analysis

To identify the five collected samples, we performed the phylogenetic analysis including the five taxa and other related species of *Medicago* using nucleotide sequence data of *GA3ox1* and *trnK-matK* (Steele *et al.,* 2010). In total, 44 GenBank-derived sequences of *Medicago* were used as ingroups for phylogenetic analyses.GenBank data for four taxa of*M. indicus, M. albus, T. elliptica,* and *T. anguina* were used as outgroups (Appendix 1). The *GA3ox1* and *trnK-matK* sequence alignments contained 1,270 and 2,526 characters, 436 (34.3%) and 538 (21.3%) variable sites, and 256 (20.2%) and 256 (10.1%) parsimony informative sites, respectively (Table 1). Phylogenetic analysis of the *GA3ox1* and *trnK-matK* datasets resulted in one equally parsimonious tree (tree length=921 and 770; CI=0.61 and 0.80; RI=0.71 and 0.85, respectively).

The cluster of *Medicago* was significantly supported (bootstrap value=100) in the phylogenetic trees constructed using *GA3ox1* and *trnK-matK* sequences (Fig. 2). Generally, the same species formed a clade; the relationship between each clade is not clear. In the MP tree using *GA3ox1* sequences, except for *M. sativa*, four different clades of four species were obtained (Fig. 2A). For *M. minima* and *M. lupulina*, the collected samples and reference sequences formed clades with 100% bootstrap value, respectively. *M. ruthenica* and *M. polymorpha* were supported with 98% and 85% bootstrap values, respectively. The phylogenetic tree based on the *trnK-matK* region showed higher resolution than that obtained using *GA3ox1* (Fig. 2B). *M. polymorpha, M. minima,* and *M. lupulina* were separated with 100% bootstrap value. *M. sativa* and *M. ruthenica* also formed two clades with 86% and 82% bootstrap values, respectively. In the two trees, the five *Medicago* species formed distinct clades with over 80% bootstrap value. Therefore, it was confirmed that the five *Medicago* species were distributed in South Korea. In addition, this indicated that identification of the five taxa using plant material for developing detection methods is highly specific.

Table 2. List of designed primers and results to identify the five species

Primer name	Primer sequence (5′-3′)	Product size (bp)	Amplification results				
			Medicago sativa	*Medicago polymorpha*	*Medicago ruthenica*	*Medicago minima*	*Medicago lupulina*
matK_1_F	CCAAAAACTCGATTTCTATTTTTTTCAAAA	463	+	+	+		
matK_1_R	TGACTCCGTACCACTGAACG						
matK_2_F	TGGGCCGATTCATCCGATTT	269				+	+
matK_2_R	TGAATTGCATTCATTATTCCAATAATTGG						
CP_1_F	TACAGCGAAGGGTGAACAAA	396	+		+	+	
CP_1_R	CTGATCTCAATGTTTGCAAATAG						
CP_2_F	CATTGAAGCGGCTAATCCTATGC	401				+	
CP_2_R	GGCTGACTATACTGCTTTTGGGC						
CP_3_F	GTTGTTTATGAATGTCTCATCTGAA	284			+		
CP_3_R	TAGACATAGTATAGGGCGGATGTAG						

Development of a species-specific detection method

Novel primer sets were developed to identify the five *Medicago* taxa, including *M. sativa, M. ruthenica, M. polymorpha, M. minima,* and *M. lupulina*, which are distributed in South Korea (Table 2). All primers were designed using the chloroplast genome of each species to increase specificity (Fazekas *et al.,* 2008). The PCR product sizes were selected over 200 bp for visual analysis on agarose gel. Consequently, five primer pairs were successfully obtained for the five *Medicago* taxa (Table 2). To increase the primer accuracy for species specificity, they were all designed to be contained within conserved and highly variable regions. The two primer sets, named matK_1 (463 bp) and matK_2 (269 bp), were designed using the *matK* region and separated the five species into two groups (group 1: *M. sativa, M. ruthenica,* and *M. polymorpha*; group 2: *M. minima* and *M. lupulina*). The other three primers were also constructed using a non-coding region with highly variable sequences between the two conserved genes. The CP_1 primer set (396 bp) was developed using the nucleotide sequences between ribosomal protein S4 and tRNA-Ser to detect three species (*M. sativa, M. ruthenica,* and *M. minima*). The CP_2 (401 bp) and CP_3 (284 bp) primer sets consisted of sequences in the Clp protease proteolytic subunit to tRNA-Asn and NADH dehydrogenase subunit 5 to tRNA-His regions, which can identify *M. minima* and *M. ruthenica,* respectively.

To detect multiple primer sets using a single simultaneous PCR assay in the five *Medicago* taxa, various PCR parameters such as annealing temperature and extension time were tested (data not shown). Consequentially, the primer concentration and PCR condition were determined to increase the specificity of PCR amplification. The agarose gel image showed high efficiency for the developed primer pair as only the expected band was detected for each species (Fig. 3). Further, these results confirmed that all PCR product bands were of their expected size. These results showed that a particular species could be distinguished using a combination of the five designed primer pairs. Therefore, the developed PCR method was considered suitable for qualitative analysis of *Medicago* species in South Korea.

Discussion

Little is known about the investigation of phylogenetic analysis and species-specific DNA markers in the five *Medicago* plant species speculated to be distributed in South Korea. Therefore, this study applied molecular phylogeny based on the nuclear gene *GA3ox1* and chloroplast region *TrnK-matK* and developed species-specific markers for five *Medicago* taxa collected from South Korea. Distribution maps of *Medicago* plants in South Korea were constructed based on specimen information and previous studies (Fig. 1). In this study, we obtained three seed specimen samples of *M. sativa, M. minima,* and *M. lupulina* from NIBR and two wild plant samples of *M. polymorpha* and *M. ruthenica*. Previous studies have shown that sequences of *trnK-matK* and *GA3ox1* regions have a phylogenetic resolution at the species level in *Medicago* (Chen *et al.,* 2021; Hu *et al.,* 2014; Steele *et al.,* 2010). Molecular phylogenetic analyses indicated that the phylogenetic trees constructed using nucleotide sequences (*trnK-matK* and *GA3ox1*) showed significant separation between the five taxa of *Medicago*. The *GA3ox1*-based tree could not distinguish *M. sativa* (Fig. 2A). Unlike the *GA3ox1*-based tree, *trnK-matK*-based analysis could distinguish all five taxa (Fig. 2B). Previous study results strongly supported our research data (Steele *et al.,*

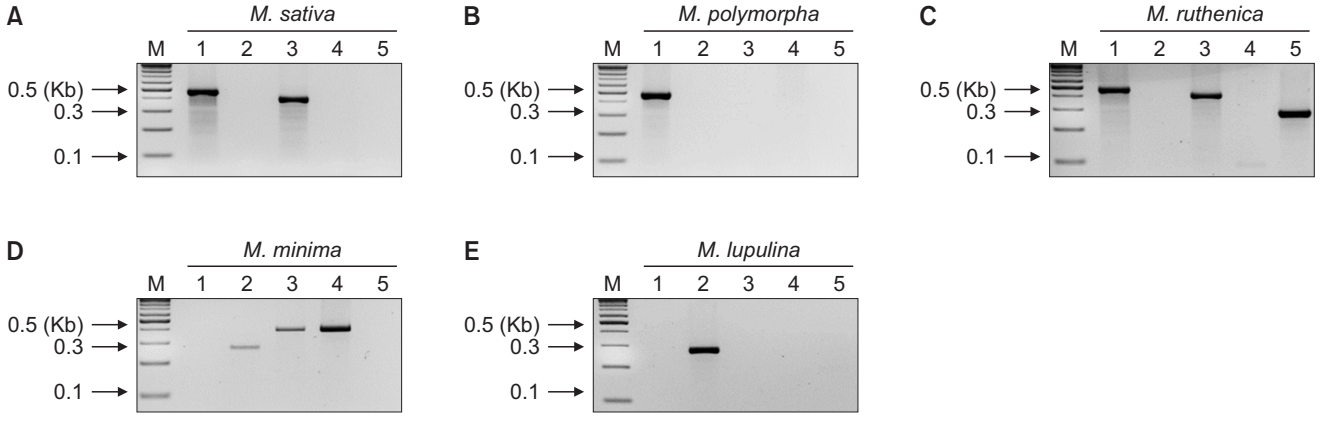

Fig. 3. Specificity test of the polymerase chain reaction primers designed for *Medicago* species in South Korea. Agarose gel images correspond to (A) *Medicago sativa*, (B) *Medicago polymorpha*, (C) *Medicago ruthenica,* (D) *Medicago minima*, and (E) *Medicago lupulina*, respectively. Lane M: 100 bp DNA ladder marker; Lane 1: matK_1 primer; Lane 2: matK_2 primer; Lane 3: CP_1 primer; Lane 4: CP_2 primer; Lane 5: CP_3 primer.

2010). Nucleotide sequences of the five taxa from previous studies and our collected samples formed clusters for the same species.

The five *Medicago* taxa distributed in South Korea could be identified using plastid DNA sequences (*trnK-matK*), but not nuclear DNA sequences (*GA3ox1*). The discrimination efficiency of the developed PCR markers for the five species can be increased based on differences in plastid DNA sequences. Plastid genomes have been used to develop species-specific markers in various fields, such as identifying species in the family Orchidaceae, genera *Pinus* and *Dendrobium,* and invasive aquatic plants (Asahina *et al.,* 2010; Li *et al.,* 2021; Scriver *et al.,* 2015; Wachowiak *et al.,* 2004). As expected, bands of the developed PCR markers in the agarose gel image also showed a clear distinction between the five *Medicago* species (Fig. 3). This method will facilitate similar future studies on distinguishing domestic and foreign species of *Medicago*.

In conclusion, we clarified that five species of *Medicago* are distributed in Korea based on molecular phylogenetic analysis. Our novel PCR-based detection method is suitable for distinguishing *Medicago* taxa with similar morphologies distributed in South Korea and can be applied to other foreign *Medicago* species in a timely and cost-effective manner.

Author Contributions

IRK and WC contributed to the conception and design of the study. All authors performed the experiments. IRK, A-MY, and WC analyzed the data. HSL and SL prepared figures and tables. IRK and WC authored or reviewed drafts of the paper. IRK, JRL, and WC participated in valuable discussions. All the authors read and approved the final draft.

Conflict of Interest

The authors declare that they have no competing interests.

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Appendix 1. List of GenBank accession numbers used in this study

No.	Name	*GA3ox1*	*trnK-matK*
1	*Medicago arabica*	HM211102.1	HM159554.1
2	*Medicago arborea*	HM211103.1	HM159555.1
3	*Medicago biflora*	HM211104.1	AF522091.2
4	*Medicago brachycarpa*	HM211106.1	AF522092.2
5	*Medicago cancellata*	HM211107.1	HM159557.1
6	*Medicago ciliaris*	HM211108.1	HM159559.1
7	*Medicago constricta*	HM211109.1	HM159560.1
8	*Medicago daghestanica*	HM211110.1	HM159561.1
9	*Medicago granadensis*	HM211112.1	HM159563.1
10	*Medicago heyniana*	HM211113.1	AF522093.2
11	*Medicago hybrida*	HM211114.1	HM159564.1
12	*Medicago hypogaea*	HM211115.1	AF522094.2
13	*Medicago intertexta*	HM211116.1	HM159565.1
14	*Medicago italica*	HM211117.1	AF522095.2
15	*Medicago lanigera*	HM211118.1	AF522096.2
16	*Medicago littoralis*	HM211119.1	HM159567.1
17	*Medicago littoralis*	HM211120.1	HM159568.1
18	*Medicago lupulina*	HM211121.1	KC333395.1
19	*Medicago lupulina*	MW242212.1	MW241884.1
20	*Medicago marina*	HM211123.1	HM159570.1
21	*Medicago medicaginoides*	HM211124.1	AF522097.2
22	*Medicago minima*	HM211125.1	HM159571.1
23	*Medicago minima*	MW242329.1	MW241965.1
24	*Medicago monantha*	HM211127.1	HM159573.1
25	*Medicago murex*	HM211128.1	HM159574.1
26	*Medicago orbicularis*	HM211130.1	AF522101.2
27	*Medicago papillosa*	HM211131.1	HM159575.1
28	*Medicago pironae*	HM211132.1	HM159576.1
29	*Medicago platycarpa*	HM211133.1	AF522102.2
30	*Medicago polyceratia*	HM211134.1	AF522103.2
31	*Medicago polymorpha*	MW242319.1	MW241955.1
32	*Medicago praecox*	HM211136.1	HM159577.1
33	*Medicago prostrata*	HM211137.1	AF522105.2
34	*Medicago radiata*	HM211138.1	AF522106.2
35	*Medicago rigidula*	HM211139.1	HM159579.1
36	*Medicago ruthenica*	HM211140.1	AF522107.2
37	*Medicago ruthenica*	MW242235.1	MW241907.1
38	*Medicago sativa*	HM211141.1	AF522108.2
39	*Medicago sativa*	MW242289.1	MW241984.1
40	*Medicago saxatilis*	HM211145.1	HM159583.1
41	*Medicago secundiflora*	HM211146.1	HM159585.1
42	*Medicago shepardii*	HM211147.1	HM159586.1
43	*Medicago soleirolii*	HM211148.1	HM159587.1
44	*Medicago turbinata*	HM211151.1	HM159590.1
45	*Melilotus albus*	HM211152.1	AF522110.2
46	*Melilotus indicus*	HM211153.1	AF522111.2
47	*Trigonella anguina*	HM211154.1	HM159593.1
48	*Trigonella elliptica*	HM211163.1	HM159594.1