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Original Research Article

Molecular Authentication of Magnoliae Flos Using Robust SNP Marker Base on *trn*L-F and *ndh*F Region

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Abstract - Magnoliae Flos (Sini in Korean) is one of the most important oriental medicinal plants. In the Korean Herbal Pharmacopeia, the bud of the all species in *Manolia denudate* and *Manolia* genus were regarded as the botanical sources for 'Sini'. Most the dried bud of *Manolia denudata*, *Manolia biondii* and *Manolia sprengeri* were used as 'Xin-yi' in China. Therefore, the purpose of this study was to determine and compare the '*Magnolia*' species, four species including *Manolia denudata*, *M. biondii*, *M. liliiflora* and *M. Kobus* were analysis of sequencing data revealed DNA polymorphisms. The based on tRNA coding leucine/phenylalanine (*trn*L-F) and NADH-plastoquinone oxidoreductase subunit 5 (*ndh*F) sequences in chloroplast DNA. For the identification of '*Magnolia*' species, polymerase chain reaction (PCR) analysis of chloroplast DNA regions such as *ndh*F have proven an appropriate method. A single nucleotide polymorphism (SNP) has been identified between genuine "Sini" and their fraudulent and misuse. Specific PCR primers were designed from this polymorphic site within the sequence data, and were used to detect true plants via multiplex PCR.

Key words - Magnolia, Magnoliae Flos, *trn*L-F/*ndh*F, SNP, Multiplex PCR

Introduction

Magnoliae Flos (Sini in Korean), the dried bud of *Manolia denudate* including *Manolia* genus is one of the most widely used herbal medicinal plants in Korean Herbal Pharmacopeia (KHP, 4th). Among the "*Magnolia*" species, four species, *Manolia denudata*, *M. biondii*, *M. liliiflora* and *M. kobus* were most used as publicly certified medicinal materials in Korean Herbal Pharmacopeia (KHP, 5th).

Magnoliae Flos has an astringent effect, which can improve local blood circulation and promote the absorption of secretions, thereby diminishing inflammation, clearing nasal passage, and relieving or eliminating symptoms (Wang *et al.,* 1996), it also has an anti-allergic effect, which can effectively fight against the nasal itching, sneezing, runny nose, and other symptoms caused by allergic rhinitis (Kim *et al.,* 2002). However, it is the absence of the test component for each of the four species. Also, the dried bud of four species are always misidentified as Magnoliae Flos due to their morphological similarities, most of medicinal plant products in the markets are packaged of powders of slices and Processed things that no longer bear the original morphological features of the plants (Park *et al.,* 2006; Jigden *et al.,* 2010; Jin *et al.,* 2013).

Traditional authentication methods, which have relied on morphological and histological differences, are limited and quite often unreliable. Chemical analysis are significantly affected by environmental growth conditions as well as storage conditions that in comparison, DNA analysis by molecular techniques is not influenced by growth stage and environmental conditions of plants (Zhu *et al.,* 2004).

Several molecular methods, such as arbitrarily primed polymerase chain reaction (AP-PCR) (Cheung *et al.,* 1994), random amplified polymorphic DNA (RAPD) (Cui *et al.,* 2003; Shim *et al.,* 2003; Shaw and But, 1995), loop-mediated isothermal amplification (LAMP) (Sasaki *et al.,* 2008), sequence characterized-amplified region (SCAR) (Choi *et al.,* 2008; Wang *et al.,* 2001), restriction fragment length polymorphism

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(RFLP) (Ngan *et al.,* 1999), amplification fragment length polymorphism (AFLP) (Ha *et al.,* 2002), and DNA microarray (Zhu *et al.,* 2008) have previously been described. The main drawback of RAPD are lack of reliability and reproducibility. RFLP and AFLP need stringent reaction conditions and tedious operation, and they are not suitable for identification of processed sample due to the degradation of genomic DNA.

In this study, we describe general ways to differentiate medicinally important plants in a more reproducible and robust approach by analyzing Single nucleotide polymorphisms (SNP). For the SNP analysis, used regions include ribosomal genes, chloroplast genes and mitochondrial genes.

In this case, in order to determine the base sequence of *trn*L (tRNA-Leu) and *ndh*F intron region were targeted for molecular analysis and these regions were proved to be useful for discrimination of *M. denudata*, *M. biondii*, *M. liliiflora* and *M. kobus*.

Chloroplast DNA (*cp*DNA) intron and intergenic spacer sequence is used for evolutionary and phylogenetic study (Olmstead and Palmer 1994). The cpDNA *trn*L (tRNA-Leu) and *ndh*F intron region in land plants comprising the *trn*L (UAA) intron and *trn*L (UAA)- *trn*F (GAA) noncoding intergenic spacer is one of most widely used chloroplast markers for phylogenetic analysis in plants (Quandt and Stech, 2004). It has been used successfully to infer phylogenetic relationships within and among angiosperm families (Olmstead *et al.,* 1992)

It was possible to test paternity of medicinal herbs, as it is possible to identify true *Bos taurus coreanae* and young antlers of deers, using genetic base sequencing (An *et al.,* 2006). To identify plants using DNA base sequencing, chloroplast DNA or *trn*L-F (tRNA-Leu/Phe) /*ndh*F (NADH dehydrogenase subunit F) is frequently used as it is found only in plants (Lang *et al.,* 2006).

A marker that recognizes four species of the genus *Magnolia* (*Manolia denudata*, *M. biondii*, *M. liliiflora* and *M. kobus*) for herbal use was developed after determining the *trn*L-F region and base sequencing *ndh*F gene and verifying its SNP.

Although traditional herbal medicine industry enrooted in medicinal plants as resources is one of the major medical organizations partnering with Western medicine, it currently imports more than 90% of raw materials from China, and there are cases of adverse reactions because of mixture of unsorted ingredients, improper applications and misunderstandings due to lack of manufacture and distribution management system. Therefore, the aim of this research is to trace the origin of Magnoliae Flos through analysis of DNA base sequence, and to develop identifying technology such as SNP markers so that there will be standardization of distribution of the herbal Magnoliae Flos.

This research promotes to verify authenticity of Magnoliae Flos and to develop identification methods, as demanded by Korea Food and Drug Administration-linked Center for Herbal Medicine.

Materials and Methods

Plant

The collected samples of Magnoliae Flos are shown in Table 1. Ten samples of herbal Magnoliae Flos currently distributed in Korea and China were purchased. And the standard ten samples were collected from the following places, The Ok-cheon medicinal plants authentication center of Korea Food and Drug Administration (http://agri.oc.go.kr), Kyung Hee University in Su-won, Po-cheon, and Young-cheon. Other eight samples were collected from substance separation and analysis team at Andong National University. All samples were correctly identified by the sequence of *trn*L-F and *ndh*F of NCBI-registered *Magnolia* plants were screened and confirmed.

DNA extraction

A total of twenty eight collected samples were frozen using liquid nitrogen, ground using porcelain mortar and pestle, and the genomic DNA were extracted using G-spin™ Genomic DNA Extraction Kit (iNtRON, Seongnam, Koera) and each dried sample was isolated using a modified cetyltrimethylammonium bromid (CTAB) method (Murray *et al.,* 1980).

PCR amplification of the *trn*L (tRNA-Leu) and *ndh*F (*ndhF*) intron region

In order to determine the base sequence of *trn*L-F, universal primers *trn*LF-c (forward) and *trn*LF-f (reverse) of the *trn*L-F

Table 1. List of 'Magnoliae Flos (Sini)' samples used in this study

No.	voucher number	Place	Nation	Note
1	Sini01	Local market	China	dried
2	Sini02	Ok-cheon Medicinal Plants Authentication Center	Korea	fresh
3	Sini03	Ok-cheon Medicinal Plants Authentication Center	Korea	fresh
4	Sini04	Andong Univ.	China	dried
5	Sini05	Local market	China	dried
6	Sini06	Local market	China	dried
7	Sini07	Local market	China	dried
8	Sini08	Local market	China	dried
9	Sini09	Local market	China	dried
10	Sini10	Local market	China	dried
11	Sini11	Local market	China	dried
12	Sini12	Local market	China	dried
13	Sini13	Local market	China	dried
14	Sini14	Andong Univ.	Korea	dried
15	Sini15	Andong Univ.	Korea	dried
16	Sini16	Andong Univ.	Korea	dried
17	Sini17	Andong Univ.	Korea	dried
18	Sini18	Andong Univ.	Korea	dried
19	Sini19	Andong Univ.	China	dried
20	Sini20	Andong Univ.	China	dried
21	Sini21	Kyung-Hee Univ.	Korea	fresh
22	Sini22	Kyung-Hee Univ.	Korea	fresh
23	Sini23	Yung-Chun	Korea	fresh
24	Sini24	Suwon	Korea	fresh
25	Sini25	Suwon	Korea	fresh
26	Sini26	Pochun	Korea	fresh
27	Sini27	Pochun	Korea	fresh
28	Sini28	Pochun	Korea	fresh

region were used in PCR amplification. The base sequence of the primers were *trn*LF-c (5'-CGA AAT CGG TAG ACG CTA-3') and *trn*LF-f (5'-ATT TGA ACT GGT GAC ACG AG-3') (Taberlet *et al.,* 1991).

The conditions of running PCR were set as the following: pre-denaturation at 96℃ for 2 minutes; denaturation at 96℃ for 30 seconds; annealing at 57℃ for 30 seconds; and extension at 72℃ for 2 minutes, for 36 cycles. Universal primers *ndh*F-F1 (forward) and *ndh*F-R1 (reverse) of the *ndh*F region were used in PCR amplification to determine the base sequence of *ndh*F. The base sequence of the primers were *ndh*F-F1 (5'-TTG GGA ATT GGT GGG AAT GTG-3') and *ndh*F-R1 (5'-TTC CTA TGG ACC CAA CGA AC-3') (Zhang *et al.,* 2003), and the conditions of running PCR were set as the following: pre-denaturation at 95℃ for 3 minutes; denaturation at 95℃ for 30 seconds; annealing at 61℃ for 30 seconds; and extension at 72℃ for 2 minutes, for 36 cycles. Each PCR amplification was performed in a volume of 20㎕, and the reaction mixture consisted of each of the primers at a concentration of 0.5 ㎛, 20 ng of template DNA. The PCR products were migrated on a 1.0% agarose gel electrophoresis and detected by ethidium bromide staining under UV.

Sequencing and DNA sequence analysis

The PCR products were purified using a GENEALL PCR SV Purification Kit (GeneAll Biotechnology, Seoul, Koera) per the manufacturer's instructions and then sequenced by Genotech, Inc (Genotech, Daejeon, Korea). The DNA sequence of the *trn*L (tRNA-Leu) and *ndh*F (*ndh*F) intron regions obtained in sequencing experiments were compiled using SeqMan software, and the sequence were edited with BioEdit program (Hall *et al.,* 1999). Multiplex sequence alignments were performed using online Clustal W2 program (http://www.ebi.ac.uk/Tools/clustalw2/).

Design of specific primers

Specific primer were designed for *M. denudata*, *M. liliiflora*, *M. Kobus* and *M. biondii,* respectively, on the basis of the DNA polymorphisms detected (Table 2). In order to design specific primers to recognize the four species of *Magnolia* (*M. denudata*, *M. liliiflora*, *M. kobus*, *M. biondii*) through DNA obtained from plants and herbal Magnoliae Flos used in this research, SNP unique to *ndh*F of each species were determined.

There was no SNP unique to *M. kobus*, and therefore "SIkbnF" specific primer was designed to recognize *M. kobus* and *M. biondii*, a specific primer "SIbinF" was designed only to identify *M. biondii*. Likewise, no unique SNP could be verified in *M. denudata* that after designing a specific primer "SIdlnF" to recognize *M. denudata* and *M. liliiflora*, a specific primer "SIlinF" was designed only to identify *M. liliiflora*.

Table 2. Specific primer design adjacent to SNP position using the mismatching technology

Species	GenBank accession of *ndh*F	Sequences (5'->3')	Specific primer design	PCR product size (bp)	Target species
M. biondii	KP742960	CTCTGTGTGCCTCCCTAC	CTCTGTGTGCCTCCATAC (SIbinF)	1,032	*M. biondii*
M. kobus	KP7429611	CTCTGTGTGCCTCCCTAT			
M. denudata	KP742958	CTCTGTGTGCCTCCCTAT			
M. liliiflora	KP742959	CTCTGTGTGCCTCCCTAT			
M. kobus	KP7429611	ACAGTCATACCTTACATAATGAATCTA	ACAGTCATACCTTACATAATGAAACTA (SIkbnF)	860	*M. kobus* and *M. biondii*
M. biondii	KP742960	ACAGTCATACCTTACATAATGAATCTA			
M. denudata	KP742958	ACAGTCATACCTTACATAATGAATCTC			
M. liliiflora	KP742959	ACAGTCATACCTTACATAATGAATCTC			
M. denudata	KP742958	TCAATTGGGTTATATTATGTTAGCTCT	TCAATTGGGTTATATTATGTTAGATCT (SIdlnF)	738	*M. denudata* and *M. liliiflora*
M. liliiflora	KP742959	TCAATTGGGTTATATTATGTTAGCTCT			
M. biondii	KP742960	TCAATTGGGTTATATTATGTTAGCTCC			
M. kobus	KP7429611	TCAATTGGGTTATATTATGTTAGCTCC			
M. liliiflora	KP742959	TTTCAAAATTACAGTGGCACTAAAAAG	TTTCAAAATTACAGTGGCACTAATAAG (SIlinF)	328	*M. liliiflora*
M. denudata	KP742958	TTTCAAAATTACAGTGGCACTAAAAAT			
M. biondii	KP742960	TTTCAAAATTACAGTGGCACTAAAAAT			
M. kobus	KP7429611	TTTCAAAATTACAGTGGCACTAAAAAT			

Specific primers were designed through "mismatching" technology (Kim *et al.,* 2005; Shiokai *et al.,* 2008; Hayashi *et al.,* 2004).

Multiplex PCR

Molecular authentication of *M. denudata*, *M. liliiflora*, *M. Kobus* and *M. biondii* was performed using multiplex-PCR. Six primers (*ndh*F-F1, SIbinF, SIkbnF, SIdlnF, SIlinF and *ndh*F-R1) were used simultaneously in multiplex PCR amplification. The reaction mixture was identical to the one described earlier except the concentrations of *ndh*F-F1, SIkbnF, SIdlnF, SIlinF and *ndh*F-R1 were 0.63 ㎛, 0.31 ㎛, 0.63 ㎛, 1.13 ㎛, 0.5 ㎛ and 0.81 ㎛ respectively. PCR amplification was performed in a total volume of 20㎕. The conditions for DNA amplification were set as the following: pre-denaturation at 95℃ for 3 minutes; denaturation at 95℃ for 30 seconds; annealing at 61℃ for 30 seconds; and extension at 72℃ for 2 minutes, for 33 cycles. The PCR products were visualized on a 1% agarose gel.

Results and Discussion

Chloroplast DNA (*cp*DNA) sequence variations are now widely used to investigate interspecific relationships among angiosperms and other plants (palmer *et al.,* 1988).

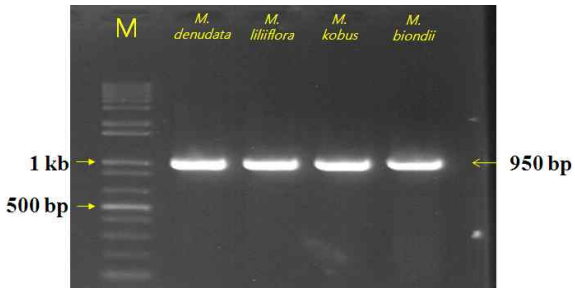

Fig. 1. Amplification of four species 'Magnoliae Flos (Sini)' DNA sequence (*trn*L-F).

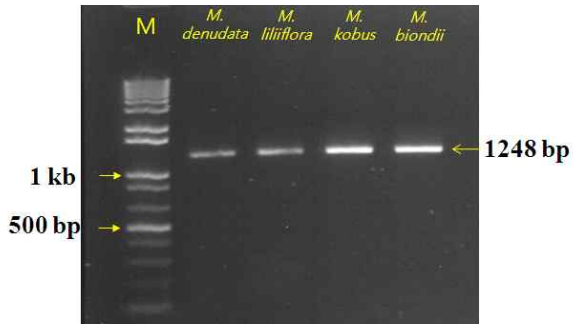

Fig. 2. Amplification of four species 'Magnoliae Flos (Sini)' DNA sequence (*ndh*F).

In this study, each *cp*DNA was separated and the regions of *trn*L-F and *ndh*F were amplified using PCR, in order to

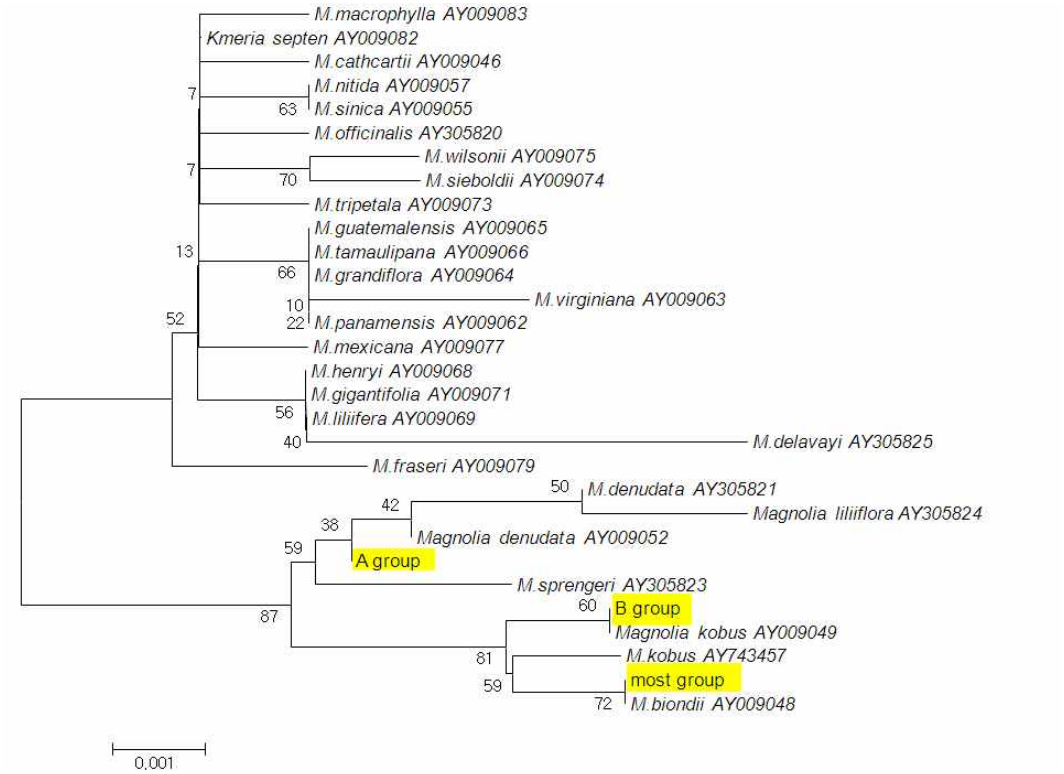

Fig. 3. The Neighbor-joining tree of *trn*L-F sequences of species belonging to the genus *Magnolia*.

Table 3. The base of SNP position and classification of Magnoliae Flos (Sini)

voucher number	Base of SNP position							
	*trn*L-F			*ndh*F				
	333 bp	835 bp		213 bp	395 bp	517 bp	926 bp	
Sini01	T	G	most group	C	A	C	T	most group
Sini02	T	G	most group	C	A	C	T	most group
Sini03	T	G	most group	C	A	C	T	most group
Sini04	T	G	most group	C	A	C	T	most group
Sini05	T	G	most group	C	A	C	T	most group
Sini06	T	G	most group	C	A	C	T	most group
Sini07	T	G	most group	C	A	C	T	most group
Sini08	T	G	most group	C	A	C	T	most group
Sini09	T	G	most group	C	A	C	T	most group
Sini10	T	G	most group	C	A	C	T	most group
Sini11	T	G	most group	C	A	C	T	most group
Sini12	T	G	most group	C	A	C	T	most group
Sini13	T	G	most group	C	A	C	T	most group
Sini14	T	T	A group	T	C	T	T	A group
Sini15	T	T	A group	T	C	T	T	A group
Sini16	G	G	B group	T	A	C	T	B group
Sini17	T	T	A group	T	C	T	G	C group
Sini18	T	T	A group	T	C	T	T	A group
Sini19	T	G	most group	C	A	C	T	most group
Sini20	T	G	most group	C	A	C	T	most group

Table 3. Continued

voucher number	Base of SNP position							
	*trn*L-F			*ndh*F				
Sini21	T	T	A group	T	C	T	T	A group
Sini22	T	T	A group	T	C	T	G	C group
Sini23	T	T	A group	T	C	T	T	A group
Sini24	T	T	A group	T	C	T	G	C group
Sini25	T	T	A group	T	C	T	T	A group
Sini26	T	T	A group	T	C	T	G	C group
Sini27	T	T	A group	T	C	T	T	A group
Sini28	T	T	A group	T	C	T	G	C group

verify the information on the base sequence of the collected 28 samples of Magnoliae Flos. As a result, each sample yielded 950 bp and 1,248 bp of *trn*L-F band and *ndh*F band, respectively (Fig. 1, Fig. 2). As a result of analysis of base sequencing of *trn*L-F region, the samples were divided into three groups by the different base of SNP position (333 bp and 835 bp).

Twenty eight plants samples of Magnoliae Flos *trn*L-F region DNA sequences were collected form NCBI, and aligned in Bioedit program with ClustalX program and a phylogenetic tree was built using Mega4 program. The A group, B group and most group were classified cousin relationship with *M. denudate, M. Kobus* and *M. biondii* is classified distant relationship (Fig. 3).

In contrast, the analysis of base sequencing of *ndh*F domain divided the samples into four groups by the different base of SNP position (213 bp, 395 bp, 517 bp and 926 bp, respectively) (Table 3).

The *t*RNA coding sequences (*trn*L-F), the regions cannot be used for authentication of Magnoliae Flos due to their less polymorphism. In comparison, the *ndh*F (NADH dehydrogenase subunit F noncoding region was targeted for molecular analysis and differentiation among Magnoliae Flos. These regions can be used for authentication of *M. denudata*, *M. liliiflora*, *M. Kobus* and *M. biondii*.

The *ndh*F noncoding regions of four species were PCR-amplified using the *ndh*F-F1 and *ndh*F-R1 universal primer set has 1,248 bp of band. All the herbal Magnoliae Flos that are distributed currently in Korea and China are verified as *M. biondii*. *M. denudata* and *M. liliiflora* are related closely in their familial lines, and this could be verified through the analysis of divergence between the *Magnolia* species. The difference came out to be only 1 bp out of 1,143 bp of the aligned sequence for divergence analysis (Table 4).

Twenty eight plants samples of Magnoliae Flos *ndh*F region DNA sequences were collected form NCBI, and aligned in Bioedit program with ClustalX program and a phylogenetic tree was built using Mega4 program. The A group , B group, C group and most group were classified cousin relationship with *M. denudate, M. Kobus, M. liliiflora* and *M. biondii* is classified distant relationship (Fig. 4).

The *ndh*F universal band at 1,248 bp was used as control in order to verify the presence or absence of PCR amplification. Their sequences were deposited in GeneBank (KP742958-KP74296).

Multiplex alignment of *ndh*F noncoding region of *M. denudata*, *M. liliiflora*, *M. Kobus* and *M. biondii* were performed with CLUSTALX program. Specific primer were designed for *M. denudata*, *M. liliiflora*, *M. Kobus* and *M. biondii* based on the detected SNP sites. Molecular discrimination of Magnoliae Flos was conducted using multiplex PCR with the six primers described. The combination of six specific primers described, as shown in Fig. 5, yield expected amplicons for different species. The specific primer "SIbinF" developed a specific band at 1,032 bp, which is amplified only in *M. biondii*; "SIkbnF" showed a specific band at 860 bp, amplified only in *M. kobus* / *M. biondii*; "SIdlnF" expressed at 738 bp, amplified uniquely in *M. denudata* / *M. liliiflora*; and "SIlinF" band appeared at 328 bp, amplified only in *M. liliiflora* (Fig. 5).

Table 4. Nucleotide divergences of *ndh*F regions

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *M. denudata_AF107949*	—	0.999	0.995	0.997	0.996	0.998	0.992	0.994	0.997	0.992	0.996	0.997	0.997	0.997	0.997
2. *M. liliiflora_AF107960*	1	—	0.994	0.996	0.995	0.997	0.991	0.993	0.996	0.992	0.995	0.996	0.996	0.996	0.996
3. *M. sprengeri_AF107951*	6	7	—	0.994	0.993	0.995	0.989	0.992	0.994	0.99	0.993	0.994	0.994	0.994	0.994
4. *M. kobus_AF107954*	3	5	7	—	0.997	0.997	0.991	0.993	0.996	0.992	0.995	0.996	0.996	0.996	0.996
5. *M. biondii_AF107953*	5	6	8	3	—	0.996	0.99	0.992	0.995	0.991	0.994	0.995	0.995	0.995	0.995
6. *M. fraseri_AF216256*	2	3	6	3	5	—	0.993	0.996	0.999	0.994	0.998	0.999	0.999	0.999	0.999
7. *M. delavayi_AF107917*	9	10	13	10	11	8	—	0.995	0.992	0.988	0.992	0.992	0.992	0.992	0.992
8. *M. gigantifolia_AF107944*	7	8	9	8	9	5	6	—	0.995	0.991	0.994	0.995	0.995	0.995	0.995
9. *M. mexicana_AF216263*	3	5	5	5	6	1	9	6	—	0.993	0.997	0.998	0.998	0.998	0.998
10. *M. panamensis_AF216255*	7	7	11	7	10	7	14	10	8	—	0.994	0.995	0.995	0.995	0.993
11. *M. virginiana_AF107939*	5	6	8	6	7	2	9	7	3	7	—	0.999	0.999	0.999	0.997
12. *M. grandiflora_AF107940*	3	5	7	5	6	1	9	6	2	6	1	—	1	1	0.998
13. *M. tamaulipana_AF107943*	3	5	7	5	6	1	9	6	2	6	1	0	—	1	0.998
14. *M. uatemalensis_AF10794*	3	5	7	5	6	1	9	6	2	6	1	0	0	—	0.998
15. *M. cathcartii_AF107945*	3	5	7	5	6	1	9	6	2	8	3	2	2	2	—

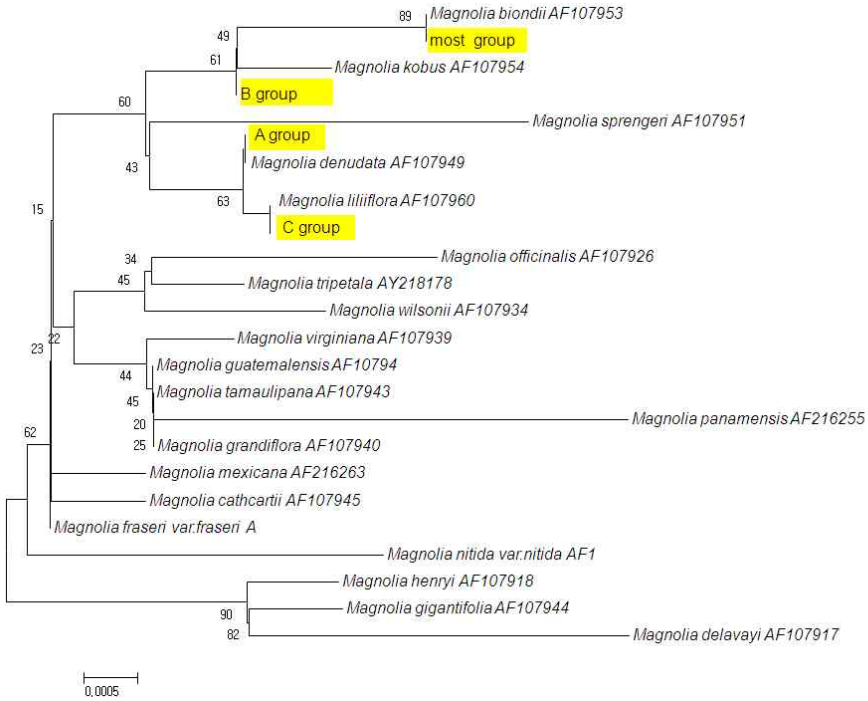

Fig. 4. The Neighbor-joining tree of *ndh*F sequences of species belonging to the genus *Magnolia*.

The specific primer "SIbinF" developed a specific band at 1,032 bp, which is amplified only in *M. biondii*; "SIkbnF" showed a specific band at 860 bp, amplified only in *M. kobus* / *M. biondii*; "SIdlnF" expressed at 738 bp, amplified uniquely in *M. denudata* / *M. liliiflora*; and "SIlinF" band appeared at 328 bp, amplified only in *M. liliiflora*.

Therefore, authentic Magnoliae Flos can be identified through its specific band from the influx of commercially available herbal plants, distinguished by its SNP markers (specific primers) using multiplex-PCR. The results obtained

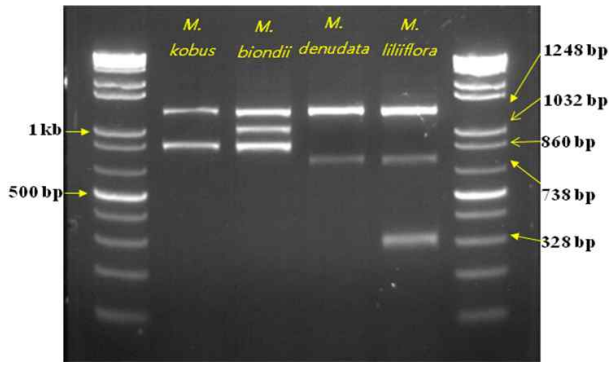

Fig. 5. Multiplex PCR for identification of four species of Magnoliae Flos (Sini).

through this research can be expected not only to verify the origin of Magnoliae Flos that is circulated in today's market but also to recognize its species, as well as to separate from inauthentic and misused plants.

The described method has important implications in both the production and sale of this medicinal products, allowing for the Prevention of fraud and misuse, and also revealing the possible presence of other with cheaper plant material. This method is reliable, efficient, and can be used for numerous repeated tests of many medicinal plants, and the methodology presented in this study can be adapted for authentication of other medicinal materials.

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