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Research Article
Identification of DNA Barcode Sequence and Genetic Relationship among Some Species of *Magnolia* Family

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Abstract

Background and Objective: *Magnoliaceae* is one of the arguable botanist families in classification by conventional taxonomic methods. DNA barcoding has been proposed as a powerful tool for identifying, confirming species and genetic relationship among species. Using DNA barcoding can overcome problems of morphological-based species identification. The objective of this study was to isolate, analyze and determine the DNA barcode regions for species identification and analysis of genetic relationship among some species of *Magnolia* family. **Materials and Methods:** The leaf specimens were collected and dried *in situ* with silica gel and then stored in -20°C freezer. Total DNA extraction and PCR amplification were performed with standard chemicals and Kit and these DNA fragments were sequenced by Sanger method. The data analysis was supported by software programs such as Mega6, BioEdit, GeneDoc. **Results:** In this study, four candidate DNA barcode sequences including *mat*K, *rbc*L, *trn*H-*psb*A and *ycf*1b were isolated and sequence analysed. The result showed that their size were 714, 553, 509-514 and 909-962 bp, respectively. The result of genetic distance analysis showed that only *trn*H-*psb*A fragment is best discrimination between four studied plant species. The combinations of *ycf*1b+*trn*H-*psb*A, *mat*K+*trnH-psbA* or *matK*+*ycf*1b+*trn*H-*psb*A also were potential choices to be used as DNA barcode for these four species. The phylogenetic trees were built based on *trn*H-*psb*A sequences and the combinations above revealed the genetic relationship of these four species, in which *Magnolia chevalieri* (Dandy) V.S.Kumar was closer with *Michelia braianensis* Gagnep. than *Michelia tonkinensis* A.Chev and *Michelia baillonii* (Pierre) Finet and Gagnep. **Conclusion:** Among 4 candidate DNA barcodes in this study, the *psb*A-*trn*H region is the most efficient DNA barcode sequence. Furthermore the combinations (*mat*K+*trn*H-*psb*A, *rbc*L+*trn*H-*psb*A, *ycf*1b+*trn*H-*psb*A, *mat*K+*ycf*1b+*trn*H-*psb*A, *mat*K+*rbc*L+*ycf*1b+*trn*H-*psb*A) could be used as the DNA barcode for species identification and analysis of genetic relationship among some species of *Magnolia* family.

Key words: DNA barcode, *Magnolia*, *Magnoliaceae*, *matK*, *rbcL*, *trnH-psbA*, *ycf1*b

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Magnoliaceae is a family in order *Magnoliales*, which has a large number of species. Some of them are valuable economic timber trees[1]. In this study, four plant species of *Magnolia* family were chosen including *Magnolia chevalieri* (Dandy) V.S.Kumar, *Michelia tonkinensis* A. Chev., *Michelia baillonii* (Pierre) Finet and Gagnep and *Michelia braianensis* Gagnep. The wood of these species has good quality to be used in house furniture, interior decoration and handicraft production[2]. However, they are found very little in natural forest and these species are on the list of plants that need to be protected and conserved. Therefore, the study on classification, conservation and development of their genetic resources is essential. Previously, identification and classification of plants were mainly based on morphological methods[3-6]. Although there have been improvements in the application process, there are still many difficulties as in cases that the samples have been distorted (nature, colour changes), processed samples can not be identified. Recently, the development of DNA barcode technology application, which is an advanced method using the sequence of short DNA strands featured in genome of organisms to identify and distinguish species, brings high efficiency in a short time, contributes to improve the drawbacks of the previous method[7-10].

In fact, the identification of some species belong to *Magnolia* family based on morphological indicator sometimes faces difficulties and get confusedness because of the similarity in their features. Therefore, the new classification methods need to applied to overcome these limitations[11-15]. In this study, conducted a selection of 4 candidate DNA barcode regions (*mat*K, *rbc*L, *trn*H-*psb*A and *ycf1*b). They are all located in the chloroplast genome, in which the sequences have high conservation, suitable to be DNA barcode in plant[16-19]. Hence, the study on creation of DNA barcode sequence data used as indicative standard DNA molecule method in order to serve the identification species and the study of genetic relationship is necessary, contributes to natural resources management, national precious gene sources conservation and development.

MATERIALS AND METHODS

The study was conducted at Vietnam National University of Forestry (VNUF) and completed on July, 2017.

Materials

Plant materials: The leaf specimens were collected from 4 plant species from different populations. Three specimens were collected from 3 different individuals of each species. The all specimens were labeled with full information, dried *in situ* with silica gel and then stored in -20°C freezer until DNA extraction to avoid DNA degradation. Symbols of these samples were taken in accordance with their abbreviations and scientific names of species.

Scientific name of species	Symbol
Magnolia chevalieri (Dandy) V.S.Kumar	M1
Michelia tonkinensis A.Chev.	M2
Michelia baillonii (Pierre) Finet and Gagnep	M3
Michelia braianensis Gagnep	M4

Chemical materials: The chemicals used to isolate the total DNA from leaf samples: Plant DNA Isolation Kit of Norgen, Canada, Chemicals for PCR cloning fragments of DNA barcode: Master mix of iNtRON Biotechnology, Korea, PCR Purification Kit of Norgen, Canada, Chemicals for electrophoresis on Agarose gel: Agarose, 1 kb DNA ladder, Redsafe nucleic acid staining solution provided by Norgen, Canada. The primers were designed for amplification of DNA barcode sequences as in Table 1.

Methods

Total DNA isolation: Total DNA was isolated from dried leaf samples of 4 plant species under the guidance of Plant DNA Isolation Kit, Norgen, Canada. Concentration, purity and integrity levels of total DNA are determined by spectrophotometric and electrophoresis methods on 1.0% agarose gel.

Table 1: List of primers to amplify the DNA barcode regions

Forward/reverse primers	Primer sequence (dimensional 5'-3')	DNA barcode regions	References
*mat*KF	5'-TTCCATGGCCTTCTTTBCATTTGTTGC-3'	*mat*K	
*mat*KR	5'-TTCCATGGTTTTTTGAGGATCCGCTGT-3'		
rP1F	5'-ATGTCACCACAAACAGAGACTAAAGC-3'	*rbc*L	Levin *et al.*[23]
rP1R	5'-GTAAAATCAAGTCCACCRCG-3'		Kress *et al.*[17]
trnPF1	5'-GTTATGCATGAACGTAATGCTC-3'	*trn*H-*psb*A	Sang *et al.*[4]
psbPR1	5'-CGCGCATGGTGGATTCACAATCC-3'		Tate *et al.*[25]
ycf1bF	5'-TCTCGACGAAAATCAGATTGTTGTGAA-3'	*ycf*1b	Dong *et al.*[19]
ycf1bR	5'-ATACATGTCAAAGTGATGGAAAA-3'		

PCR amplification of candidate DNA barcodes: The 4 candidate DNA barcode fragments (*mat*K, *rbc*L, *trn*H-*psb*A and *ycf*1b) were amplified by PCR technique on PCR 9700 Thermal Cycler Applied Biosystems (USA), each PCR reaction was performed in a total volume of 20 µL, including: H_2O deion (7 µL), 2x PCR Master mix Solution (10 µL), 10 pmol μL^{-1} of forward primers (1.0 µL), 10 pmol μL^{-1} of reverse primers (1.0 µL) and 50 ng μL^{-1} of DNA template (1 µL). The PCR reaction program: 94°C in 3 min (94°C: 30 sec, 59°C: 30 sec, 72°C: 1 min) repeating 40 cycles, 72°C in 5 min, PCR product preservation was at 4°C. Primers annealing temperature in PCR reactions were different depended on primers. Each PCR reaction was repeated 3 times for each sample. The PCR results were tested by electrophoresis on 1.2% agarose gel and observed under ultraviolet light (UV). The PCR products were purified according to instructions of PCR Purification Kit of Norgen, Canada.

Sequencing of candidate DNA barcode regions: The amplified and purificated PCR products were sequenced by Sanger's method, using Kit BigDye® Terminator v3.1 Cycle Sequencing. These sequences were processed and analyzed using specialized softwares such as Mega6, BioEdit. The completed sequences of these 4 candidate DNA barcode loci would be deposited on the Vietnam DNA Database Bank.

Genetic distance and phylogenetic analysis: After sequencing, the DNA barcode sequences would be aligned using the MUSCLE algorithm. The whole 4 DNA barcode sequences and their combinations were evaluated using 2 different methods, genetic distance and phylogenetic tree. The genetic distance was caculated based on Kimura 2-parameter method in MEGA6 software. Phylogenetic trees were constructed following Neighbor Joining estimation, Kimura 2-parameter model, boostrap value 1000.

RESULTS

Total DNA extraction: Total DNA extracted from dried leaves of *Magnolia chevalieri* (Dandy) V.S.Kumar, *Michelia tonkinensis* A. Chev., *Michelia baillonii* (Pierre) Finet and Gagnep. and *Michelia braianensis* Gagnep were tested by electrophoresis on 1.0% agarose gel to preliminarily evaluate the content and quality. The result of electrophoresis test showed that the all DNA bands were relatively sharp, with little breakage, it proves that the total DNA is quite intact (Fig. 1). The concentration and purity of the DNA solution were determined by spectrophotometric method at $A_{260\,nm}$ and $A_{280\,nm}$ wavelengths. The result indicated that the total DNA solution extracted from leaves of these 4 species were ready to amplify 4 DNA fragments.

PCR amplification: Total DNA products after extracting and diluting to appropriate concentration would be used directly as templates for amplification of *mat*K, *rbc*L, *trn*H-*psb*A and *ycf*1b fragments using specific pairs of primers. The composition and reaction cycle are described in the method section. Each PCR reaction was repeated 3 times on each sample. PCR products were examined by electrophoresis on 1.2% agarose gel, using DNA marker 1 kb ladder (Fig. 2). Electrophoresis result showed that the corresponding DNA bands had the size as expected: 750, 600, 500 and 950 bp for *mat*K, *rbc*L, *trn*H-*psb*A and *ycf*1b, respectively. The DNA bands were bold, sharp, with no byproducts proving that PCR products were specific amplified, could be purified and directly used in subsequent research steps.

DNA sequencing: The purified PCR product of 4 candidate DNA barcode fragments were proceeded to determine the nucleotide sequence. The result of sequencing analysis showed that *mat*K, *rbc*L, *trn*H-*psb*A and *ycf*1b fragments had the lengths of 714, 553, 509-514 and 909-962 bp, respectively. This result indicated that the size of these 4 DNA fragments

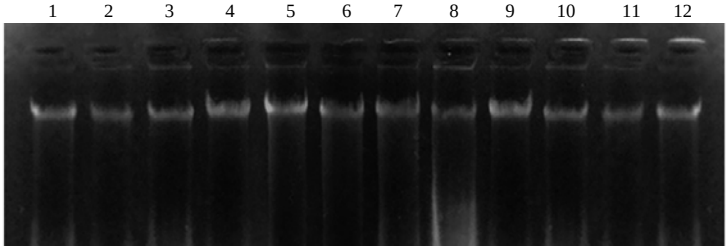

Fig. 1: Electrophoresis of total DNA from 12 specimens of 4 species
The using high ranger 1 kb DNA ladder (Cat. 11900, Norgen, Canada)

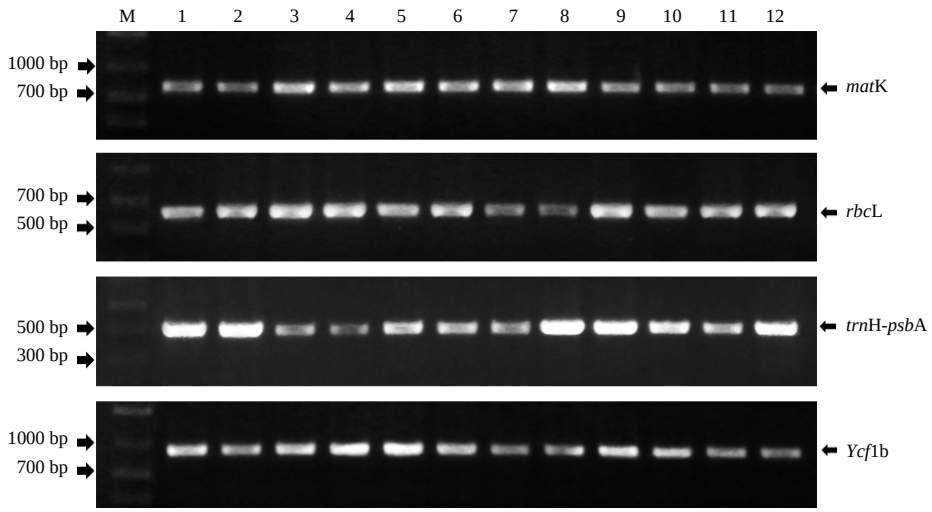

Fig. 2: PCR result of cadidate DNA barcode regions
The using high ranger 1 kb DNA ladder (Cat. 11900, Norgen, Canada)

Table 2: Evaluation of four DNA barcode regions

	DNA barcode regions			
	*mat*K	*rbc*L	*trn*H-*psb*A	*ycf*1b
Number of individuals	12	12	12	12
PCR success (%)	100	100	100	100
Sequencing success (%)	100	100	100	100
Sequence length	714	553	509-514	909-962
Aligned length	714	553	514	964
Number of variable sites	9	1	18	17
Number of indels	0	0	5	56
Number of informative sites	0	0	4	4

were suitable to expected size and the bands size on electrophoresis result. Comparing DNA sequences in all 3 iterations, found no significantly difference with all 4 fragments. Though *trn*H-*psb*A was the shortest sequence, it had the most variable sites, followed by *ycf*1b, *mat*K and *rbc*L (Table 2). The number of infomative sites was proportional to number of variable sites, with 4 in both *trn*H-*psb*A and *ycf*1b while none in *mat*K and *rbc*L. The completed sequences after analysis were deposited on the Vietnam DNA Database Bank.

Genetic distances: The pairwise distances were calculated to estimate the discrimination of 4 DNA barcode loci and their combinations. Because there was no difference among 3 samples for each fragment in the same species, interspecific distance was measured instead of both intra-and inter-specific distance. According to evaluation result, the pairwise distance in these 4 loci ranged from 0-3.2%, the mean ranged from 0.09% (*rbc*L) to 2.23% (*trn*H-*psb*A). The fragment had the highest genetic distance was *trn*H-*psb*A, followed by *ycf1*b, *mat*K and *rbc*L (Fig. 3, 4). This result was suitable with the number of variable sites in each sequence had shown above. In all 4 sequences, it was realized that *trn*H-*psb*A was the only one could discriminate all four species, which had minimum of interspecific distance was 0.79% while three others was 0%. It showed that *trn*H-*psb*A was a potential DNA locus to discriminate species in *Magnolia* family. We continued calculating the pairwise distance of these four fragments combinations and found out several possible candidates had the minimum interspecific distance greater than 0% such as *mat*K+*trn*H-*psb*A (0.33%), *rbc*L+*trn*H-*psb*A (0.38%), *ycf*1b+*trn*H-*psb*A (0.28%) for combination of 2 sequences, or *mat*K+*ycf*1b+*trn*H-*psb*A (0.19%) for 3 sequences (Table 3).

Phylogenetic tree analysis: To construct phylogenetic tree analysis, author had downloaded several sequences of other species in *Magnoliaceae* from GenBank (Table 4). Four phylogentic trees were built correspond to four DNA loci as *mat*K, *rbc*L, *trn*H-*psb*A, *ycf*1b (Fig. 5). In general, these 4 species had the closed relationship with some other species like *Magnolia officinalis*, *Magnolia tripetala*, *Magnolia yunnanensis*. The result indicated that *Magnolia chevalieri* (Dandy) V.S. Kumar and *Michelia braianensis* Gagnep. were

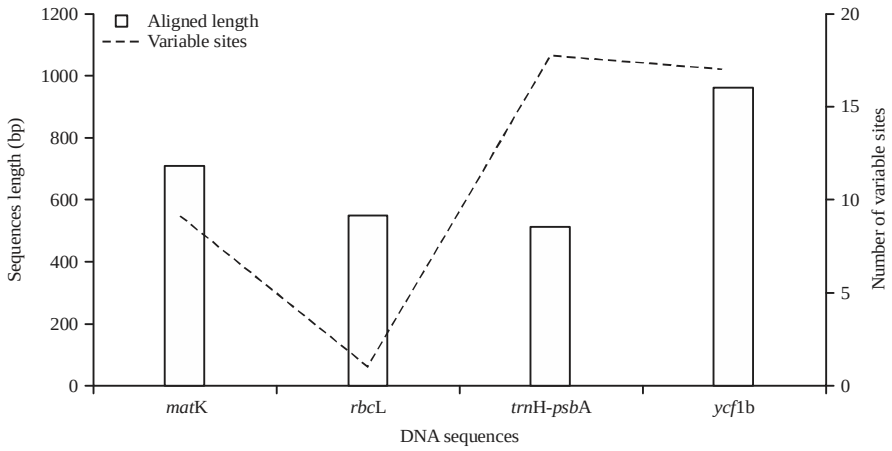

Fig. 3: Comparison of sequence characteristics

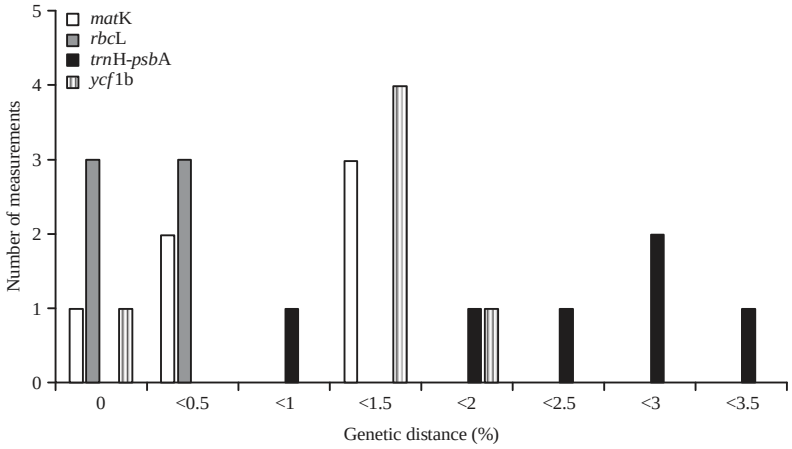

Fig. 4: Genetic distance of four DNA barcode loci

Table 3: Genetic distance of four DNA barcode loci and their combinations using Kimura 2-parameter

	Interspecific distance (%)		
Barcode loci and combinations	Min	Max	Mean
*mat*K	0.00	1.27	0.63
*rbc*L	0.00	0.18	0.09
*trn*H-*psb*A	0.79	3.20	2.23
*ycf*1b	0.00	1.56	1.11
*mat*K+*rbc*L	0.00	0.79	0.40
*mat*K+*trn*H-*psb*A	0.33	1.99	1.29
*mat*K+*ycf*1b	0.00	1.43	0.90
*rbc*L+*trn*H-*psb*A	0.38	1.62	1.11
*rbc*L+*ycf*1b	0.00	1.03	0.70
*ycf*1b+*trn*H-*psb*A	0.28	1.86	1.48
*mat*K+*rbc*L+*trn*H-*psb*A	0.23	1.42	0.92
*mat*K+*rbc*L+*ycf*1b	0.00	1.16	0.72
*mat*K+*ycf*1b+*trn*H-*psb*A	0.19	1.71	1.22
*rbc*L+*ycf*1b+*trn*H-*psb*A	0.20	1.38	1.08
*mat*K+*rbc*L+*ycf*1b+*trn*H-*psb*A	0.15	1.39	0.98

closed while *Michelia tonkinensis* A. Chev. and *Michelia baillonii* (Pierre) Finet and Gagnep. were further. It will also be observed in phylogenetic trees built based on the potential combinations has shown above: *mat*K+*trn*H-*psb*A, *rbc*L+*trn*H-*psb*A, *ycf*1b+*trn*H-*psb*A, *mat*K+*ycf*1b+*trn*H-*psb*A and *mat*K+*rbc*L+ *ycf*1b+*trn*H-*psb*A (Fig. 6).

DISCUSSION

The study was conducted on 4 species in *Magnoliaceae* family, including *Magnolia chevalieri* (Dandy) V.S. Kumar, *Michelia tonkinensis* A. Chev., *Michelia baillonii* (Pierre) Finet and Gagnep. and *Michelia braianensis* Gagnep. with 4 candidate DNA fragments *mat*K, *rbc*L, *trn*H-*psb*A and *ycf*1b[19-20]. These are the valuable in classification and identification that used extensively in plant. The *mat*K is one of the most potential segments to be DNA barcode for plant, which are useful in constructing the system of higher taxa like order or family. However, amplifying *mat*K segments has got trouble because there are no available pair of primers can be used for all species[21]. To overcome this disadvantage,

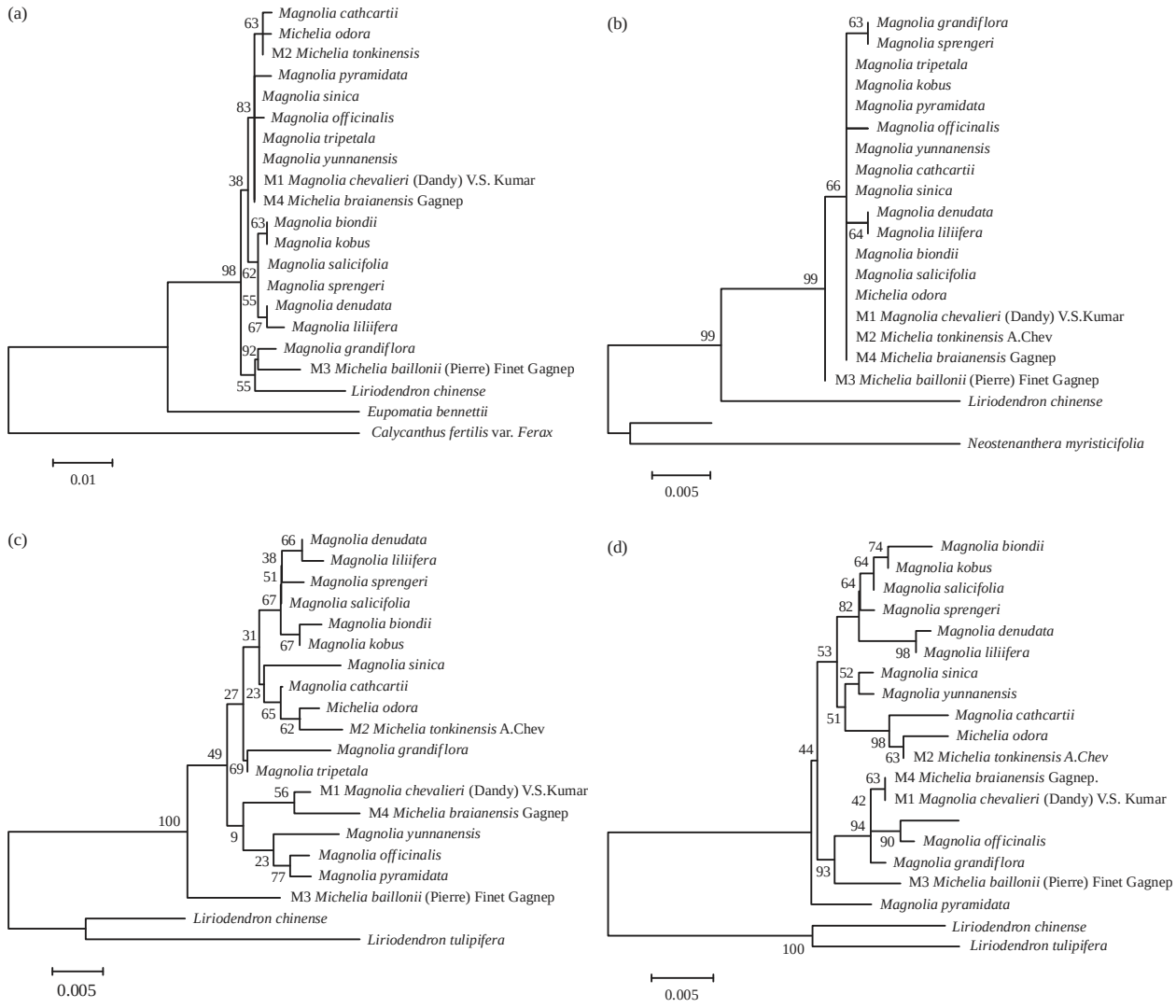

Fig. 5(a-d): Phylogenetic trees constructed based on sequence of four DNA barcode loci and sequences from GenBank, (a) *mat*K, (b) *rbc*L, (c) *trn*H-*psb*A and (d) *ycf*1 b

researchers recommended to use the core combination of *rbc*L+*mat*K. *rbc*L is a fragment in chloroplast genome with high amplification and sequencing. Though its unremarkable discriminating power, *rbc*L is regarded as a standard segment in phylogenetic construction, especially at family and genus level. Along with *rbc*L, *trn*H-*psb*A also exhibits high PCR success reaches to over 90% by standard primers. This non-coding region is appreciated even when be used as single locus or combinated with others[22]. Apart from three fragments above, Dong's[19] research has proposed another powerful locus with 75% discrimination possibility which can be used to identification lower taxa. As the 2nd largest gene in chloroplast genome, this locus contains 2 region *ycf*1 a and *ycf*1 b, is the most variable plastid genome region and can be the core barcode for land plant[19].

In this study, amplification using the specific pair of primer correspond to each candidate loci and sequencing got 100% successfully. However, the result of sequence analysis revealed that not all fragments were suitable to be DNA barcode for 4 species *Magnolia chevalieri* (Dandy) V.S. Kumar, *Michelia tonkinensis* A.Chev., *Michelia baillonii* (Pierre) Finet and Gagnep. and *Michelia braianensis* Gagnep. *rbc*L was the lowest discrimination power locus with only one variable site and the maximum genetic distance was 0.18%. Followed *rbc*L by *mat*K with 9 variable sites, but this difference did not afford to discriminate four species. With low divergence in sequence, the combination of these two candidates as CBOL proposal did not bring a satisfactory result. Different from *rbc*L and *mat*K, *ycf*1 b and *trn*H-*psb*A showed the potential discrimination with the maximum genetic distance obtained

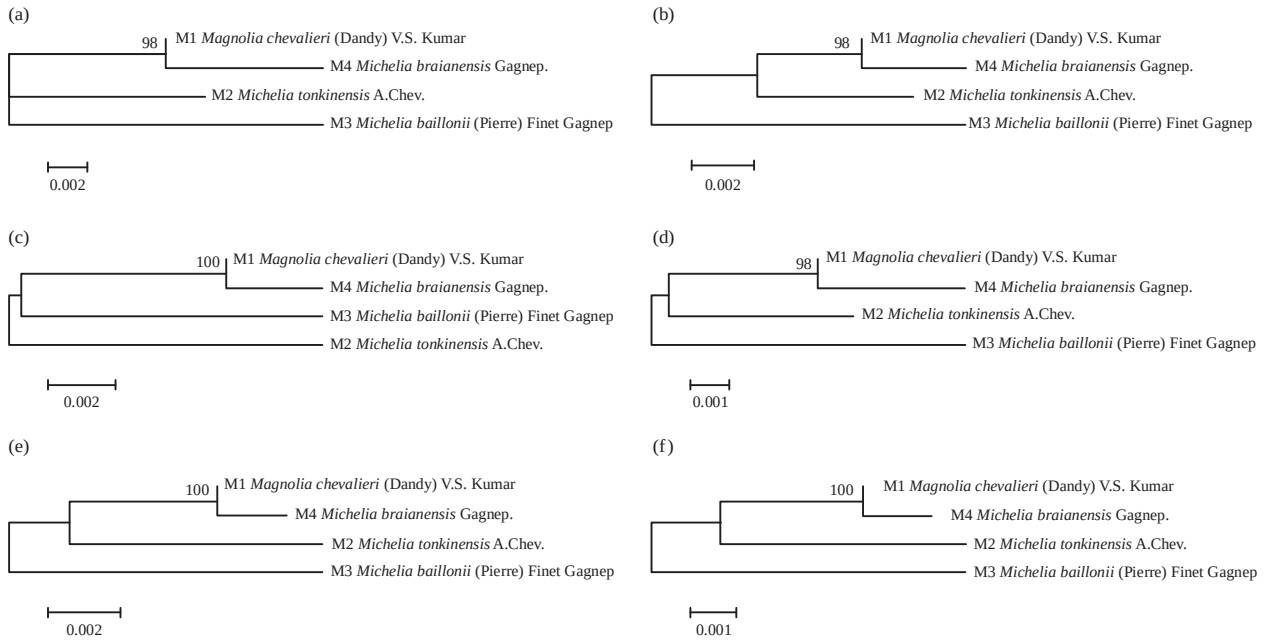

Fig. 6(a-f): Phylogenetic trees constructed based on several potential combinations of four DNA barcode loci, (a) *trn*H-*psb*A, (b) *mat*K+*trn*H-*psb*A, (c) *ycf*1b+*trn*H-*psb*A, (d) *rbc*L+*trn*H-*psb*A ,(e) *mat*K+*ycf*1b+*trn*H-*psb*A and (f) *mat*K+*rbc*L+*ycf*1b+*trn*H-*psb*A

Table 4: Reference DNA sequences from NCBI GenBank

DNA barcode loci	Species	Genbank accession
*mat*K, *rbc*L, *trn*H-*psb*A, *ycf*1b	*Magnolia biondii*	KY085894.1
	Magnolia cathcartii	JX280392.1
	Magnolia denudata	KY085917.1
	Magnolia grandiflora	JN867584.1
	Magnolia kobus	JX280396.1
	Magnolia liliifera	JX280397.1
	Magnolia officinalis	KY085916.1
	Magnolia pyramidata	JX280395.1
	Magnolia salicifolia	JX280399.1
	Magnolia sinica	JX280400.1
	Magnolia sprengeri	JX280401.1
	Magnolia tripetala	KJ408574.1
	Magnolia yunnanensis	KF753638.1
	Michelia odora	JX280398.1
	Liriodendron chinense	KU170538.1
*mat*K	*Calycanthus fertilis var. Ferax*	AJ428413.1
	Eupomatia bennettii	DQ401341.1
*rbc*L	*Malmea dimera*	AY841631.1
	Neostenanthera myristicifolia	AY743448.1
*trn*H-*psb*A, *ycf*1b	*Liriodendron tulipifera*	DQ899947.1

1.56 and 3.2%, respectively. The *Ycf*1b-the longest fragment in this study had seventeen variable sites and mean of genetic distance was 1.11% but the minimum was 0%, mean that it was disability to distinguish these species. Meanwhile, *trn*H-*psb*A the shortest fragment had the maximum divergence with genetic distance from 0.79-3.2%. This result revealed that not only discriminated successfully all four species in this study, *trn*H-*psb*A fragment but also performed the promised ability to identify other species in *Magnoliaceae* family by the safe distance in sequence. In fact, many researches have shown the similar result. In 1999, the study conducted by Azuma *et al.*[11] with 25 *Magnolia*, two *Michelia* and two *Liriodendron* taxa, analyzed three DNA sequences as *trn*K intron (including *mat*K coding region), *trn*H-*psb*A and *atp*B-*rbc*L indicated that *trn*H-*psb*A had the shortest fragment but exhibited the highest sequence divergence[12]. Although, at

this period of time, the definition "DNA barcode" have not been proposed, the idea of using short DNA sequence was applied extensively. In *Magnolia* family, there were several other outstanding researches using this effective tool such as study of Azuma *et al.*[11] indicated that *trn*H-*psb*A got the biggest rate of variable and informative charaters among three candidate *mat*K, *trn*H-*psb*A and *atp*B-*rbc*L, Kim *et al.*[13] research provided the new look in classification at genus level in *Magnoliaceae* based on *ndh*F sequence[13]. Another study of Kim has carried out with ten chloroplast regions from 48 taxa to address phylogenetic question in *Magnolia* family in 2013, including *ndh*F, *rbc*L, *mat*K, ORF350, *trn*L intron, *trn*L-*trn*F, *trn*H-*psb*A, *rbc*L-*atp*B, *trn*K 5′ intron and *trn*K 3′ intron. In 2014, Yu Hua *et al.*[15] published their study of *Magnoliaceae* species identification using seven candidate DNA region *psb*A-*trn*H, *mat*K, *rbc*L, ITS, ITS2, *rpo*B and *rpo*C1. These researches have obtained several results, but they actually were not sufficient to ascertain the relationship within *Magnolia* family, required further studies to construct the most expedient phylogenesis of this family.

Research in classification and identification of *Magnoliaceae* were executed in the early of the 20th century, firstly by morphological method with research of Dandy *et al.* proposed 10 genara into 2 tribes[1]. The classification based on morphological marker had got lots of argument[3-6], it was necessary to have another means to resolve this question. Therefore, method using molecular marker in general and DNA barcode in particular has attracted many researchers not only for family *Magnolia* family but also all organisms system. This study for 4 species *Magnolia chevalieri* (Dandy) V.S. Kumar *Michelia braianensis* Gagnep., *Michelia tonkinensis* A. Chev. and *Michelia baillonii* (Pierre) Finet and Gagnep. species that have not received much attention in previous researches has contributed in the campaign of building classification system for *Magnolia* family by DNA barcode. With outstanding advantage compare to traditional method that not require standard specimen, this technique is a powerful tool complement the morphological method in classification and identification, especially for complicated and controversal clusters.

CONCLUSION

Four candidate DNA barcodes (*mat*K, *rbc*L, *trn*H-*psb*A and *ycf*1b) were successfully amplified and sequenced from 4 plant species of *Magnolia* family. The result of sequencing indicated that the lengths of *mat*K, *rbc*L, *trn*H-*psb*A and *ycf*1b fragments were 714, 553, 509-514 and 909-962 bp, respectively. These nucleotide sequences have been registered on the Vietnam DNA Data Bank. The study result showed that among four candidates DNA barcodes were studied, the *psb*A-*trn*H region is the most efficient DNA barcode sequence with maximum genetic distance reached 3.2%. Furthermore, the combinations including *mat*K+*trn*H-*psb*A, *rbc*L+*trn*H-*psb*A, *ycf*1b+*trn*H-*psb*A, *mat*K+*ycf*1b+*trn*H-*psb*A, *mat*K+*rbc*L+*ycf*1b+*trn*H-*psb*A could be used as the DNA barcode for species identification and analysis of genetic relationship among some species of *Magnolia* family.

SIGNIFICANCE STATEMENTS

This study develops DNA barcodes for selected species within *Magnolia* family. Results of this study are not only supported species identification and analysis of genetic relationship among some species of *Magnolia* family but also contributed to the conservation and commercialization of these important forest tree species.

ACKNOWLEDGMENT

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