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Expedient identification of Magnoliaceae species by DNA barcoding

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Abstract

Magnoliaceae species have high ornamental and medicinal importance, but they are morphologically similar. DNA barcoding has been regarded as a rapid and effective approach for species identification. To determine the efficiency of expedient identification in Magnoliaceae species by DNA barcoding, in this study, we collected 83 samples belonging to 68 species in 10 genera of Magnoliaceae. Candidate DNA regions (i.e., *psbA-trnH*, *mat*K, *rbcL*, ITS, ITS2, *rpoB*, and *rpoC1*) were amplified and sequenced for the evaluation of their PCR amplification, sequencing efficiency, intra- and inter-specific divergence and barcoding gap by sequence alignment and Kimura 2-Parameter (K2P) distance analysis, and the rate of correct identification was assessed by BLAST analysis. The results showed that *psbA-trnH* and *mat*K exhibited high performance in efficiency of PCR amplification and the rate of successful sequencing, followed by *rbcL*. Associated with the analysis of 199 sequences for 96 species in 9 genera of Magnoliaceae retrieved from GenBank, it was discovered that *psbA-trnH* was highest in inter-specific divergence and rate of correct identification, indicating its efficiency in the identification of Magnoliaceae species. Besides, *mat*K was also easy to amplify and had high rate of correct identification, suggesting its potential to distinguish Magnoliaceae species. This study indicates that DNA barcoding provides an effective technique for the expedient identification of morphologically similar species, and it is a powerful aid to the conventional methods for species identification.

Keywords: candidate DNA region; DNA barcoding; intra- and inter- specific divergence; Magnoliaceae species; plant identification.

Abbreviations: *psbA-trnH*_chloroplast *psbA-trnH* intergenic spacer region; *mat*K_maturase coding gene; *rbcL*_ ribulose-1,5-bisphosphate carboxylase / oxygenase large subunit gene; ITS_the internal transcribed spacer of nuclear ribosomal DNA; ITS2_the second internal transcribed spacer of nuclear ribosomal DNA; *rpoB*_RNA polymerase β-subunit gene; *rpoC1*_RNA polymerase γ-subunit gene; PCR_polymerase chain reaction; K2P_Kimura 2-parameter distance; MEGA_molecular evolutionary genetics analysis; BLAST_basic local alignment search tool.

Introduction

Characterized by their spirally arranged flower parts (e.g., sepals, petals, stamens, and pistils), Magnoliaceae species have long been recognized as primitive flowering plants and as such have played an important role in understanding the origin and diversification of angiosperms (Law, 1984; Matsui et al., 1993). The family comprises approximately 16 genera worldwide, represented by 270 species of evergreen and deciduous trees and shrubs (Luo et al., 2006; Nie et al., 2008). Their native distribution is centered mainly in the temperate and tropical regions of Southeast Asia, with outlying species in the south-eastern states of North America (Azuma et al., 2001). Many species of Magnoliaceae are used worldwide for ornamental and medicinal purposes; examples of the latter include the bark of *Magnolia officinalis* Rehd. & E.H.Wilson, which is a traditional remedy for cramps, while the flower-buds of *M. liliiflora* Desr. are used to treat chronic respiratory infections (Shi et al., 2000). Their economic importance, disjunct distribution and phylogenetics have been attracting much recent study (Nie et al., 2008; Zhang et al., 2010).

Many Magnoliaceae species are morphologically similar. It is not only because Magnoliaceae is a primitive group with low divergence, but also due to their geographical distribution is a result of intercontinental disjunction which has isolated some species while keeping the others in close contact (Kim et al., 2001; Li and Conran, 2003), leading to the considerable overlap in morphological characteristics of Magnoliaceae species (Shi et al., 2000; Nie et al., 2008). Therefore, the lack of an accurate identification system remains the major obstacle to correctly identify an unknown specimen to species. For traditional taxonomic identification, it is difficult to identify and differentiate specimen into species level especially when diagnostic morphological features are absent (Hardy et al., 2008)

Table 1. Sequences of seven candidate DNA barcodes and the conditions of their PCR amplification.

Barcode	DNA region	DNA sequence (5'-3')	Condition of PCR amplification	Literature
ITS	5 forward 4 reverse	CCTTATCATTTAGAGGAAGGAG TCCTCCGCTTATTGATATGC	94 °C 5 min 94 °C 1 min, 50 °C 1 min 72 °C 1.5 min + 3 sec/cycle, 30 cycles 72 °C 7 min	Kress et al., 2005
ITS2	2 forward 3 reverse	ATGCGATACTTGGTGTGAAT GACGCTTCTCCAGACTACAAT	94 °C 5 min 94 °C 30 sec, 56 °C 30 sec 72 °C 45 sec, 40 cycles 72 °C 10 min	Chen et al., 2010
*mat*K	390 forward 1326 reverse	CGATCTATTCATTCAATATTTC TCTAGCACACGAAAGTCGAAGT	94 °C 1 min, 48 °C 30 sec 72 °C 1 min, 26 cycles 72 °C 7 min	Cuénoud et al., 2002
psbA -trnH	PA forward TH reverse	GTTATGCATGAACGTAATGCTC CGCGCATGGTGGATTCACAATCC	94 °C 5 min 94 °C 1 min, 55 °C 1 min 72 °C 1.5 min, 30 cycles 72 °C 7 min	Kress et al., 2005
rbcL	1 forward 724 reverse	ATGTCACCACAAACAGAAAC TCGCATGTACCTGCAGTAGC	95 °C 2 min 94 °C 1 min, 55 °C 30 sec 72 °C 1 min, 34 cycles 72 °C 7 min	Kress et al., 2005
rpoB	1 forward 4 reverse	AAGTGCATTGTTGGAACTGG GATCCCAGCATCACAATTCC	94 °C 4 min 94 °C 30 sec, 53 °C 40 sec 72 °C 40 sec, 40 cycles 72 °C 7 min	Hollingsworth et al., 2009
rpoC1	2 forward 4 reverse	GGCAAAGAGGGAAGATTTCG CCATAAGCATATCTTGAGTTGG	94 °C 4 min 94 °C 30 sec, 53 °C 40 sec 72 °C 40 sec, 40 cycles 72 °C 7 min	CBOL Plant Working Group, 2009

Therefore, molecular cladistic analysis has been employed to identify Magnoliaceae species (Shi et al., 2000), and it stimulates the debate on the diagnosis of morphologically similar group based on conventional taxonomic methods (Benemann et al., 2012; Zhu et al., 2012).

Regarded as an effective approach for species identification, in recent years DNA barcoding has been receiving increasing attention (Tautz et al., 2003; von Cräutlein et al., 2011). The approach in principle relies on the use of a standardized DNA region used as a tag for rapid and accurate species identification (Packer et al., 2009; Valentini et al., 2009) and, in particular, can be used to discriminate closely related species regardless of the absence of morphological features (Newmaster et al., 2008; Shen et al., 2013). As such, the approach can not only by-pass identification challenges where there is phenotypic plasticity or lack of morphological traits (Hardy et al., 2008), but can use any plant part and regardless of its stage of maturity. This is a distinct advantage over morphological identification alone (Hebert and Gregory, 2005; Bhargava and Sharma, 2013). To explore a universal barcoding region is central to the efficacy of DNA barcoding (Luo et al., 2011), and a DNA barcode sequence must not only easily be amplified with universal primers and reaction conditions, but also contain enough variation to generate unique identifiers at both species and genus levels (Hebert and Gregory, 2005).

As a primitive group with the involvement of some recently evolved species, Magnoliaceae provides a challenging task for DNA barcoding of plant species. Many regions have been proposed as promising barcode sequences for plant identification, such as, internal transcribed spacer regions ITS and ITS2, chloroplast non-coding spacer *psbA-trnH*, and transcribed regions *mat*K, *rbcL*, *rpoB*, and *rpoC1*. However, DNA barcoding with these markers can still be problematic for a particular family with closely related species (Chen et al., 2010; Roy et al., 2010). In this study, to detect the species discriminatory power of expedient identification by DNA barcoding, we evaluated the genetic divergence and species identification efficiency of potential barcoding regions (i.e., ITS, ITS2, *psbA-trnH*, *mat*K, *rbcL*, *rpoB*, and *rpoC1*) in the morphologically similar but ecologically important Magnoliaceae species, and also discussed the importance of DNA barcoding as an expedient identification measure in species diagnosis of morphologically similar species.

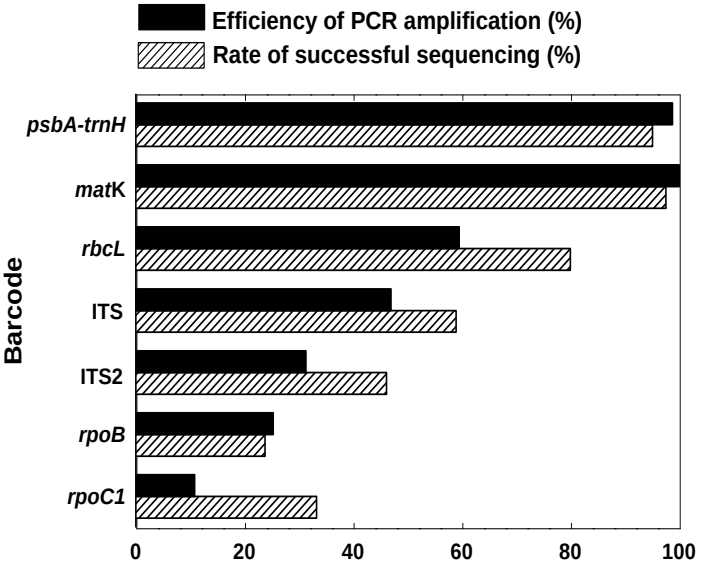

Fig 1. Efficiency of PCR amplification (in dark) and rate of successful sequencing (in hatched) for Magnoliaceae species by seven candidate regions.

Table 2. Inter- and intra-specific divergences of *psbA-trnH*, *mat*K, and *rbcL*.

Parameter	Candidate barcode		
	psbA-trnH	*mat*K	*rbcL*
Average inter-specific distance	0.0175 ± 0.0079	0.0156 ± 0.0097	0.0040 ± 0.0023
Theta prime	0.0147 ± 0.0056	0.0132 ± 0.0098	0.0037 ± 0.0013
Minimum inter-specific distance	0.0045 ± 0.0064	0.0039 ± 0.0084	0.0008 ± 0.0013
Average intra-specific distance	0.0064 ± 0.0071	0.0018 ± 0.0025	0.0012 ± 0.0024
Theta	0.0065 ± 0.0063	0.0012 ± 0.0013	0.0021 ± 0.0032
Coalescent depth	0.0090 ± 0.0087	0.0016 ± 0.0022	0.0023 ± 0.0033

Table 3. Wilcoxon signed-rank tests for inter- and intra-specific divergences among *psbA-trnH*, *mat*K, and *rbcL*.

ivergence	W+	W-	Relative ranks, *n*, *P* value	Result
Inter-specific	*psbA-trnH*	*mat*K	W+=455875, W-=42626, n=998, p=6.213×10^{-24}	*psbA-trnH* >> *mat*K
	psbA-trnH	*rbcL*	W+=17444, W-=134, n=187, p=1.657×10^{-31}	*psbA-trnH* >> *rbcL*
	*mat*K	*rbcL*	W+=4430, W-=13148, n=187, p=1.315×10^{-10}	*mat*K > *rbcL*
Intra-specific	*psbA-trnH*	*mat*K	W+=491, W-=5, n=31, p =1.916×10^{-5}	*psbA-trnH* > *mat*K
	psbA-trnH	*rbcL*	W+=120, W-=8, n=7, p =3.102×10^{-3}	*psbA-trnH* > *rbcL*
	*mat*K	*rbcL*	W+=19, W-=2, n=6, p =0.073	*mat*K = *rbcL*

Results

PCR amplification efficiency and sequence quality

Among the seven candidate DNA regions (i.e., *psbA-trnH*, *mat*K, *rbcL*, ITS, ITS2, *rpoB*, and *rpoC1*), *psbA-trnH* and *mat*K exhibited the highest efficiency of PCR amplification, and showed the highest rates of successful sequencing, followed by *rbcL* (Fig.1). The other regions (i.e., ITS, ITS2, *rpoB*, and *rpoC1*) were excluded since they demonstrated relatively low efficiency of PCR amplification in the pilot study (< 60%, Fig.1).

Inter- and intra-specific divergences and barcoding gap analysis

Three parameters (i.e., average inter-specific distance, theta prime, and the minimum inter-specific distance) were employed to characterize inter-specific divergence, and the others (i.e., average intra-specific distance, mean theta, and coalescent depth) were used to indicate intra-specific variation (Table 2). By comparison on the inter-specific divergences of three candidate DNA regions (i.e., *psbA-trnH*, *mat*K, and *rbcL*), *psbA-trnH* had the highest inter-specific divergence with the maximum values in average inter-specific distance, theta prime, and the minimum inter-specific distance, followed by *mat*K and *rbcL* respectively (Table 2 and 3). There showed a similar pattern for their intra-specific variation. Specifically, intra-specific variation of *psbA-trnH* was higher than those of both *mat*K and *rbcL*, but there existed no significant difference between those of the later two candidate DNA regions (Table 2 and 3).

Using the Wilcoxon test, there was significant difference between the inter- and intra-specific divergences of the candidate DNA regions (i.e., *psbA-trnH*, *mat*K, and *rbcL*), with their inter-specific divergences significantly higher than their related intra-specific variations (P < 0.05, Table 4). For the analysis of DNA barcoding gap, *psbA-trnH*, *mat*K and *rbcL* showed different patterns (Fig.2). Specifically, the distribution pattern of inter- and intra-specific divergences of *psbA-trnH* exhibited a barcoding gap, but those of *mat*K and *rbcL* had significant overlaps without any gap (Fig.2).

Efficiency of species identification

BLAST analysis on the identification efficiency of three candidate DNA regions (i.e., *psbA-trnH*, *mat*K, and *rbcL*) was performed based on their related DNA sequences including those generated from the experiment (199 sequences of 68 species in 10 genera) and those retrieved from GenBank (199 sequences of 96 species in 9 genera), with a total of 398 sequences for 135 species in 13 genera (Table 5 and Supplementary Table 1). The results showed that *psbA-trnH* exhibited the highest rates of correct identification at both species level and genus level, followed by *mat*K and *rbcL* respectively (Table 5). By comparison, *rbcL* had the lowest rates of correct identification at both species level and genus level, either for the sequences generated from the experiment or for those retrieved from GenBank (Table 5).

Discussion

This study was undertaken in order to test the robustness of different DNA barcode regions to discriminate between species in the Magnoliaceae family. Results showed that the potential barcoding regions *psbA-trnH* and *mat*K could easily be amplified and sequenced, with *psbA-trnH* providing the highest rates of correct identification at both species and genus level; they also provided significant discrimination inter- and intra-specifically. As one of the most variable markers, *psbA-trnH* contains more comprehensive information, and it can be more universally amplified than the other regions (Kress and Erickson, 2007; Storchova and Olson, 2007). Although it is criticized for being prone to read errors at the sequencing stage, *psbA-trnH* has been tested to be powerful and effective in the identification of plant species (Gonzalez et al., 2009; Chen et al., 2010; von Cräutlein et al., 2011). For example, *psbA-trnH* can correctly identify various *Dendrobium* species and effectively distinguish them from other adulterant species (Yao et al., 2009). In line with Kress and Erickson (2007), this study confirmed that *psbA-trnH* has sufficient discrimination and it can be used as a prime candidate for barcoding the Magnoliaceae species.

DNA regions used as a barcode must be routinely amplifiable, easily sequenced and sufficiently variable to separate the congeneric species (Chen et al., 2010; von Cräutlein et al., 2011). In this study, the results demonstrate that *mat*K could be easily sequenced and had considerable high rate of correct identification, indicating its potential for the identification of Magnoliaceae species. However, amplifying and sequencing were not sufficiently smooth for *rbcL*, and it had lower identification efficiency than that of *psbA-trnH* and *mat*K. It was likely because Magnoliaceae species is a primitive group with low divergence and *rbcL* was not variable enough to identify the closely related species (Gonzalez et al.,

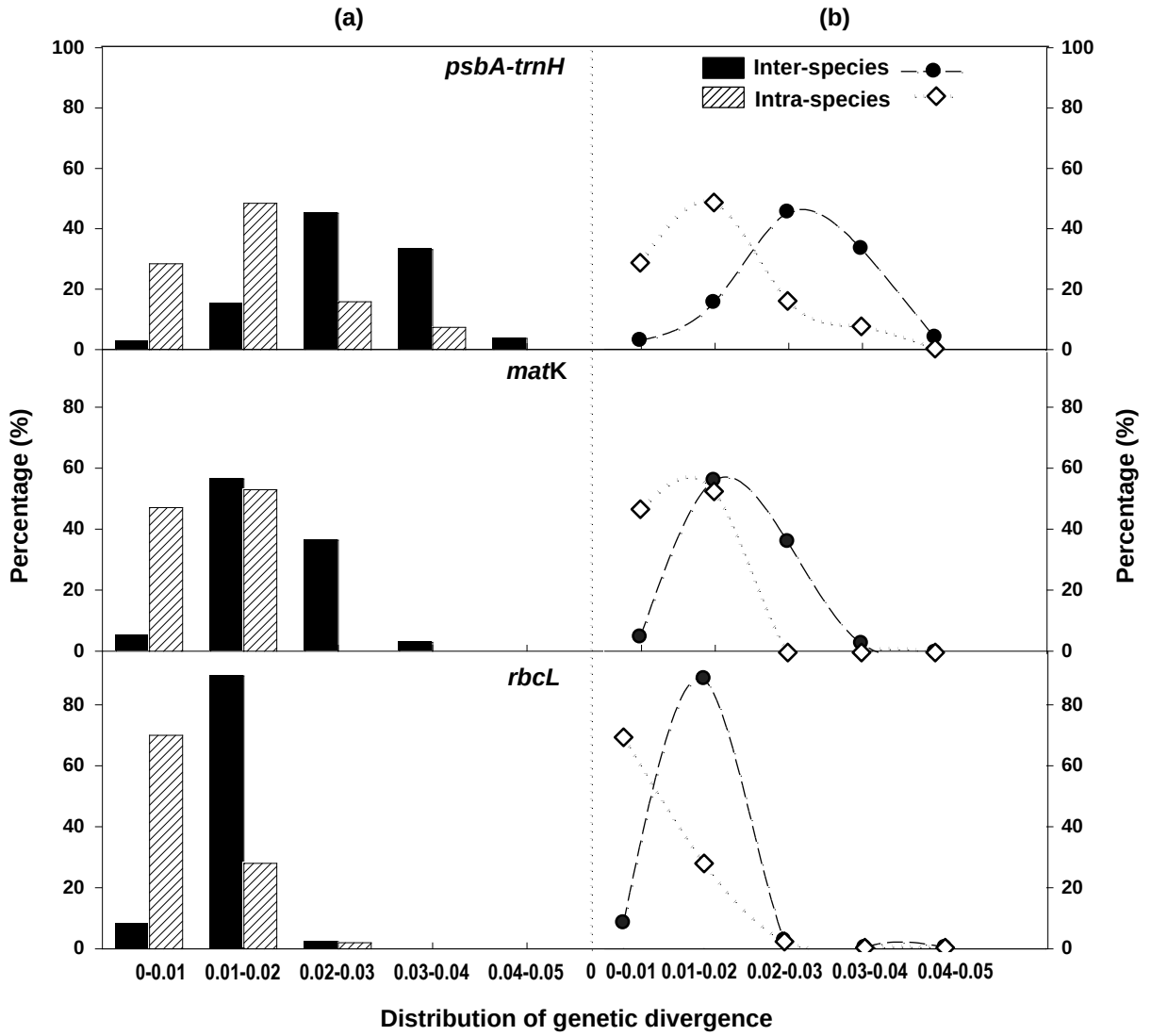

Fig 2. Distribution of genetic divergence for Magnoliaceae species by *psbA-trnH*, *mat*K, and *rbcL*. It includes inter-specific variation (in dark) and intra-specific divergence (in hatched) with bars (a) and spots (b) respectively.

2009). However, the other candidate DNA regions (i.e., ITS, ITS2, *rpoB*, and *rpoC1*) exhibited a low performance and were excluded for further analysis either because they failed in PCR amplification, or because they had very low rates of successful sequencing. By comparison, *mat*K with high PCR reliability and species discrimination is worth further consideration for recognition of Magnoliaceae species.

DNA barcoding may also provide insights into the phylogenetic classification of Magnoliaceae species. Because of the extensive homogeneity and considerable overlap in morphological characters, the phylogenetic relationships of Magnoliaceae species have been controversial (Xu and Rudall, 2006). After Dandy (1927) proposed the first comprehensive taxonomic scheme of family Magnoliaceae with ten genera distributed in two tribes (i.e., Liriodendreae and Magnolieae), Law (1984) divided it into two subfamilies (i.e., Magnolioideae and Liriodendroideae) based on the external morphology, wood anatomy and palynology. However, molecular evidence from DNA regions (e.g., *mat*K and *ndhF* regions) challenges the current alignment of Magnoliaceae species, and it stimulates the debate on the morphological-based classifications (Shi et al., 2000; Kim et al., 2001; Xu and Rudall, 2006). However, there is no statement on whether or not DNA barcoding has sufficient phylogenetic signal to solve the phylogenetic position of Magnoliaceae species, further studies concerning on the genera delimitation of Magnoliaceae species by DNA barcoding are needed.

Due to genetic variation between species exceeding that within species, DNA barcoding based on a short standardized sequence can rapidly and accurately distinguish the individuals of species (Hajibabaei et al., 2007), and it provides an effective technique for the expedient identification of Magnoliaceae species. Different from the conventional taxonomic method requiring the collection of morphological characters, DNA barcoding can be used to identify plant debris and atypical specimen. Although it is difficult to designate unknown species which is poorly studied or has no existing record in the barcode database, it can be performed to quickly sort specimens into genetically divergent groups (Moritz and Cicero, 2004; Hajibabaei et al., 2006). Overview, DNA barcoding provides a rapid and effective technique for species identification, and it is a powerful complement to conventional taxonomic methods in the identification of taxonomically complex and morphologically problematic species.

Table 4. Wilcoxon two-sample test for inter- versus intra-specific divergences of *psbA-trnH*, *mat*K, and *rbcL*.

Barcode	Number of inter-specific	Number of intra-specific	Wilcoxon W	*P* value
psbA-trnH	5	22	79	0.001
*mat*K	11	42	86	0.011
rbcL	5	21	95	0.027

Table 5. Rates of correct identification for *psbA-trnH*, *mat*K, and *rbcL* on Magnoliaceae species using BLAST analysis.

Barcode	Data source [a]	Sample number	Species number	Genus number	Rate of correct identification	
					Species level (%)	Genus level (%)
psbA-trnH	Experiment	78	64	8	98.7	100
	GenBank	39	28	4	97.4	100
	Total	117	82	9	98.3	100
*mat*K	Experiment	81	68	10	97.5	98.8
	GenBank	97	75	8	95.9	97.9
	Total	178	116	12	96.6	98.3
rbcL	Experiment	40	32	5	92.5	95.0
	GenBank	63	50	7	88.9	93.7
	Total	103	70	8	90.3	94.2

[a] Data include those acquired from this research (Experiment), retrieved from GenBank (GenBank), and from both this research and GenBank (Total).

Materials and methods

Sampling

In this study, a total of 83 samples belonging to 68 species in 10 genera of Magnoliaceae were collected from the Botanical Garden at the South China Institute of Botany, Chinese Academy of Sciences (23.2^o N, 113.4^o E) in Guangdong province, and Longli Botanical Garden of Rare Species (26.5^o N, 106.9^o E) in Guizhou province (Supplementary Table 1). The voucher samples were deposited in the Herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences (40.0^o N, 116.3^o E). Besides, 403 sequences of Magnoliaceae species containing the "*psbA-trnH*", "*mat*K" or "*rbcL*" were retrieved according to their annotations in GenBank (March 1st, 2011). After quality control of each sequence was performed as the previous studies (Chen et al., 2010), 199 sequences representing 96 species in 9 genera were kept for further analysis on their efficiency in species identification of Magnoliaceae species (Supplementary Table 1).

PCR amplification and sequencing of candidate DNA regions

The total DNA of each plant sample was extracted from 10 mg of dried leaf tissue using the universal genomic DNA extraction kit (Tiangen Biotech Co., China). The sequences of candidate DNA regions (i.e., *psbA-trnH*, *mat*K, *rbcL*, ITS, ITS2, *rpoB*, and *rpoC1*) were amplified under the PCR reaction conditions (Table 1) and following methods described in previous studies (Song et al., 2009; Chen et al., 2010). Purified PCR products were sequenced in both forward and reverse directions with the primers used for PCR amplification on a 3730XL sequencer (Applied Biosystems, USA). Both forward and reverse sequences were assembled using CodonCode Aligner 3.7 (CodonCode Co., USA), and a quality evaluation of the generated sequences was performed following the approach by Chen et al (2010).

Data analysis

The sequences of candidate DNA regions were aligned with Clustal W, and Kimura 2-Parameter (K2P) distance were analyzed with MEGA 5.0 (Tamura et al., 2011). To evaluate the intra-specific divergence, the average intra-specific distance, mean theta and coalescent depth were analyzed using the K2P model. To indicate inter-specific variation, the average inter-specific distance, theta prime and the minimum inter-specific distance were employed (Chen et al., 2010; Yao et al., 2010). The distribution of intra- versus inter-specific divergence was used to illustrate as DNA barcoding gap (Lahaye et al., 2008). BLAST analysis of acquired sequences was conducted as previously described (Chen et al., 2010) and the Wilcoxon test for inter- and intra-specific divergences was conducted using SPSS 15.0 software (SPSS Inc, Chicago, USA).

Conclusion

Species identification within the Magnoliaceae family, using morphological characters alone, is a challenging task since there are few diagnostic characters to reliably separate individual species. This study evaluated the role of DNA barcoding as an alternative and/or complementary tool to address this challenge. Of the candidate DNA regions assessed, *psbA-trnH* best demonstrated inter-specific divergence and species identification accuracy, followed by *mat*K which demonstrated high PCR reliability and species discrimination. Both regions therefore provided robust evidence of their efficiency to identify Magnoliaceae species using DNA barcoding and notably to discriminate closely related species in this family. As such, this method provides a robust technique that complements conventional methods for the identification of taxonomically complex and morphologically problematic species.

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