Molecular Analysis of the Genus *Mitragyna* Existing in Thailand Based on rDNA ITS Sequences and Its Application to Identify a Narcotic Species: *Mitragyna speciosa*

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In Thailand, there are four *Mitragyna* species; *M. speciosa*, *M. hirsuta*, *M. diversifolia*, and *M. rotundifolia*. One, *M. speciosa*, is a narcotic plant and has medicinal importance for its opium-like effect. Since the use of *M. speciosa* has been forbidden in Thailand, the leaves of *M. diversifolia* or others are frequently used as substitutes but are not considered as effective. Therefore, accurate authentication of *M. speciosa* is essential for both medicinal and forensic purposes. The nucleotide sequences of internal transcribed spacers (ITS) and the 5.8S coding region of nuclear ribosomal DNA (rDNA) of the *Mitragyna* species were analyzed. The whole length of ITS1-5.8S-ITS2 region was 608 bp in *M. speciosa*, 607 bp in the other species. Nineteen sites of nucleotide substitutions and 3 sites of 1-bp indels were observed, and *M. speciosa* showed specific sequence differed from the others. Based on the ITS sequences, a distinctive site recognized by a restriction enzyme *Xma* I in *M. speciosa* was found and then PCR-restriction fragment length polymorphism (RFLP) analysis was established to differentiate *M. speciosa* from the others. By the method, a 409-bp PCR fragment of ITS1-5.8S (partial) rDNA region from *M. speciosa* was cleaved into two fragments of 119 bp and 290 bp while the other species remained undigested. This method provides an effective and accurate identification of *M. speciosa*.

Key words *Mitragyna*; Kratom; rDNA internal transcribed spacer (ITS); PCR-restriction fragment-length polymorphism (RFLP); identification

Mitragyna KORTH. is a small genus in family Rubiaceae consisting of ten species in the world. Six species, *M. speciosa* (KORTH.) HAVIL., *M. tubulosa* (ARN.) HAVIL., *M. parvifolia* (ROXB.) KORTH., *M. hirsuta* HAVIL., *M. diversifolia* (WALL. ex G. DON) HAVIL., *M. rotundifolia* (ROXB.) O. KUNTZE, widely grow in Thailand, Vietnam, Philippines, Malay Peninsula, Sumatra, Borneo, and New Guinea islands.[1,2] In Thailand, *M. hirsuta*, *M. diversifolia* and *M. rotundifolia* are seen commonly. *M. speciosa* distributed from central to southern part, is a narcotic plant, so called "Kratom," but with specific medicinal importance.[1,3] The leaves of *M. speciosa* have been chewed habitually by Thai natives for the opium-like effect and coca-like stimulant to overcome the burdens of their hard work. In Thai traditional medicine it has also been used to treat diarrhea, stop coughing and relieve injury pain, and in modern it has been used as a substitute for opium and for treatment of addiction to morphine.[4] Phytochemical studies revealed that mitragynine, an indole alkaloid, was the major constituent of this plant, accounting for about half of the total alkaloid contents. Its analogues, speciogynine, speciociliatine, and paynanthine *etc.* were also found.[5,6] Recent pharmacological studies reported the antinociceptive effect of this species due to containing of mitragynine and its analogues. Especially, 7-hydroxymitragynine, a minor constituent, revealed significantly potent antinociceptive activity through opioid receptors.[5—7] Its antinociceptive activity was many times more potent than morphine when administrated orally and subcutaneously.[8,9]

The use of *M. speciosa* has been forbidden in Thailand due to its narcotic effects. The Thai government passed the law to make planting of *M. speciosa* illegal. However, since the species is indigenous to Thailand and widely distributed, the law is hard to raise its efficiency. On the other hand, the leaves of *M. diversifolia* or others are frequently used as substitutes but are not considered as effective. Therefore, authentication of *M. speciosa* is essential for both forensic and medicinal purpose.

In recent years, molecular identification of herbal drugs by nucleotide sequence of various DNA regions has been demonstrated to be a powerful way.[10—16] Especially, DNA regions with high evolutionary rate have been widely used to discriminate species and investigate phylogenetic relationship among closely-related taxa, such as chloroplast *trn*K/*mat*K,[11] *rpl*14-16,[12] *trn*T-F,[13] and nuclear internal transcribed spacers (ITS),[13—15] 5S spacer region,[16] *etc.* In a preliminary study, we found that there was no nucleotide difference in chloroplast *mat*K gene and nuclear 18S rRNA gene regions between *M. speciosa* and *M. hirsuta* (unpublished data). In the present study, we investigated the sequence of ITS region for characterizing four *Mitragyna* species in Thailand and further to discriminate *M. speciosa* from the other species.

MATERIALS AND METHODS

Plant Materials Total of seventeen specimens of four *Mitragyna* species were collected from Thailand, including ten vouchers of *M. speciosa*, three vouchers each of *M. diversifolia* and *M. hirsuta*, and one voucher of *M. rotundifolia*. Due to the morphological variation of leaf, *M. speciosa* specimens were grouped into 3 types, red-veining leaf (Kan daeng in Thai), white-veining leaf (Tang gua), and leaf with a pair of very small teeth exerted near apex (Yak yai). All specimens were identified by Assoc. Prof. Dr. Nijsiri Ruan-

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Table 1. Plant Materials of Four Species Used in This Study and the Accession Numbers of These Species for ITS1-5.8S-ITS2 Sequences in GenBank

Species	Code	Place of collection (Thailand)	Date	Voucher No.	Notes	GenBank Accession
Mitragyna speciosa (Korth.) Havil.	MS-05	Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok	2005.10	TTP-Suchada-051001	Leaf: red-veined (Kan daeng)	AB249645
	MS-07	Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok	2005.10	TTP-Suchada-051002		
	MS-08	Bangkok	2005.10	NSR-090294-597		
	MS-09	Bangkok	2005.10	NSR-090294-598		
	MS-10	Pathumthani Province	2005.10	NSR-090294-599		
	MS-11	Bangkok	2005.10	NSR-090294-600		
	MS-12	Bangkok	2005.10	NSR-090294-601		
	MS-13	Bangkok	2005.10	NSR-090294-602	Leaf: white-veined (Tang gua)	
	MS-14	Bangkok	2005.10	NSR-090294-603		
	MS-15	Bangkok	2005.10	NSR-090294-604	Leaf: a pair of small teeth exerted near apex (Yak yai)	
M. diversifolia (Wall. ex G. Don) Havil.	MD-01	Buffalo House, Supaburi Province	2004.11	Suchada-041101	—	AB249646
	MD-02	National Institute of Thai Traditional Medicine, Bangkok	2005.10	NSR-090294-596		
	MD-06	Medicinal Plant Garden, Nakornprathom Province	2005.09	TTP-050901		
M. hirsuta Havil.	MH-04	Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok	2005.10	TTP-050902	—	AB249647
	MH-17	Sukothai Province	2005.11	TTP-051103		
	MH-18	Kampaengpet Province	2005.11	TTP-051104		
M. rotundifolia (Roxb.) O. Kuntze	MR-16	Lablae, Uttaradit Province	2005.11	TTP-051105	—	AB249648

grungsi and Assoc. Prof. Thatree Phadungcharoen of Chulalongkorn University and preserved at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. Details of the plant materials are shown in Table 1.

Genomic DNA Extraction Collected plant materials kept in silica gel were transported to the lab where they were ground into fine powder in liquid nitrogen by a mortar and a pestle. Extraction of DNA utilized the DNeasy^{TM} Plant Mini Kit (QIAGEN, Germany). DNA quality and quantity were determined by electrophoresis on 0.8% agarose gel stained by ethidium bromide. The extracted total DNA was stored at −20 °C for further use as template in PCR amplification.

PCR Amplification The primers used for PCR amplification of approximately 650 bp region including ITS1-5.8S-ITS2 region were oligonucleotide 18S-25S-5′F (forward primer), 5′-GTA GGT GAA CCT GCA GAA GGA TCA-3′, and 18S-25S-3′R (reverse primer), 5′-CCA TGC TTA AAC TCA GCG GGT-3′. The forward primer located in 3′ end of 18S rDNA region and the reverse one located in 5′ end of 25S rDNA region were designed according to the known sequences of tomato and mung bean[17,18] (Fig. 1). PCR amplification was performed using 50—100 ng of total DNA as a template in 50 μl of reaction mixture, consisting of 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.25 μM of each primer, and 1.5 U of Taq Polymerase (Promega, U.S.A.). A Takara thermal cycler (Takara, Japan) was used to carry out PCR amplification under cycling profile of a preliminary denaturation at 95 °C for 3 min, 39 cycles of 95 °C for 40 s, 55 °C for 40 s, and 72 °C for 1 min, and a final extension at and 72 °C for 10 min.

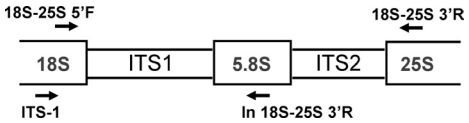

Fig. 1. Map of ITS1-5.8S-ITS2 Region with Used Primers Indicated

The length of ITS1, 5.8S and ITS2 in *Mitragyna* species are 228, 163 and 216/217 bp, respectively.

Sequence Analysis and Phylogenetic Analysis The purified PCR products by a QIA quick PCR purification kit (QIAGEN, Germany) served as template, sequencing reaction were performed by using the Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems, U.S.A.) with the same primers as in PCR amplification. The thermal cycling condition was 96 °C for 1 min, 25 cycles of 96 °C for 30 s, 50 °C for 5 s and 60 °C for 4 min. After removal of unincorporated fluorescent reagents from the produced mixture using AutoSeq^{TM} G-50 columns (GE healthcare Biosciences, U.K.), sequencing products were analyzed on an automated DNA sequencer (ABI Prism 3100-Avant Genetic Analyzer, Applied Biosystems, U.S.A.). The obtained sequences were edited and aligned by the AutoAssemble program (Version 1.3.0, Applied Biosystems, U.S.A.) and Genetyx-SV/RC version 11.0 (Software Development Co., Ltd., Tokyo, Japan). The nucleotides sequence data of ITS1-5.8S-ITS2 region were deposited in the DDBJ, EMBL, and GenBank nucleotide sequence database with the accession numbers shown in Table 1. The borders of ITS1, 5.8S, ITS2 regions

were determined by comparison with the known sequences of tomato, mung bean and rice.[17—19]

The phylogenetic trees were constructed using the computer program PAUP* (Version 4.0 beta 10a, Sinauer Assoc. Inc., U.S.A.). Parsimony analysis was performed using the Heuristic search method, with tree-bisection-reconnection (TBR) branch-swapping, MULPARS, a random addition sequence of 100 replicates. Besides sequences of the four plant species of Thailand determined in our present study, other five sequences obtained from GenBank were also included for tree reconstruction. *Uncaria rhynchophylla* (Miq.) Jackson (AJ346900) belonging to the same Rubiaceae family was used as outgroup. Bootstrap (1000 replications) analysis was performed to estimate the confidence of topology of the consensus tree.

PCR-RFLP Analysis of the Partial ITS Region On the basis of nucleotide difference at position 42 between *M. speciosa* and other three *Mitragyna* species, *Xma* I restriction site that can discriminate the substitution at this site was found and applied for PCR-RFLP analysis. A smaller fragment of 409 bp in length was generated with a pair of new primers, ITS-1 (5′-TCC ACT GAA CCT TAT CAT TTA G-3′)[20] and In-18S-25S-3′R (5′-GAC TCG ATG GTT CAC GGG ATT CT-3′, designed on the basis of determined sequence of *Mitragyna* species). The locations of primers are shown in Fig. 1. The resulting PCR products from all specimens were digested with a restriction enzyme, *Xma*I (Bio-Lab, Inc., U.S.A.) at 37 °C for 4 h. The digested fragments were detected by 2.5% agarose gel electrophoresis and visualized by staining with GelRed™ Nucleic Acid Gel Stain (Wako Chemicals, Japan).

RESULTS

Comparison of ITS1-5.8S-ITS2 Sequences The ITS region is ubiquitous in all organisms, presents as repeated units in high copy numbers and has both conserved and variable segments.[21] These qualifications aid in PCR amplification of certain DNA regions and also in molecular identification of various plant species. We determined nucleotide sequence of the ITS1-5.8S-ITS2 region of 17 specimens from four *Mitragyna* species distributed in Thailand. All specimens of the same species showed completely identical sequence despite of differences in collection places and morphological variations in leaf, revealing species specific property. The sequence alignment was shown in Fig. 2 and the border of each region was indicated. The ITS sequences of *M. diversifolia*, *M. hirsuta*, and *M. rotundifolia* were of 607 bp, while the 608 bp was specific to *M. speciosa*. The sequence of ITS1 was 228 bp in length and ITS2 was 216 bp or 217 bp. The length of the 5.8S coding region is 163 bp for all four species, ranging from nucleotide positions 230 to 392. Including 5.8S rDNA region, there were 22 variable sites among the four species, which included 19 sites of nucleotide substitutions and 3 sites of 1-bp indels. The sequence divergence among these species ranged from 1.82 to 2.64%. The same ITS sequences were obtained from *M. hirsuta* and *M. diversifolia*. However, the sequence divergence of *M. speciosa* from other three provided useful molecular marker for identification of this narcotic species. Nucleotides of ITS1 region at positions 42 and 101, as well as ITS2 region at positions 564 and 565 can distinguish completely *M. speciosa* from the other three species.

Phylogenetic Relationship in the Genus *Mitragyna* Based on the ITS sequences of the four species determined in the present study and five sequences obtained from GenBank, parsimony analysis produced six most parsimonious trees of 118 steps, with a consistency index (CI) of 0.8390 and a retention index (RI) of 0.6200. As shown in Fig. 3, strict consensus tree of the 6 most parsimonious trees divided the seven *Mitragyna* species into two main groups. The four Thailand species and *M. inermi* from Africa formed a big group, which separated from the other small group composed of *M. robrostipulata* and *M. stipulosa*, the two African species. *M. speciosa* placed in the former group as an individual branch.

Identification of a Narcotic Species, *Mitragyna speciosa* by PCR-RFLP Method Further to develop a simple method by which rapid examination can be performed in the forefront of drug control administrations, we tried to search for a restriction enzyme which can recognize the specific nucleotides in *M. speciosa*. A restriction enzyme *Xma* I was found to be able to recognize 6-bp sequence (**C**CCGGG) including the cytosine at position 42 from 5′-end of ITS1 regions in *M. speciosa*, while the other species with a transversion of guanine at this position resulted in absent of this recognize site (Fig. 4). Since in many samples, DNA was highly degraded into small pieces probably due to the oxidative and hydrolytic process during preservation period,[22,23] longer PCR products were difficult to be obtained. Therefore, a new primer set (ITS-1F located in 18S rDNA region and In-18S-25S-3′R located in 5.8S rDNA region) was designed, which produced a short fragment, to allow identification of crude drug samples. The PCR products from all species were generated with new primer set, giving a band of 409 bp. Subsequently, the PCR products were digested with *Xma* I. As shown in Fig. 4, only the PCR products from *M. speciosa* were cleaved into two fragments of 119 bp and 290 bp in length, while the other three species showed single band of 409 bp. Despite of the three types of *M. speciosa* specimens based on morphological variations of leaf, presented as red vein, green vein, and two spikes near the leaf apex, they all showed unambiguous digested patterns of *M. speciosa*.

DISCUSSION

The phylogenetic relationship constructed in the present study was consistent with that obtained by Razafimandimbison and Bremer[13] and further provided precise resolution within this genus. The two African species, *M. robrostipulata* and *M. stipulosa* from *Mitragyna* sensu lato which were formerly treated under genus *Hallea*,[24] revealed far relations from other species of *Mitragyna* sensu stricto which are mainly distributed in Asia except *M. inermis*, restricted to Sudanian regions, Africa. It was interested that *M. inermis*[13] showed close relation to the Asian species but far from African group. The ITS sequences of *M. inermis* (AJ346873) and *M. rotundifolia* (AJ346874) deposited in GenBank were identical to each other, whereas the ITS sequence of *M. rotundifolia* in our study was different from that by 13 bp substitutions. The similar intra-species difference was also observed between the sequences of *M. diversifolia*, *i.e.*, 11 sites

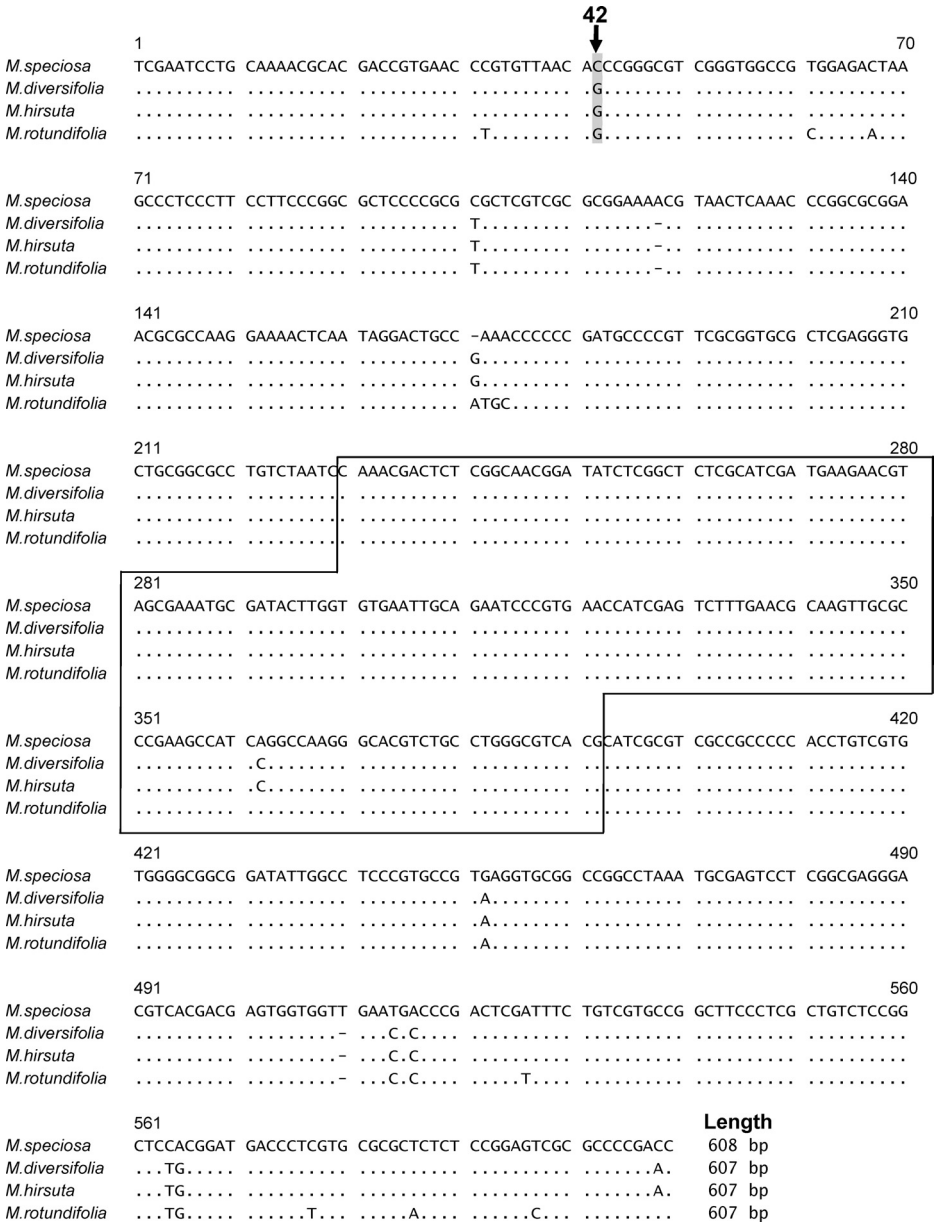

Fig. 2. The Sequence Alignments of ITS1-5.8S-ITS2 Region of *M. diversifolia*, *M. hirsuta*, *M. rotundifolia*, and *M. speciosa*

Position 1 is the 5′ end of ITS1. For *Mitragyna* species, the 5.8S region corresponds to positions 230—392 (enclosed in box); ITS1 1—229 and ITS2 393—609. The same sequences are indicated by dot (•). Gaps (–) are introduced for the best alignment.

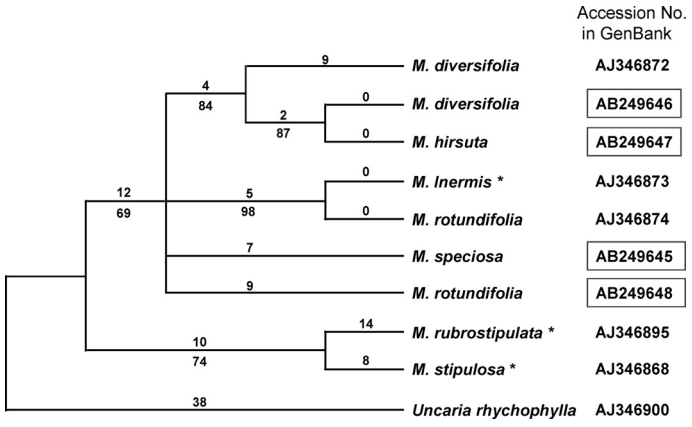

Fig. 3. Strict Consensus Tree of Six Most Parsimonious Trees Reconstructed on the Basis of ITS Sequence from *Mitragyna* Species

Tree length=118, CI=0.8390, RI=0.6200, RC=0.5202. Number above line is branch length, and number below line is the bootstrap value with 1000 replicates. Boxed accession number has been obtained by the present study. The African species are shown with asterisks.

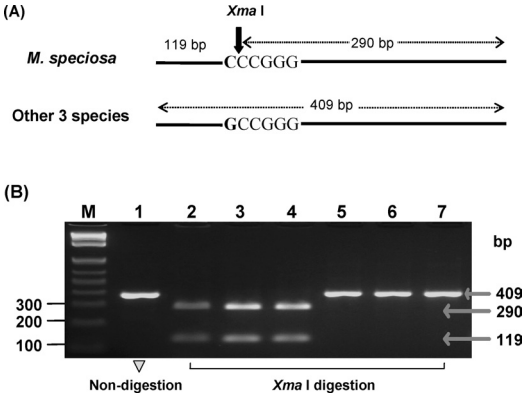

Fig. 4. PCR-RFLP Analysis Using Restriction Enzyme *Xma* I on Partial 18S-ITS1-5.8S Region

(A) *Xma* I restriction site in *Mitragyna speciosa* and other three *Mitragyna* species. Nucleotide with bold face indicates the defined marker nucleotide at position 42. (B) Agarose gel electrophoretogram of PCR product generated by primers ITS-1F and In-18S-25S-3′R, and then digested with *Xma* I (lanes 2—7); lane 1: *M. speciosa* (MS-11, Non-digestion), lane 2: *M. speciosa* (MS-11, Red-veined: Kan daeng), lane 3: *M. speciosa* (MS-14: Green-veined: Tang gua), lane 4: *M. speciosa* (MS-15, a pair of small teeth exerted near apex: Yak yai), lane 5: *M. diversifolia* (MD-2), lane 6: *M. hirsuta* (MH-18), lane 7: *M. rotundifolia* (MR-16), M: 1 kb plus DNA ladder.

of differences presented between our data and that deposited in GenBank (AJ346872). The specimens of *M. rotundifolia* and *M. diversifolia*, by which Razafimandimbison and Bremer[13] determined the sequences (Accession No. AJ346874 and AJ346872), have been collected from Thailand. These sequence variations might be due to intra-species polymorphism, however, more detailed investigations are needed to find out reasons behind these polymorphisms, including wide sampling of both species in Thailand, and the specimen verification between two research groups might need to be conducted.

Generally, three types of *M. speciosa* have been differentiated by the color of veins in leaf or by leaf morphology. It is said that the green-vein type has a stronger effect than red-vein type[25] and alkaloids content also varies among different batches and from place to place. The causes due to genetic diversity of the plant itself or geographical variant or other factors have not been undertaken yet. To investigate genetic diversity among populations as well as its relations to chemical compositions, further study should be applied to survey whole genome information by using such as AFLP, RAPD-PCR markers for discrimination of the different types of *M. speciosa*. However, nine samples of *M. speciosa* with typically morphological difference analyzed in the present study revealed the completely identical ITS sequence regardless of different phenotypic appearances and various locations, which provide molecular evidence to identify *M. speciosa* from other species.

In conclusion, we determined the ITS sequences of the four *Mitragyna* species existing in Thailand to obtain a molecular basis for authentication, and further established a convenient and rapid PCR-RPLF method for identification of the narcotic species, *M. speciosa*. The method is expected to be powerful for both medicinal and forensic uses.

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