THE STUDY OF ITS, *rbc*L, AND *trn*T-F REGIONS IN 'KRATOM' (*MITRAGYNA SPECIOSA* KORTH.) FOR FORENSIC IDENTIFICATION BY DNA

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Introduction

Mitragyna speciosa Korth., known as 'Kratom', is a indigenous narcotic plant to Thailand. *M. speciosa* has long been known to possess narcotic properties (1), which the leaf part was ingested for opium-like and coca-like stimulant ability to treat fatigue symptoms and to enhance tolerance for hard work under a scorching sun. This plant is used in folk medicine to stop diarrhea. It has also been used as a substitute in cases of opium and morphine addiction (1-3). There are two kinds of *M. speciosa* that are popular among Thai addicts, i.e., the red vein-type and the green vein-type Kratom. Both have been reported as having the same types of alkaloid (4). The pharmacological activities mitragynine, a major indole alkaloid, and its derivatives have been studied and reported that these compounds have opioid and morphine-like action (5-7).

There are few reports on the detection and identification of *M. speciosa*. The morphology is used for physical examination in the gross level. The analytical techniques to identify mitragynine, are thin layer chromatography (TLC), gas chromatography (GC) (8), and gas chromatography-mass spectrometry (GC-MS) (9). Razafimandimbison and Bremer reported the classification of the genus '*Mitragyna*' which belongs to the tribe Naucleeae (Rubiaceae) in 2002 by using molecular data (10). The study was based on the non-coding internal transcribed spacer (ITS) regions of nuclear rDNA, the protein coding *rbc*L and non-coding *trn*T-F regions of chloroplast DNA. The DNA sequence of the *M. speciosa*'s ITS region and *rbc*L gene were recently available in GenBank database, not the *trn*T-F region.

In this study, we propose three regions for DNA analysis in *M. speciosa* according to Razafimandimbison and Bremer (10). Universal primers of the ITS, *rbc*L and *trn*T-F regions are used for developing DNA markers by PCR amplification.

Objective

To develop DNA markers for identifying Kratom (*Mitragyna speciosa* Korth.) by isolating and evaluating the ITS, *rbc*L, and *trn*T-F regions.

Materials and methods

Plant materials

Kratom leaves were collected from various natural sources in Thailand, i.e., Sing Buri, Pathum Thani, Chumphorn, and Surat Thani province. Fresh leaves were frozen by liquid nitrogen and stored at -80°C prior to DNA extration. The use of plant was restricted to research purpose only.

DNA extraction and quantification

Total Kratom DNA was extracted using DNeasy Plant Mini Kit (QIAGEN, USA) following the manufacturer's protocol. Then, the quantity of extracted DNA was estimated by using GeneSnap software (Syngene, UK).

DNA amplification

Three universal primer pairs of the ITS regions for nuclear rRNA gene, *rbc*L and non-coding *trn*T-F regions of chloroplast DNA were used in this study to amplify the ITS, *rbc*L, and *trn*T-F regions for Kratom DNA analysis. The PCR amplifications were conducted in a GeneAmp PCR system 9700 (Applied Biosystems, Sweden).

ITS regions

The ITS1, 5.8S rRNA gene, and ITS2 were completely amplified using the forward primer: ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and the reverse primer: ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (11). PCR for the ITS was performed in a total volume 25 µl reaction containing 15 ng of genomic DNA, 0.2 mM of dNTPs (Promega, USA), 1X PCR buffer (Applied Biosystems, Sweden), 1.5 mM of $MgCl_2$ (Applied Biosystems, Sweden), 1 unit of AmpliTaq® DNA polymerase (Applied Biosystems, Sweden), 10 pmol of each primer, and sterile deionized water. The following thermocycle condition was applied an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, final extension was carried out at 72°C for 10 min. The amplified product size was about 700 bp

*rbc*L gene

The *rbc*L gene region was amplified using the forward primer (5′-TGT CAC CAC AAA CAG AAA CTA AAG CAA GT-3′) and the reverse primer (5′-CTT TTA GTA AAG ATT GGG CCG AG-3′), correspond to the first part and a region approximately 100 bp downstream from the coding region (12). PCR amplificaton was performed in a total volume of 50 µl reaction containing 1 ng of genomic DNA, 0.4 mM of dNTPs (Promega, USA), 1X PCR buffer (Applied Biosystems, Sweden), 1.5 mM of $MgCl_2$ (Applied Biosystems, Sweden), 1.25 unit of AmpliTaq® DNA polymerase (Applied Biosystems, Sweden), 20 pmol of each primer, and sterile deionized water. The following thermocycle condition was applied an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1.30 min, and extension at 72°C for 2 min, and final extension was carried out at 72°C for 7 min. The amplified product was approximately 1.5 kb.

*trn*T-F regions

The *trn*T-F region was amplified by using the forward primers: *trn*T-F_aF (5′-CAT TAC AAA TGC GAT GCT CT-3′) and the reverse primer: *trn*T-F_fR (5′-ATT TGA ACT GGT GAC ACG AG-3′) (13). The PCR amplification was performed in a total volume of 50 µl reaction containing 50 ng of genomic DNA, 0.4 mM of dNTPs (Promega, USA), 1X PCR buffer (Applied Biosystems, Sweden), 2.5 mM of $MgCl_2$ (Applied Biosystems, Sweden), 1.25 unit of AmpliTaq® DNA polymerase (Applied Biosystems, Sweden), 1X Q-solution (QIAGEN, German), 50 pmol of each primer, and sterile deionized water. The following thermocycle condition was applied an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1.30 min, and extension at 72°C for 1.30 min, and final extension was carried out at 72°C for 7 min. The amplified product size was approximately 1.8 kb.

DNA sequencing

The PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, USA) before submitting for DNA sequencing. The PCR products were sequenced by BioService Unit (BSU, Bangkok) or Mahidol University-Osaka University Collaborative Research Center for Bioscience and Biotechnology (MU-OU: CRC, Bangkok). Both forward and reverse primers were used as sequencing primers for all three regions. In addition, *trn*T-F_iR (5′-CCA ACT CCA TTT GTT AGA AC-3′) primer was used as an internal sequencing primer for the *trn*T-F region.

DNA sequence analysis

ClustalW alignment algorithm at BioEdit v 7.0.7 program were used to multiple aligned the sequences of the three regions, i.e., ITS, *rbc*L and *trn*T-F. The sequences were also compared with other sequences of the same genus in GenBank database.

Results

DNA amplification

DNA amplifications of ITS, *rbc*L, and *trn*T-F regions were obtained for studied samples. The fragments of PCR products are shown in Figure 1. The size of amplified fragments of ITS, *rbc*L, and *trn*T-F regions were approximately 700, 1500, and 1800 bp, respectively.

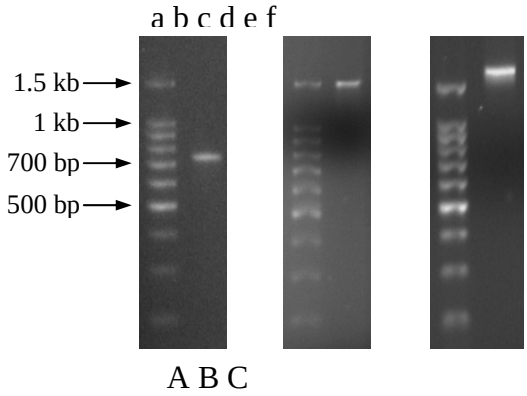

Figure 1. 2% (w/v) Agarose/ TBE gel depicting 3 PCR-amplified products. Figure A shows the 700-bp PCR product of ITS region in lane b; 1.5-kb PCR product of *rbc*L region in lane d (Figure B), and the 1.8-kb PCR products of *trn*T-F region in lane f (Figure C). Lane a, c, and e are the 100-bp DNA ladder.

Sequence analysis

Comparison of ITS regions

One of each of the 608-bp ITS sequences of the red-vein and green-vein *M. speciosa* were compared. Sequence alignment showed 100% similarity between the two samples. The positions corresponding to ITS1, 5.8S, and ITS2 regions are positions 1 to 228 (228 bp), 229 to 391 (163 bp), and 392 to 608 (217 bp), respectively. The GC content is 63.65%. These two sequences were compared to the *M. speciosa* ITS sequence which was recently deposit in GenBank by Sukrong *et al*.(14) Sequence comparison also showed 100% similarity between the 608 bp of three *M. speciosa* ITS sequence.

ITS sequence of *M. speciosa* was then compared with sequences of other members of the genus *Mitragyna*, i.e, *M. diversifolia* (Accession No. AB249646 and AJ346872), *M. hirsuta* (Accession No. AB249647), *M. inermis* (Accession No. AJ346873), *M. rotundifolia* (Accession No. AB249648 and AJ346874), *M. rubrostipulata* (Accession No. AJ346895), *M. speciosa* (Accession No. AB249645), and *M. stipulosa* (Accession No. AJ346868). The length of the ITS sequences are in the range of 604-608 bp, with GC content between 63.25-63.92%. Pairwise alignment scores ranged from 95 to 99, indicating that there are differences within the ITS region between members of the genus. There are 65 separate polymorphisms within the ITS region, which consisted 5 indels and 60 nucleotide substitutions. It is shown that there are 5 polymorphisms when compared *M. speciosa* with other members of the genus. These are presented at positions 42 and 101 of ITS1 region and positions 452, 516, and 564 of ITS2 region, see Figure 2.

		.|.... 40	..|... 100	.|.... 450	|....|. 520	.|....|. 560
M.speciosa	Red-vein leaf	ACACCC	GCGCGC	CGTGAG	GACCCGA	GGCTCCAC
M.speciosa	Green-vein leaf	.....	.....	.....	.....	.....
M.speciosa	AB249645	.....	.....	.....	.....	.....
M.diversifolia	AB249646	...G..	...T..	...A..	.C....	.....TG.
M.diversifolia	AJ346872	...G..	...T..	...A..	.C....	.....TG.
M.hirsuta	AB249647	...G..	...T..	...A..	.C....	.....TG.
M.inermis	AJ346873	...G..	...T..	...A..	.C....	..G..T..
M.rotundifolia	AB249648	...G..	...T..	...A..	.C....	.....TG.
M.rotundifolia	AJ346874	...G..	...T..	...A..	.C....	..G..T..
M.rubrostipulata	AJ346895	...G..	...T..	...T..	.C....	.....TG.
M.stipulosa	AJ346868	...G..	...T..	...T..	.C....	.....TG.

Figure 2. The sequence alignment of ITS region of members of the genus *Mitragyna,* including the red-vein and green-vein *M. speciosa*. Comparing the sequences to the consensus sequence, nucleotide substitutions are shown at positions 42, 101, 452, 516, and 564.

Comparison of *rbc*L gene

Each of the red-vein and green-vein *M. speciosa rbc*L PCR-amplified product were sequenced. Length of the sequences were 1368 and 1405 bp, with a GC content of 44.30 and 44.06%, respectively. These were only part of the *rbc*L gene region. Sequence comparison showed a single nucleotide gap at position 16 in the green-vein type, and nucleotide substitutions at position 1250 and 1283 of red-vein and green-vein samples, respectively (see Figure 3).

The two *rbc*L sequences were also compared with the *rbc*L sequences of members of the genus *Mitragyna* in GenBank, i.e., *M. diversifolia* (Accession No. AJ346985), *M. inermis* (Accession No. AJ346986), *M. rotundifolia* (Accession No. AJ346987), *M. rubrostipulota* (Accession No. AY538486 and X83640), *M. speciosa* (Accession No. AJ346988), and *M. stipulosa* (Accession No. AJ346981). The sequences were 1407 and 1411 bp in length, with GC content between 43.66-44.08%. There are 27 nucleotide polymorphisms within the *rbc*L region of *Mitragyna*. When compared the sequences between the three *M. speciosa* sequences, there were two single-nucleotide polymorphisms at positions 1171, 1289 (see Figure 3).

		.|....|.	.|...	..|..
		20	1170	1290
M.speciosa	Red-vein leaf	--------	GTACT	TTCTG
M.speciosa	Green-vein leaf	AA-GCTGG	.....	.G...
M.speciosa	AJ346988	AAAGCTGG	..C..	.G...
M.diversifolia	AJ346985	AAAGCTGG	.....	.....
M.inermis	AJ346986	AAAGCTGG	.....	.G...
M.rotundifolia	AJ346987	AAAGCTGG	.....	.G...
M.rubrostipulata	X83640	AAAGCTGG	.....	.G...
M.rubrostipulata	AY538486	AAAGCTGG	.....	.G...
M.stipulosa	AJ346981	AAAGCTGG	.....	.G...

Figure 3. Sequence comparison of the *rbc*L gene region of members of the genus *Mitragyna*, including the red-vein and green-vein types. Sequence comparison showed an indels at positions 16, and nucleotide substitutions at 1171 and 1289.

Comparison of *trn*T-F regions

The *trn*T-F region is approximately 1.8-kb long. Sequences obtained from the forward and reverse primers did not overlap; hence an internal sequencing primer was then used to retrieve the sequences within the gap left in one sample. The nucleotide sequence of a red-vein sample of *M. speciosa* was 1,689 bp long, with a GC content of 31.44%. The other 5 samples (2 red- and 3 green vein types) were sequenced from the 5' and 3' end, leaving approximately 200-base gap between the two regions. Sequence comparison of the 5'- and 3'- regions indicated a single base indels at position 107 in five samples, and a 6-bp indels from 676-681 in one of each green- and red-vein type sample. In addition, nucleotide substitutions were presented at positions 1012, 1386, and 1440 (Figure 4). These demonstrated the variation of nucleotide sequences within the species (intra-species variation).

The 1689-bp *trn*T-F sequence of *M. speciosa* was then compared with other members of the genus *Mitragyna* available in GenBank, i.e., *M. diversifolia* (Accession No. AJ346931), *M. inermis* (Accession No. AJ346932), *M. rotundifolia* (Accession No. AJ346933), *M. rubrostipulota* (Accession No. AJ346957), and *M. stipulosa* (Accession No. AJ346927). The length of sequences were between 1731-1734 bp and GC contents were between 31.85-32.06%. Sequence alignment showed 98-99% similarity. There were 35 polymorphic sites within *trn*T-F region, which included 13 indels and 22 nucleotide substitutions. There were 7 nucleotides in *M. speciosa*, which were different from other species; a single base indels presented at positions 107, and a 6-bp long indels presented at 676 to 681 (see Figure 5).

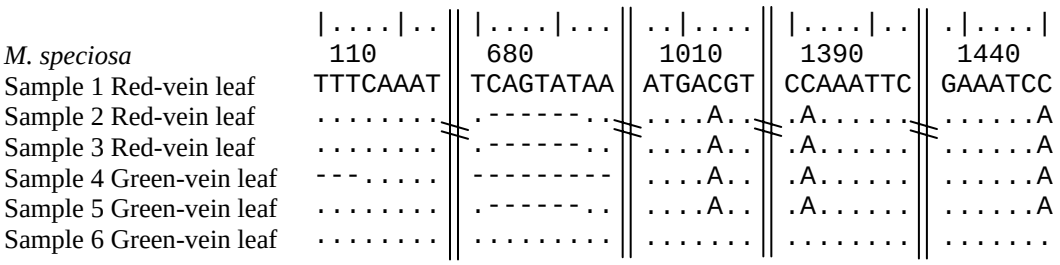

Figure 4. The sequence alignments of *trn*T-F region of red-vein and green-vein samples of *M. speciosa*, showed the consensus base at position 107, the 6-bp indels at positions 676-681, and the single base substitutions at positions 1012, 1386, and 1440 of nucleotide sequences.

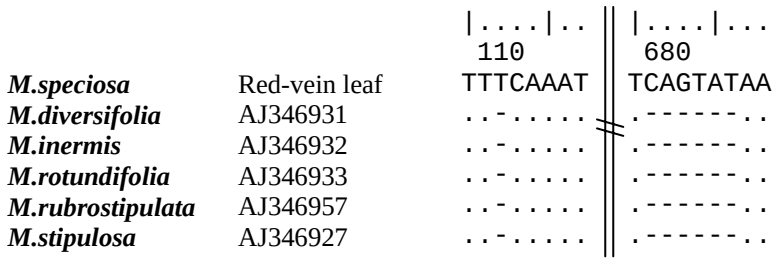

Figure 5. The sequence alignments of *trn*T-F region of *Mitragyna* species between studied samples, red-vein sample and other *Mitragyna* species available in GenBank with Accession No. AJ346931, AJ346932, AJ346933, AJ346957, and AJ346927, showed a single base indels at position 107 and 6-bp of indels at positions 676-681.

Discussion

Sequences of the ITS, *rbc*L and *trn*T-F regions of members of the genus *Mitragyna* showed approximately 95% similarity in all three regions. The 5 nucleotide polymorphism in the ITS region of *M. speciosa* are specific to the species. Recently, Sukrong *et al.* (14) utilized one of the polymorphic site for identification of *M. speciosa*, by identifying a restriction enzyme to cut within the amplified ITS1 PCR-product to differentiate *M. speciosa* from the other members of the genus. The *rbc*L gene region is highly conserved, this may be because of the low evolutionary rate of nucleotide substitution, which is a characteristic of chloroplast DNA sequence (13), and the difference of origin (location) which the samples were collected. There is one single base indels present in the *trn*T-F region of 6 individual Kratom samples seems to be an informative polymorphic position. Moreover, the intra-species polymorphisms were found in these two regions, though sequence from more than one individual sample was analyzed. These suggested that when a polymorphism is detected within a sequence, it is necessary to determine if the polymorphic sequence is conserved within the species, not being just an intra-species variation. As a consequence, in order to determine DNA loci for species determination, it is necessary to test the loci against a number of individuals within the species.

Conclusion and Suggestion

The ITS, *rbc*L, and *trn*T-F regions of *M. speciosa* could be amplified by the universal primers. Sequence comparisons revealed no distinguishing character between the red- and green-vein Kratom types. The single nucleotide polymorphisms are detected to be spreading along the length of the loci of interest. However, species-specific primers could not be developed for identifying *M. speciosa* from these 3 regions. Nevertheless, single nucleotide polymorphisms (SNPs) technique may be applied to develop N-labeled primers from these nucleotide positions, which showed specificity in *M. speciosa* ITS and trnT-F regions.

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