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DNA barcoding for authentication of the endangered plants in genus *Aquilaria*

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ABSTRACT

Introduction: Agarwood is a type of wood that is used in traditional Thai medicines and has been applied in several recipes; in particular, this wood is considered as a costly resinous non-wood product. However, the morphological characteristics of the plant in the genus *Aquilaria* are quite similar, and so the authentication steps need a botanical expert for the purposes of identification and then also require an efficient tool for species-level discrimination. DNA barcoding is an approved molecular technique which uses a short sequence as a barcoding region specific for identified species. **Materials and Methods:** In this study, we established the DNA barcoding sequences from six candidate of barcoding loci (ITS, *mat*K, *rbc*L, *rpoC*1, *psb*A-*trn*H intergenic spacer, and ycf1) from three species of *Aquilaria*, and the outgroup (*Enkleia siamensis* (Kurz) Nervling). The phylogenetic tree of each locus was reconstructed and the genetic distances were also determined using a maximum likelihood method. **Results:** Our results showed that all samples in each locus were successfully amplified and sequenced with universal primers. The ITS was only performed with suitable markers for *Aquilaria* species identification, which were considered from ML phylogenetic tree reconstruction and the optimum length of genetic distance. Other cpDNA barcoding regions; *mat*K, *rbc*L, *rpoC*1, *ycf*1 and *trn*H-*psb*A, showed lower discrimination power, so these loci were deemed inappropriate for species identification. Notwithstanding, each barcoding locus can be suitable for genus discrimination in the Thymelaeaceae family. **Conclusion:** From our findings, DNA barcoding is able to be used as an efficient and reliable tool in *Aquilaria* species authentication, which can be applied for quality control in the agarwood in the global market.

Keywords: *Aquilaria*, authentication, DNA barcode, phylogenetic tree, Thymelaeaceae

INTRODUCTION

Aquilaria is a genus of endangered flowering plants which are a part from Thymelaeaceae family. These plants have been utilized in Ayurvedic medicine, Traditional Chinese Medicine, and Traditional Thai Medicine for centuries and are renowned for fragrant production from its wood. Plants in genus *Aquilaria* accounted for 21 species and are only found in China and Southeast Asian countries, such as Malaysia, Thailand, Vietnam, and Indonesia;[1] however, the Flora of Thailand reported that there are only four *Aquilaria* species available in Thailand.[2] At present, the environmental source of agarwood typically impacts consumer preference. This is most suggestive of the species maturing in specific areas and, as a result, the expected quality of agarwood resin. Since merchants and potential buyers think agarwood is derived from a distinctive *Aquilaria* species and has characteristic fragrance with therapeutic features, the market prices are settled accordingly. As an example, the pleasantness of agarwood from *Aquilaria crassna* (AC) Pierre ex Lecomte when burnt is favored for certain ceremonies,[3] while those living in the Middle East tend to prefer essential oils taken from *Aquilaria malaccensis* (AM) Lam. due to its robust smell compared to other species.[4] The *Aquilaria subintegra* (AS) Ding Hou was also mercenarily cultivated in the eastern area of Thailand. However, its quality of essential oil, in terms of fragrances, is lower than that of AC.[5] Thus,

a vital act for ensuring quality for a client is the validation step. Furthermore, typically employed for plant validation together with chemical profile assessments are morphological examinations and cytological determinations.[6,7] Then again, CITES has been proposed for improvement of the *Aquilaria* species classification process, which is primarily based on floral and fruit attributes compared to other methods and relies on a fast and precise detection system.[8]

The molecular techniques are utilized productively to support the morphological information for the authentication process.[9] DNA barcoding is an approved molecular technique which uses a short region as a barcoding region for species discrimination.[10,11] DNA barcoding is recognized as a powerful technique for species discrimination in plants and also enables experts to resolve the relationships among taxa, forensic identification, and species authentication of endangered species, and medicinal plant materials.[12,13] Regarding the Metazoa, the mitochondria gene cytochrome c oxidase I (*CO*I) is widely affirmed as the universal barcode used for all species.[10] On the other hand, the *CO*I gene was notified as an improper region for higher plants because of the species hybridization and slower mutation rate of mitochondria DNA.[14,15] For the land plant, the chloroplast gene region; the ribulose-bisphosphate carboxylase (*rbc*L) gene and the maturase K (*mat*K) gene have been properly suggested as a core region for plant barcoding analysis.[16] In addition, for the non-coding region, the nuclear internal transcribed spacer (ITS) region and the plastid *psb*A-*trn*H intergenic spacer are potentially practicable regions for angiosperm barcoding.[14] Lately, the *ycf*1 plastid gene was also recommended for land plants discrimination.[17] Based on various research studies on plants, the barcode which was generated from the combination of several loci was able to exhibit greater discrimination power than the single-locus barcode.[18] Hence, our research aimed to investigate the efficient DNA barcoding loci and suggested which ones are the most suitable for *Aquilaria* species identification.

MATERIALS AND METHODS

Plant Materials

Eight specimens of three *Aquilaria* species were collected from various sources and *Enkleia siamensis* (ES), the plant in the Thymelaeaceae family was included to serve as outgroups. All samples were authenticated by Associate Professor Nijsiri Ruangrungsi, Ph.D. Information of the collected samples was listed in Table 1. Plant specimens were preserved at College of Public Health Sciences, Chulalongkorn University, Bangkok, Thailand.

Genomic DNA Extraction

Total genomic DNA was extracted by the DNeasy™ plant Mini Kit (Qiagen, Germany) and the extraction process followed the manufacturer's recommendations. In summary of the process, fresh leaves were ground in liquid nitrogen using mortar and pestle. The lysis buffer was added and then incubated at 65°C for 1 h. The lysate was applied to a spin column to remove precipitates and cell debris by centrifugation. The flow-through fraction was brought to a mini-spin column. The mini-spin column was centrifuged, washed and eluted with AE buffer. Finally, the DNA quality and quantity were ascertained using agarose gel electrophoresis. All DNA samples were preserved at −20°C before the further experiment.

Polymerase Chain Reaction (PCR) Amplification and DNA Sequencing

DNA amplification was performed for the standard barcoding region. Primer sequences and the references of each locus were tabulated in Table 2. These universal primers were used for amplifying four coding cpDNA loci; *rbc*L, *mat*K, *rpo*C1, and *ycf*1, one non-coding cpDNA loci; *psb*A-*trn*H intergenic spacer, and the nDNA loci; ITS regions. The 25 μl PCR reaction mixtures were comprised ×1 PCR buffer with 2 mM $MgCl_2$, 0.4 mM dNTPs, 1.0

Table 1: Plant materials and their respective accession numbers

Species	Geographical location	Voucher No.	Accession number					
			ITS	*mat*K	*rbc*L	*rpo*C1	*psb*A-*trn*H intergenic spacer	*ycf*1
AC Pierre ex Lecomte	Bangkok, Thailand	AQWT01	LC384009	LC383997	LC383710	LC383849	LC384006	LC384001
	Rayong, Thailand	AQWT02						
	Nan, Thailand	AQVT03						
AM Lam.	Pattalung, Thailand	AMNN01	LC384010	LC383998	LC383712	LC383850	LC384005	LC384002
	Tanglin, Singapore	AMWT02						
	Tanglin, Singapore	AMWT03						
AS Ding Hou	Trat, Thailand	ASTS01	LC384011	LC383999	LC383711	LC383851	LC384007	LC384003
	Trat, Thailand	ASTS02						
ES (Kurz) Nervling	Loei, Thailand	ESKL01	LC384012	LC384000	LC383713	LC383852	LC384008	LC384004

AC: *Aquilaria crassna,* AM: *Aquilaria malaccensis,* AS: *Aquilaria subintegra,* ITS: Internal transcribed spacer, *mat*K: Maturase K, *rbc*L: Ribulose-bisphosphate carboxylase

Table 2: Information of PCR primers used for DNA barcoding study

Region	Name of primer	Sequence (5'→ 3')	References
ITS	ITS5_F	GGA AGT AAA AGT CGT AAC AAG G	[20]
	ITS4_R	TCC TCC GCT TAT TGA TAT GC	
*mat*K	3F-KIM f	CGT ACA GTA CTT TTG TGT TTA CGA G	[21]
	1R-KIM r	ACC CAG TCC ATC TGG AAA TCT TGG TTC	
*psb*A-*trn*H intergenic spacer	psbA3f	GTT ATG CAT GAA CGT AAT GCT C	[22]
	trnHf-05	CGC GCA TGG TGG ATT CAC AAT CC	[23]
*rbc*L	rbcL-aF	ATG TCA CCA CAA ACA GAG ACT AAA GC	[24]
	rbcL-aR	CTT CTG CTA CAA ATA AGA ATC GAT CTC	[25]
*rpo*C1	2F	GGC AAA GAG GGA AGA TTT CG	[26]
	4R	CCA TAA GCA TAT CTT GAG TTG G	
*ycf*1	ycf1bF	TCT CGA CGA AAA TCA GAT TGT TGT GAA T	[17]
	ycf1bR	ATA CAT GTC AAA GTG ATG GAA AA	

PCR: Polymerase chain reaction, ITS: Internal transcribed spacer, *mat*K: Maturase K, *rbc*L: Ribulose-bisphosphate carboxylase

unit of Invitrogen® *Taq* DNA polymerase (Invitrogen, USA), and 20 ng of genomic DNA. The PCR reaction mixture was incubated using the following conditions: 95°C for 5 min; 30 cycles of 95°C for 30 s, 52°C for 1 min, and 72°C for 1 min (for ITS, *trn*H-*psb*A, *rpo*C1, and *ycf*1) or 2 min (for *mat*K and *rbc*L); and final extension at 72°C for 10 min. The amplicons were examined using agarose gel electrophoresis in TAE buffer and were visualized by SYBR® safe staining. The amplified specific regions were purified using AccuPrep® PCR Purification Kit (Bioneer, Republic of Korea) before undergoing sequencing procedure. The successful PCR products were subjected to sequence investigation on an ABI 3730XL DNA analyzer. DNA sequences in FASTA format from each locus were ascertained and edited using ATOM text editing software and also used MUSCLE in Seaview software version 4.6.5 as a tool for alignment process.[19] The annotated sequences were then registered to DDBJ. The accession numbers of the sequences are noted in Table 1.

Sequence Interpretation

The obtained sequences from each region were aligned using a multiple sequence alignment tool; MUSCLE (Multiple Sequence Comparison by Log-Expectation).[27] These six barcoding regions and their combination were determined in terms of genetic distance, phylogenetic relationship, and also the species discrimination level. The genetic distances were computed using the Kimura 2-parameter distance method in MEGA7.0.[28,29] The phylogenetic trees were created using the maximum likelihood (ML) method based on the Kimura 2-parameter model[30] with 1,000 bootstrap re-samplings in MEGA 7.0; in addition, the highest log likelihood was calculated from this program to show the suitably of the tree. The efficiency of the candidate loci was investigated through phylogenetic tree-based analysis and sequence determination.

RESULTS

Sequence Analysis of the Barcoding Region

The six barcoding regions of each sample were successfully amplified and sequenced. All specimens of the selfsame species performed entirely identical sequences in spite of different places of collection. The 24 nucleotide sequences of these three species in genus *Aquilaria*, and the outgroup; ES was obtained, and the alignment information in each locus was displayed in supplementary data. The DNA barcoding amplifications for ITS, *mat*K, *rbc*L, *rpo*C1, *psb*A-*trn*H intergenic spacer, and *ycf*1 showed the length of the amplicons to be in the ranges of approximately 682–745 bp, 812–818 bps, 1203 bp, 529–531 bp, 441–468 bp, and 846–850 bp, respectively [Table 3]. The variable sites and gaps of these four species were found in all barcoding regions.

Determination of Genetic Distance and Phylogenetic Tree Analysis

The genetic distances (D value) of each locus were tabulated in Table 4, while the distances of combination among three plastid regions (*mat*K + *psb*A-*trn*H intergenic spacer + *ycf*1) were shown in Table 5. The ML tree based on barcoding sequences in each of the regions was displayed in Figure 1. In addition, the combination of three plastid regions was constructed using ML method and was shown in Figure 2. Our phylogenetic tree data could be divided into two groups: Clustering and non-clustering group. The clustering group which consisted of three *Aquilaria* species could classified into two clades. The first clade comprised AC and AS, while AM was classified in the other clade. This clustering group composed of the cladogram from ITS sequences, *mat*K gene, *rbc*L gene, *psb*A-*trn*H intergenic spacer, and *ycf*1 gene. For non-clustering group, the cladogram of *rpo*C1 gene sequence was arranged in this group because all three *Aquilaria* species were considered as one clade.

DISCUSSION

Nowadays, the application of traditional medicine is internationally recognized as an effective treatment for many ailments. Thailand's practice dates back to prehistoric times where indigenous, regional practices were performed, and its development can be traced through history until the present day. In addition, herbal products have long been promoted

Table 3: Evaluation of the six DNA barcode loci

Locus	Parameter assessed					
	Full/partial	Sequence length	Aligned length	No. of variable site	% Variation	Log likelihood of tree
ITS region	Partial	682–745	745	20	2.92	−1476.87
*mat*K gene	Partial	812–818	818	5	0.62	−1359.39
*rbc*L gene	Partial	1203	1203	3	0.25	−1834.40
*rpo*C1 gene	Partial	529–531	531	0	0.00	−805.16
*psb*A-*trn*H intergenic spacer	Partial	441–468	468	1	0.23	−787.18
*ycf*1 gene	Partial	846–850	850	2	0.24	−1525.74

ITS*:* Internal transcribed spacer, *mat*K: Maturase K, *rbc*L: Ribulose-bisphosphate carboxylase

Table 4: Genetic distances of all studied samples based on each locus using MEGA 7.0

ITS					***mat*K**				
	AC	**AM**	**AS**	**ES**		**AC**	**AM**	**AS**	**ES**
AC	-				AC	-			
AM	0.0326	-			AM	0.0062	-		
AS	0.0016	0.0308	-		AS	0.0000	0.0062	-	
ES	3.0483	3.1453	3.0413	-	ES	0.0512	0.0580	0.0512	-
***rbc*L**					***rpo*C1**				
	AC	**AM**	**AS**	**ES**		**AC**	**AM**	**AS**	**ES**
AC	-				AC	-			
AM	0.0008	-			AM	0.0000	-		
AS	0.0017	0.0025	-		AS	0.0000	0.0000	-	
ES	0.0211	0.0220	0.0211	-	ES	0.0251	0.0251	0.0251	-
***psb*A-*trn*H intergenic spacer**					***ycf*1**				
	AC	**AM**	**AS**	**ES**		**AC**	**AM**	**AS**	**ES**
AC	-				AC	-			
AM	0.0024	-			AM	0.0024	-		
AS	0.0000	0.0024	-		AS	0.0012	0.0036	-	
ES	0.0952	0.0918	0.0952	-	ES	0.1195	0.1195	0.1210	-

Analysis was conducted using the maximum composite likelihood model. AC: *Aquilaria crassna,* AM: *Aquilaria malaccensis,* AS: *Aquilaria subintegra,* ES: *Enkleia siamensis,* ITS*:* Internal transcribed spacer, *mat*K: Maturase K, *rbc*L: Ribulose-bisphosphate carboxylase

Table 5: Estimates of evolutionary divergence between combination sequence of chloroplast genome

Combination of chloroplast barcoding regions (*mat*K+p*sb*A-*trn*H intergenic spacer+*ycf*1)			
	AC	**AM**	**AS**	**ES**
AC	-			
AM	0.0039	-		
AS	0.0005	0.0044	-	
ES	0.0785	0.0809	0.0791	

The number of base substitutions per site from between sequences was shown. Analysis was conducted using the maximum composite likelihood model. AC: *Aquilaria crassna,* AM: *Aquilaria malaccensis,* AS: *Aquilaria subintegra,* ES: *Enkleia siamensis, mat*K: Maturase K

in terms of dispensing in the Thai health-care system and, in support of this; the Thai FDA has recorded a list of medicinal plants in the National List of Essential Medicines 2017. However, the major problem of using medicinal plant products has been the process of authentication. For several decades, the identification procedure of plant materials was evaluated using morphological characteristics, but some of the herbal raw materials were substituted, adulterated or contaminated with forged materials to minimize costs. Thereby, these low-quality materials might influence the degree of therapeutic effectiveness which leads to problems regarding patient safety. Thus, the authentication step is a crucial process to control quality and certify the efficacy and safety of such herbal products. It is also worth noting that molecular techniques are used as an approach for supporting in this essential step through various DNA fingerprinting techniques, such as PCR-RFLP, AFLP, microsatellite, and DNA barcoding.

DNA barcoding is one of the molecular methods which use small DNA regions for organism discrimination. For plant creatures, there are various recommended regions which can be used as barcodes for identifying plants. First of all, the CBOL

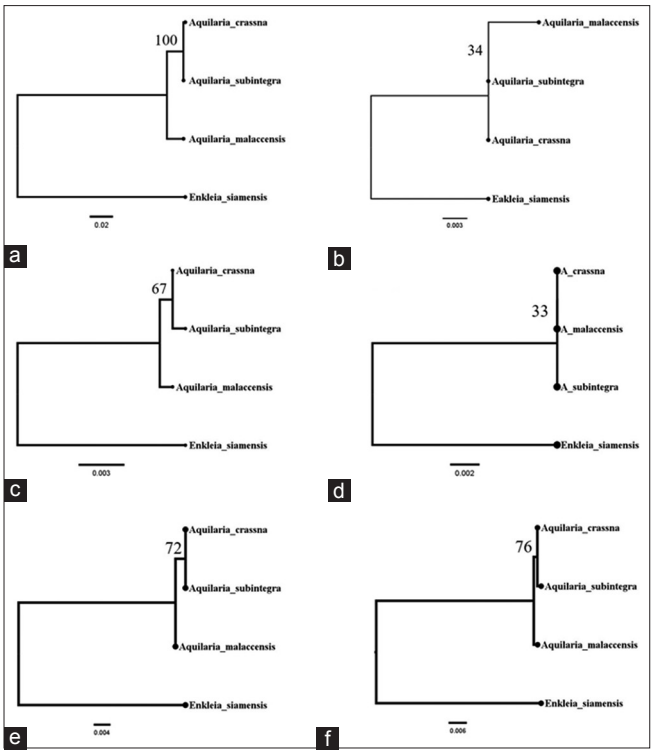

Figure 1: Maximum likelihood tree constructed from the sequences of ITS region (a), *mat*K gene (b), *rbc*L gene (c), *rpo*C gene (d), *psb*A-*trn*H intergenic spacer (e), and *ycf*1 gene (f)

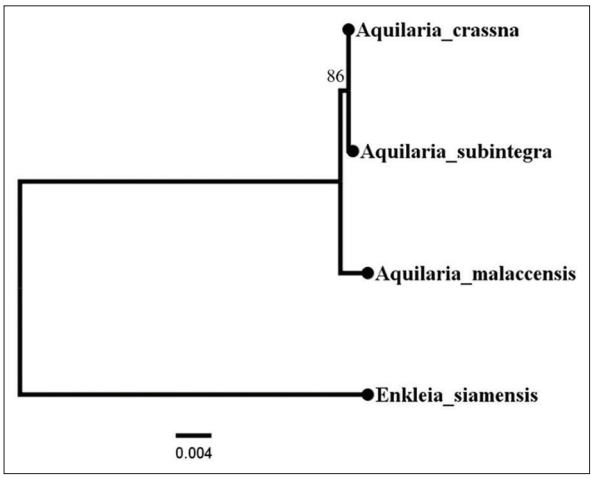

Figure 2: Maximum likelihood tree based on the combined sequences data of *mat*K, *trn*H-*psb*A, and *ycf*1. The tree with the highest log likelihood (−6,319.66) was shown

Plant Working Group recommended *rbc*L and *mat*K gene as a plant barcode to identifying plants at species-level.[16] In our study, the results from the phylogenetic tree reconstruction of the *rbc*L gene among *Aquilaria* species found that AM was separated from those two species, but the genetic distances were insignificantly different. Thus, the *rbc*L gene barcode was unsuitable for species identification because of its low discrimination power. While the genetic distance of the *mat*K gene sequences in AC and AS, which was calculated based on a sequences comparison, could explain why these two species were so closely related (D value = 0.000). However, AM displayed a slight difference from those two species (D value = 0.002). These two recommended barcoding regions were unsuitable for use as *Aquilaria* species identification. In addition, this implication conformed to Jiao *et al*.'s conclusion,[31] which noted that the *rbc*L and *mat*K sequence data among *A. sinensis* and close species were unsuitable for cladogram reconstruction as well as species identification.

The non-coding *psb*A-*trn*H spacer region was also endorsed as a global land plant barcode that could be utilized for discrimination at species-level.[32] Our results revealed that the genetic distance of the intergenic spacer of *psb*A-*trn*H was similar to the result from the *mat*K gene, but the phylogenetic tree characteristic was different due to the percentage variable regions. In addition, the positions of the variable site and their percentages were critical for phylogenetic tree reconstruction.[33] Hence, our results implied that *psb*A-*trn*H intergenic spacer was unsuitable for *Aquilaria* species identification due to its low discrimination power.

In addition, Chase *et al*. (2007) suggested that the combination of two plastid barcoding regions among the *mat*K gene, non-coding region of *psb*A-*trn*H, *rpo*B, and the *rpo*C gene was suitable for land plants.[34] It was mentioned that the *rpo*C1 gene sequences could be utilized for the determination of universality and/or sequence quality.[16] In our work, all *Aquilaria* sequences were also identical in each one of the nucleotides. The *rpo*C1 region was unsuitable for species authentication because of its non-existent discrimination power; however, it could be used as a quality control marker from a high percentage of successfully amplified and sequenced properties.

Recently, the *ycf*1 gene has been proposed as an effective barcoding marker because this gene was conserved but showed a high amount of variables in angiosperm.[35] This gene could potentially be used for phylogenetic determination in various plants, such as pines,[36] orchids.[37] In ML tree analysis, each of the species was separated into branches with a high bootstrap value. This gene was an interesting cpDNA barcoding region in *Aquilaria* species discrimination due to the variable regions, but the genetic distances among *Aquilaria* species were too low. Hence, only the cpDNA barcode region is inappropriate to use for identifying *Aquilaria* plants which are in agreement with the previous findings.[18] The combination of three barcoding regions; *mat*K, *psb*A-*trn*H intergenic spacer, and *ycf*1, determined the genetic relationship using ML tree, but it exhibited low genetic distances. Hence, we could suggest that the only one loci or combination loci of *mat*K, *trn*H-*psb*A, and *ycf*1 region can be classified into only two groups; AM and a group of both AC and AS.

The nuclear DNA not only performs as a barcoding loci but it is also used for molecular systematic investigation in species-level identification.[14] The ITS region among the nuclear ribosomal cistron is recommended as probable barcoding in plants, fungi, and bacteria.[38,39] In our work, there were some variable sites which could be utilized in the specification of different species which were able to distinctly separate AM apart from those two species. The genetic distance from AC and AS sequences, in comparison, was too low, but some variable sites could be used for distinguishing at species-level. Thus, the ITS region was an optimum tool for resolving the *Aquilaria*

species-level discrimination according to previous reports.[31,40] Nevertheless, there were some concerns about using ITS as a barcoding region because ITS region could quickly evolve and required neighboring locus (5.8S) for the determination of the sequence comparative relation.[14] However, each barcoding locus could be suitable for genus discrimination (among *Aquilaria* and *Enkleia*) in Thymelaeaceae family because there was a high genetic distance and the branch of *Enkleia* in each tree was isolated from the group of *Aquilaria*.

CONCLUSION

The six candidate DNA barcode loci of *Aquilaria* plants were prosperously amplified and sequenced. We suggested that the DNA barcoding could be used for *Aquilaria* species discrimination through the ITS region. The establishment of the *Aquilaria* species DNA barcoding database is beneficial to the application of the authentication step of traditional medicine uses; thus, it also increases the overall confidence medical practitioners have in terms of therapeutic outcomes and safety profiles.

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