DNA BARCODE CHARACTERIZATION OF MISTLETOE INFESTATION IN TEAK CLONAL SEED ORCHARD (CSO) IN PADANGAN, EAST JAVA PROVINCE, INDONESIA

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ABSTRACT

For effective teak plantation management, early detection system in controlling teak mistletoe requires various basic information, including degree of infestation and accuracy of the species names. Mistletoe infestations in teak and mistletoe species name have been reported, but there are still problems in identifying the correct species or subspecies due to morphological similarity. The objective of this study was to clarify the species identity of hemi-parasitic mistletoe plants, which were found in teak Clonal Seed Orchard (CSO) in Padangan, East Java Province, Indonesia using DNA barcodes. Species identification of teak mistletoe based on leaf morphological characteristics and universal DNA barcode regions (i.e. *mat*K and *rbc*L) were carried out. The results showed that the Canonical Discriminant Analysis (CDA) could differentiate *Dendrophthoe pentandra* and *Macrosolen tetragonus* based on leaf morphological characteristics. Variables having high correlation to distinguish both species were length of petiole, width of the widest leaf, number of secondary leaf veins, leaf base shape, aspect ratio, form factor and perimeter ratio of diameter. The results of DNA barcoding showed that the two DNA barcode regions presented good amplification and sequence results. Both DNA barcode regions successfully differentiated two species i.e. *D. pentandra* and *M. tetragonus* which belong to Loranthaceae family and have similar leaf morphological characteristics. Those regions were also able to identify *Viscum articulatum* and other species belonging to Santalaceae family. These results suggested that the two DNA regions could become recommended universal DNA barcode for identifying teak mistletoe.

Keywords: Clonal Seed Orchard, DNA barcode, *mat*K, *rbc*L, teak mistletoe

INTRODUCTION

Mistletoe infestation in the Clonal Seed Orchard (CSO) of Perum Perhutani, in Padangan teak plantation forests causes the decrease on quantity and quality of seed and wood production. Mistletoe infestation level in CSO Padangan was reported by Muttaqin *et al.* (2016) by describing the ratio of infected trees to the total number of trees as assessed in Observation Sample Plot (OSP). The ratios were divided into several ranges i.e. 1. range of 0.32 – 0.43 indicating light intensity which corresponded to TMR value (True Mistletoe Rating) of 0.86 – 1.21 (rather light); 2. range of 0.50 – 0.65 indicating light-rather moderate which corresponded to TMR value of 1.29 -3.27 (light-rather moderate); and 3. range of 0.74 – 0.83 indicating heavy intensity which corresponded to TMR value of 1.80 – 3.58 (light-rather moderate). Total TMR value was in the range of 0.86 – 3.58. There was no heavy intensity (TMR 5 – 7).

Preventive parasites control program in Perum Perhutani are carried out through early detection system to anticipate the widespread of the parasites. The first attempt to assess parasite attack patterns and distribution in sampled compartments of teak stands and a Clonal Seed Orchard (CSO) was conducted by Muttaqin *et al.*

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(2016). In addition, dominant mistletoe species have also been identified. However, correct identification of the species is still problematic due to morphological similarities. Hasanbahri *et al.* (2014) reported that there were two mistletoe species of *Scurrula parasitica* and *Dendrophthoe pentandra* belonging to Loranthaceae family in the teak plantation of Krandegan Ranger Resort, Begal Forest Sub-District, Ngawi Forest District. However, it is quite difficult to distinguish mistletoe species in the field. Currently, the identification is based on a reference to herbarium collection which usually requires complete parts of plants. In certain cases, leaves are the only available part of the plants that can be used for identification. This situation could lead to incorrect classification of the targeted mistletoe species. Species identification based on morphological characteristics frequently resulted to incorrect identification due to cryptic species phenomenon and species siblings. This phenomenon can cause synonymous substitution in the name of the species or vice versa.

This study was carried out by employing DNA barcode method to complement the traditional plant identification. This method is more rapid and accurate than the morphological marker. Usually, DNA barcoding is based on short sequences (< 800 bp) (Valentini 2009) that can identify and characterize different species which cannot be distinguished morphologically (Tudge 2000). Moreover, each mistletoe species that has potential as medicinal plant and is widely used for the treatment of certain diseases should have a specific barcode DNA marker to guarantee the correctness of medical treatment (Kwanda *et al.* 2013). Also, Purushothaman *et al.* (2014) and Mishra *et al.* (2016) affirm that DNA barcoding is an efficient and authentic tool to differentiate medicinal plant.

Several studies showed that *rbc*L (*ribulase-1, 5-biphosphate carboxylase*) and *mat*K (*maturase* K) regions are the two-locus barcode generally used in plants due to their easiness in sequencing and high amplification for many plant species (Hollingsworth *et al.* 2011). Thus, the objective of this study was mainly to clarify the identity of hemi-parasitic mistletoe plant, which are now found in teak Clonal Seed Orchard (CSO) Padangan, Perum Perhutani, East Java Province, Indonesia, using DNA barcodes.

MATERIALS AND METHODS

Plant Materials

Leaf samples (young or old leaves) of mistletoe that grow on branch or twig of teak crown were collected from compartments of teak stands in Padangan Clonal Seed Orchard (CSO). Those compartments were assigned as Observation Sample Plot (OSP) and Observation Measurement Plot (OMP). Four OMPs (50 x 50 m for each plot) were established inside the OSP units that were classified based on the level of

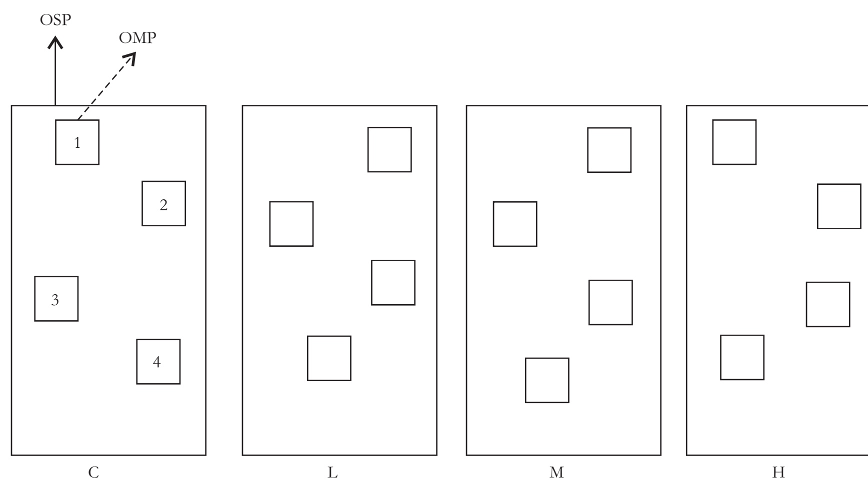

Figure 1 Design of OSP and OMP positions in an OSP unit, modified from CRC990/EFForTS (Drescher *et al.* 2016; Muttaqin *et al.* 2016)
(Note: 1, 2, 3, 4 = number of each OMP, respectively; C = control, L = low, M = medium, H = high)

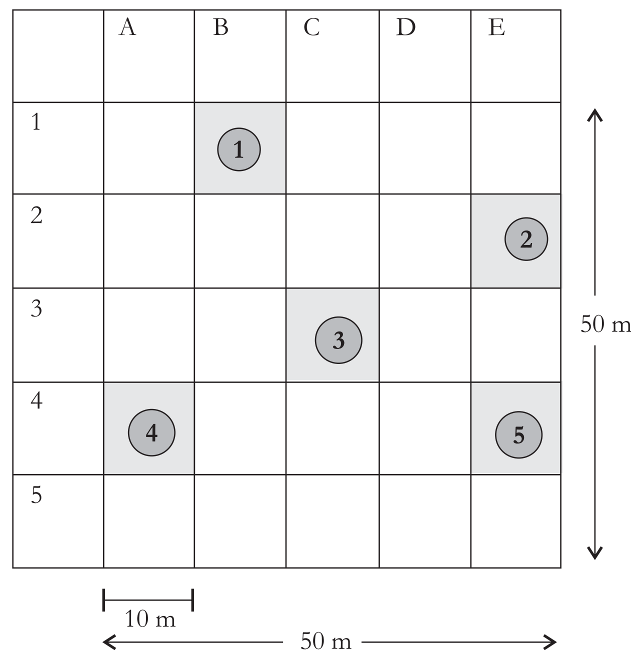

Figure 2 Design of sub-OSPs in an OSP unit (Drescher *et al.* 2016; Muttaqin *et al.* 2016); A, B, C, D, E and 1, 2, 3, 4, 5 as order of column and row of sub-OSPs

parasite attacks i.e. low, medium, high and control (no parasite attacks) (Barbu 2012). If there were no samples obtained for the assigned criteria, the sample collection was then conducted around the corresponding OMP. In this study, OSP and OMP were designed by modifying plots presented in the guidelines of EFForTS/CRC990 project (Drescher *et al.* 2016) (Fig. 1 & 2). Molecular analysis was carried out in the Laboratory of Plant Biotechnology, Center of Research Biological Resources and Biotechnology, Institut Pertanian Bogor (IPB) as well as in the Laboratory of Genetics and Forestry Molecular, Silviculture Department, Faculty of Forestry, IPB.

Leaf Morphology

Based on a preliminary study of the voucher specimen identification on teak mistletoe in the field and verification through the herbarium identification in Herbarium Bogoriense (BO), Research Center for Biology LIPI, Bogor, three species were known, namely *Dendrophthoe pentandra* (Loranthaceae), *Macrosolen tetragonus* (Loranthaceae) and *Viscum articulatum* (Santalaceae). In this study, 62 individual leaf samples were collected for *D. pentandra.* Thirty seven (37) samples of *M. tetragonus* were collected from five host trees (sub-OMPs) in 12 OMPs for further identification of leaf morphology. Specimens collected for each of *D. pentandra* and *M. tetragonus* were twigs in which each twig has three to five mistletoe leaves. The specimens were directly sterilized by swabbing 70% ethanol onto the leaves using cotton balls. The sterilized specimens were then covered with newspaper. Each sterilized specimen was tagged using a hanging label. Information written on the hanging label was sequential number of the sample, name of mistletoe species, sampling date and sequential number of the host tree.

Leaf morphology identification was conducted at unit sample of three leaves having sufficient phenotype in reference to Kremer *et al.* (2002) with several modifications following simplified procedures of Gembong (2001), Wu *et al.* (2007), Ellis *et al.* (2009) and Kadir *et al.* (2012). Measured variables for each leaf samples were dimensional characteristics such as lamina length (LL), petiole length (PL), the width of the widest part of leaf (WL), the length of the largest leaf to the base of the leaf (LB), arch of leaf veins (SD), angle between the midvein with the right or left branch of leaf veins. The number of secondary leaf veins (NV) was also recorded. The number of leaves (NL) was not used as variables in this study, because the calculation of the number of mistletoe leaves was inconsistent due to the loss of some leaves.

Other observed variables were apex shape (AS) and base shape (BS), which were observed with visual assessment from 1 to 8 (Ellis *et al.* 2009). Calculated variables were: 1. leaf area (LA), which was calculated using the ellipse area formula: ½ x (3.14) x (WL x LL); 2. leaf circumference (LC), which was calculated using the ellipse circumference formula: ½ x (3.14) x (WL + LL); 3. aspect ratio (AR), defined as the ratio of leaf length and width that is used to estimate the shape of the leaf blade; 4. form factor (FF), describes the shape of leaf the roundness of the leaf blade, which form factor (FF) was calculated by the formula: $4\pi \times LA/LC^2$; 5. perimeter ratio of diameter (PR), determines the ovalness of the leaf, which can be calculated by the formula: LC/WL (Wu *et al.* 2007).

The 13 leaf morphological variables were then analyzed using three different multivariate analyses, namely Principal Component Analysis (PCA), Canonical Discriminant Analysis (CDA), Multiple Correspondence Analysis (MCA) (Kremer *et al.* 2002; Anwar 2015) of the SPSS 17 program (SPSS Inc. 2007). Furthermore, t-test analysis was performed on the average of the 13 variables of *D. pentandra* and *M. tetragonus* using Minitab 15 software (Minitab Inc. 2007).

DNA Barcode

For DNA barcode, three replications were applied and analyzed for both *mat*K and *rbc*L regions of *D. pentandra*, *M. tetragonus* and *V. articulatum*, respectively. Thus, there were nine host trees. There were 18 samples examined for DNA barcode analysis (Table 1). For each mistletoe species, a sample unit consisted of three leaves. Those leaves were directly sterilized by swabbing 70% ethanol onto the leaves using cotton balls. Each sample unit was then cut into pieces of 2 x 2 cm. The cuttings of each sample unit were then put inside a tea bag of 9 x 7 cm. One tea bag was designated for one sample unit. Silica gel granules were put inside the respective tea bags to keep the samples dried in field condition (Fazekaz *et al.* 2012). Three tea bags representing the three replications for each mistletoe species for each DNA marker were put into one airtight plastic bag which was then labeled according to the sample code. The sample code consisted of sequential number of sample, name of mistletoe species, sampling date and sequential number of the host tree. Subsequently, all samples were carefully packed and transported to the laboratory for DNA extraction.

Total genomic DNA was extracted from each sampled leaf using *DNeasy Plant Mini Kit* protocol (http://www.qiagen.com) with catalog number 6235. Two DNA barcode regions (*mat*K and *rbc*L), which are recommended as universal DNA barcodes for land plants, were used for PCR amplification (Hollingsworth *et al.* 2009). The PCR amplification was carried out using PCR machine of *AB Applied Biosystem Veriti*™ Thermal Cycler (www.appliedbiosystem.com). Primer sequences used in this study are shown in Table 2.

The process of PCR begins with the dilution of DNA and primer. Firstly, DNA extract was diluted 100 times using double-distilled water (Bidest. water). Secondly, primer dilution was conducted by directly taking 10 μL of high concentrated *mat*K and *rbc*L primers, respectively, which was then added with 90 μL nuclease free water. Substance composition for PCR reaction consisted of 2.5 μL H_2O, 7.5 μL green go taq master mix, 1.5 μL primer F and R and 2 μL diluted DNA (Fazekaz *et al.* 2012). The DNA amplification results were sent to Genetika Science Indonesia (http://www.base-asia.com) for automated sequencing. Additional sequence data of *mat*K and *rbc*L regions for each species including Out Group were collected from NCBI

Table 1 List of leaf samples of teak mistletoe used in this study

No.	Species	Number of samples collected for		
		Leaf morphology characteristics determination	DNA barcode determination	
			*mat*K	*rbc*L
1	*Dendrophthoe pentandra*	62	3	3
2	*Macrosolen tetragonus*	37	3	3
3	*Viscum articulatum*	-	3	3

Table 2 Nucleotide sequence of the primers used in this study

No.	Region	Primer name	Sequence (5' – 3')	Length of PCR product (bp)	Temperature (°C)	Reference
1	*mat*K	[*mat*K-3F, *mat*K-3R]	F: AAGATGCCTCTTCTTTGCAT R: GATCCGCTGTGATAATGAGA	620	50	Marazzi *et al.* (2006)
2	*rbc*L	[*rbc*L-barP-1F, *rbc*L-barP-724R]	F: ATGTCACCACAAACAGAAAC R: TCGCATGTACCTGCAGTAGC	677	50	Dev *et al.* (2014)

GenBank database (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov) and BOLD System (http://boldsystems.org/).

DNA sequences i.e. the length of sequence data (bp) and nucleotide composition were checked visually, edited manually based on chromatogram and aligned manually using ClustalW implemented in MEGA 6.06 program (Tamura *et al.* 2013). Sequence data were then analyzed using BLAST in NCBI database to identify the three targeted species. Furthermore, barcoding gap and phylogeny trees were checked using MEGA 6.06 program. Phylogeny trees were constructed using Neighbor Joining (NJ) method with 1,000 replicates of bootstrap based on Kimura-2-parameter (K2P) distance.

RESULTS AND DISCUSSION

Characteristic of Leaf Morphology

Results of species identification based on observation and leaf dimension measurement supported by standard reference and complete identity verification using herbarium specimen are presented in Table 3.

Table 3 Differences in morphological characteristics of leaf sampled from three species of teak mistletoe

Morphological characteristics	Loranthaceae		Santalaceae
	Dendrophthoe pentandra	*Macrosolen tetragonus*	*Viscum articulatum*
Leaf arrangement	leaves alternate and small leaves subopposite	leaves opposite	leaves rudimenter and resemble small bractea, leaves alternate and subopposite
Leaf length	8.42±1.65 cm	8.56±1.36 cm	2.60±0.60 cm
Leaf width	4.15±0.87 cm	3.77±0.73 cm	0.30±0.10 cm
Petiole length	1.37±0.29 cm	0.28±0.26 cm	-
Amount of secondary veins	8.98±0.86	12.03±1.44	-
Shape of leaf base	developed-rounded (scale 2.54±0.32)	rounded (scale 2.84±0.28)	slightly convex
Shape of leaves (aspect ratio)	lengthwise (scale 2.06±0.29)	lengthwise (scale 2.32±0.31)	spatulate
Shape of leaf rounded (form factor)	round (1.76±0.09)	slightly round (1.68±0.08)	0.8±0.20
Leaf ovalness (perimeter ratio of diameter)	slightly oval (4.81±0.47)	oval (5.22±0.48)	very oval (15.6±4.60)

Variation in Leaf Morphology

In this study, the three multivariate analyses (PCA, MCA, and CDA) were conducted using the first synthesis variables, which were expected to contribute the highest rate of total variance, followed by the second synthesis variables, as presented in Table 4.

Table 4 shows that the three multivariate analyses (PCA, MCA, and CDA) were able to explain more than 50% of the total variance, representing the wide distribution of leaf morphology grouping of *D. pentandra* and *M. tetragonus* (Loranthaceae). Furthermore, the results of three multivariate analyses (PCA, MCA, and CDA) are shown in Figure 3.

Table 4 Proportion of total variance explained by the first and second synthesis variable of three multivariate analyses (PCA, MCA, CDA) in grouping *D. pentandra* and *M. tetragonus*

	Total variance explained by each multivariate analysis (%)		
	PCA	MCA	CDA
Synthesis variable 1	37.40	41.32	100
Synthesis variable 2	29.00	33.44	0
Total	66.40	74.76	100

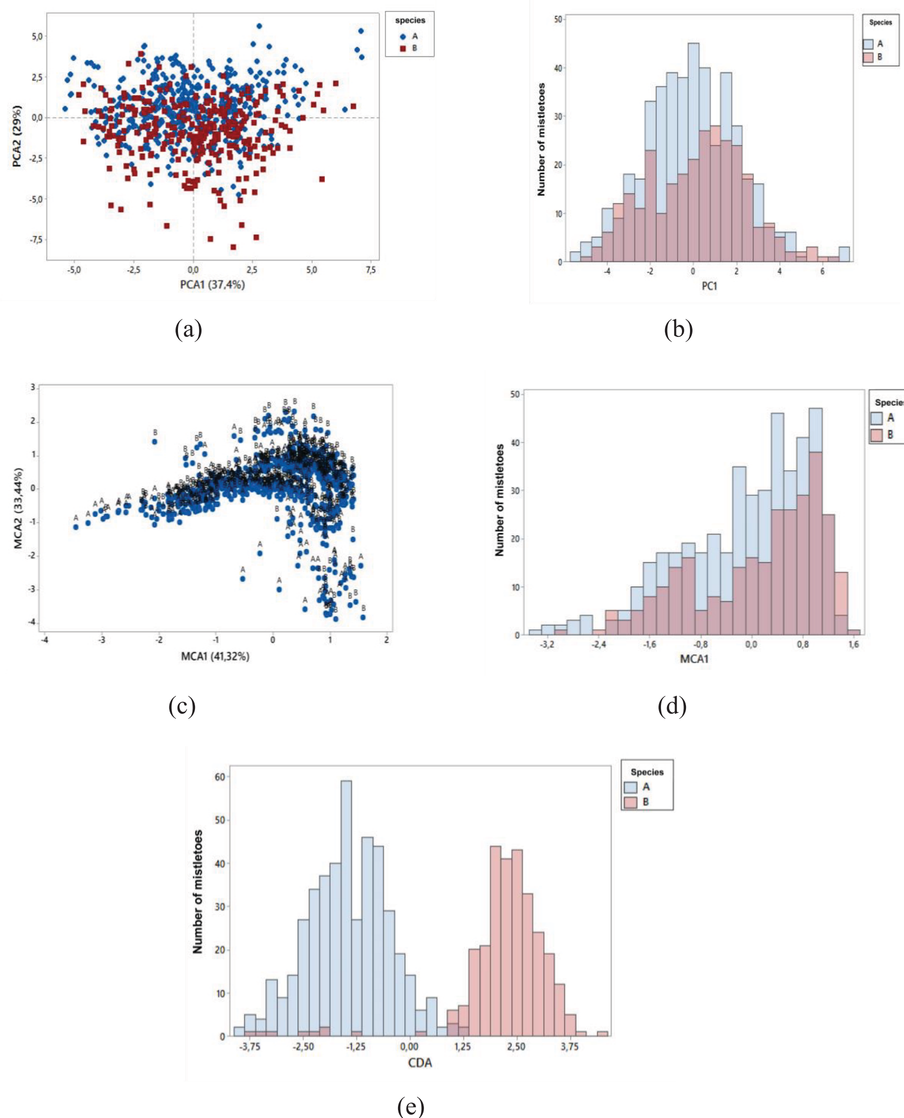

Figure 3 Results of multivariate analysis (a) scatter plot of PCA; (b) histogram of PCA; (c) scatter plot of MCA; (d) histogram of MCA and (e) histogram of CDA
(Note: A = *D. pentandra*; B = *M. tetragonus*)

Results of these three multivariate analyses showed that only CDA analysis could differentiate *D. pentandra* and *M. tetragonus* based on leaf morphological characteristics. Figure 3b and 3d (PCA and MCA), however, show that there were some samples, which were not included in the *D. pentandra* and *M. tetragonus* groups. Those are unstable as transition group between the two mistletoe species. Based on correlation analysis between the leaf dimensional variable and the synthesis 1 (global analysis) of *D. pentandra* and *M. tetragonus* (Loranthaceae), it was found out that variables having high correlation to distinguish both species using CDA analysis were long petiole, width of the widest leaf, number of secondary leaf veins, leaf base shape, aspect ratio, form factor and perimeter ratio of diameter (Fig. 3e).

Characteristics of Leaf Variables for *D. pentandra* and *M. tetragonus*

Results of t-test for each leaf morphological variable of *D. pentandra* and *M. tetragonus* showed that several variables having significant difference were PL, NV, BS, AR, FF, PR ($p < 0.01$) and WL ($p < 0.05$).

DNA Barcode

Amplification and sequence results

Obtaining optimum annealing temperature is necessary in order to get a successful PCR amplification process. This study indicated that annealing temperature of 50 °C was the best temperature for *mat*K and *rbc*L, which showed a very clear single band when visualized by agarose gel. This temperature was lower than the temperature suggested by Stoeckle *et al.* (2011) for *mat*K and *rbc*L markers, which was 52 °C and 56 °C, respectively. The results showed that 17 samples were successfully amplified and sequenced with primers *mat*K and *rbc*L. However, one sample could not be well amplified in *mat*K for *Viscum articulatum*.

DNA analysis and nucleotide base composition

Size of *mat*K and *rbc*L regions were 744 – 764 bp and 738 – 740 bp, respectively (Table 5). The result of sequence bases showed that the average percentage of nucleotides C + G on the three teak mistletoe species was 30.0 - 44.4%. Moreover, it is known that *mat*K region has a longer average sequence length than *rbc*L region. Study reported by Trang *et al.* (2015) showed that sequence alignment can be used for examining the evolution process from the common ancestor, and thus, can be used for the three mistletoes species in the present study. The sequence length difference is helpful as early identification for species difference and correlated with differences in the composition of nucleotide bases (Dick & Kress 2009). Calculation of base substitution among three species in the same family was done after sequence alignment. In this study, an average sequence length of *rbc*L region was 738 - 740 bp. This value was higher than the length reported by Kwanda *et al.* (2013) who used the same *rbc*L region, but different primer base pairs (5'-ATGTCACCACAAACAGAGACTAAAGC-3' and 5'-GTAAAATCAAGTCCACCRCG-3'), showing 550 bp for Loranthaceae family (*D. pentandra, D. lanosa, Scurrula atropurpurea, M. cochinchinensis, M. bradisianus, Helixanthera parasitica*) and Santalaceae family (*V. articulatum* and *V. ovalifolium*).

Barcoding gap

Barcoding gap is defined when the interspecific diversity is higher than intraspecific diversity. Ideal barcode of genetic locus can be determined by looking at smaller intraspecific

Table 5 Sequence length (bp) in *mat*K and *rbc*L for the three studied mistletoe species

No.	Species	*mat*K		*rbc*L	
		n[a]	bp[b]	n	bp
1	*D. pentandra*	3	760, 761, 760	3	738, 740, 739
2	*M. tetragonus*	3	763, 764, 763	3	738, 739, 739
3	*V. articulatum*	2	745, 744	3	739, 739, 737
	Mean		757.5		738

Note: n[a] = number of sequence, exception of 1 sample which could not be amplified well in *mat*K for *Viscum articulatum*
bp[b] = base pair

Table 6 Average value of intraspecific and interspecific distances calculated using a Kimura-2-parameter model

Family	Average of intraspecific distance		Average of interspecific distance	
	*mat*K	*rbc*L	*mat*K	*rbc*L
Loranthaceae	0.012±0.017	0.003±0.006	0.128±0.091	0.064±0.029
Santalaceae	0.010±0.013	0.010±0.013	0.156±0.087	0.042±0.013

Note: Data are presented as mean±SD

diversity compared to the interspecific diversity (Lahaye *et al.* 2008). On sequence total, gap is marked with dotted line due to insertion or deletion. Variance of intraspecific and interspecific must be compared depending on the determined barcode of genetic locus (Tamura *et al.* 2013). Thus, barcoding gap between intra and interspesific diversities was determined using graphic and distribution of intra and interspesific diversities on Kimura-2-parameter (K2P) distance.

Generally, Table 6 shows that the average value of interspecific distances are higher than intraspecific distances in accordance with previous study of Lahaye *et al.* (2008). The present study showed that the average value of interspecific and intraspecific distances were higher for the *mat*K region than that for *rbc*L region. Furthermore, the intraspecific variation of *mat*K region for Loranthaceae was 0.012, slightly different from Santalaceae (0.010). Intraspecific variation of *rbc*L region for Loranthaceae was 0.003 and slightly different from Santalaceae (0.010). Interspecific variations of *mat*K region were 0.128 and 0.156 for Loranthaceae and Santalaceae, respectively. On the other hand, interspecific variations of *rbc*L region were 0.064 and 0.042 for Loranthaceae and Santalaceae, respectively. Value of interspecific distances of *rbc*L region for Loranthaceae and Santalaceae reported in this study were similar to the results reported by Kwanda *et al.* (2013), which was 0.032 - 0.067.

The present study showed that nucleotide variations in intraspecific and interspecific species were applicable whenever the tags were compared. Analysis of intra and interspecific distances showed a barcoding gap in Santalaceae (*V. articulatum*) using *mat*K region (Fig. 4) and Loranthaceae using *rbc*L region (Fig. 5). However, the finding of barcoding gap using genetic locus of *mat*K and *rbc*L still needs improved accuracy by increasing the number of samples.

According to Liu *et al.* (2011), the more overlap occurs between intraspecific and interspecific distances, the less effective of barcode region that can be chosen as a candidate DNA barcode due to the condition of the region. Only the region that has a smaller intraspecific variation than interspecific can be a candidate DNA barcode. In the case of interspesific variety, *mat*K and *rbc*L regions used in this study for Loranthaceae and Santalaceae were considered to have good distinctive ability, as shown from the gap.

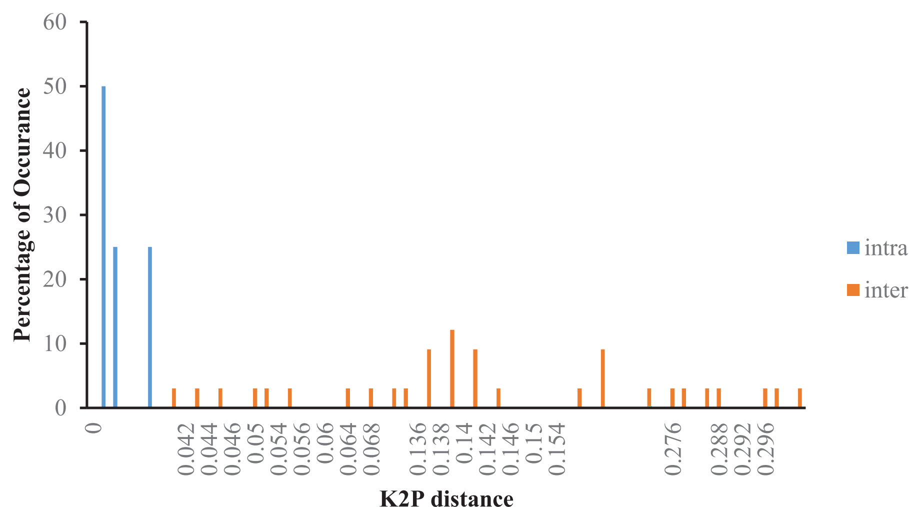

Figure 4 Distibutions of intraspecific K2P and interspecific K2P in *mat*K region showing barcoding gap in Santalaceae family

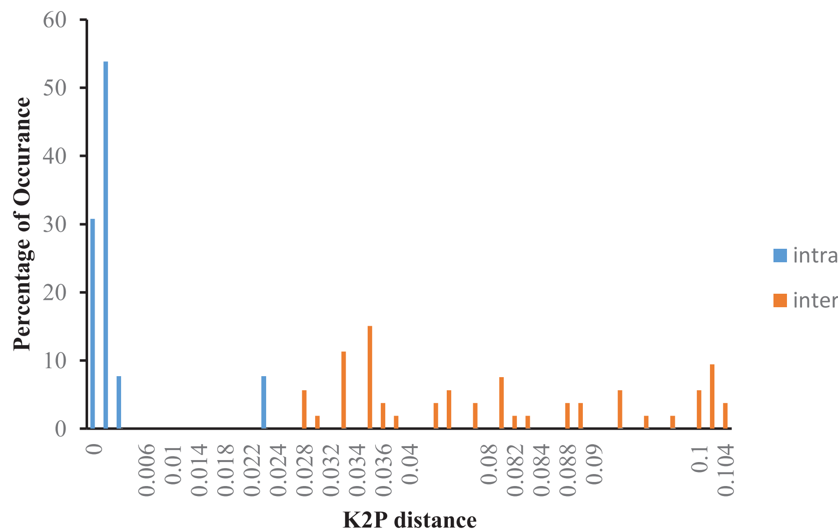

Figure 5 Distibutions of intraspecific K2P and interspecific K2P in *rbc*L region showing barcoding gap in Loranthaceae family

Phylogenetic Analysis

The aim of phylogenetic analysis is to confirm the kinship of individual samples. It is expected that the individual samples can be grouped based on taxonomical similarity, at the species, genus or family levels and be confirmed with high value of Consistency Index (CI). The present study showed that preliminary results of BLAST sequences matched with the same DNA sequence or has a close resemblance to teak mistletoe species (Table 7).

Results of species identification conducted using BLAST analysis (Table 7) were different from results of species identification conducted based on morphological characteristics (Table 3); for example *Dendrophthoe curvata* (Table 7) is

Table 7 Identification results of teak mistletoe using DNA barcode

No.	Code[a]	Region	BLAST analysis result	Identification (%)	Accession code (NCBI)
1	D1	*mat*K	*Dendrophthoe curvata*	99	EU544424.1
		*rbc*L	*Dendrophthoe pentandra*	100	HQ317759.1
2	M1	*mat*K	*Macrosolen cochinchinensis*	99	KP093836.1
		*rbc*L	*Macrosolen cochinchinensis*	99	HQ317768.1
3	V1	*mat*K	*Viscum articulatum*	99	EF464496.1
		*rbc*L	*Viscum articulatum*	99	KF496504.1
4	D2	*mat*K	*Dendrophthoe pentandra*	99	AB924787.1
		*rbc*L	*Dendrophthoe pentandra*	100	HQ317759.1
5	M2	*mat*K	*Macrosolen cochinchinensis*	99	KP093836.1
		*rbc*L	*Macrosolen cochinchinensis*	99	HQ317768.1
6	V2	*mat*K	*Viscum articulatum*	99	EF464496.1
		*rbc*L	*Viscum liquidambaricola*	99	GQ436640.1
7	D3	*mat*K	*Dendrophthoe curvata*	99	EU544424.1
		*rbc*L	*Dendrophthoe pentandra*	100	HQ317759.1
8	M3	*mat*K	*Macrosolen cochinchinensis*	99	KP093836.1
		*rbc*L	*Macrosolen cochinchinensis*	99	HQ317768.1
9	V3	*mat*K[b]	*Viscum articulatum*	-	-
		*rbc*L	*Viscum liquidambaricola*	99	EQ436640.1

Note: [a] refers to species code: D1 = *Dendrophthoe pentandra* – first replication, M1 = *Macrosolen tetragonus* – first replication, V1 = *Viscum articulatum* – first replication and so on
[b] indicates that the amplification was not successful in *mat*K region (primer *mat*K-3F, *mat*K-3R)

Table 8 Degree of accuracy of species identification based on *mat*K and *rbc*L regions using BLAST analysis

No.	Code[a]	Region	BLAST analysis result	Identification (%)	Accession code (NCBI)
1	D1	*mat*K	*Dendrophthoe curvata*	99	EU544424.1
		*rbc*L	*Dendrophthoe pentandra*	100	HQ317759.1
2	M1	*mat*K	*Macrosolen cochinchinensis*	99	KP093836.1
		*rbc*L	*Macrosolen cochinchinensis*	99	HQ317768.1
3	V1	*mat*K	*Viscum articulatum*	99	EF464496.1
		*rbc*L	*Viscum articulatum*	99	KF496504.1
4	D2	*mat*K	*Dendrophthoe pentandra*	99	AB924787.1
		*rbc*L	*Dendrophthoe pentandra*	100	HQ317759.1
5	M2	*mat*K	*Macrosolen cochinchinensis*	99	KP093836.1
		*rbc*L	*Macrosolen cochinchinensis*	99	HQ317768.1
6	V2	*mat*K	*Viscum articulatum*	99	EF464496.1
		*rbc*L	*Viscum liquidambaricola*	99	GQ436640.1
7	D3	*mat*K	*Dendrophthoe curvata*	99	EU544424.1
		*rbc*L	*Dendrophthoe pentandra*	100	HQ317759.1
8	M3	*mat*K	*Macrosolen cochinchinensis*	99	KP093836.1
		*rbc*L	*Macrosolen cochinchinensis*	99	HQ317768.1
9	V3	*mat*K[b]	*Viscum articulatum*	-	-
		*rbc*L	*Viscum liquidambaricola*	99	EQ436640.1

Note: [a] refers to species code: D1 = *Dendrophthoe pentandra* – first replication, M1 = *Macrosolen tetragonus* – first replication, V1 = *Viscum articulatum* – first replication, and so on.
[b] indicates that the amplification was not successful in *mat*K region (primer *mat*K-3F, *mat*K-3R)

different from *Dendrophthoe pentandra* (Table 3), *Viscum liquidambaricola* (Table 7) is different from *Viscum articulatum* (Table 3). Table 7 also shows different species name from the samples, i.e. D1 and D3 at *mat*K region and V2 and V3 at *rbc*L region. Degree of accuracy of species identification using BLAST analysis is presented in Table 8.

Analysis of DNA barcode using *mat*K region could identify *D. pentandra* up to species level (one individual) with homology value of 99% and up to genus level (two individuals) with homology value of 99%. *mat*K region could also identify *M. tetragonus* up to genus level (three individuals) with homology value of 99%, but could not identify *M. tetragonus* up to species level due to unavailable sequence data of *M. tetragonus* in the *Gen Bank* database and BOLD system which were used as the references. Furthermore, this region could identify *V. articulatum* up to species level (two individuals) with homology value of 99%.

On the other hand, analysis of DNA barcode using *rbc*L region could identify *D. pentandra* up to species level (three individuals) with homology value of 100%, *M. tetragonus* up to genus level (three individuals) with homology value of 99% and *V. articulatum* up to species level (one individual) and genus level (two individuals) with the same homology values of 99%. Thus, Table 8 shows the degree of similarity (homology) obtained from BLAST analysis having homology values ranged from 99 to 100%. Scientific names of the three mistletoe species are listed in the Plant List (http://www.theplantlist.org) in which *Dendrophthoe pentandra* has 'accepted' status with confidence level **; *Macrosolen tetragonus* has 'unresolved' status with confidence level *; *Viscum articulatum* has 'accepted' status with confidence level **.

Table 8 also shows that DNA barcoding using *mat*K and *rbc*L regions is more accurate to distinguish samples up to genus and species levels compared to morphological characteristics. However, the present study showed that *mat*K region has better ability to discriminate than *rbc*L region. This finding is consistent with studies reported by Hollingsworth *et al.* (2009) and Novianty (2016). *rbc*L region has high amplification success and is easily sequenced for many species. On the other hand, *mat*K region is difficult to amplify, but has high accuracy to distinguish up to species level (Koch *et al.* 2008; Trang *et al.* 2015). The present study showed that amplification of sample V3 was not successfully conducted using *mat*K region (Table 7).

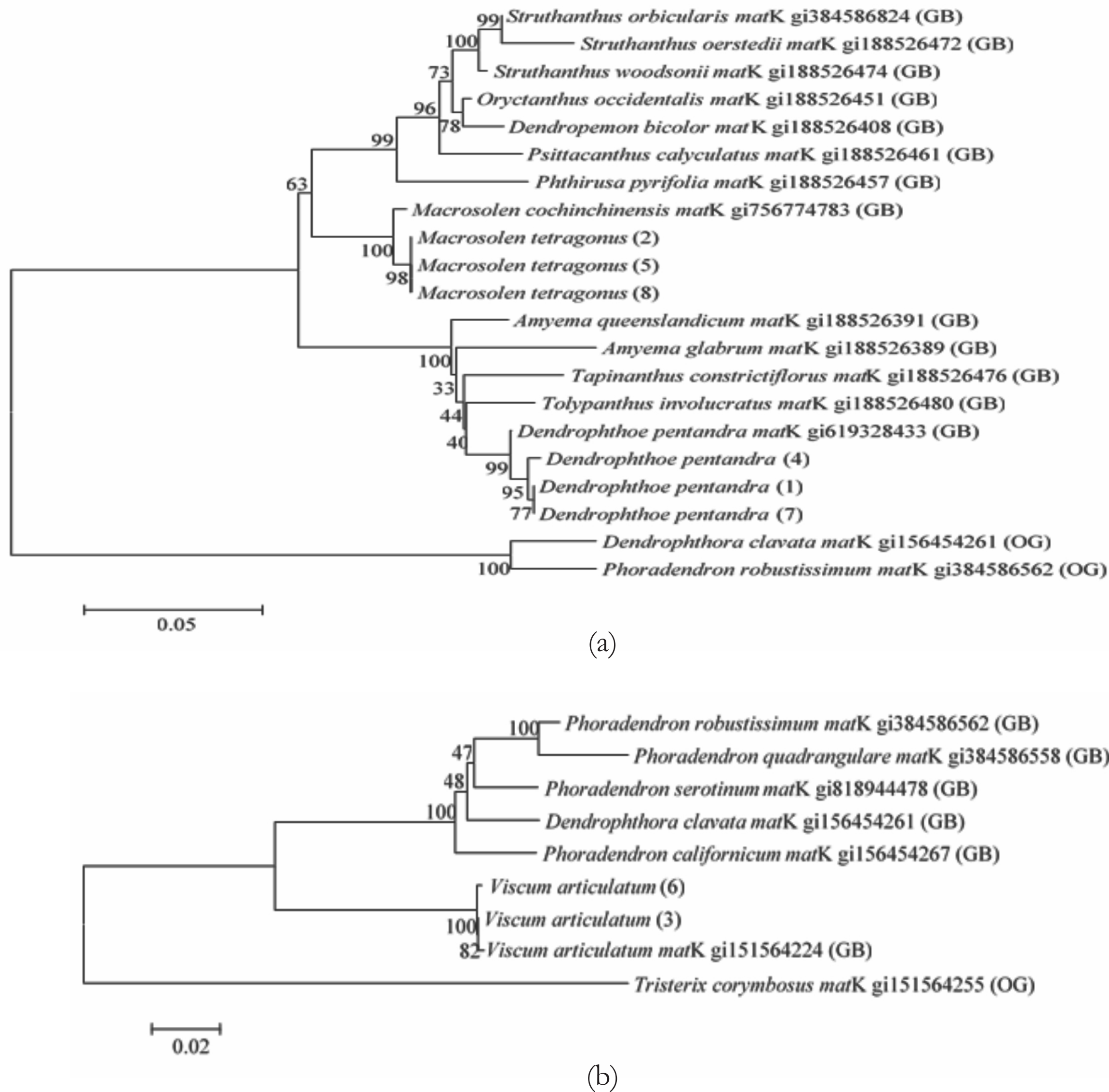

Figure 6 Phylogeny tree based on *mat*K region at family level using Neighbor-Joining (NJ) tree method (Note: (a) Loranthaceae family; (b) Santalaceae family; GB = GenBank; OG = Out group)

Studying family of certain species can be explained by reconstructing phylogeny tree of the family. In the cladogram, species belong to one family are located in an adjacent clade. Small clade is suspected of having very small differences in base sequence (Zuazo & Agnarson 2010). Phylogenetic trees of Loranthaceae and Santalaceae families are presented in Figure 6 and 7. Both figures show consistency value of bootstrap that diverse on every locus of phylogeny tree. *mat*K region has bootstrap value range of 33 – 100 for Loranthaceae family and range of 47 – 100 for Santalaceae family. *rbc*L region has bootstrap value range of 28 – 100 for Loranthaceae family and range of 69 – 100 for Santalaceae family. Higher bootstrap value indicates higher stability of phylogeny tree branch.

*mat*K and *rbc*L regions of the three samples of the respective *D. pentandra* and *M. tetragonus* (Loranthaceae) form separate clades of both species and it is called *monophyletic*. The same result interpretation can also be applied on the two samples of *V. articulatum* (Santalaceae) which included as *monophyletic*. Figure 6a & 7a shows that *D. pentandra* and *M. tetragonus* form separate clades. These figures also show that the two mistletoe species were truly different species based on both *mat*K and *rbc*L regions.

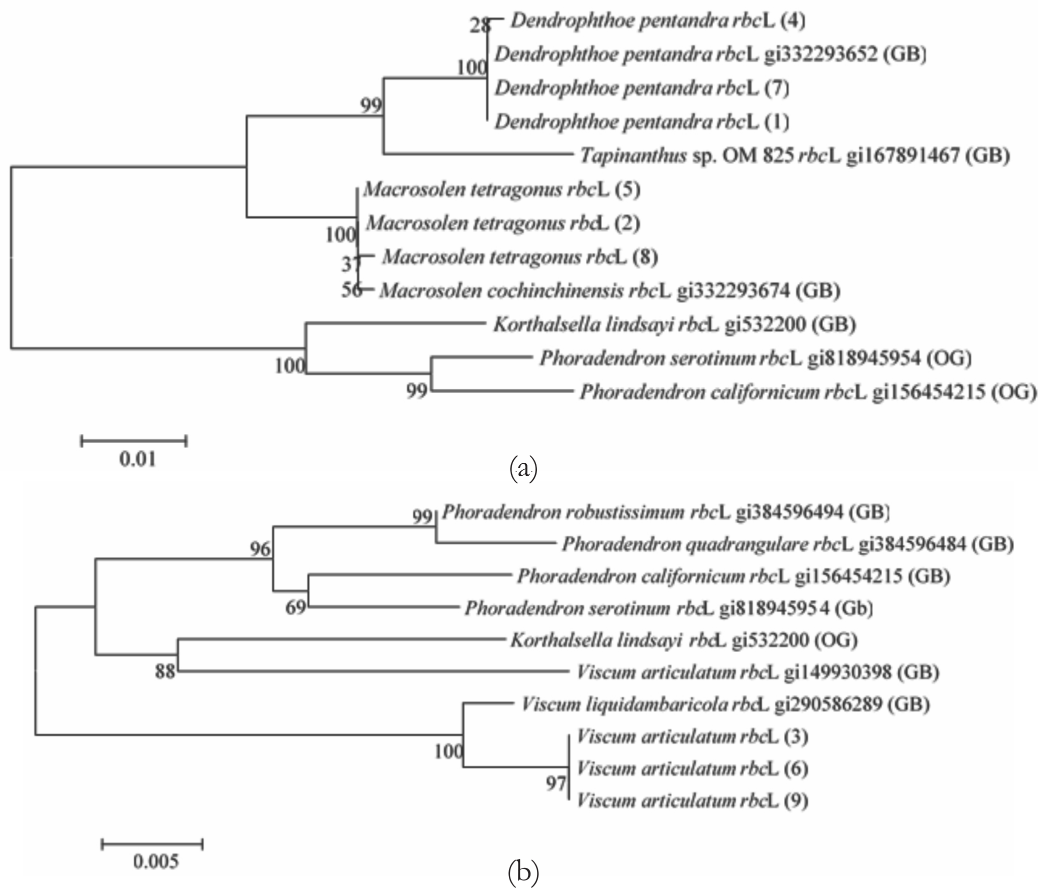

Figure 7 Phylogeny tree based on *rbc*L region at family level using Neighbor-Joining (NJ) tree method (Note: (a) Loranthaceae family; (b) Santalaceae family; GB = GenBank; OG = Out group)

CONCLUSIONS

DNA barcoding gap results obtained in this study showed that *mat*K and *rbc*L regions had an average value of interspecific distances that were higher than intraspecific one. The results suggested that those regions could be accurate candidates of DNA barcode for distinguishing the two mistletoe species i.e. *Dendrophthoe pentandra* and *Macrosolen tetragonus* having similar leaf morphological characteristics and belonging to Loranthaceae family. These regions could also identify *Viscum articulatum* and other species belonging to Santalaceae family. Application of *mat*K and *rbc*L regions was able to distinguish those three mistletoe species up to species and genus levels.

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