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Molecular phylogenetics of Malesian *Diospyros* (Ebenaceae) based *trnL-F* spacer sequences

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Abstract. *Wanda IF, Djuita NR, Chikmawati T. 2021. Molecular phylogenetics of Malesian* Diospyros *(Ebenaceae) based* trnL-F *spacer sequences. Biodiversitas 22: 4106-4114. Diospyros* is a genus composed of potential species as an economic commodity with high diversity. However, there is limited information on the phylogenetic relationship of this genus in the Malesian region. This study aimed to provide information on the species diversity through a DNA barcoding approach and revealing the phylogenetic information of *Diospyros* spp. in the Malesian region. This study used 20 *Diospyros* accessions from Bogor Botanical Garden collections, 40 *Diospyros* accessions, and four outgroup accessions obtained from the NCBI database. The DNA barcoding primer utilized comes from plastids, *trnL-F* intergenic spacer. The phylogenetic trees were constructed using the Maximum-Parsimony method. A total of 20 accessions of *Diospyros* were validated with sequence data on the genebank. The result showed that all accessions had relationships with 44 other *Diospyros* species globally. Here, we reported 10 new *trnL-F* intergenic spacer sequences of Malesian *Diospyros* species. A phylogenetic tree grouped 64 monophyletic *Diospyros* species into seven clades. The phylogenetic results supports the biogeographic hypothesis: the Malesian region, the Malesian-Caledonian Region, and Cosmopolite species in almost all bioregions.

Keywords*: Diospyros,* ebony, phylogenetics, Malesia, *trnL-F* intergenic spacer

INTRODUCTION

Diospyros spp. is the largest genus of the Ebenaceae, with more than 700 species scattered in tropical and subtropical forest areas (POWO 2019). One of the diversity centers of *Diospyros* spp. is the continents of Asia and the Indo-Pacific, especially the Malesian region (Singh 2005; APG IV 2016). There are an estimated 167 *Diospyros* species in the Malesian region (GBIF 2019). The number of *Diospyros* species reduced from the previously reported by Bakhuizen van den Brink (1933), 181 species. This reduction is due to the revision of several *Diospyros* names based on the results of the latest taxonomic studies. *Diospyros* spp. are most widely distributed in the Indonesian archipelago (Borneo, Celebes, Sumatra, Papua) and the Philippines' islands. Many *Diospyros* species are found in in-situ and ex-situ conservation areas such as the Bogor Botanical Gardens (BBG). BBG has at least 32 species of *Diospyros* originating from the Malesian region (Ariati et al. 2019; Wanda et al. 2019). Several *Diospyros* species were not even identified, and three species have the status of near threatened (*D. philippinensis*), endangered (*D. molissima*), and vulnerable (*D. celebica*) (IUCN 2019).

Several *Diospyros* species are well known by the community and have the potential as an economic commodity (Tang et al. 2014; Yang et al. 2015; Zhang et al. 2016; Fu et al. 2016; Li et al. 2018; Guan et al. 2020). Eboni (*D. celebica*), *D. ebenum*, and *D. japonica* have excellent quality and expensive wood, while *D. discolor*, *D. lotus*, *D. virginiana*, and persimmon (*D. kaki*) produce edible fruit. *Diospyros* species are also known as a source of antioxidants, antidiabetic, antibacterial, anti-diarrheal, antifungal, anti-protozoan, anti-inflammatory, and tannins (Mallavadhani et al. 1998; Wallnöfer 2001a; Singh 2005; Howlader et al. 2012; Yang et al. 2015; Rauf et al. 2017; Guan et al. 2019; Tang et al. 2019). Unfortunately, despite having high diversity and economic potential, *Diospyros* has not been widely explored and studied because the differences between *Diospyros* species are still unclear and complicated to be distinguished (Bakhuizen van den Brink 1936; Duangjai et al. 2009).

Identifying species in the genus *Diospyros* requires generative organs (flowers and fruit) as distinguishing features between species (Hiern 1873; Wallnöfer 2001b; Duangjai et al. 2006; Jessup 2014). However, in *Diospyros*' living collections, those not identified are rarely found flowering and fruiting, complicating the identification process. However, identifying species in this genus can be replaced by using vegetative organs, such as leaves. Leaves have the most information, including morphology, anatomy, phytochemistry, cytology, and genetics (Stuessy 2009). In addition, leaf genetic information can be analyzed using barcoding DNA to determine *Diospyros* species.

Barcoding DNA is a short sequence that has been universally standardized to quickly identify living species (Costion et al. 2011; Techen et al. 2014; Li et al. 2015; Coissac dan Peter 2016). DNA sequences used in barcoding come from the nucleus (ITS 1, ncpGS, PHYA), chloroplasts

(*rbcL*, *atpB*, *matK*), and mitochondrial DNA. In plants, mitochondrial genes do not fit DNA barcoding because they exhibit low mutation rates (Hebert dan Gregory 2005; Kress et al. 2005). Barcoding DNA can also be performed at all levels or phases of living things and only requires a small part of the organs as material for analysis. In the *Diospyros* spp, barcoding markers tried and used successfully consist of ITS1 (Tang et al. 2014), ncpGS and PHYA (Turner et al. 2013), and plastid regions (Vijayan dan Tsou 2010; Alaklabi et al. 2014; Turner dan Paun 2016). According to Duangjai (2009), DNA barcoding can analyze and trace *Diospyros* species on the island of New Caledonia. This study used DNA sequences from eight plastid regions (*rbcL*, *atpB*, *matK*, *ndhF*, *trnK intron*, *trnL intron*, *trnL-F spacer*, and *trnS-G spacer*) and included 119 *Diospyros* species. DNA barcoding studies on *Diospyros* based on polymorphic molecular analysis of SNPs from DNA sequence data has been used to study the evolution, phylogeny, and ecological adaptation of *Diospyros* from New Caledonia (Samuel et al. 2019, Turner et al. 2013, Duangjai et al. 2006).

DNA markers often and widely used in *Diospyros* are plastid markers such as *rbcL*, *matK*, *trnL-F* spacer. The *trnL-F* intergenic spacer sequence is a noncoding region in the chloroplast genome located between the transfer RNA genes $trnL_{UAA}$ and $trnF_{GAA}$ (Quandt et al. 2004). The *trnL-F* intergenic spacer gene has the highest substitution speed compared to *matK* and *rbcL*, has a high rate of evolution and mutation, is relatively short in size, and is easily amplified (Chen et al. 2013). The characters make the *trnL-F* intergenic spacer gene quite good as a DNA barcode marker. Therefore, the approach through DNA barcoding is expected to assist the confirmation, evaluation, and identification of living specimens of *Diospyros* spp. In addition, it can validate the species of *Diospyros* found in the Malesian region.

The use of six markers of plastid DNA sequence in revealing the phylogenetic relationships among *Diospyros* from the Pantropical region strongly supported the presence of monophyly of Ebenaceae *s.l.* The family can be divided into two main clades related to the Lissocarpoideae and Ebenoideae sub-families (Duangjai et al. 2006). Unfortunately, the relationships of Malesian *Diospyros* have not known since most studies have not analyzed the Malesian *Diospyros*. In several studies, primer *trnL-F* spacer effectively determines *Diospyros* even with a relatively short sequence length. In addition, there is not much data on *Diospyros* sequences that use this primer. Therefore, this study aimed to profile the *trnL-F* intergenic spacer sequences of the Malesian *Diospyros* and used the sequences to reveal their phylogenetic relationships.

MATERIALS AND METHODS

Plant materials

The research was conducted in May 2020 - February 2021 at the Molecular Laboratory of the Research Center for Plant Conservation and Botanic Gardens, Indonesian Institute of Sciences (LIPI). Young and healthy leaves of 20 accessions representing 17 *Diospyros* species were taken from the Bogor Botanical Gardens collection, then stored and put in plastic clips containing silica gel to dry quickly and avoid DNA damage (Rugayah et al. 2004). The observed species were *D. andamanica*, *D. aurea*, *D. buxifolia*, *D. coriacea*, *D. korthalsiana*, *D. lolin*, *D. macrophylla*, *D. malaccensis*, *D. nigra*, *D. pilosanthera*, *D racemose*, *D. ridleyi*, *D. subrhomboidea*, *D. sumatrana*, *D. celebica*, *Diospyros* sp1, and *Diospyros* sp2. One species, *D. discolor*, consisted of three accessions that came from three locations. *Diospyros* were mainly found in Java (6 accessions) and Celebes (5 accessions) (Figure 1), whereas the least number of species (1accession) was in Sumatra and the Philippines.

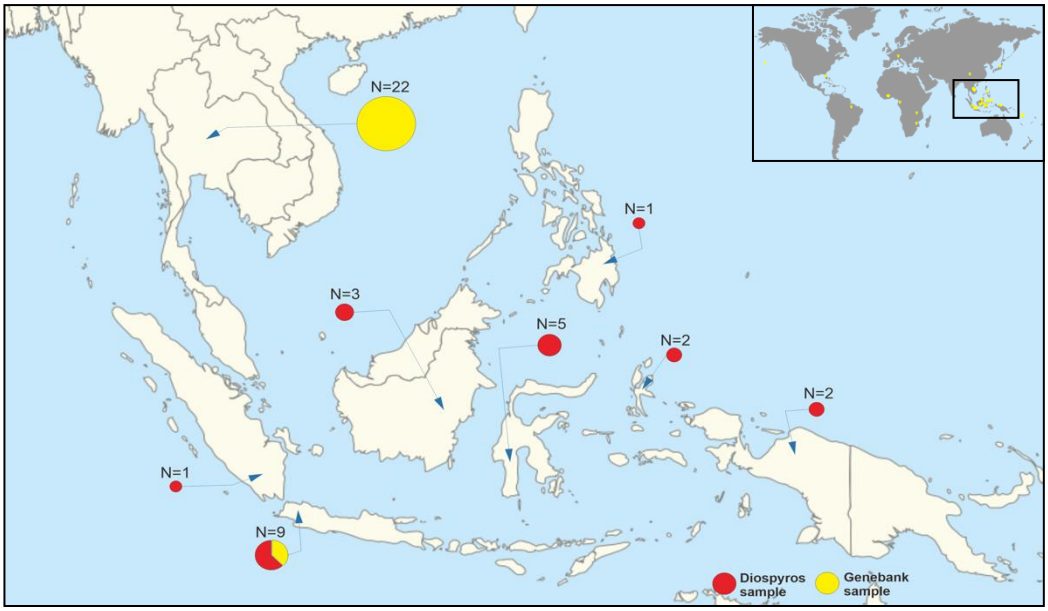

Figure 1. Accession distribution of *Diospyros* spp. Red circle = number of *Diospyros* accessions sequenced in this study, yellow circle = number of *Diospyros* accessions from GenBank

DNA extraction, amplification, and sequencing

DNA extraction followed the standard procedure of the Tiangen Plant Genomic DNA kit. The DNA was then amplified in a specific target area limited by a pair of *trnL-F* intergenic spacer primers (forward and reverse primers). Barcoding sequence amplification is done through the PCR technique. The reagent used is the ThermoScientific DreamTaq Green PCR Master Mix (2X), following the standard procedures that have been available. The *trnL-F* sequences used were cpDNA primers forward 5'-GGTTCAAGTCCCTCTATCCC-3' and cpDNA reverse 5'-ATTTGAACTGG TGACACGAG-3' (Taberlet et al. 1991). The PCR conditions consisted of initial denaturation at 95 °C for 3 minutes, denature at 95 °C for 1 minute, annealing at 54 °C for 45 seconds, DNA elongation at 72 °C for 1 minute, and post extension at 72 °C for 5 minutes. The PCR results were then visualized using an electrophoresis technique in 0.8% agarose, adding 1-3 µl of GelRed dye to the TAE 1X buffer solution. The electrophoresis process was carried out using the Mupid EXU Chamber with 100 v for 30 minutes for DNA and 45 minutes for PCR products. The DNA ladder used Thermo Scientific GeneRuler 1 kb. DNA visualization and was performed using the Bio-Rad Gel Doc ™ EZ Imager. The PCR product was then sequenced in the 1st Base Singapura.

Data analysis

DNA sequence data were analyzed using BioEdit software for the consensus sequence construction process (Hall 1999). Online data mining BLAST was carried out to identify similar sequences based on the National Center for Biotechnology Information (NCBI). Multiple alignment sequences were done through multiple sequence comparison by log-expectation (MUSCLE) (Edgar 2004). Phylogenetic tree construction used the Maximum-Parsimony (MP) method with the Tree-Bisection-Regrafting (TBR) algorithm (Nei dan Kumar 2000), which was carried out using MEGA X software with pairwise deletion options (Kumar et al. 2018), and 1000X bootstrap (Felsenstein 1985). Phylogenetic tree construction was carried out on 20 sequenced *Diospyros* spp accessions, 40 BLAST accessions from *Diospyros* spp, and four outgroups obtained from the NCBI database. The outgroup is the genus closest to *Diospyros*, namely *Euclea* and *Lissocarpa*. *Euclea* and *Lissocarpa* are still in the Ebenaceae family.

RESULTS AND DISCUSSION

Profile of the *trnl-F* Intergenic spacer sequences of the Malesian *Diospyros*

The *trnL-F* intergenic spacer *Diospyros* sequences obtained were then carried out by BLAST data mining to identify the sequence similarity based on the NCBI database to ensure that the sequence obtained was the *Diospyros* sequence. Each *Diospyros* species was taken as many as one to five species with a similar sequence to the sample DNA sequence based on the highest percentage of query cover and percentage identity (Table 1). The highest cover query value was 100% (*D. andamanica*), and the lowest was 94% (*D. nigra*). The highest identity value was 100% (*D. discolor, D. nigra, D. celebica*), and the lowest was 96.77% (*D. buxifolia*). Most of the DNA sequences of *Diospyros* species in this study are new data from NCBI so that there are only little similar sequences with the same species name. A total of 68 DNA sequences (64 *Diospyros* spp. and four outgroups) was constructed of their phylogeny tree to see their relationships.

Phylogenetic relationships of the Malesian *Diospyros*

Characteristics and statistical data from the Maximum-Parsimony (MP) analysis are presented in Table 2. Based on the 367 characters obtained, there are 299 characters (81.5%) that are constant, 68 characters (18.5%) that are variable or parsimony-uninformative, and 35 parsimony-informative characters (9.5%). Based on the results of the maximum-parsimony study, the resulting phylogenetic tree had a consistency index (CI) of 0.82, a retention index (RI) of 0.89, and a parsimony-informative consistency index (iCI) of 0.71. Phylogeny analysis of *Diospyros* species using the maximum-parsimony method with a bootstrap of 1000 replications produced a cladogram (Figure 2).

Discussion

Maximum-parsimony analysis for phylogenetic analysis of *Diospyros* in the Malesian region show sites are parsimony-informative if they contain at least two types of nucleotides, and two of them occur with a frequency of at least two. This site shows differences in the number of substitutions in the branching patterns to explain variations at these sites (Kumar et al. 2018). Thus, parsimony-informative sites help reconstruct parsimony phylogenetic trees, while parsimony-uninformative characters do not provide information about variations. However, specific parsimony-uninformative character sequences can also be used for species identification using the DNA barcoding method (Hapsari et al. 2018). Consistency-index (CI) and retention-index (RI) were compared to assess homoplasy present in the result tree from the analysis. A good phylogeny tree is characterized by a CI value close to 1. CI values close to or equal to 1 indicate that the tree does not have homoplastic characteristics. The retention index value is a stability index which is the proportion of synapomorphic characteristics that can be accepted as actual synapomorphic characteristics (Klingenberg and Gidaszewski 2010). The characters used in reconstructing the phylogeny tree were considered consistent when the RI value was one or close to 1 ($RI \leq 1$). A good consistency and retention index combined with a bootstrap of 1000 times can produce the most parsimony tree phylogeny (Soltis dan Soltis 2003; Duangjai et al. 2006).

Table 1. BLAST results of the nucleotide base samples at GeneBank

GenBank acc. no.	Species	Bed No. in BBG	Locality: voucher	Sequence similarity to	GenBank acc no.	Locality: voucher	% query cover	% per. ident
OK040108	*D. andamanica* (Kurz) Bakh.*	IV.C.101a	Indonesia: Borneo	*D. toposia* Buch.-Ham.	EU981041	Thailand	100	97.90
				D. frutescens Blume	EU981012	Thailand	100	97.90
				D. borbonica I.Richardson	EU981000	Thailand	100	97.90
				D. ferrea (Willd.) Bakh.	DQ924234	Thailand	100	97.60
				D. diepenhorstii Miq.	DQ924229	Thailand	100	97.60
OK040109	*D. aurea* Teijsm. & Binn.*	IV.D.199a	Indonesia: Sumatra	*D. mollifolia* W.W.Sm.	AF534665	China	96	98.72
OK040110	*D. buxifolia* (Blume) Hiern	X.G.130b	Indonesia: Celebes	*D. buxifolia* (Blume) Hiern	EU981001	Thailand	95	98.20
				D. kaki L.f.	AB115365	Japan	98	96.77
OK040111	*D. discolor* Willd.*	IV.D.180a	Indonesia: Papua	*D. philippinensis* A.DC.	DQ924264	Indonesia: Java	90	100.00
				D. mespiliformis Hochst. ex A.DC.	DQ924256	Austria	90	99.21
OK040112	*D. coriacea* Hiern*	IV.D.211	Indonesia: Papua	*D. samoensis* A.Gray	EU981032	USA: Hawaii	95	100.00
				D. castanea (Craib) H.R.Fletcher	DQ924217	Thailand	95	99.74
				D. dictyonema Hiern	EU981005	Thailand	95	99.48
				D. venosa Wall. ex A.DC.	EU981047	Thailand	95	99.21
OK040113	*D. discolor* Willd.*	II.Q.2	Philippines	*D. bejaudii* Lecomte	DQ924212	Thailand	95	98.95
				D. scalariformis H.R.Fletcher	EU981037	Thailand	95	98.43
OK040114	*D. korthalsiana* Hier*	XXIV.A.90b	Indonesia: Borneo	*D. cf. ulo*	EU981042	Brunei	95	99.21
				D. oblonga Wall. ex G.Don	DQ92426	Thailand	95	99.21
				D. filipendula Pierre ex Lecomte	DQ924236	Thailand	95	98.95
OK040115	*D. lolin* Bakh.*	XXIV.A.263	Indonesia: Moluccas	*D. curranii* Merr.	DQ924226	Thailand	95	98.72
OK040116	*D. macrophylla* Blume*	XXIV.A.330	Indonesia: Java	*D. consolatae* Chiov.	DQ924223	Mozambique	95	99.21
				D. phengklai	MG924497	Thailand	95	98.95
OK040117	*D.* sp1	XXIV.A.270	Indonesia: Celebes	*D. mindanaensis* Merr.	DQ924257	Brunei	95	98.37
				D. malabarica f. Atrata (Desr.) Kostel.	DQ924251	Indonesia: Java	95	98.63
OK040118	*D. nigra* (J.F.Gmel.) Perr.	XXIV.A.215	Indonesia: Java	*D. digyna* Loudon	DQ924230	USA	94	100.00
				D. tenuiflora A.C.Sm.	DQ92424	Brazil	94	100.00
OK040119	*D. pilosanthera* Blanco	XXIV.A.200	Indonesia: Maluccas	*D. melocarpa* F.White	DQ924255	Gabon	95	98.95
				D. mannii Hiern	DQ924253	Ghana	95	98.95
				D. dasyphylla Kurz	DQ924227	Thailand	95	98.95
OK040120	*Diospyros toposia* Buch.-Ham.	IV.C.74	Indonesia: Java	*D. pubicalyx* Bakh.	DQ924267	Thailand	95	99.74
				D. macrocarpa Hiern	DQ924249	New Caledonia	95	99.48
OK040121	*D. ridleyi* Bakh.	XIX.B.41	Indonesia: Celebes	*D. cooperi* (Hutch. & Dalziel) F.White	DQ924224	Ghana	95	98.70
				D. vieillardii (Hiern) Kosterm.	EU981048	New Caledonia	95	98.44
OK040122	*D. subrhomboidea* King & Gamble *	XXIV.A.234	Indonesia: Java	*D. pilosula* G.Don	DQ924265	Thailand	95	99.21
				D. glandulosa Lace	DQ924240	Thailand	95	98.95
OK040123	*D. sumatrana* Miq.	XIX.B.37	Indonesia: Celebes	*D. abyssinica* (Hiern) F.White	DQ92420	Africa	95	98.43
				D. oubatchensis Kosterm.	EU981020	New Caledonia	95	98.16
OK040124	*D. celebica* Bakh.*	IV.D.190a	Indonesia: Celebes	*D. celebica* Bakh.	DQ924221	Indonesia: java	96	100.00
				D. apiculata Hiern	EU980998	Thailand	96	97.40
OK040125	*D. discolor* Willd.*	V.C.90b	Indonesia: Java	*D. brassica* F.White	DQ924216	New Caledonia	95	97.64
OK040126	*D.* sp2	IV.C.106	Indonesia: Borneo	*D. montana* Roxb.	DQ924259	Thailand	95	99.22
				D. ehretioides Wall. ex G.Don	DQ924231	Thailand	95	99.22
OK040127	*D. cauliflora* Blume	XXIV.A.200	Indonesia: Java	*D. borneensis* Hiern	DQ924214	Thailand	95	99.10
				D. cauliflora Blume	DQ924218	Thailand	95	99.21

Note: * New sequence data in the NCBI database.

Table 2. Summary of characteristics and statistics of maximum-parsimony analysis for phylogenetic analysis of *Diospyros* in the Malesian region

Phylogenetic information	*trnL-F* Intergenic spacer
Number of ingroup taxa	64
Number of outgroup taxa	4
Sequence length	367
Number of characters constant (%)	81.5
Number of uninformative characters (%)	18.5
Number of potentially parsimony-informative character	9.5
Consistency index (CI)	0.82
Retention index (RI)	0.89

The phylogenetic tree showed that 64 *Diospyros* species were monophyletic and divided into seven clades (Figure 2). The topology of the phylogenetic tree *Diospyros* species formed in this study is monophyletic, dichotomous, and indicates support bootstrap value. The bootstrapping values demonstrate how many times the same branch was observed when repeating the phylogenetic reconstruction on a re-sampled data set. The bootstrap value of "70%" indicates the support of a phylogenetic tree (Hillis dan Bull 1993). The monophyletic formation is an indication that the use of *trnL-F* intergenic spacer primer is good enough to distinguish and see the phylogeny of *Diospyros* spp. Several studies have also used *trnL-F* intergenic spacer primers to reveal monophyletic *Diospyros* spp (Duangjai et al. 2018; Samuel et al. 2019). *TrnL-F* intergenic spacer indicates a good primer in *Diospyros* phylogenetic studies with values of consistency index and retention index (0.69, 0.71, respectively) although with a relatively short sequence length (163-387 bp) (Duangjai et al. 2006). All group members are separated by unique characters in a particular clade and all their derivatives (apomorphic) (Slobodian dan Pastana 2020).

Clades I to clade VI contain *Diospyros* species grouped into the sub-genus Eudiospyros by Bakhuizen van den Brink (1933). In Clade VIII, there is *D. ferrea* which is grouped into the sub-genus Maba. *Diospyros* in South-East Asia (include Malesia) and Pacific was grouped into five subgenera, i.e., subgenus Cargillia, Diospyros (Eudiospyros), Hierniodendron, Maba, and Mabacea. The sub-genus Eudiospyros was grouped into 32 sections, while Maba was into four sections. Eudiospyros have morphological characters: 4-5-7- (8-10) -merous flowers; sepals and the petals imbricate in growing bud; ovaries 4-5-, 8-10- 16- or 20; ovule 4-5, 8-10, and 16-20. Eudiospyros are generally distributed in America and Asia. The sub-genus of Maba have characters: flowers 3-merous, rarely 4-5 -merous; ovules 3-6 cell; sepals and petals valvate in bud stage (Bakhuizen van den Brink 1936).

Clade I contains the most *Diospyros* from the Malesian region. Clade I has variations at the 15 nucleotide sites (Table 2). Most of the clade I was identical to the clade "K" in the study of Duangjai et al. (2006). This study added five *Diospyros* species to clade K, namely three accessions of *D. discolor* (Java, Papua, and the Philippines), *D. korthalsiana*, *D. lolin*, *D. pilosanthera*, and *D. scalariformis*. Clade I is also similar to the clade "VII" in Duangjai et al. (2009) and added four *Diospyros* species, namely *D. discolor*, *D. korthalsiana*, *D. lolin*, and *D. pilosanthera*. Based on Bakhuizen van den Brink (1933), *Diospyros* in clade I belong to the sub-genus Eudiospyros. Sub-genus Eudiospyros include sections of Brachycylix (*D. philippinensis*), Campanulata (*D. bejaudii*), Confertiflora (*D. curranii*), Ebenaster (D. *discolor* and *D. celebica*), Melonia (*D. mespiliformis*), Ptychocylix (*D. oblonga*), and Stelechantha (*D. cauliflora*). Biogeographically, *Diospyros* species of clade one are scattered in Malesia, Asia, Africa, and Central America. *Diospyros discolor* is the one that is widely distributed to the four regions, although the centre of origin of *D. discolor* is in the Malesian Region (GBIF 2019). *Diospyros* species in clade I have some of the same characteristics: oblong-lanceolate, opposite, spiral; inflorescences cymose, bracteola alternate, petals 4-5-merous (Hiern 1873; Bakhuizen van den Brink 1936). *Diospyros* in clade I have fruit that can be eaten (edible) by mammals and at the same time become dispersing agents of these species (Duangjai et al. 2006). Also, the group in this clade is a producer of high-quality wood commodities, i.e., *D. celebica*, *D. mespiliformis*, *D curranii* and *D. lolin*.

Clade II consists of *Diospyros* species that originated from Asia, America, Africa, and Europe. Several members of clade II were grouped into different clade in the previous study. *D. kaki* and *D. glandulosa* were grouped in the "P" and "IX" clades. *D. andamanica*, *D. buxifolia*, and *D. dasyphylla* were grouped into the "Q" and "XI" clades (Duangjai et al. 2006; Duangjai et al. 2009). These five *Diospyros* species were split into one clade due to more *Diospyros* samples from the same area used in the phylogenetic tree reconstruction. Clade I and clade II are sisters in subclade *Ib.1.a* (figure 2) with 32 nucleotide base characters. In Clade II, *D. macrophylla* is a new record on the two previously published phylogenetic trees of *Diospyros* spp. *D. buxifolia* is at the nodes some distance away from *D. mollifolia*, *D. glandulosa*, and *D. kaki*. This condition is presumably because *D. buxifolia* has distributed in tropical areas. At the same time, *D mollifolia* lives in temperate regions (GBIF 2019). *Diospyros* in clade II has several characteristics: tree; leaves ovate-elliptical-lanceolate, opposite; inflorescence 1-3 flowers, style glabrous. Clade III supports the hypothesis that *D. glandulosa* is the ancestor of *D. kaki* (Ng 1978) shown by placing *D. glandulosa* and *D. kaki* as sister groups.

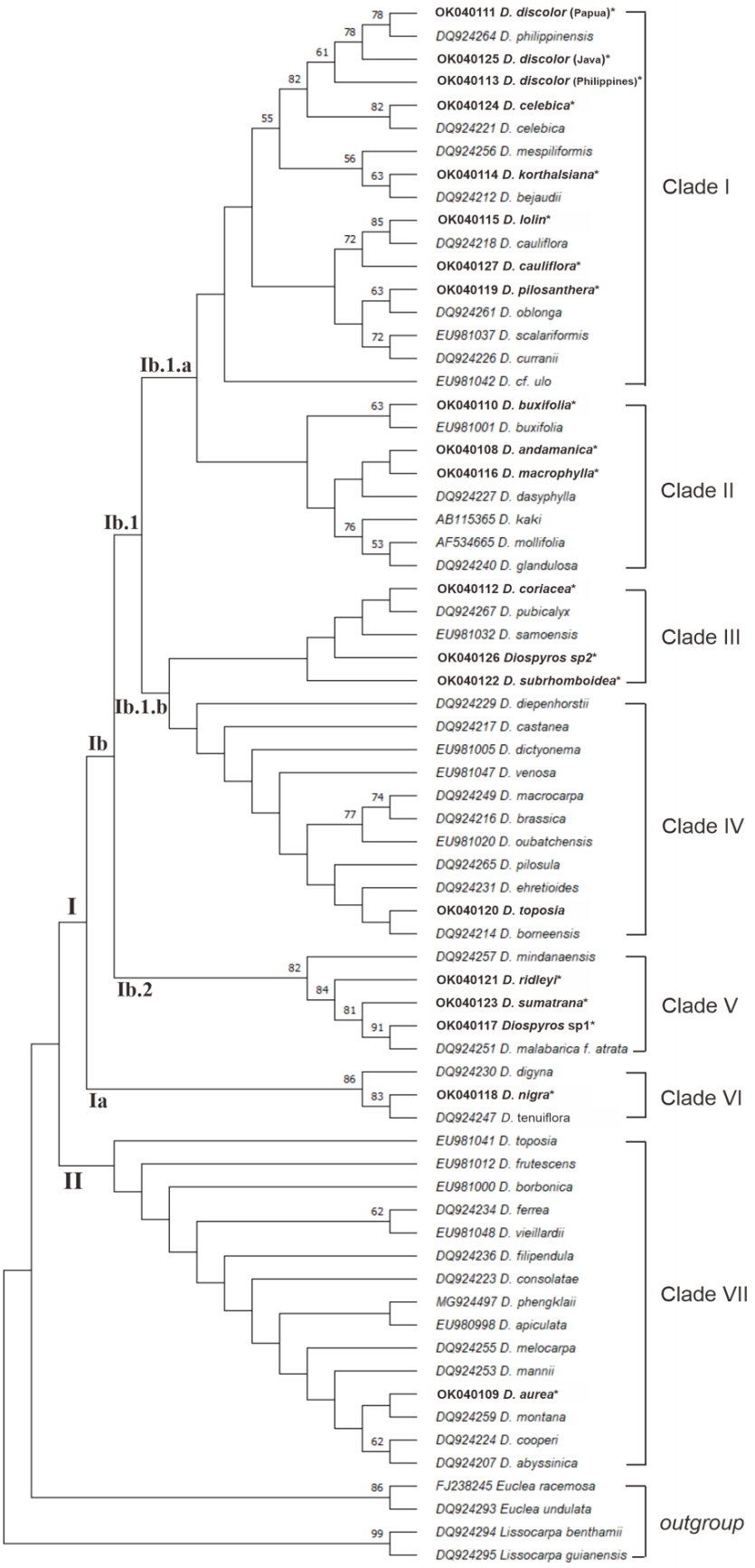

Figure 2. Phylogenetic tree based on *trnL-F* intergenic spacer sequence data reconstructed using the maximum-parsimony method. Branches were analyzed by bootstrapping 1000 replications. Bootstrap values on nodes that are less than 50% are not displayed.

Clade III consists of five *Diospyros* species distributed in the Malesian to Caledonian Region. *Diospyros samoensis* originated from Caledonia, phylogenetically adjacent to *D. coriacea* and *D. pubicalyx* from the Malesian region. Duangjai et al. (2006) proposed that *D. samoensis* and *D. pubicalyx* are in the same clade. In clade III, *Diospyros* has the same characteristics, such as shrub-tree; leaves are oval-ovate, leaf base obtuse; stamens 8-17. In Clade III, *Diospyros* sp2 is the sister of three *Diospyros*, namely *D. samoensis*, *D. pubicalyx*, and *D. coriacea*, with a bootstrap node value below 50. A bootstrap value of less than 70 indicates bias and possible clade arrangement changes (Falsenstein 2004).

Clade IV consists of *Diospyros* species spread from Thailand, Malesia to New Caledonia. Clade IV and clade III are sisters in subgroup *Ib.1.b*. Clade IV unites the three clades formed by Duangjai et al. (2006). These three clades are the clade "G" (*D. macrocarpa*, *D. brassica*), the Clade "I" (*D. borneensis*), and the clade "Q" (*D. diepenhorstii*, *D. castanea*, *D. venosa*, *D. pilosula*, *D. ehretioides*). In another study, *D. diepenhorstii*, *D. venosa*, *D. pilosula* were also separated in different clusters from *D. macrocarpa*, *D. brassica, and D. borneensis* (Duangjai et al. 2009). In this clade, *D. racemosa* is a newly published record of the hypothetical phylogenetic tree.

Clade V consists of *Diospyros* from Malesia's western and central parts, including Peninsular Malaysia, Sumatra, Java, Borneo, and Celebes. In clade V, two species of *Diospyros* are also placed in the same clade in other publications. The two species of *Diospyros* are *D. mindanaensis* and *D. malabarica f. atrata* that is in the clade "P" (Duangjai et al. 2006). Besides, this clade also unites three species of *Diospyros* that were previously placed in different clades, namely *D. sumatrana* (clade "XI), *D. malabarica f. atrata* (clade "X"), and *D. ridleyi* (clade VII). In addition to the barcoding DNA approach, the biogeographic similarity is an assumption that can be put forward to unite or separate these species. The bootstrap value in this clade is relatively high, in the range of 81-91. In clade V *Diospyros* has the same characteristics: tree-shrub; leaves oblong-elliptical, leaf base obtuse; style 4-6. *Diospyros* sp1, which has not been identified morphologically, shows similar DNA with *D. malabarica* f. (percentage identity value of 98.63%, and query cover 95%).

Clade VI and VII consist of *Diospyros*, which have the widest distribution from Malesia, Caledonia, China, the Indian Peninsula, Africa to America. Clade VII only consists of three species of *Diospyros*. In this clade, *D. nigra* originating from Java Island has a close relationship with *D. tenuiflora* and *D. digyna*, which have distribution in North America - South America. Based on GBIF data, *D. nigra* also has a reasonably wide distribution spread from the Oceania Islands to America. On the other hand, *Diospyro*s sensu lato dominates clade VIII (White 1980). These *Diospyros* came from Africa (including the Madagascar Islands), namely *D. borbonica*, *D. consolatae*, *D. melocarpa*, *D. mannii*, *D. cooperi*, and *D. abyssinica* (White 1988). In this study, the *Diospyros* from the Malesian region and Africa were grouped into one clade, while in another study, they were grouped into different clades, clade M and O (Duangjai et al. 2006). *Diospyros* from this region of Malesia is *D. aurea*, *D. toposia*, *D. frutescens*, *D. apiculata*, *D. ferrea*, and *D. montana*. *Diospyros aurea* from Sumatra is close to *D. montana*, which is distributed in Malesia to China. Besides *Diospyros* Africa and Malesia, there is also *D. vieillardii* from the Caledonia Islands.

In conclusion, the diversity of *Diospyros* in the Malesian region has been validated on 20 accessions representing 18 *Diospyros* species using sequence data on the genebank. They are related to 44 other *Diospyros* species in the world. Ten *Diospyros* species are new sequence information published in the Malesian region. Based on the phylogenetic analysis using the maximum-parsimony method, 64 *Diospyros* species are monophyletic and grouped into seven clades. These seven clades update the phylogenetic data of *Diospyros* in the Malesian region. The biogeographic proximity hypothesis supports the phylogenetic results. Thus, this study supports that the distribution of clades can be grouped based on the distribution of *Diospyros* as well as the similarity of nucleotide bases: Malesian region (clades I and V), Malesian-Caledonian region (clade III and IV), cosmopolitan in almost all bioregions (Clade II, VI, and VII).

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