



MOLECULAR IDENTIFICATION OF *Shorea johorensis* IN KETAMBE RESEARCH STATION, GUNUNG LEUSER NATIONAL PARK

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Abstract. *Shorea johorensis* is one of the red meranti plants in Ketambe Research Station, Gunung Leuser National Park, Aceh Tenggara. Currently, *Shorea johorensis* also is well known as a major source of valuable commercial timber. This research aims to analyze the phylogenetic of *Shorea johorensis* based on chloroplast and nuclear DNA in Ketambe Research Station so that it can be known the relationship of *Shorea johorensis* with other species of Dipterocarpaceae in GenBank database. The research was conducted from July 2015 to August 2016 in Ketambe Research Station and Forestry and Forest Genetics Laboratory of Molecular, Bogor Agricultural University. The method used quadrat sampling technique with purposive sampling and experimental laboratory that consisted of DNA extraction, PCR, electrophoresis, and sequencing. The data analysis was done using BLAST, BioEdit, and MEGA6. The results showed that the phylogenetic tree of *Shorea johorensis* based on the *rbcL* and *matK* showed that *Shorea johorensis* was closely related with some species of *Hopea*; but the phylogenetic tree based on *psbA-trnH*, 5.8S rRNA, ITS2, and 28S rRNA showed that *Shorea johorensis* was closely related with *Shorea robusta*.

Keywords: Chloroplast DNA, Ketambe Research Station, Nuclear DNA, *Shorea johorensis*

I INTRODUCTION

Gunung Leuser National Park is one of Nature Conservation Areas in Indonesia covering an area of 1.094.692 hectares. This national park is located in Aceh and Sumatera Utara provinces. It is known as one of the World Heritage Sites for the rainforest which has several ecosystems from the coast to the high mountains [1]. One of the research stations in Gunung Leuser National Park is Ketambe Research Station. Ketambe Research Station has high and unique biodiversity. It lies in Aceh Tenggara district. A few Dipterocarpaceae species is found growing in Ketambe Research Station [2]. Dipterocarpaceae is the most typical family of tropical forest trees in the Malesian region with a geographical distribution that extends to South America and Africa. The family comprises approximately 500 species in 17 genera. It is subdivided into three subfamilies: Dipterocarpoideae, Monotoideae, and Pakaraimoideae. Dipterocarpoideae comprises 470 species in 13 genera [3]. The most Dipterocarpaceae are large trees with towering top reaching 70-80 m. Dipterocarpaceae dominate in tropical lowland forest [4]. The

non-timber products of dipterocarp are used by some wildlife in the forest for their survival therefore, ecologically it is essential. The wood of Dipterocarpaceae is well known as a major source of valuable commercial timber. Currently, the dipterocarps predominate the international tropical timber market and therefore play an important role in the economy of many of the Southeast Asian countries [5]. One of the Dipterocarpaceae species is *Shorea johorensis*. It can be found in Southeast Asian particularly in Borneo, the Peninsular of Thailand and Peninsular Malaysia, and Sumatra. In the timber trade, this *Shorea* is a type of wood which is grouped into red meranti. Its wood is light, soft, moderately durable, resistant to dry wood borers and fungi but susceptible to termites. *Shorea johorensis* has a high value of timber trade [6]. The chloroplast genome is most widely used as a source of information on the inference of the evolutionary patterns and processes of plants [7] because this genome is thought to evolve slowly, with low mutation rates and maternal inheritance in most angiosperms, along with being a conserved region in structure and gene order. A chloroplast DNA marker that is

maternally inherited shows more conserved DNA patterns compared with a nuclear gene that is biparentally inherited. Several regions of chloroplast genome are *rbcL* gene, *matK* gene, and *psbA-trnH* intergenic spacer. The *rbcL* gene is a gene encoding a large subunit of ribulose 1.5 biphosphate carboxylase (Rubisco or RuBPCase), which is important for photosynthesis. The sequence of *rbcL* gene data is extensively used in the reconstruction of the whole seed plants phylogeny because it has a fairly conservative level of evolution [8]. On the other hand, the *matK* gene is a gene encoding the maturase enzyme subunit K. In plant systematics, *matK* appears as a valuable gene because it has a high phylogenetic signal than another gene [9]. The *psbA-trnH* intergenic spacer is an evolutionary plastid region and employed as a phylogenetic marker [10].

In addition, nuclear DNA is also generally used in evolutionary as well as phylogenetic studies. Nuclear DNA is transmitted from parent to offspring by nuclear division through sexual or asexual reproduction [11]. Since a nuclear genome is biparentally inherited, it is expected to provide more information than a chloroplast or mitochondrial genome on species identity, including hybridization. One of the most useful types of nuclear DNA sequences is the Internal Transcribed Spacer

(ITS) region, which contains multiple DNA copies. The ITS region lies between 16S and 28S nuclear ribosomal DNA (nrDNA). Several years ago, ITS regions were often used by experts for molecular phylogenetic analysis on plants in order to understand diversity and answer some issues phylogenetic. This is because the ITS region has superior characteristics namely, has small size (approximately 700 base pairs) and a lot of copying in the nuclear genome [12]. These characteristics cause the ITS region to be easy to be isolated, amplified, and analyzed. Therefore, this research aims to analyze the phylogenetic of *Shorea johorensis* based on chloroplast and nuclear DNA in Ketambe Research Station so that the relationship of *Shorea johorensis* with some species in Dipterocarpaceae can be determined.

II METHODOLOGY

Study Area

The research was conducted in Ketambe Research Station, Gunung Leuser National Park, Aceh Tenggara and Forestry and Forest Genetic Laboratory of Molecular, Bogor Agricultural University. The research was begun on July 2015 to August 2016. The location of the research area is showed from the map (Figure 1).

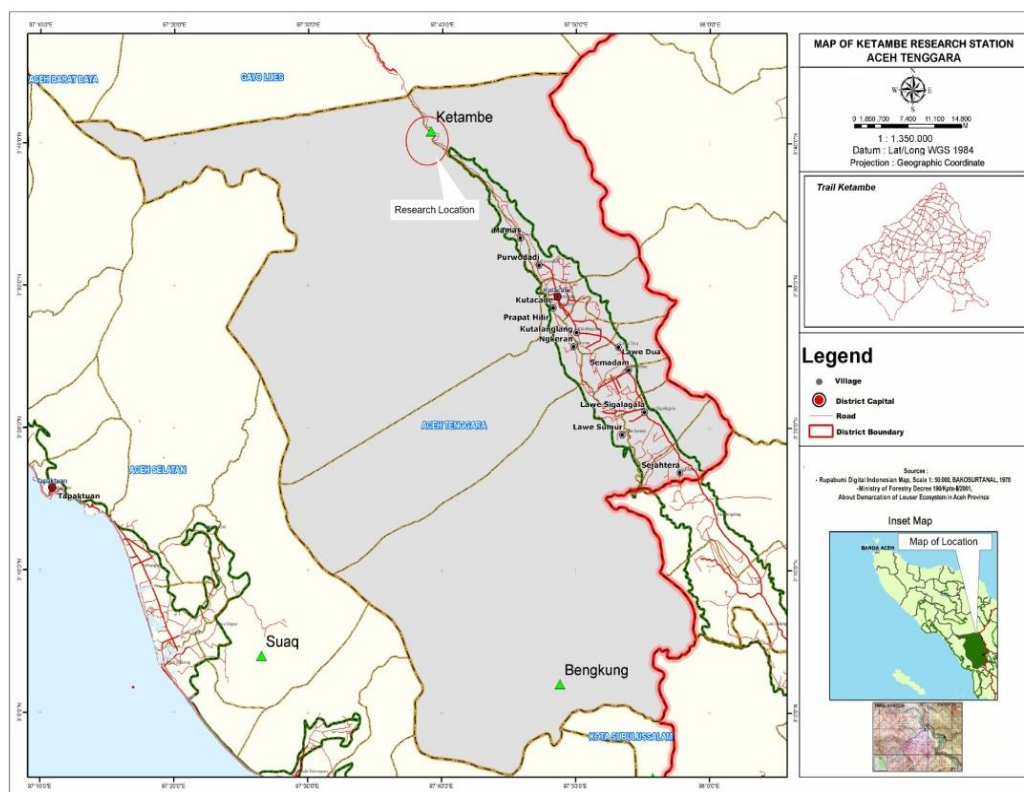


Figure 1 Map of Research Sites in Ketambe Research Station, Gunung Leuser National Park, Aceh Tenggara

Samples collection

Samples of Dipterocarpaceae were collected from Ketambe Research Station, Gunung Leuser National Park, Aceh Tenggara (Figure 1). Samples were collected using Quadrat Sampling Technique. Intake of vegetation data by sampling plot was done by purposive sampling. There were 25 plots with an area of each plot 20 x 20 m [13]. The total area of the plots was 1 ha 10.000 m². Samples consisted of leaves from sampling stage, pole stage, or tree of Dipterocarpaceae. Three individuals per species were collected represent Ketambe Research Station location. The three individual samples had the same ID number, and they were numbered individually. Three sets of specimen leaves were collected from each individual sample: (a) two sets of leaves for the herbarium (leaves must have important taxonomic characters such as leaf tip, leaf surface, stipule, petiole/leaf stalk). Specimens for herbarium were put on paper sheets, and moistened with 70% alcohol. The specimens were dried and glued on herbarium paper pairs. Specimens were labeled information such as ID numbers, collector name, collection date, and taxonomy. Identification of samples was done using Dipterocarpaceae identification book; and (b) a set of leaves for DNA extraction (soft, fresh, and young leaf tissue). Specimen for DNA extraction: leaf sheets were cleaned with the dry cloth. Specimens were placed into an existing sac containing another sac filled with silica gel (ratio of silica gel 5-10: 1). The ID number of the specimens were written on the outside of the bag using a permanent marker. All packets/bags of the specimen were stored in containers [14].

DNA Extraction

DNA extraction was carried out using Cetyltrimethyl Ammonium Bromide (CTAB) method developed by Doyle and Doyle [15]. Young leave of 200 mg was ground in a mortar with liquid nitrogen. The leaf powder was put into 2 mL tubes, 500 extraction buffer solutions and 100 µL polivinilpirodin (PVP) solution were added. The mixture was vortexed

and then incubated in a water heater (waterbath) for 60 min at a temperature of 65°C and every 15 min once reversed. After cooling to room temperature for 15 min, the mixture was added with 500 µL chloroform-isoamyl alcohol (24:1) and centrifuged for 10 min at 10,000 rpm. The upper layer (water phase/supernatant) was separated from the organic phase by using the micropipette into the new tube. Chloroform-isoamyl alcohol was added twice. The Supernatant was added with 500 µL cold isopropanol and NaCl of 300 µL. Samples were incubated overnight in the freezer. The precipitation result was centrifuged at 10,000 rpm for 10 min. The DNA pellet was washed twice using 96% ethanol of 300 µL and dried in a desiccator for 15 min. The dried DNA was added with 50 µL TE buffer (5 M Tris-HCl pH 8.0; 0.5 M EDTA pH 8.0). The DNA then was flicked and centrifuged at 10,000 rpm for 2 min. DNA was stored at -20°C in the freezer.

Polymerase Chain Reaction (PCR)

For chloroplast DNA, The final conditions of each PCR reaction were 16 µL consisted of 8 µL Green GoTaq DNA polymerase, 2 µL Nuclease-Free Water, 2 µL primer (forward), 2 µL primer (reverse), and 2 µL DNA template. The primers were *rbcl*, *matK*, and *psbA-trnH*. The temperature settings of the thermocycler were the initial denaturation at 95°C for 4 min and then 35 cycles with denaturation at 94°C for 30 s, annealing at 58°C (for *rbcl* and *psbA-trnH*) and at 56°C (for *matK*) for 1 min, extension at 72 °C for 1 min and rest at 4°C [16]. For nuclear DNA, amplification segments of DNA were conducted in 20 µL PCR reactions (Green goTaq PCR and PCR MasterMix). All of the PCR components consisted of 10 µL (1X Green goTaq), 1 µL forward primer, 1 µL reserve primer, 3 µL DNA template, and 5 µL Nuclease-Free Water were mixed into one tube. The temperatures for PCR machine were started by initial denaturation at 94°C for 3 min; 30 cycles 3 stages: (at 94°C for 30 s), annealing (at 58°C for 30 s), and extension (at 72°C for 1 min); and elongation stage at 72°C for 10 min. The primers in this study were ITS1 and ITS4 (Table 1).

Table 1 Primers Data in This Study

DNA region	Primer	Sequence (5'---- 3')	Reference
<i>rbcl</i>	<i>rbcl</i> F	ATGTCACCACAAACAGAGACTAAA	[17]
	<i>rbcl</i> R	GTAAATCAAGTCCACCRCG	[17]
<i>matK</i>	<i>matK</i> 472F	CCCRTYCATCTGGAAATCTTGTTTC	[18]
	<i>matK</i> 1248R	GCTRTATAATGAGAAAGATTTCTG	[18]
<i>psbA-trnH</i>	<i>psbA</i> F	GTTATGCATGAACGTAATGCTC	[19]
	<i>trnH</i> R	CGCGCATGGTGGATTCACAATCC	[20]
ITS	ITS1	TCCGTAGGTGAACCTGCGG	[21]
	ITS4	TCCTCCGCTTATTGATATGC	[21]

Gel electrophoresis

The result of PCR (DNA) was visualized by 1% agarose gel electrophoresis. Gel electrophoresis procedure consisted of making agarose gel, sample loading into the gel, running electrophoresis, and observation of electrophoresis running with UV transilluminator. For the DNA extraction, gel electrophoresis was done by using electric current with a voltage of 100 volt for 45 min and for the PCR, the process of running electrophoresis was for 30 min with a voltage of 100 volt. The results of running electrophoresis were observed using UV transilluminator.

Sequencing

The nucleotide sequence of the amplicon was identified using the Sanger method carried out by 1st BASE Sequencing INT in Malaysia. The sequencing process was done twice with different directions (forward and reverse). The sequencing data were used for the construction of phylogenetic trees.

Data analysis

The result of sequencing were analyzed with the following stages: (i) Annotation of ITS2 Sequence using ITS2 Database [22], (ii) BLAST (Basic Local Alignment and Search Tool) sequence using GenBank Database, (iii) Sequence alignment using Bioedit program [23], (iv) The result of sequence alignment was used to develop phylogenetic tree by Neighbor Joining (NJ) method with MEGA (Molecular Evolutionary Genetics Analysis) version 6 [24], (v) The reliable test of the tree was done by the bootstrap method 1000 times.

III RESULT AND DISCUSSION

A. The result of Electrophoresis Gel

DNA Extraction

DNA extraction is a method of separating DNA from other cell components. The extraction of DNA Dipterocarpaceae was performed to obtain DNA from the genome total of *Shorea johorensis* which were used as DNA template for PCR amplification process. The most methods for DNA extraction used CTAB buffer solution as cell wall degradation because it has advantages i.e., easy to do and the possibility of DNA degrading enzymes is smaller than other methods [25]. Based on the visualization of electrophoresis result in Figure 2, there were DNA bands of the three samples. All of DNA bands look thick, so the process of DNA extraction was successful.

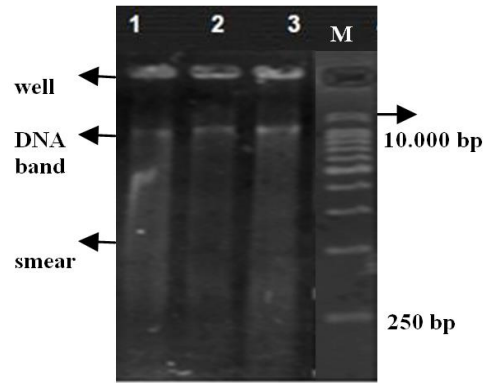


Figure 2 The Results of Gel Electrophoresis of DNA Extraction *Shorea johorensis* (M = Marker 1 kb DNA Ladder; 1 = *Shorea johorensis* 1; 2 = *Shorea johorensis* 2; 3 = *Shorea johorensis* 3)

PCR Amplification

PCR Amplification was performed to multiply *Shorea johorensis* genomic DNA strands with the target of the *rbcL*, *matK*, *psbA-trnH*, and ITS region. The result of electrophoresis is presented in Figure 3.

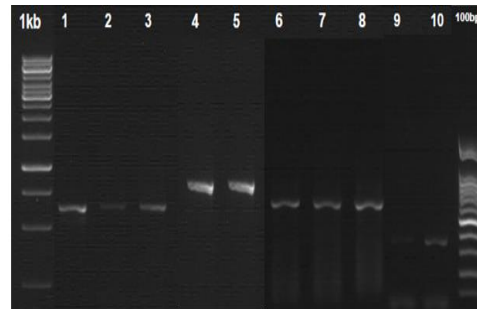


Figure 3 The results of Gel Electrophoresis of PCR Amplification (1-3 = (*Shorea johorensis* 1, *Shorea johorensis* 2, and *Shorea johorensis* 3; *rbcL* sequences); 4-5 = (*Shorea johorensis* 1 and *Shorea johorensis* 2; *matK* sequences); 6-8 = (*Shorea johorensis* 1, *Shorea johorensis* 2, and *Shorea johorensis* 3; ITS sequences); 9-10 = (*Shorea johorensis* 1 and *Shorea johorensis* 2; *psbA-trnH* sequences); 1kb DNA Ladder (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000; 100bp DNA Ladder (bp): 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500)

The DNA bands of *Shorea johorensis* were between the 500-750bp (base pair) for *rbcL*, *matK*, and ITS region. However for *psbA-trnH*, the DNA bands of *Shorea johorensis* were 300 bp. For *rbcL* and ITS region, all of the three samples were

Table 2 The result of BLAST Analysis of *Shorea johorensis*

No.	Region	Species from GenBank Database	Max Identity (%)	Query Coverage (%)	E-Value	Accession
1.	<i>rbcL</i>	<i>Hopea ponga</i>	99	97	0.0	JX163308.1
		<i>Shorea brevipetiolaris</i>	99	96	0.0	KX944295.1
		<i>Hopea bracteata</i>	99	95	0.0	KY973134.1
		<i>Hopea dryobalanoides</i>	99	95	0.0	KY973136.1
		<i>Parashorea macrophylla</i>	99	95	0.0	KY973159.1
		<i>Shorea parvifolia</i>	99	95	0.0	KY973239.1
		<i>Shorea robusta</i>	99	91	0.0	KX010597.1
2.	<i>matK</i>	<i>Hopea nervosa</i>	98	99	0.0	KY972948.1
		<i>Hopea bracteata</i>	98	99	0.0	KY972938.1
		<i>Hopea dryobalanoides</i>	98	99	0.0	KY972940.1
		<i>Hopea hongayensis</i>	98	99	0.0	KJ611239.1
		<i>Parashorea chinensis</i>	99	96	0.0	KJ611235.1
		<i>Shorea robusta</i>	99	95	0.0	KJ973059.1
3.	<i>psbA-trnH</i>	<i>Shorea robusta</i>	95	80	2e-111	JX856942.1
		<i>Shorea robusta</i>	95	75	9e-106	JX856943.1
		<i>Parashorea chinensis</i>	98	64	5e-98	KR534161.1
4.	5,8 S rRNA	<i>Shorea robusta</i>	90	84	0.43	KM51467.3
		<i>Parashorea chinensis</i>	93	60	6.9	KR532475.1
		<i>Hopea hainanensis</i>	100	40	27	AY328172.1
5.	ITS2	<i>Shorea robusta</i>	90	84	0.43	KM51467.3
		<i>Parashorea chinensis</i>	93	60	6.9	KR532475.1
		<i>Hopea dryobalanoides</i>	89	83	8e-62	AY026705.1
		<i>Hopea mengarawan</i>	88	83	4e-60	AY026705.1
6.	28 S rRNA	<i>Shorea robusta</i>	100	100	3e-08	KM51467.3
		<i>Parashorea chinensis</i>	96	100	7e-6	KR532475.1
		<i>Parashorea chinensis</i>	96	100	7e-6	KR532476.1

successfully done but for *matK* and *trnH-psbA* only two samples were successfully amplification and sequencing. One of the successes of amplification is affected by the primer. In this study, all primers are universal primers for recognizing regions of Angiospermae plant. All of the ten samples of DNA bands look quite thick.

B. BLAST Analysis

The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families [26]. The result of BLAST Analysis of *Shorea johorensis* can be seen in Table 2. The results of BLAST analysis of *Shorea johorensis* based on *rbcL* showed that this *Shorea* had similarity with some species of *Shorea* and *Hopea*. *Shorea johorensis* had the highest similarity with *Hopea ponga*. *Shorea johorensis* had the maximum identity value 99%, the query coverage 97%, and the E-value 0.0 with *Hopea ponga*. According to Ref. [27], maximum identity is the highest value of the percentage of identity or compatibility

between a query sequence (sequence research) with sequence from GenBank database.

Query coverage is the percentage of the nucleotides length aligned with the existing database on BLAST. According to Ref. [28] the value of an E-value is an alleged value that gives statistically significant sizes to the second sequence. The higher value of E-value indicates the lower homology between sequences, while the lower E-value indicates the higher homology between sequences. An E-value of 0 indicates the both of sequences are identical. For *matK*, *Shorea johorensis* had similarity with some species of *Shorea* and *Hopea*. *Shorea johorensis* had the highest similarity with *Hopea nervosa*. *Shorea johorensis* had the maximum identity value 99%, the query coverage 98%, and the E-value 0.0 with *Hopea nervosa*. *Shorea Johorensis* based on *psbA-trnH* had the highest similarity with *Shorea robusta*. *Shorea johorensis* had the maximum identity value 95%, the query coverage 80%, and the E-value 2e-111 (far enough from 0) with *Shorea robusta*. It caused there were only a few sequences *psbA-trnH* of Dipterocarpaceae in GenBank database.

ITS1 and ITS4 primers can amplify ITS region from 16S until 28S nuclear ribosomal DNA. In this study, there were 3 parts of ITS region for phylogenetic analysis namely, 5.8S rRNA gene, ITS2, and 28S rRNA gene. The results of

BLAST of *Shorea johorensis* based on 5.8 S rRNA showed that there were only 3 sequences of 5.8 S rRNA available in the NCBI database i.e., *Shorea robusta*, *Parashorea chinensis*, and *Hopea hainanensis*. *Shorea johorensis* had the highest similarity to *Shorea robusta*. *Shorea johorensis* had the maximum identity value 90%, the value of query coverage 84%, and the E-value 0.43 (almost close to 0) with *Shorea robusta*. The three sequences of 5.8S rRNA have different genera. So it is clear that *Shorea robusta* had a similarity to *Shorea johorensis* due to still in one genus. *Hopea hainanensis* showed the E-value high enough, so it had low homology level with *Shorea johorensis*. For ITS2, this *Shorea* showed similarity with some species of *Shorea* and *Hopea*. However, only 4 sequences were selected, i.e., *Shorea robusta*, *Parashorea chinensis*, *Hopea dryobalanoides*, and *Hopea mengarawan*. The highest similarity was owned by *Shorea robusta*. *Shorea johorensis* had the maximum identity value 90%, the query coverage 84%, and the E-value 0.43 with *Shorea robusta*.

The result of BLAST analysis based on ITS2 showed the highest similarity with the same species in 5.8S rRNA (*Shorea robusta*). The other species showed low homology level with *Shorea johorensis*. For 28S rRNA, this *Shorea* showed that there were only 3 sequences of 28S rRNA available in the NCBI database i.e., *Shorea robusta*, *Parashorea chinensis* (KR532475.1), and *Parashorea chinensis* (KR532475.1). *Shorea johorensis* had the highest similarity to *Shorea robusta*. *Shorea johorensis* had the maximum identity value and the query coverage value 100% with *Shorea robusta*.

The E-value between of these sequences 3e-08. The result of BLAST analysis based on 28S rRNA showed the highest similarity with the same species in 5.8S rRNA and ITS2 (*Shorea robusta*), but it had the difference of E-value with them. Overall, Sequence data from *Shorea johorensis* based on *rbcL*, *matK*, *psbA-trnH*, 5.8S rRNA, ITS2, and 28S rRNA have not been found in the NCBI database, so this *Shorea* had the highest similarity to other Dipterocarpaceae species. In addition, chloroplast DNA analysis of Dipterocarpaceae showed the difficulty of distinguishing between closely related species in the genera level primarily to indicate the species of *Shorea*. In fact, several types in the same tribe have identical sequences [29].

C. PHYLOGENETIC TREE

Phylogenetic Tree of *Shorea johorensis* based on *rbcL*

The construction of phylogenetic tree was conducted using MEGA 6 program with Neighbor Joining (NJ) method. The construction of phylogenetic tree aims to determine the relationship of among several Dipterocarpaceae species. Based on Figure 4, there were two groups (clades) namely group 1 and group 2. The first group had bootstrap value 75, consisted of *Hopea ponga*, *Shorea brevipedicularis*, *Hopea bracteata*, *Shorea johorensis* 1, *Shorea johorensis* 2, *Shorea johorensis* 3, *Hopea dryobalanoides*, and *Shorea robusta*. The second group had bootstrap value 90 consisted of *Shorea robusta*, *Parashorea macrophylla*, and *Shorea parvifolia*.

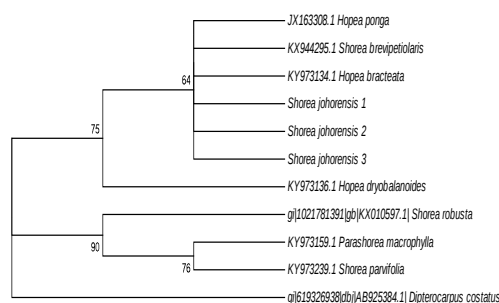


Figure 4 The phylogenetic tree of *Shorea johorensis* based on *rbcL* gene using the neighbor joining method

Each group formed a monophyletic group. A group of species is a monophyletic if all of the species present in the branches come from one common ancestor [30]. In this phylogenetic tree, *Shorea johorensis* had a closer relationship with *Shorea brevipedicularis* and some of *Hopea* than *Shorea parvifolia*, *Shorea robusta*, and *Parashorea macrophylla*. This is in accordance with the research by Ref. [31], *Shorea johorensis* formed a separate group with *Shorea parvifolia*. In addition, chloroplast DNA analysis by Ref. [14 and 32] also explained that *Shorea johorensis* and *Shorea parvifolia* each formed the monophyletic group. Based on this phylogenetic tree, *rbcL* gene was not able to separate *Shorea*, *Hopea*, and *Parashorea* to different monophyletic. This is relevant to the cpDNA analysis by Ref. [14 and 33] *Parashorea* clustered with *Shorea*. Molecular data from Ref. [14, 34, 35, and 36] also indicated that *Parashorea* was a very close relative of *Shorea*. This incongruence may suggest interspecific hybridization or ancestral polymorphisms. In addition, research by Ref. [37], based on *rbcL*, *Hopea* formed monophyletic to several species in the tribe of Shoreae.

Dipterocarpus costatus was an outgroup because it had a distant relationship with Dipterocarpaceae research samples. According to Ref. [38], in the analysis of phylogenetics, outgroup lead to the polarization of characters or characteristics, namely apomorphic and plesiomorphic characters. The apomorphic character is the changed and derived characters which were found in the ingroup (species which were studied), whereas the plesiomorphic character is the primitive character found in the outgroup. The synapomorphic character is a derived character in the monophyletic group.

Phylogenetic Tree of *Shorea johorensis* based on *matK*

The phylogenetic tree of *Shorea johorensis* based on *matK* is presented in Figure 5. There were two monophyletic groups. The first group had bootstrap value 100, consisted of *Shorea johorensis*, *Hopea bracteata*, *Hopea dryobalanoides*, and *Hopea nervosa*. The second group had bootstrap value 89, consisted of *Parashorea chinensis* and *Shorea robusta*.

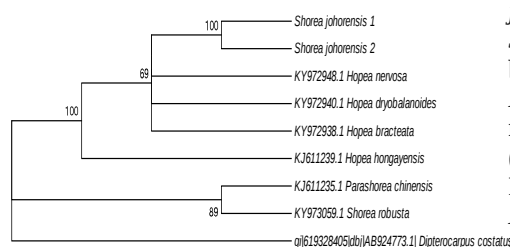


Figure 5 The phylogenetic tree of *Shorea johorensis* based on *matK* gene using the neighbor joining method

Shorea johorensis had a closer relationship with some of *Hopea* than *Parashorea chinensis* or *Shorea robusta*. Based on this phylogenetic tree, the *matK* gene also was not able to separate *Shorea*, *Hopea*, and *Parashorea* to different monophyletic. The phylogenetic analysis using *trnL-trnF*, *trnL*, and *matK* from several species of Dipterocarpoidea also showed that *Hopea* formed monophyletic with several of *Shorea* [39]. *Dipterocarpus costatus* was an outgroup because it had a distant relationship with Dipterocarpaceae research samples.

Phylogenetic Tree of *Shorea johorensis* based on *psbA-trnH*

Based on Figure 6, *Shorea johorensis* formed the monophyletic group with *Shorea robusta* (bootstrap value 100). It means this *Shorea* had a closer relationship with *Shorea robusta* than *Parashorea chinensis*. Based on morphology data, *Shorea robusta* showed some similarities

in morphological characters with *Shorea johorensis*. *Shorea robusta* is a large, deciduous tree up to 50 m tall (but these are exceptional sizes), and under normal conditions *Shorea robusta* trees have a height of about 18-32 m and girths of 1.5-2 m; the bole is clean, straight and cylindrical, but often bearing epicormic branches; the crown is spreading and spherical. The bark is dark brown and thick, with longitudinal fissures deep in poles, becoming shallow in mature trees [40]. *Dipterocarpus zeylanicus* was an outgroup.

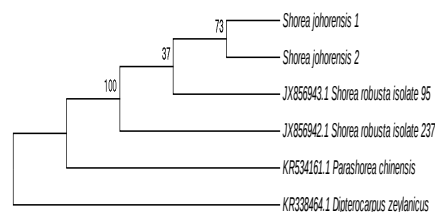


Figure 6 The phylogenetic tree of *Shorea johorensis* based on *psbA-trnH* using the neighbor joining method

Phylogenetic Analysis Based on 5.8S rRNA

All of the three individuals of *Shorea johorensis* formed a monophyletic group with *Shorea robusta* (first group). This group had bootstrap value 66. *Hopea hainanensis* and *Parashorea chinensis* also formed a monophyletic group with bootstrap value 73 (second group). There were 3 genera of Dipterocarpaceae i.e., *Shorea*, *Parashorea*, and *Hopea*. *Shorea* was separate with *Parashorea* (Figure 7). This is in accordance with the analysis of nuclear DNA conducted by Ref. [36], *Shorea* and *Parashorea* were separate and not belonging to a monophyletic group.

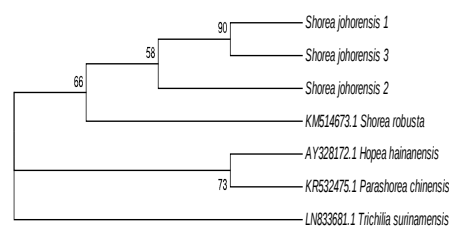


Figure 7 The phylogenetic tree of *Shorea johorensis* based on 5.8S rRNA using the neighbor joining method

In addition, *Shorea* also formed a separate group with *Hopea*. This is in contrast to *rbcL* and *matK* genes where *Shorea* formed a monophyletic group with *Hopea*. It means that ITS2 was able to separate *Shorea* with *Parashorea* and *Hopea* but not able to separate *Parashorea* with *Hopea*. *Trichilia surinamensis* was an outgroup because it had a distant relationship with other samples.

Phylogenetic Analysis Based on ITS2

The three individuals of *Shorea johorensis* formed a monophyletic group with *Shorea robusta* and *Parashorea chinensis* with bootstrap value 81 (Figure 8). This is in accordance with chloroplast DNA analysis research by Ref. [14 and 33], *Parashorea* formed a monophyletic group with *Shorea*. In addition, molecular data from Ref. [34, 35, and 36] also explained that *Parashorea* was relatively close to several species of *Shorea*.

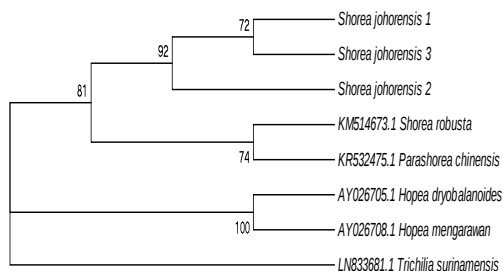


Figure 8 The phylogenetic tree of *Shorea johorensis* based on ITS2 using the neighbor joining method

Hopea mengarawan and *Hopea dryobalanoides* formed a monophyletic group with bootstrap value 100. Based on this phylogenetic tree it appears that *Shorea* and *Parashorea* formed a separate group with *Hopea*. It means that ITS2 was able to separate *Shorea* with *Hopea* but not able to separate *Shorea* with *Parashorea*. *Trichilia surinamensis* was an outgroup because it had a distant relationship with other samples.

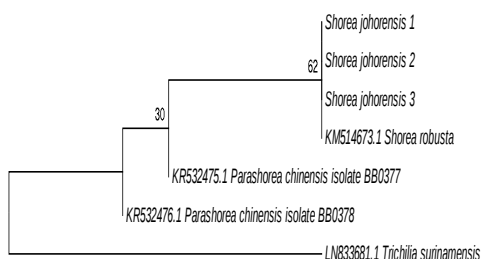


Figure 9 The phylogenetic tree of *Shorea johorensis* based on 28S rRNA gene using the neighbor joining method

Phylogenetic Analysis Based on 28S rRNA

Based on Figure 9, all of the three individuals of *Shorea johorensis* formed a monophyletic group with *Shorea robusta* (bootstrap value 62). This *Shorea* formed the separate group with *Parashorea chinensis*. This is in accordance with the nuclear DNA analysis performed by [36], where *Parashorea* and *Shorea* were separated and not belonging to a monophyletic group. *Trichilia surinamensis* was an outgroup.

CONCLUSION

The phylogenetic tree of *Shorea johorensis* based on the *rbcL* and *matK* showed that *Shorea johorensis* was closely related with some species of *Hopea*; but the phylogenetic tree based on *psbA-trnH*, 5.8S rRNA, ITS2, and 28S rRNA showed that *Shorea johorensis* was closely related with *Shorea robusta*.

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