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Molecular phylogeny of trees species in Tripa Peat Swamp Forest, Aceh, Indonesia inferred by 5.8S nuclear gen

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Abstract. *Authors. 2018. Molecular phylogeny of trees species in Tripa Peat Swamp Forest, Aceh, Indonesia inferred by 5.8S nuclear gen. Biodiversitas 19: 1186-1193.* Tripa peat swamp forest is protected areas that have high biodiversity. Nevertheless, in some areas, the damage occurred due to conversions of land function to oil palm plantations. The impact of conversions of peat swamp forest to oil palm plantations has led to biodiversity decreased. Hence, it is important to identify the remain tree species in Tripa peat swamp forest. This study aimed to determine of trees species diversity in Tripa peat swamp forest by using of 5.8S rRNA nuclear gene. Research was conducted at Forest Genetics and Molecular Forestry Laboratory, Faculty of Forestry, IPB from September 2015 to August 2016. Molecular identification consisted of DNA extraction, PCR analysis, and sequencing. The data were analyzed using Bioedit, MEGA 6, BLAST, and ITS2 database. Molecular identification using ITS 1 and ITS 4 primer successfully amplified (the ITS region ITS1-5.8S-ITS2) of 16 trees species from 9 families. BLAST analysis results indicate the presence of 16 species has similar bases sequence with the GeneBank DNA database. The plant species are *Branckenridgea palustris* (Ochnaceae), *Gonystylus* sp. (Thymelaeaceae), *Tristaniopsis whiteana* (Myrtaceae), *Syzygium* sp.1 (Myrtaceae), *Macaranga triloba* (Euphorbiaceae), *Syzygium garciniifolium* (Myrtaceae), *Knema intermedia* (Myristicaceae), *Palaquium ridleyi* (Sapotaceae), *Palaquium* sp. (Sapotaceae), *Dyera lowii* (Apocynaceae), *Elaeocarpus petiolatus* (Elaeocarpaceae), *Ficus* sp. (Moraceae), *Syzygium leptostemon* (Myrtaceae), *Chilocarpus suaveolens* (Apocynaceae), *Alstonia pneumatophora* (Apocynaceae), and *Alstonia* sp. (Apocynaceae). Phylogeny tree reconstruction using the Neighbor-Joining Method (NJ) showed that 5.8S rRNA nuclear gene was successful as marker for 16 trees species from 9 different families. In addition, the 5.8S also successful for resolving phylogenetic relationships at genus level i.e. *Alstonia*, *Palaquium*, *Syzygium*, *Tristaniopsis*, *Macaranga*, *Elaeocarpus*, and *Ficus*.

Keywords: Tripa peat swamp forests identification, phylogenetic relationships, tree species, 5.8S rRNA nuclear gene

INTRODUCTION

Tripa peat swamp forest is a protected peat swamp forest area of ± 63.228 hectares and part of the Leuser Ecosystem. It locates in Aceh Barat Daya District to Nagan Raya District, Aceh Province, Indonesia. The Tripa peat swamp forest is one of megadiversity center and also the largest carbon storage site in Aceh (YLI-AFEP 2008). Peatland has extremely poor soils which are acidic and lower minerals as well as nutrients content. The unique and specific conditions of peatland created unique species diversity compared to another forest. According to Thomy et al. (2016) there were 17 families of trees species that can be found in Tripa peat swamp forest, i.e., Myrtaceae, Apocynaceae, Sapotaceae, Anacardiaceae, Sterculiaceae, Moraceae, Euphorbiaceae, Rubiaceae, Stemonuraceae, Thymelaeaceae, Ochnaceae, Rhizophoraceae, Annonaceae, Dipterocarpaceae, Myristicaceae, Elaeocarpaceae, and Arecaceae. WWF and LIPI (2007) reported that the endemic species in peat swamp ecosystem are *Dyera lowii* (Apocynaceae), *Gonystylus bancanus* (Thymelaeaceae), *Kompassia malaccensis* (Fabaceae), *Alstonia pneumatophora* (Apocynaceae), *Campnosperma* spp. (Anacardiaceae), *Callophylum* spp. (Calophyllaceae), *Palaquium* spp. (Sapotaceae), and *Lagerstroemia speciosa* (Lythraceae). Moreover, Djufri et al. (2016) reported that there were 41 species of herbs, seven species of shrubs and 24 species of trees in deforested peat-swamp forest of Tripa.

Tripa was mostly covered by forest as many as 67.000 hectares or 65% of the area, of which most was peat swamp forest. In the most recent year of observation, forest cover was 19.000 hectare (18% of the area). The largest forest conversion took place from 2005 to 2009, with the loss of approximately 4.000 hectares per year. Historical analyses can be used to support efforts to protect the remaining peat swamp forest in Tripa. In addition, forest degradation, conversion of forest area into oil palm plantations, illegal logging, forest fire, reduced Tripa peat swamp forest area as well as biodiversity, especially tree species. Hence, it is important to identify trees species that still found in the remain forest area. Database about the trees species will support the restorations program in the future (Widayati et al. 2012).

The 5.8S nuclear rRNA gene lies between Internal Transcribed Spacer 1 (ITS1) and Internal Transcribed Spacer 2 (ITS2). The 5.8 S rDNA sequences contain three conserved motives in their nucleotide sequences that are essential for the correct folding of secondary structure (Harpke and Peterson 2008a,b). According to Gomes et al. (2002) and; O'Brien et al. (2005) the 5.8S rRNA gene has a

very high conserved level and commonly used as a marker for plant identification. Hribova et al. (2011) also reported that the 5.8S rDNA sequence region had a conserved length of 155 bp or 154 bp, its GC content varied from 49.68 to 57.48% and it can be used for molecular phylogeny. Alvarez and Wendel (2003) also reported that 5.8S sequence region is one of the most popular loci used in molecular phylogenetic studies. Therefore this study was conducted to identify peat swamp trees species in Tripa swamp forest using of 5.8S rRNA nuclear gene for conserving and restoring effort in the future.

MATERIALS AND METHODS

Study area

The research was conducted in Tripa peat swamp forest, Darul Makmur Sub-district of Nagan Raya District, Aceh, Indonesia and Laboratory of Forest Genetics and Molecular Forestry, Faculty of Forestry, Bogor Agricultural University, Bogor, West Java, Indonesia. The research was begun from September 2015 to August 2016.

Procedures

Sample collection

This research used purposive sampling method. The total area for sampling site is 18.000 ha. Species sample was taken as much as 2% per plot in the three locations, i.e., primary, secondary, and tertiary forest. Forty-five plots were designed in the study area and each location was divided into fifteen plots. The plot size was 20 m x 20 m for tree sampling (tree diameter > 20 cm and tree height > 10 m).

DNA extraction, amplification, and sequencing

Total genomic DNA from each 16 trees species was extracted from young leaves using the Qiagen DNeasy Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The concentration and quality of DNA were checked by 2% agarose gel electrophoresis and visualized by UV transilluminator after red gel staining.

Amplification segments of DNA were conducted using 20 µL Polymerase Chain Reaction (PCR) reactions (Kapa *Taq* PCR MasterMix). All of the components consists of 10 µL (1X Kapa *Taq*), 1 µL forward primer, 1 µL reverse primer, 3 µL DNA template, and 5 µL nuclease-free water. The temperature for PCR condition start to initial denaturation at 94˚C for 3 minutes, 30 cycles of denaturation (at 94˚C for 30 s), annealing (at 58˚C for 30 s), and extension (at 72˚C for 1 minutes), and ends with an elongation stage at 72˚C for 10 minutes. The primer used in this study was ITS 1 and ITS 4 (Table 1).

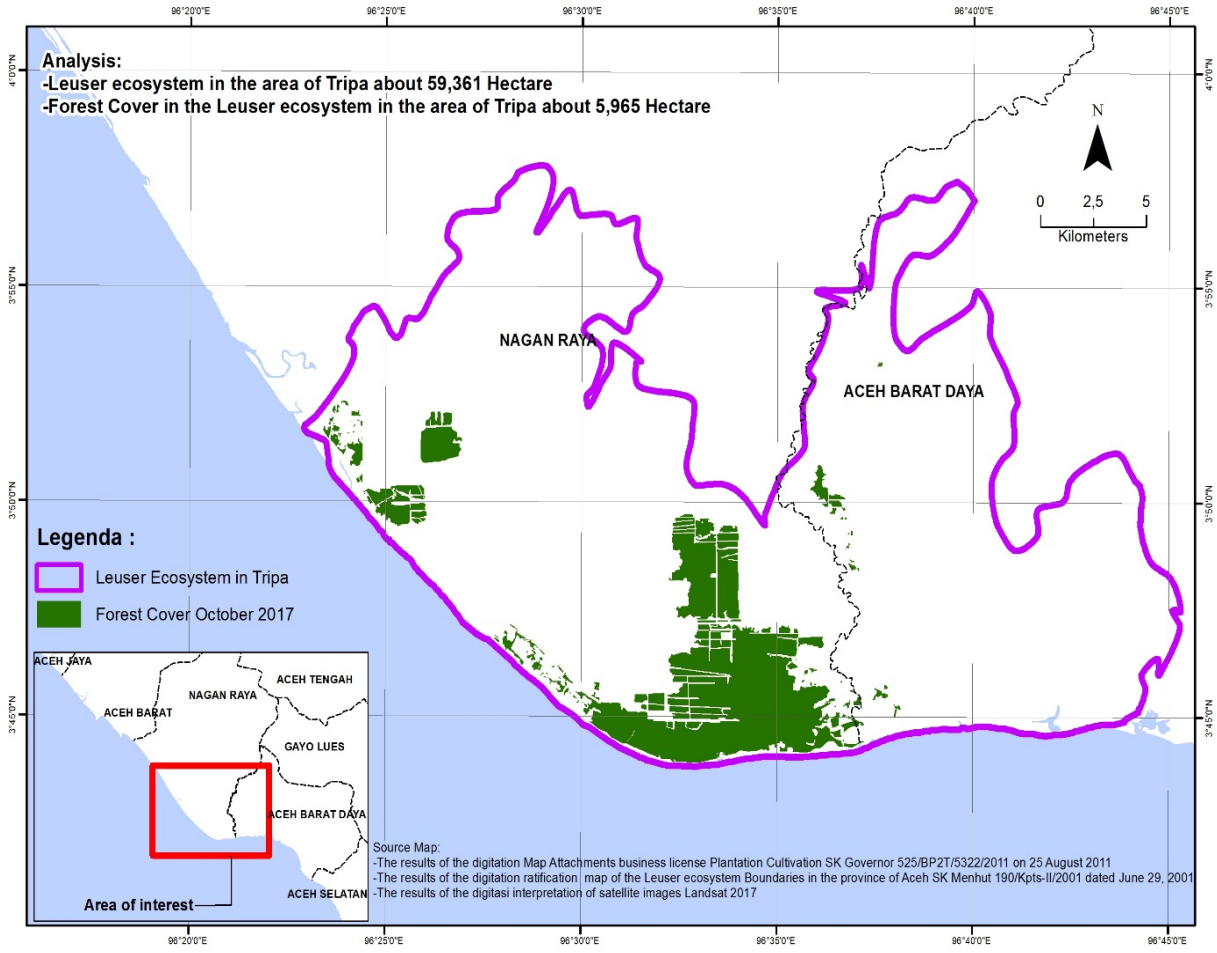

Figure 1. The forest cover map in The Leuser Ecosystem in the area of Tripa peat swamp forest, Aceh, Indonesia

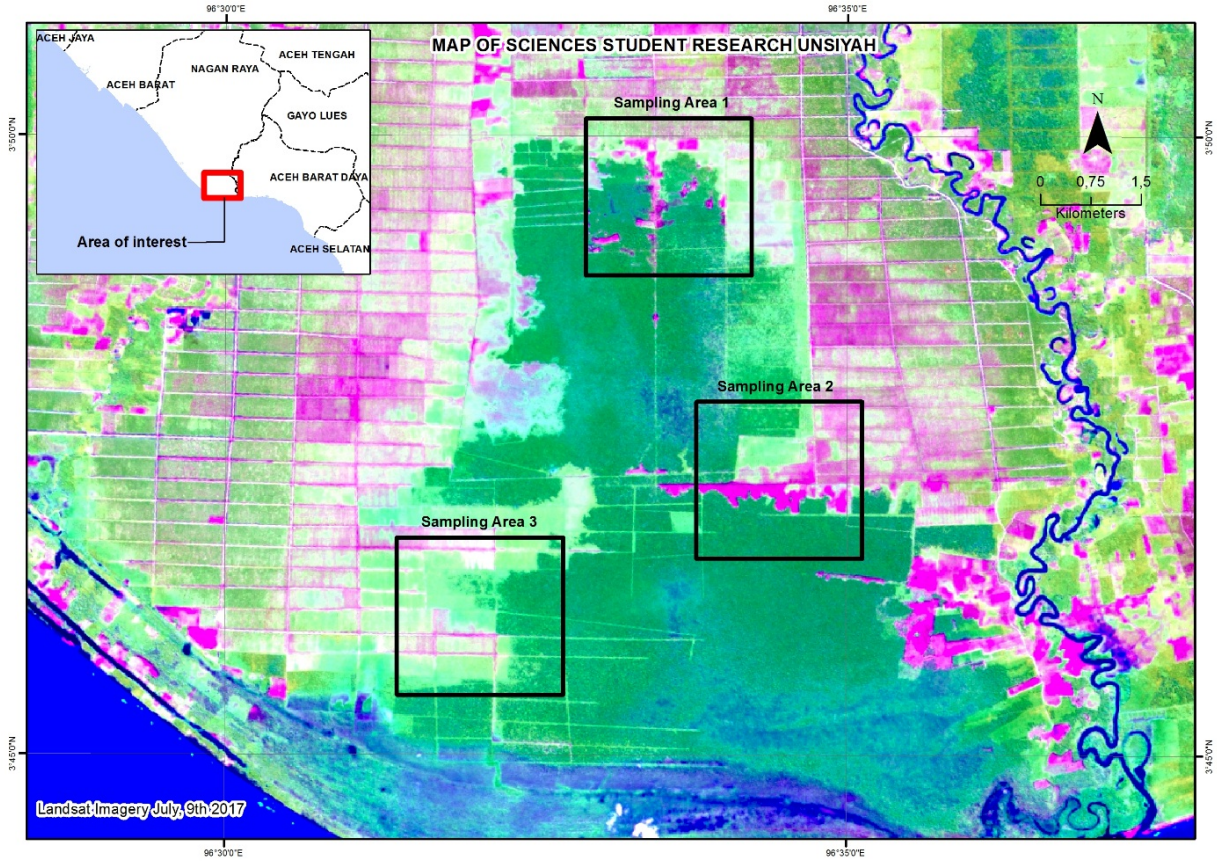

Figure 2. Location of sample collection in Tripa peat swamp forest, Aceh, Indonesia

Table 1. Bases sequence of ITS 1 and ITS 4 primers

Genom	DNA region	Primer name	Bases Sequence (5'-3')	Reference
Nuclear	ITS1	ITS 1	TCCGTAGGTGAACCTGCGG	White et al. (1990)
	5.8S rRNA			
	ITS2	ITS 4	TCCTCCGCTTATTGATATGC	White et al. (1990)

Sequencing process is conducted after amplicon was checked by 2% agarose gel electrophoresis. The amplicons were sequenced based on the selective incorporation of chain-terminating dideoxynucleotides method according to the manufacturer's instructions and run on an ABI-3100 automatic sequencer (Applied Biosystems) (Sanger and Coulson 1997). DNA strands were fully sequenced.

Editing and sequence alignment

The data was analyzed using BioEdit, BLAST and MEGA 6. Manual data review was performed by using BioEdit version 7.0.5.2 (Hall 1999). The Basic Local Alignment Search Tool (BLAST) from the NCBI homepage (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) was then used to compare these sequences with in-house sequences and GenBank database sequences. The software of Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 (Tamura et al. 2013) was used to predict phylogenetic relationships among trees species in Tripa peat swamp forest. The reconstructing of phylogenetic tree using Neighbor-Joining method (NJ) bootstrap 1000x (Saitou and Nei 1987).

RESULTS AND DISCUSSION

The 5.8S motifs for the identifications of peat swamp tree

The ITS2 database gives information about the sequence, structure, and taxonomic classification in GenBank. ITS2 region was delimited in the database http://its2.bioapps.biozentrum.uni-wuerzburg.de/cgibin/index.pl?annotator. The ITS2 database also looked for several motifs in Internal Transcribed Spacer (ITS), i.e., 5.8S, ITS2, and 28S (Keller et al. 2009). The presence of all these motifs suggests that all ITS sequences obtained in this study are functional. An example of annotating result

of a complete sequence of the ITS region in *Palaquim ridleyi* is presented in Figure 4.

In the following years, the ITS2 database was further expanded from a data repository to a rather full-featured interactive workbench (Selig et al. 2008; Koetschan et al. 2010, 2012; Wolf et al. 2014). This figure also explained that one of species originated from Tripa peat swamp forest has a complete sequence. But in the several cases, not all species have a complete sequence. This research focused on the 5.8S rRNA as the functional part of the ITS region as marker for revolving phylogenetic relationships at the genus/species level. Generally, the region of 5.8S motifs has a length of 24 base pair (bp). However, the length of base pair (bp) could vary in other species. The 5.8S rRNA nuclear gene is the short sequence which is often used to identify and predict phylogenetic in plants and fungi. The 5.8S motifs for tree species identification in Table 2.

In tree phylogenetic, ITS1 and ITS2 can be used for distantly related species whereas 5.8S gene can be used for closely related species include plants and fungi. Conserved motifs for the 5.8S gene are poorly described in fungi. However, three motifs of the 5.8S gene are conserved for angiosperms M1 (5'-CGAUGAAGAACGUAGC-3'), M2 (5'-GAAUUGCAGAAUCC-3') and M3 (5'-UUUGAACGCA-3') (Harpke et al. 2008). The 5.8S ribosomal RNA (5.8S rRNA) is a non-coding RNA component of the large subunit of the eukaryotic ribosome and plays an important role in protein translation. Thus, the 5.8S locus can serve as a critical alignment-free anchor point for search algorithms that make sequence comparisons for both phylogenetic and barcoding purposes.

Basic Local Alignment Search Tool (BLAST)

BLAST analysis was conducted to compare the DNA sequence of the peat swamp tree and the DNA sequence in GenBank DNA database. BLAST analysis also finds the similarity regions between biological sequences. Hence, it is assisting for reconstruction of the phylogenetic tree. Furthermore, it could explain the position of the species in taxonomy. The BLAST analysis result of the 16 trees species from 9 families in Tripa peat swamp forest is presented in Table 3.

Figure 4. The annotated of 5.8S motifs in *Palaquim ridleyi*

Table 2. The 5.8S motifs of the tree species in Tripa peat swamp forest, Aceh, Indonesia

Name of species	Locus	Sequences	Length (bp)
Palaquium ridleyi	5.8 S	CGAGGGCACGCCTGCCTGGGCGTCT	25
Palaquium sp.	5.8 S	CGAGGGCACGCCTGCCTGGGCGTCT	25
Alstonia sp.	5.8 S	CGAGGGCACGTCTGCCTGGGCGTCA	25
Alstonia pneumatophora	5.8 S	CGAGGGCACGTCTGCCTGGGCGTCA	25
Chilocarpus suaveolens	5.8 S	CGAGGGCACGTCTGCCTGGGCGTCA	25
Dyera lowii	5.8 S	GGAGGGCGCGTCTGCCTTGGGGGTCA	26
Elaeocarpus petiolatus	5.8 S	CGAGGGCACGTCTGCCTGGGGTCA	24
Ficus sp.	5.8 S	CGAGGGCACGTCTGCCTGGGCGTCA	25
Syzygium sp.	5.8 S	CGAGGGCACGTTTGCCTGGGTGTCA	25
S. leptostemon	5.8 S	TGAGGGCACGTTTGCCTGGGTGTCA	25
S. garciniifolium	5.8 S	TTAGGGCACGTTTGCCTGGGTGTCA	25
Tristaniopsis whiteana	5.8 S	AGAGGGCACGCTTGCCTGGGTGTCA	25
Macaranga triloba	5.8 S	CAAGGGCACGTCTGCCTGGGTGTCA	25
Branckenridgea palustris	5.8 S	CGAGGGCACGCCTGCCTGGGCGTCA	25
Knema intermedia	5.8 S	TGAGGGCACGTCTGCCTGGGCGTCA	25
Gonystylus sp.	5.8 S	CGAGGGCACGCCTGCCTGGGTGTCA	25

Table 3. BLAST analysis of the 16 trees species from Tripa peat swamp forest, Aceh, Indonesia based on 5.8S rRNA nuclear gene

Species name	Families	Process ID	*Quary cover*	*Ident*	*E-value*
Palaquium ridleyi	Sapotaceae	>KF686291.1 P. *xanthochymum*	100%	100%	3e-08
Palaquium sp.	Sapotaceae	>KF686291.1 P. *xanthochymum*	100%	100%	3e-08
Alstonia sp.	Apocynaceae	>KC960438.1 *Alstoniascholaris*	100%	100%	2e-07
Alstonia. pneumatophora	Apocynaceae	>KC960438.1 *Alstoniascholaris*	100%	100%	2e-07
Chilocarpus. suaveolens	Apocynaceae	>KC960438.1 *Alstoniascholaris*	100%	100%	2e-07
Dyera lowii	Apocynaceae	>JX856398.1 *Alstonia macrophylla*	96%	92%	0.014
Elaeocarpus petiolatus	Elaeocarpaceae	>KX365744.1E. *floribundus*	83%	100%	9e-06
Ficus sp.	Moraceae	>KX572966.1 F. *carica*	100%	100%	4e-07
Syzygium sp.	Myrtaceae	>KR532633.1 *Syzygium oblatum*	100%	100%	1e-06
S. leptostemon	Myrtaceae	>KX079334.1 *Syzygium cumini*	100%	100%	1e-06
S. garciniifolium	Myrtaceae	>JF682809.1 *S. aqueum*	100%	100%	1e-06
Tristaniopsis whiteana	Myrtaceae	>KM064872.1 *T. laurina*	100%	100%	1e-06
Macaranga triloba	Euphorbiaceae	>AF361166.1 *M. bancana*	100%	100%	3e-06
Branckenridgea palustris	Ochnaceae	>KF263225.1 B. *zanguebarica*	100%	100%	1e-08
Knema intermedia	Myristicaceae	>KR532228.1 K.*globularia*	100%	100%	5e-09
Gonystylus sp.	Thymelaeaceae	>GQ205178.1 *Pimelea williamsonii*	100%	100%	3e-08

All of the trees species from Tripa peat swamp forest has a similarity at the genus level with the sequences from GenBank DNA database. The *Query Cover* for 16 trees species has values in the range of 83% to 100%. It has a high degree of alignment to BLAST sequences. The *E-value* of 0.0 indicates the number of alignments with scores equivalent to or greater than that is expected to occur in a database by chance. Therefore the lower the *E-value* the more significant the score is and a better quality of the alignment BLAST search. The *E-value* was high in this study due to the locus sequence as the marker is very short. In this study, the 5.8S nuclear gene has a length of about 24-26 bp. Hence, searching for similarity to a query sequence limited. According to Claverie and Notredame (2003), the DNA sequence has a high similarity if the *Query Cover* value is close to 100% and the E-*value* is close to 0.0.

Phylogenetic relationships based on 5.8S rRNA nuclear gene

The reconstructions of the phylogenetic tree (Figure 5) explained about phylogenetic relationships within the 9 families of the plants, i.e., Apocynaceae, Sapotaceae, Myrtaceae, Euphorbiaceae, Elaeocarpaceae, Moraceae, Ochnaceae, Thymelaeaceae, and Myristicaceae. In our study, from 16 different species, the 5.8S nuclear gene were clearly differentiated species from genus *Alstonia* (Figure 5.A), *Palaquium* (Figure 5.B), *Syzygium* (Figure 5.C), *Tristaniopsis* (Figure 5.C), *Macaranga* (Figure 5.D), *Elaeocarpus* (Figure 5.E), and *Ficus* (Figure 5.F). This marker is attractive because it has been used in previous barcoding studies of the eukaryotic with success (Nguyen and Seifert 2008; Schoch et al. 2012). However, the 5.8S as marker was not successful to resolve phylogenetic relationships in genus *Chilocarpus* (Figure 5.A), *Dyera* (Figure 5.A), *Branckenridgea* (Figure 5.G), *Gonystylus* (Figure 5.H), and *Knema* (Figure 5.I). According to Stern et al. (2012) ITS region is one of the difficult markers technically because it is present in multiple distinct copies. ITS region also has the high level of intra and intergenomic variability in species that can make alignment difficult.

The 5.8S is the small and large subunit ribosomal RNA genes are separated by the Internal Transcribed Spacer (ITS) units 1 and 2 as well as-as a marker using a wide variety of the tree species from peat swamp forest. The molecular phylogeny studies about plants in the peat swamp forest are very limited. It is therefore important to develop of molecular phylogenetic to determine the best of DNA barcode markers for the identification. The best performance of 5.8S occurred in Figure 5.A, whereby the 5.8S was successful grouped genus *Alstonia* (TPSF) with *Alstonia* from GenBank while there are four genera there. (Burge et al. 2013) reported that the complete sequence of ITS (ITS1, 5.8S, ITS2) was used to predict molecular phylogeny of the plant within genus *Pachypodium* (Apocynaceae).

The most dominant species in Tripa peat swamp forests belong to Myrtaceae. According to Manshor and Manshor (2014), most species in peat swamp forests belong to the Myrtaceae and Dipterocarpaceae. Among all phylogenetic trees, the 5.8S was very successful for resolving phylogenetic relationships within families Myrtaceae. Caused all member of families Myrtaceae (Figure 5.C) grouped clades based on the same genus i.e. *Syzygium* (TPSF) with S*yzygium* (GenBank) as well as *Tristaniopsis* (TPSF) with *Tristaniopsis* (GenBank). Even though 5.8S is highly conserved within plants, but this marker can be used to differentiate among species within families Myrtaceae. The 5.8S was used for reconstructing phylogenetic relationships among species of *Myrceugenia* (Myrtaceae) (José Murillo-A et al. 2012). Furthermore, the 5.8S nuclear gene is recommended as universal barcodes for plants, especially within families Myrtaceae.

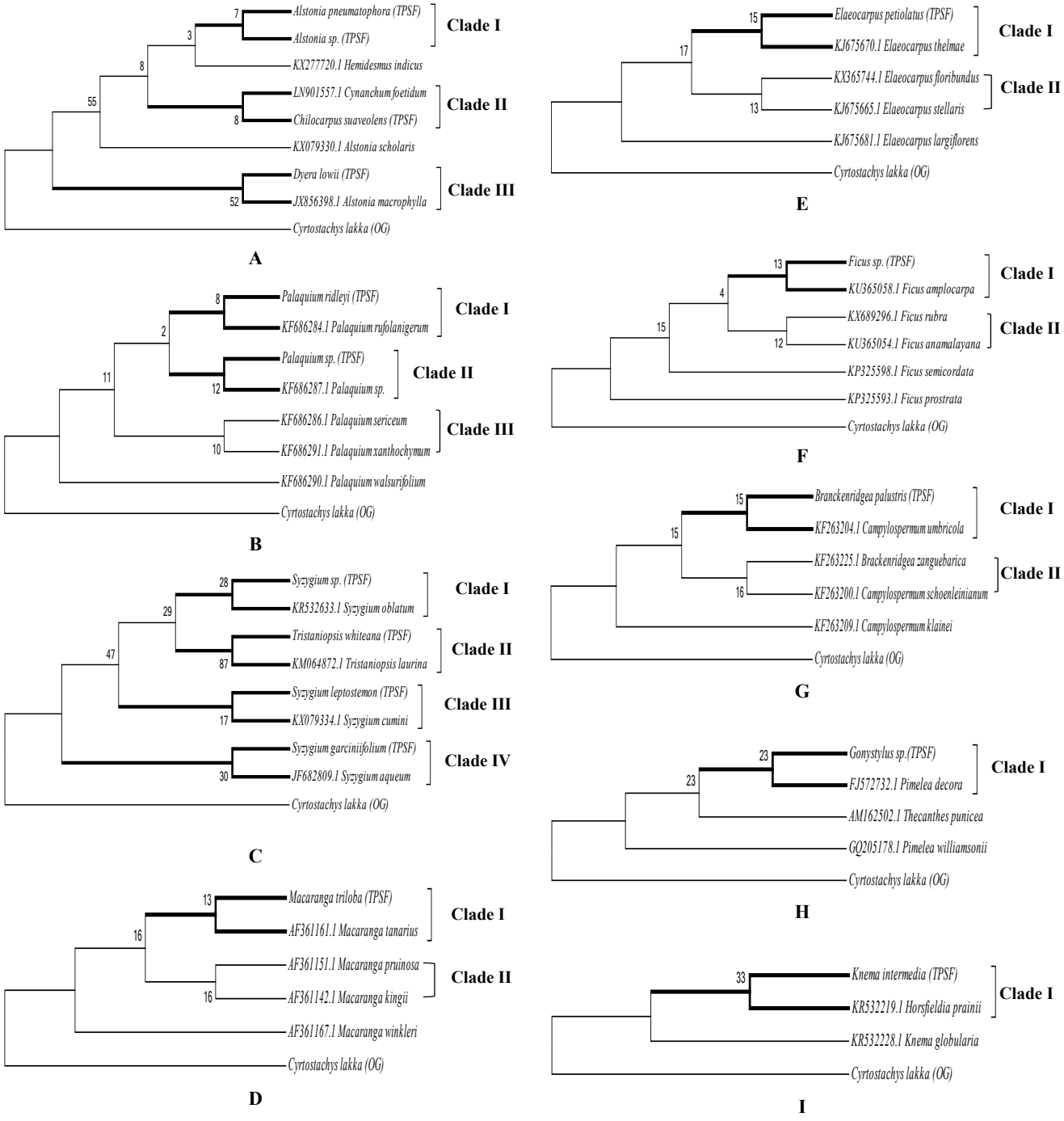

Figure 5. Phylogenetic tree based on 58s rRNA nuclear gene within families: A. Apocynaceae; B. Sapotaceae; C. Myrtaceae; D. Euphorbiaceae; E. Elaeocarpaceae; F. Moraceae; G. Ochnaceae; H. Thymelaeaceae; I. Myristicaceae using Neighbor-Joining (NJ) method. The phylogenetic tree is the consensus result. The bootstrap value shows in every node. TPSF: Tripa Peat Swamp Forest, OG: Out Group

Species endemic, which is discovered in Tripa peat swamp forest i.e. *Palaquium, Palaquium ridleyi*, *Gonystylus* sp., *Dyera lowii,* and *Alstonia pneumatophora*. Generally, all the species are classified as timber forest products. According to (Yamada 1997) reported that the dominants of mixed swamp forest common both to Sumatra and Kalimantan are *Alstonia pneumatophora*, *Campnosperma coriacea*, *Durio carinatus*, *Dyera lowii*, *Gonystylus bancanus, Koompassis malaccensis, Lophopetalum multinervium, Mezzettia leptopoda, Palaquium burckii, Parastemon urophyllum, Shorea platycarpa, S. teysmanniana* and *S. uliginosa*.The timber production in peat swamp forest is low when compared to the lowland or mix dipterocarp forests. Therefore, the problem faced by peat swamp forest is due to increased human activity on tropical peatlands has resulted in much

of the destruction of the peat swamp forest ecosystem. The remaining pristine peat swamp forests need to be protected and managed wisely to prevent further losses of valuable endemic species. We concluded that 5.8S is strongly supported within genus *Palaquium* (Figure 5.B). Prior to, there were several studies used the 5.8S for the identification within families Sapotaceae. Swenson et al. (2007a) reported that the 5.8S as one of a marker was used to molecular phylogeny studies of *Planchonella* (Sapotaceae) as well as eight new species from New Caledonia. Bartish et al. (2005) also reported that the 5.8S is one of the sequences used for resolving phylogenetic relationships among New Caledonian Sapotaceae.

In addition, there are several species include as the compiler of peat swamp vegetation in Tripa peat swamp forest, i.e., *Elaeocarpus petiolatus, Macaranga triloba*, *Ficus* sp., *Knema intermedia*, and *Branckenridgea palustris*. According to Kuniyasu and Tetsuya (2002) reported that one of the species compositions of Sumatran peat swamp forests is *Knema intermedia*. There were approximately 40 species of *Macaranga* including both peat swamp and non-peat swamp environments (Siregar and Sambas 2000). Molecular identification using the 5.8S as a marker is not recommended for several of the compiler species such as *Branckenridgea palustris* (Figure 5.G) and *Knema intermedia* (Figure 5.I). Due to searching the similarity of the 5.8S rRNA belonging to the species *B. palustris* and *K. intermedia* with the query sequence in NCBI limited. We concluded that the phylogenetic relationships among that species (Figure 5.G and 5.I) are not clear. Further, needed full sequences of ITS region for resolving the phylogenetic issue. According to Vivas et al. (2014) for species discrimination, ITS region provided the best results, followed by *mat*K, *trn*H-*psbA*, and *rbc*L. Furthermore, the combined analysis of two, three or four markers did not result in higher rates of discrimination than with ITS alone. These results indicate that the ITS region is the best option for molecular identification of Sapotaceae species from the Atlantic Forest.

Conversely, the 5.8 is very recommended for species *Elaeocarpus petiolatus* (Elaeocarpaceae)*, Macaranga triloba* (Euphorbiaceae), and *Ficus* sp. (Moraceae). The previous study reported that several molecular phylogenetic studies on the families Elaeocarpaceae, as well as the genus *Elaeocarpus*, have been undertaken. The molecular studies using full sequence data from the Internal Transcribed Spacer (ITS) of nuclear ribosomal DNA (Maynard 2008). Zeng et al. (2005) reported that phylogenetic relationships within families Moraceae have also used the 5.8S rRNA to predict phylogenetic of intra-species and inter-species within genus *Morus* (Moraceae).

In conclusion, the 5.8S was successful to distinguish all species within 9 families i.e. Apocynaceae, Sapotaceae, Myrtaceae, Euphorbiaceae, Elaeocarpaceae, Moraceae, Ochnaceae, Thymelaeaceae, and Myristicaceae from Tripa peat swamp forest. In addition, the 5.8S also successful for resolving phylogenetic relationships at the genus level, especially in genus *Alstonia*, *Palaquium*, *Syzygium*, *Tristaniopsis*, *Macaranga*, *Elaeocarpus,* and *Ficus*.

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