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PHYLOGENETIC STUDY OF *MANGIFERA* FROM SUMATRA, INDONESIA USING NUCLEAR AND CHLOROPLAST DNA SEQUENCES

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SUMMARY

Although there have been several studies on the phylogeny of *Mangifera,* however, no such studies have focused on *Mangifera* in Sumatra, Indonesia. The objectives of this study were to analyze the phylogenetic relationship of the genus *Mangifera* in Sumatra, Indonesia based on molecular characters and to clarify the relationship among sections within the genus *Mangifera* to its closely related genera even to infraspecific. The phylogenetic relationships of the genus *Mangifera* with emphasis on Sumatran species were estimated using sequence data from internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (nrDNA), *trnL-F* Intergenic Spacer (IGS) and ribulose-bisphosphate carboxylase (*rbcL*) gene of chloroplast DNA (cpDNA). Forty-four sequences which represented 23 species of *Mangifera* were used for phylogenetic study including sequences obtained from NCBI GenBank. These 23 species of *Mangifera* were grouped into two subgenus, *Limus* (section *Deciduae* and *Perennes*) and *Mangifera* (section *Marchandora, Euantherae, Rawa* and *Mangifera*). The relationship of the two traditionally accepted subgenera was not well supported. Molecular study employed the sequence data of ITS region of nrDNA. Analysis of ITS region of nrDNA using maximum parsimony (MP) and Bayesian Analyses produced phylogenetic trees which revealed that sections within Kostermans's infrageneric classification were polyphyletic. The result did not support traditional classification, i.e., Kostermans's infrageneric classification. Additional analysis about relationship of genus *Mangifera* to its closely related genera based on ITS region of nrDNA, *trnL-F* IGS and *rbcL* gene of chloroplast DNA using MP methods showed that the genus *Mangifera* was monophyletic.

Key words: *Mangifera,* Sumatra, ITS, *trnL-F* IGS, *rbcL,* phylogenetic relationship

Key findings: Phylogenetic study of *Mangifera* species from Sumatra revealed that base on molecular data (ITS, trnl-F intergenic spacer and rbcL), the infrageneric classification was consistent with morphological data but but it is not accordance with sub-genera level of *Mangifera.*

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INTRODUCTION

Mango (*Mangifera)* is the most famous plant from family Anacardiaceae and as important friut in tropic, is usually regarded as comprising two sub genera, the narrow disc of *Limus* and the broad disc of *Mangifera* with an estimated 69 species (Kostermans and Bompard, 1993). Of the 69 species, 58 species were grouped in several sections: *Deciduae, Perennes*, *Marchandora, Euantherae, Rawa,* and *Mangifera*. While another 11 species are still in uncertain position due to lack of specimen.

The genus *Mangifera* is mainly distributed in tropical lowland rainforests on well-drained soils (Kostermans and Bompard, 1993). *Mangifera* species are mostly distributed below 300 m but can occur at 600-1900 m above sea level. The species are found as scattered individuals in tropical lowland rain forests on well-drained soils (Kostermans and Bompard, 1993; Fitmawati and Hayati, 2018). *Mangifera* is represented on almost all the islands in the Malesian region, particularly in the Malay Peninsula, Sumatra, Java and Borneo with about 28 species found in the region.

Since Kostermans and Bompard's treatment in 1993, thirty-four species were found in Sumatra, Indonesia. However, recent exploration conducted by Fitmawati *et al.* (2012-2017) in various types of topography, land cover and elevation, only 12 species were found in Sumatra. Of the 12 species of *Mangifera* found, there are seven rare species with narrow distribution in the mainland of Sumatra (Fitmawati and Hayati, 2018).

The characteristics of *Mangifera* species in Sumatra were tolerant to high rainfall, capable of fruiting out of season, high production and flowers resist against wet climate (Fitmawati *et al.,*2013). The species with these traits had a potential as germplasm resources. Due to high forest and land fires frequency in Sumatra (Margono *et al.,* 2012), the genus *Mangifera* in Sumatrais threatened in its natural habitat and therefore wild germplasm resources must be conserved before it is lost in the wild.

Molecular analysis using nuclear DNA represented by Internal Transcribed spacer (ITS) sequence and chloroplast DNA represented by *rbcL* and *trnL-F* IGS sequences. These two source of DNA were used to make comparison betweenthem. Whether they are complementary or contradictory or even strengthening each other to shape the best phylogenetic tree. We also conducted a combined analysis of ITS, *trnL-F* IGS and *rbcL* to find if there is a possibility analyses of combined data sets provide better phylogenetic resolutions than do individual data sets.

Internal Transcribed Spacer (ITS) of nrDNA has been used for molecular markers at specific level of Angiosperm (Baldwin *et al.,* 1995; Yonemori *et al.,* 2002; Fitmawati *et al.,* 2016a). Internal Transcribed Spacer sequences were also useful because it has conserve region, short size (±700 bp), high evolution rate,

informative and universality (Baldwin *et al.,* 1995). Although there have been several recent studies of *Mangifera,* such as Yonemori *et al.* (2002) and Fitmawati *et al.* (2016a) using ITS sequence, the relationship among several section in the genus *Mangifera* were still unrecognized. In Yonemori *et al.,* (2002) there was no representing species from section *Deciduae* while in Fitmawati *et al.* (2016a) had species from both sections of subgenus *Limus* (*Deciduae* and *Perennes*)and a section from subgenus*Mangifera* (*Mangifera*) and lack of other sections. A molecular approach were used, utilizing nuclear ITS, to determine the relationships among sections within the genus *Mangifera* to infraspecific level.

Ribulose-bisphosphate carboxylase (*rbcL*) sequence was set by Barcode of Life Database (BOLD) as a DNA barcode sequences to analyze the genetic diversity of species and is universal in all plant genes (Judd *et al.,* 2002). *rbcL* is a marker of a conservative and has a pretty good variety for use in distinguishing between species (Newmaster *et al.,* 2006). Another research about using *rbcL* sequences in *Mangifera* has been conducted by (Suparman *et al.*, 2013) for samples from Indonesia and Thailand.

The *trnL-F* IGS is one of the most frequently markers used by plant systematic. This non-coding region has a high rate of mutation, easily isolated, purified and cloned and relatively small in size. cpDNA also suitable to investigate phylogenetic relationship (Taberlet *et al.,* 1991). Most structural mutation in cpDNA is small indel from 1-10bp (Vijeberg and Bachmann 1999). Other researches about using *trnL-F* IGS region in land plants and suitable to provide a phylogenetic information in Junacaeae (Drabkova 2003), in closest relative of *Mangifera, Bouea* (Harsono *et al.,* 2017) and in *Mangifera* have been done by Fitmawati and Hartana(2010) for *Mangifera laurina* and related species in Sulawesi and Dinesh *et al*. (2015) for *Mangifera indica* relatives.

Molecular study of specific *Mangifera* based on ITS, *rbcL* and *trnL-F* IGS has not been carried out before in Sumatra, Indonesia. Therefore, a research project was conducted to study the relationship among *Mangifera* species in Sumatra. The molecular approach has benefit to find the best phylogenetic tree model which will be useful in conservation and cultivation strategies for developing *Mangifera* in Sumatra, Indonesia as a potential source in industry of pomology.

MATERIALS AND METHODS

Plant material and DNA extraction

Samples used in this study are the representative from each of section in *Mangifera* genus. Fresh leaves of *Mangifera* used in this study were collected from Eastern Sumatra, exploration by Fitmawati *et al.* (2015), Southern Sumatra exploration (Fitmawati *et al*., 2016b) and additional exploration in Riau, Indonesia with total 20 samples of 10 species. Other sequences of *Mangifera* represented each sectionwere obtained from Genbank, especially from Fitmawati *et al.* (2016a) and Yonemori *et al.* (2002) as well as two genera from Anacardiaceae were used as outgroup with total 26 samples of 22 species (Table 1). A total of 46 sequences were used for phylogenetic

Table 1. Sources of *Mangifera* ITS sequences and their geographic origin.

Species	Genbank Acc. No.	Geographic origin	Section	Reference
M. kemanga Bl. 3A	MF678503	Southern Sumatra	*Deciduae*	
M. kemanga Bl. 3B	MF990368	Southern Sumatra	*Deciduae*	
M. foetida Lour. 2A	MF678499	Eastern Sumatra	*Perennes*	
M. foetida Lour. 2B	MF678500	Eastern Sumatra	*Perennes*	
M. foetida Lour. 3A	MF678501	Southern Sumatra	*Perennes*	
M. foetida Lour. 3B	MF678505	Southern Sumatra	*Perennes*	
M. foetida Lour. 3C	MF678506	Southern Sumatra	*Perennes*	
M. odorata Griff. 2A	MF678496	Eastern Sumatra	*Perennes*	
M. odorata Griff. 2B	MF678497	Eastern Sumatra	*Perennes*	
M. odorata Griff. 3	MF678507	Southern Sumatra	*Perennes*	
M. laurina Bl. 2A	MF678495	Eastern Sumatra	*Mangifera*	
M. laurina Bl. 2B	MF678498	Eastern Sumatra	*Mangifera*	
M. laurina Bl. 3A	MF678508	Southern Sumatra	*Mangifera*	
M. indica L. 2	MF678502	Eastern Sumatra	*Mangifera*	
M. indica L. 3A	MF678509	Southern Sumatra	*Mangifera*	
M. lalijiwa Kosterm.	MF678504	Southern Sumatra	*Mangifera*	
M. quadrifida Jack. 3	MF678511	Southern Sumatra	*Mangifera*	
M. torquenda Kosterm. 3	MF990365	Southern Sumatra	*Mangifera*	
M. casturi Kosterm.	MF678493	Central Sumatra	*Mangifera*	
Mangifera sp. 2	MF678494	Eastern Sumatra	Undetermined	
M. kemanga Bl. 1*	KX347955	Central Sumatra	*Decidueae*	
M. foetida Lour. 1*	KX347956	Central Sumatra	*Perennes*	
M. odorata Griff. 1*	KX347957	Central Sumatra	*Perennes*	
M. laurina Bl. 1*	KX347963	Central Sumatra	*Mangifera*	
M. indica L. 1*	KX347960	Central Sumatra	*Mangifera*	
M. zeylanica (Bl) Hook. f. 1*	KX347962	Central Sumatra	*Mangifera*	
M. sumatrana Miq. 1*	KX347961	Central Sumatra	Undetermined	
M. quadrifida Jack 1*	KX347959	Central Sumatra	*Mangifera*	
M. torquenda Kosterm. 1*	KX347958	Central Sumatra	*Mangifera*	
Mangifera sp. 1*	KX347964	Central Sumatra	Undetermined	
M. flava Evrard*	AB071679	Thailand	*Mangifera*	
M. cochinchinensis Engler*	AB071675	Thailand	*Euantherae*	
M. griffithii Hook. f.*	AB071685	Thailand	*Rawa*	
M. gracilipes Hook. f.*	AB071686	Thailand	*Rawa*	
M. pentandra Hooker f.*	AB071684	Thailand	*Euantherae*	
M. caloneura Auct.*	AB071678	Thailand	*Euantherae*	
M. gedebe Miq.*	AB071681	Thailand	*Marchandora*	
M. macrocarpa Bl.*	AB071688	Thailand	*Perennes*	
M. oblongifolia Hook. f.*	AB071682	Thailand	*Mangifera*	
M. sylvatica Roxb.*	AB071689	Thailand	*Mangifera*	
M. laurina Bl.*	AB071687	Thailand	*Mangifera*	
M. indica L. *	AB071672	Thailand	*Mangifera*	
M. foetida Lour.*	AB071680	Thailand	*Perennes*	
M. odorata Griff.*	AB071683	Thailand	*Perennes*	
Bouea macrophylla Griff.*	AB071691	Thailand		
Anacardium occidentale L.*	AB071690	Thailand		

Note: *) data obtained from GeneBank

study. However, a total of 29 sequences were used for phylogenetic study using combination of ITS, *trnL-F* IGS and *rbcL* sequences.

Whole genome DNA were isolated from leaves of each plant after soaking in aquadest by the CTAB method of Doyle and Doyle (1987)

with a slight modification, by soaking leaf in demineralization water for 24 hours before isolation. In isolation process CIAA solution were substitute by chloroform only. DNAs were then suspended in TE buffer.

Amplification and sequencing of ITS sequence

The genomic DNA was amplified using universal primer ITS4 and ITS5 for the entire ITS regions (White *et al.,* 1990). Reaction mixture (50 µL) contained DreamTaq Buffer 10x, 2mM each dNTP Mix, 25 pmol of each primer, 20-50 ng genomic DNA, 1 units of DreamTaq DNA Polymerase and nuclease free water. Thirty five cycles of PCR were conducted using Thermal Cycle under following profiles: 94 °C for 5 minutes, 94 °C for 1 minutes, 47.4 °C for 30 seconds, 72 °C for 1 minutes 30 seconds, 72 °C for 7 minutes. PCR products were sent to First Base Laboratories, Malaysia. The amplified products were then purified by PCR Clean-Up or Gel Extraction depends on Visualization results for Single Pass DNA Sequencing. Forward sequencing reactions were performed by a Big Dye Terminator v3. 1 cycle sequencing kit using ITS5 (First Base Laboratories).

Amplification and sequencing of *rbcL*sequence

The genomic DNA was amplified by using universal primer *rbcL* F (CTTGGCATTCCGAGTA) and *rbcL* R (TCACAAGCAGCCAGTTC) (Suparman, 2013). Thirty five cycles of PCR were conducted using Thermal Cycle under following profiles: 95 °C for 4 minutes, 94 °C for 30 seconds, 53 °C for 30 second, 72 °C for 2 minutes, 72 °C for 10 minutes. PCR products were sealed by using parafilm before sending them to First Base Laboratories, Malaysia

Amplification and sequencing of *trnL-F* IGSsequence

The highest yield of polymerase chain reaction (PCR) products was achieved by using this following condition. The PCR reaction 50 µL consisted of 10-50 ng/µL genomic DNA, 10 pmol of each primer, Dream Taq Buffer 10x, and 2mM dNTP Mix. The PCR reaction was conducted according to Small *et al.* (2004) consisted of an activation step of denaturation 95°C for 4 minutes, an annealing step of 52°C for 1 minute, and an extension step of 72°C for 1 minutes 30 seconds. The PCR mixture underwent for 35 cycles. The PCR products were run on 1.2% agarose gel electrophoresis at 110 volts for 30 minutes. The PCR products were sequenced at First Base Laboratories, Malaysia.

Phylogenetic analysis

DNA sequences of ITS region, *trnL-F* IGS, and *rbcL* of *Mangifera* species and outgroup taxa were first alligned by ClustalW Multiple Allignment in Bioedit (Thompson *et al.,* 1997). The boundaries of ITS1 and ITS2 were determined by comparing the aligned sequence with previously published sequences (Yonemori *et al.,* 2002; Fitmawati *et al.,* 2016a). The 5.8S coding sequence separating the ITS1 and ITS2 regions were also used in phylogenetic analyses, although only few variations were found among species examined.

All data matrices were analyzed with parsimony approach using MEGA6 (Tamura *et al.,* 2013). Maximum Parsimony Heuristic Search were conducted with the following

setting: all characters were treated as unordered data and have equal weight; random stepwise addition; branch swapping algorithm was run by using tree-bisection-reconnection (TBR); gaps were treated as missing; a strict consensus tree was produced from the resulting trees. Clade support values were obtained by using bootstrap. Bootstrap support (BS) was categorized as strong (>85%), moderate (70%-85%), weak (50%-69%), or poor (<50%) (Kress *et al*., 2002).

Mr. Bayes version 3.0 (Ronquist and Huelsenbeek, 2003) were used for Bayesian analysis. A general time reversible model (rates = invgamma, nst = 6) was used. Markov Chain Monte Carlo (MCMC) runs of one millions generations each, starting from different random point in parameter space to verify consistency in our results. Trees were sampled every 100th cycle from chain. All samples points that occurred before stationary score was achieved were discarded as part of the burn period. Nodes with posterior probability values ≥ 95% were retained in the 50% majority role consensus tree.

RESULTS

ITS analysis of Sumatran species of *Mangifera*

The ITS sequences region proven valuable information for phylogenetic reconstruction in angiosperms and it is one of the most popular sequences for phylogenetic inference at the generic and intra-generic levels in plant (Alvares and Wendel, 2003). At this point, the variation on ITS sequence region was analyzed to determine the phylogenetic relationship of *Mangifera* in Sumatra.

The ITS sequence (ITS1-5.8S-ITS2) obtained from Sumatran Species of *Mangifera* showed variations in both length and composition (Table 2). The ITS1 spacer was ranged from 263 to 265 bp in all samples. The length of ITS2 spacers in all the accession ranged from 226 to 230. The G+C content of ITS1 ranged from 63.64 to 67.55% while ITS2 ranged from 53.71 to 64.04%. There is little variation in length for 5.8S gene region which had a conserved length of 163 bp except for *M. foetida1* which has 162 bp. The GC content of 5.8S gene varied from 54.6 to 55.83% and it was lower than ITS1 and ITS2 in length and GC content. The length of the entire ITS region ranged from 652 to 657 bp with GC content ranging between 58.84 to 63.05%.

Alignment of the entire of ITS sequences among *Mangifera* species obtained 661 bp. There were 190 polymorphic sites. Among variable sites, 109 sites were supposed to be informative for phylogenetic analysis using parsimony method. However, when the sequences of two outgroup were added to the alignment, it resulted more indels due to short length of outgroup sequences, especially in ITS1. It resulted in 667 bp of the aligned length for the entire sequence in all species including outgroup taxa. The polymorphic sites became 268 in the entire sequence in all species including outgroup taxa, and 138 sites among them were assumed to be informative for parsimony analysis.

Table 2. Length and G + C content of ribosomal DNA segments of *Mangifera* spp. and outgroup.

Species	ITS1		5.8S		ITS2		ITS Entire Region	
	%GC	Length	%GC	Length	%GC	Length	%GC	Length
M. kemanga1	66.29	264	55.21	163	59.39	229	61.13	656
M. kemanga3A	66.29	264	55.21	163	58.33	228	60.76	655
M. kemanga3B	63.64	264	55.83	163	60.96	228	60.76	655
M. foetida1	65.91	264	55.56	162	58.85	226	60.89	652
M. foetida2A	65.15	264	55.83	163	61.40	228	61.53	655
M. foetida2B	66.29	264	55.21	163	60.53	228	61.53	655
M. foetida3A	65.15	264	55.83	163	60.96	228	61.37	655
M. foetida3B	65.15	264	55.83	163	60.96	228	61.37	655
M. foetida3C	65.15	264	55.83	163	60.96	228	61.37	655
M. foetidaThai	64.77	264	55.83	163	60.53	228	61.07	655
M. odorata1	65.15	264	55.83	163	60.96	228	61.37	655
M. odorata2A	65.15	264	55.83	163	61.40	228	61.53	655
M. odorata2B	65.53	264	55.83	163	60.53	228	61.37	655
M. odorata3	65.15	264	55.83	163	61.40	228	61.53	655
M. odorataThai	63.64	264	55.21	163	59.21	228	60.00	655
Mangifera sp.1	63.64	264	55.83	163	56.14	228	59.08	655
Mangifera sp. 2	65.02	263	54.60	163	60.09	228	60.70	654
M. griffithii	64.77	264	55.21	163	59.21	228	60.46	655
M. gracilipes	67.55	265	55.83	163	61.14	229	62.40	657
M. macrocarpa	66.42	265	55.83	163	63.16	228	62.65	656
M. torquenda1	63.64	264	55.83	163	60.53	228	60.61	655
M. torquenda3	63.64	264	55.83	163	60.96	228	60.76	655
M. quadrifida1	67.42	264	55.83	163	62.72	228	62.90	655
M. quadrifida3	64.02	264	55.83	163	60.96	228	60.92	655
M. sumatrana1	63.64	264	55.83	163	60.96	228	60.76	655
M. zeylanica1	65.53	264	55.21	163	58.77	228	60.61	655
M. laurina1	66.29	264	55.21	163	60.26	229	61.43	656
M. laurina2A	65.91	264	55.21	163	58.08	229	60.52	656
M. laurina2B	66.29	264	55.21	163	59.39	229	61.13	656
M. laurina3A	65.91	264	55.21	163	58.08	229	60.52	656
M. laurinaThai	66.29	264	55.21	163	59.13	230	61.04	657
M. oblongifolia	65.15	264	55.21	163	60.09	228	60.92	655
M. indica1	66.67	264	54.60	163	58.95	229	60.98	656
M. indica2	66.29	264	55.21	163	60.26	229	61.43	656
M. indica3A	65.53	264	55.21	163	53.71	229	58.84	656
M. indicaThai	66.29	264	55.21	163	59.83	229	61.28	656
M. lalijiwa	66.67	264	55.21	163	60.53	228	61.68	655
M. casturi	66.67	264	55.83	163	64.04	228	63.05	655
M. caloneura	67.55	265	55.83	163	61.14	229	62.40	657
M. cochinchinensis	64.77	264	55.83	163	61.84	228	61.53	655
M. flava	65.28	265	55.21	163	59.83	229	60.88	657
M. gedebe	67.05	264	55.21	163	60.96	228	61.98	655
M. pentandra	67.55	265	55.83	163	60.70	229	62.25	657
M. sylvatica	65.53	264	55.21	163	58.08	229	60.37	656
B. macrophylla	71.97	264	55.56	162	66.22	225	65.90	651
A. occidentale	71.98	232	57.06	163	73.18	220	68.46	615

Molecular phylogeny of *Mangifera*

This study obtained 20 new ITS nrDNA sequences of 10 species of Mangifera from Sumatra. In addition for phylogenetic analysis, 26 sequences (24 of *Mangifera* and two of outgroup) from GeneBank were obtained. The total alignment 46 entire sequences provided an 667-bp-long matrix. Sequence length variations resulting from insertions and deletions were found among species of *Mangifera*. The aligned ITS contained 399 (50%) conserve characters, 130 (19%) parsimony-uninformative characters and 138 (20%) parsimony-informative characters. The analysis resulted in a length of 534 steps and had consistency index (CI) and retention index (RI) indices of 0.647 and 0.797, respectively. The strict consensus tree reconstructed by the parsimony method is shown in Figure 1. The trees obtained from Bayesian analysis method is shown in Figure 2. The tree obtained from the Bayesian analysis method was mostly consistent with the tree obtained from the parsimony method, except for *M. gedebe* which appeared in different positions (Figures 1 and 2).

Using *A. occidentale* and *B. macrophylla* as outgroups, one basal branch and two subclades in both maximum parsimony and Bayesian analysis were recognized. In addition, different lineages pattern of recent common ancestor within subclades II were also recognized. Corresponding to the tree topology, the basal taxa were *M. quadrifida1* from Central Sumatra. Subclade I comprised of six rare species from Sumatra (*Mangifera* sp. 2, *Mangifera* sp. 1, *M. kemanga3B, M. torquenda1, M. torquenda3, M. sumatrana1* and *M. quadrifida3*); a species endemic to Borneo (*M. casturi*), and a species (*M. griffithii*) distributed across Malay Peninsula, Borneo and Sumatra. Subclade II consisted of species from Southern Sumatra (*M. lalijiwa*); monophyletic group of a species occurring in Thailand Peninsular, Malay Peninsula, Sumatra and Borneo (*M. macrocarpa*) and a species restricted to Eastern Thailand and Vietnam (*M. cochinchinensis*); monophyletic group of subgenus *Limus*: *a* species origin to Sumatra (*M. odorata*), a rare species in Sumatra (*M. kemanga3A*) and a widely distributed species in Western part of Malesia (*M. foetida*); monophyletic group of a widely distributed species across South-east Asia, New Guinea and Solomon Island (*M. gedebe*); a species (*M. oblongifolia*) restricted to Malay Peninsula, Sumatra and Borneo; a species (*M. flava*) restricted to Thailand and S. Vietnam; a species (*M. caloneura*) restricted to S. Burma, Thailand and Indo-China; a species (*M. sylvatica*) from South Asia; a species (*M. gracilipes*) restricted to Malay Peninsula and Sumatra; a species (*M. pentandra*) distributed across Malay Peninsula, Thailand, Borneo and Riau Archipelago; a Malesian species (*M. laurina*); an endemic species (*M. zeylanica*) from Sir Lanka, the common mango (*M. indica*), and a rare species (*M. kemanga1*) from Sumatra. The main contradiction within subclades II between MP tree and Bayesian tree is position of *M. gedebe.*

Parsimony analysis of combination sequences

The results of parsimony analysis based on the sequence data of ITS region, *trnL-F* IGS and *rbcL* gene are summarized in Table 3. The analysis

presented in the parsimony cladogram with the strict consensus tree (Figure 3).

The aligned matrix for the combined analysis comprised 2,378 characters, of which 1351 were conserved region and 362 parsimony were potentially informative. We found one most parsimonious trees with a length of 1465 steps, CI of 0.58 and RI of 0.61. In *Mangifera* case, we found the character of ITS sequence gave strong implication as well as *trnL-F*IGSand *rbcL* sequences (Figures 1, 2 and3).

Table 3. Summary of parsimony analysis.

Variables	ITS	*trnL-F*IGS	*rbcL*	ITS+*trnL-F*IGS+*rbcL*
Sequence length	665	410	1303	2378
Conserved sites	405	141	805	1351
Variable sites	251	266	320	837
Parsimony informative characters	122	147	93	362
Tree length	427	401	493	1465
Consistency index (CI)	0.60	0.88	0.59	0.58
Retention index (RI)	0.79	0.86	0.59	0.61

DISCUSSION

Phylogenetic relationship within the genus *Mangifera*

In this study of ITS sequence analyses, phylogenetic trees with relatively high bootstrap supports and Bayesian posterior probabilities were obtained. This study also completed several species and accessions from the previous study of ITS (Yonemori *et al.,* 2002; Fitmawati *et al.,* 2016a). It is advantageous to use nuclear ITS regions for phylogenetic analysis in angiosperms. The ITS nrDNA has been widely used in taxonomy and molecular phylogeny. Internal Transcribed Spacer has higher degree of variation than other regions of nrDNA (White *et al*., 1990). With high variation of nucleotides, ITS has proven especially useful for elucidating relationships among species level, infrageneric level, and closely related genera, even in level of mango cultivars (Hidayat *et al.,* 2013).

The *M. quadrifida1*from Central Sumatra, Indonesia is nested at the base next to the outgroups. The well-supported clade, which includes all of *Mangifera* species shows that *Mangifera* is a monophyletic group (Figure 1). *M. quadrifida1* was found to be the most basal taxon within the genus. Fitmawati *et al.* (2016a) also reported *M. quadrifida1* from Central Sumatra as the earlier wild type species lived in Sumatra based on parsimony analysis. It can be distinguished from all other species by its ultimate colour of fruit, which is pitch black and glossy (Fitmawati *et al.,* 2013). Species *M. quadrifida1*from Central Sumatradiverged early in the history of a group and hence it did not form a monophyletic group with *M. quadrifida3* from Southern Sumatra. Analysis of ITS sequence between *M. quadrifida1*from Central Sumatra and *M. quadrifida3* from Southern Sumatra were further analyzed. In this study, there were 617 conserved sites and 39 variable sites between both *M. quadrifida* founded. Differences of environmental factor on their habitat have been able to change nucleotide base constitution on the species of *M. quadrifida.*

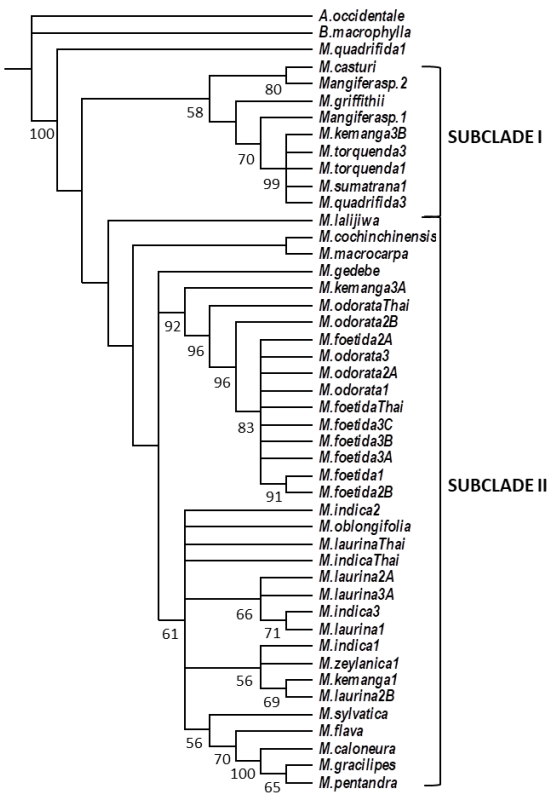

Figure 1

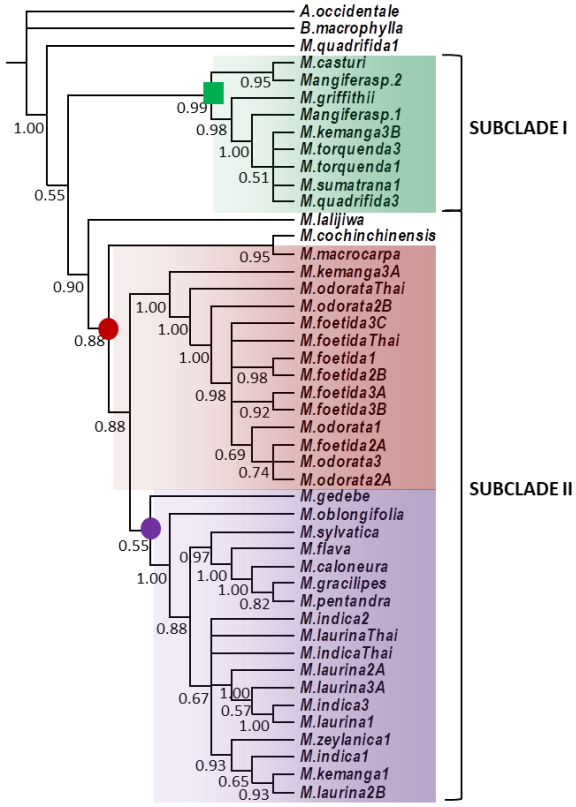

Figure 2

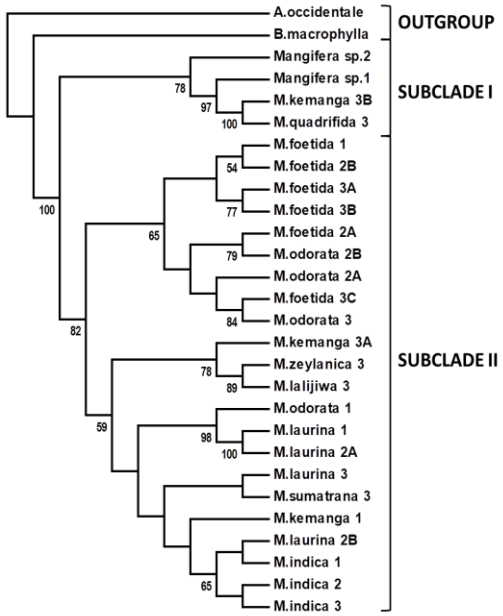

Figure 3

Figure 1. Strict consensus tree derived from maximum parsimony analysis of ITS sequences of *Mangifera* and outgroup taxa. CI=0.647, HI=0.352, RI=0.797. Numbers below branches showed bootstrap values.

Figure 2. The 50% majority-rule consensus tree derived from the Bayesian analysis of ITS sequences for *Mangifera* and outgroup taxa. Numbers below branches are Bayesian posterier probabilities. The main group color-coded: Green stands for subclade I, red stands for paraphyletic *Limus*, and purple stands for monophyletic group of *M. gedebe* and its sister group.

Figure 3. Strict consensus tree derived from Maximum Parsimony analysis of Combination ITS+*trnL-F*IGS+*rbcL* sequences of *Mangifera* and outgroup taxa. CI=0.58, RI=0.61. Numbers below branches showed bootstrap values.

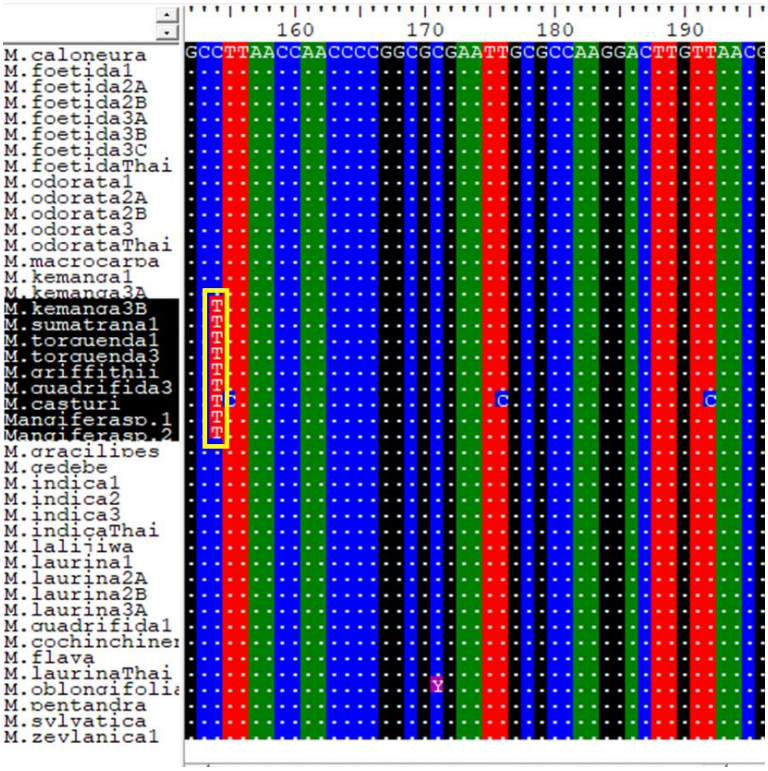

Figure 4. Unique nucleotide sequence variation of ITS region within subclade-I compared to other accessions (at base number 154, a Thymine not Cytosine).

In this study, all of the Sumatran rare species were included in subclade I. The species from this subclade are from subgenus *Mangifera* except for *M. kemanga3B* from subgenus *Limus*. Members of subclade I differ from other species/accessions of *Mangifera* at nucleotide position 154 in the nuclear ribosomal DNA of ITS region: Thymine not Cytosine (Figure 4). According to Kostermans and Bompard (1993), subgenus *Mangifera* divided into four sections, *Marchandora, Euantherae, Rawa* and *Mangifera. M. casturi, M. torquenda* and *M. quadrifida* belong to section *Mangifera. The M. griffithii* belongs to section *Rawa*. The position for three species (*M. sumatrana, Mangifera* sp. 1 and *Mangifera* sp. 2) which previously undetermined (Fitmawati *et al.,* 2016a) is now can be described. Subclade I can be found in green color-coded in Bayesian tree (Figure 2).

In this study, the lineage leading to *M. casturi* and the lineage leading to *Mangifera* sp. 2 both evolved from the common ancestor (Figures 1 and 2). That ancestor, which is now extinct, was neither *M. casturi* nor *Mangifera* sp. 2. However, its descendants include the two extant (living) species shown here, *M. casturi* and *Mangifera* sp. 2. The most recent common ancestor of the *Mangifera* sp. 2 and *M. griffithii* lived before the most recent common ancestor of the *Mangifera* sp. 2 and *M. casturi.*

Even though *M. griffithii* is more closely related to the *Mangifera* sp. 1 than *Mangifera* sp. 2, they look more like *Mangifera* sp. 2 because morphology has changed dramatically

in the *Mangifera* sp. 1 lineage. Fitmawati *et al.* (2015) reported that the species of *Mangifera* sp. 2 from Eastern Sumatra is highly similar to *M. griffithii* based on morphological characteristic.However, assumption about *Mangifera* sp. 2 and *M. griffithii* are the same species could not be stated. Another new insight can be concluded from parsimony and Bayesian analysis is both *Mangifera* sp. 1 and *Mangifera* sp. 2 are grouping in subgenus *Mangifera* of Kostermans' morphological classification.

Mangifera sp. 1 shares more recent common ancestor with *M. torquenda1, M. torquenda3, M. sumatrana1, M. quadrifida3,* and *M. kemanga3B.* Fitmawati *et al.* (2016a) reported that *Mangifera* sp. 1 from Central Sumatra as the modern species of *Mangifera* based on the longest genetic distance from other species of *Mangifera.* This rare species has unique characters such as woody coriaceous leaves, fibreless pulp of fruit and also the only species with cyclocytic stomata so far (Fitmawati *et al.,* 2013). From Bayesian and parsimony analysis the branch leading to *M. kemanga3B, M. torquenda1, M. torquenda3, M. sumatrana1* and *M. quadrifida3* could not be resolved well. It is because there was one species from subgenus *Limus* included in this branch, *M. kemanga3B* and this branch also polytomy.

The *M. sumatrana* Miq., is a unique species found in Sumatra and was treated as synonym of *M. laurina* Bl. based on morphological characters in the latest classification by Kostermans and Bompard (1993). Prior to them, this species was treated as synonym of *M. longipes* Griff. by Mukherji and Ding Hou. Phylogenetic analysis based on morphological characters also showed *M. sumatrana* Miq. has a close relationship with *M. indica* L. and *M. laurina* Bl (Fitmawati *et al.,* 2013). However, based on molecular analysis using ITS sequence by Fitmawati *et al.* (2016a) was obtained the results *M. sumatrana* Miq. did not form a clade with both of *M. indica* L. and *M. laurina* Bl.

The result of BLAST indicated that *Mangifera sumatrana* Miq. (Genbank acc. no. KX347961) has a high similarity to *M. torquenda, M. griffithii, M. macrocarpa, M. camptosperma* and *M. gedebe* with identity value ≥ 95% while the similarity to both *M. laurina* and *M. indica* is less than 95% (Table 4). Using BLAST parameters are important to determine species name (Madden, 2013). Corresponding to the tree MP and Bayesian analysis using ITS sequence and also data from BLAST parameters, *M. sumatrana* Miq.is not synonym of *M. laurina* Bl (Figures 1 and 2).

In this study, an unresolved pattern of divergence among *M. indica, M. laurina, M. zeylanica,* and *M. kemanga1* was acknowledged. Their branching pattern could not be resolved well. Moreover in MP tree, the polytomy became complicated including the position of *M. gedebe* and two large monophyletic groups within subclade II. In Bayesian tree, *M. gedebe* belongs to section *Marchandora,* became the basal taxa for the *M. oblongifolia* and its sister group. Itcan be found in purple color-coded in Bayesian tree (Figure 2).

Table 4. BLAST analysis of ITS sequence of *Mangifera sumatrana* Miq. (Genbank Acc. No. KX347961).

Description	Max score	Total score	Query cover (%)	E value	Ident (%)	Accession
Mangifera torquenda ITS1, partial sequence; 5.8S rRNA gene, complete sequence; ITS2, partial sequence	1205	1205	100	0.0	99	KX347958.1
Mangifera griffithii genes for ITS1, 5.8s rRNA, ITS2, complete sequence	1094	1094	100	0.0	96	AB071685.1
Mangifera macrocarpa genes for ITS1, 5.8s rRNA, ITS2, complete sequence	1035	1035	100	0.0	95	AB071688.1
Mangifera camptosperma genes for contains ITS1, 5.8s rRNA, ITS2, partial and complete sequence	1033	1033	100	0.0	95	AB598043.1
Mangifera gedebe genes for ITS1, 5.8s rRNA, ITS2, complete sequence	1033	1033	100	0.0	95	AB071681.1

M. oblongifolia, M. sylvatica, M. flava, M. indica, M. laurina and *M. zeylanica* belong to section *Mangifera. M. caloneura* and *M. pentandra* belong to section *Euantherae*. *M. gracilipes* belongs to section *Rawa*. *M. kemanga1* belongs to section *Deciduae* of subgenus *Limus.*

The main contradiction between MP and Bayesian trees are the position of *M. gedebe* and several polytomies. The difference of strict consensus tree may have occurred since there were two different approaches used in this study. Parsimony method refers to choosing among trees on the basis of which one requires the fewest possible mutation to explain the data. While Bayesian statistics is closely related to Maximum likelihood and focuses on the posterior probability of hypotheses. The posterior probability is proportional to the product of the prior probability and the likelihood (Holder and Lewis, 2003).

The *M. gedebe* and all of the descendants of their common ancestor is the monophyletic group consists of all of the section in subgenus *Mangifera* and one section in subgenus *Limus* based on Bayesian analysis (Figure 2). In this study, phylogenetic tree based on ITS region of nrDNA showed that sections within Kostermans' infrageneric classification were polyphyletic. Molecular phylogenetic based on ITS regions resulted in this study were not consistent with traditional classification based on morphological characters, i.e. Kostermans' classification of *Mangifera.*

According to Kostermans and Bompard (1993), subgenus *Limus* is divided into two sections, *Deciduae* and *Perennes.* Based on morphological classification by Kostermans and Bompard (1993), this subgenus share similar flower disk character.*M. kemanga* belongs to section *Deciduae* while *M. odorata, M. foetida* and *M. macrocarpa* belong to section *Perennes.* In this study, generally subgenus *Limus* can be found in red color-coded in Bayesian Tree (Figure 2). In the previous study, Yonemori *et al.* (2002) could not recognize the position of this subgenus as well as Fitmawati *et al.* (2016a). Due to the extensive sampling have been done, the future of subgenus *Limus* could be predicted.

Table 5. Variable sites in the ITS region of *M. foetida* and *M. odorata* species.

Taxa	Base sequence number																								
	1 4	3 4	3 3 8	3 3 9	8 8 8	9 9 6	1 2 4	1 5 1	2 2 9	2 3 3	2 3 8	3 1 2	3 3 7	3 4 8	4 9 7	4 4 9	5 7 7	5 3 8	5 5 6	5 7 3	5 8 2	6 9 4	6 1 1	6 1 7	6 3 4	6 5 2
M. foetida1	A	C	G	C	A	C	T	G	G	T	T	G	T	G	C	A	T	T	T	G	G	T	A	A	A	G
M. foetida2A	A	C	G	C	A	C	T	T	T	T	T	G	T	G	C	G	T	C	A	G	G	T	G	G	G	G
M. foetida2B	A	C	G	C	A	C	G	G	T	G	T	A	G	A	C	A	T	G	A	G	T	G	G	A	G	G
M. foetida3A	A	C	G	C	A	C	T	T	T	T	T	G	T	G	C	A	C	T	A	G	G	T	G	G	G	G
M. foetida3B	A	C	G	C	A	C	T	T	T	T	T	G	T	G	C	A	C	T	A	G	G	T	G	G	G	G
M. foetida3C	A	C	G	C	A	C	T	T	T	T	T	G	T	G	C	A	T	C	A	G	G	T	G	G	G	G
M. foetidaThai	A	C	K	C	A	C	T	T	T	T	T	G	T	G	C	A	T	T	A	G	G	T	G	G	G	G
M. odorata1	A	C	G	C	A	C	T	T	T	T	T	G	T	G	C	G	T	T	A	G	G	T	G	G	G	G
M. odorata2A	A	C	G	C	A	C	T	T	T	T	T	G	T	G	C	G	T	C	A	G	G	T	G	G	G	G
M. odorata3	A	C	G	C	A	C	T	T	T	T	T	G	T	G	C	G	T	C	A	G	G	T	G	G	G	G
M. odorataThai	G	T	T	G	G	G	T	T	T	T	T	G	T	G	T	A	Y	T	R	A	G	T	G	G	G	T
M. odorata2B	G	T	G	C	A	C	T	T	T	T	G	G	T	G	C	A	T	T	A	G	G	T	G	G	G	G

Our study indicates that subgenus *Limus* was paraphyletic group if only two accessions of *M. kemanga* are excluded. However, other accessions of *M. kemanga,* ie: *M. kemanga1* from Central Sumatra and *M. kemanga3B* from Southern Sumatraare scattered in several lineages hence this subgenus become polyphyletic. Initially, there were no different morphological characteristics among the accessions hence a conclusion about there are few nucleotide variations within the species of *M. kemanga* are admitted. There is a possibility that the accessions are members of different species. This movement of genes from one organism to another probably occurred and it needs further analysis. Perhaps it is related to the horizontal gene transfer event (Renner and Bellot, 2012).

In addition, an unresolved pattern of divergence between *M. odorata* and *M. foetida* was acknowledged. Their branching pattern could not be resolved well. Both MP and Bayesian tree gave information that the common ancestor of *M. odorata* lived earlier than the common ancestor of *M. foetida.* Out of 661 nucleotide sequence, almost 95% (630 bp) are conserved region. There are few nucleotide variations between *M. foetida* and *M. odorata.* With 26 variable sites, there were no differences between *M. foetida* and *M. odorata* (Table 5). This fact also somewhat similar to the statement from Hou (1978) and Corner (1940) based on morphological data. Kostermans stated that the fruit between these two species are perhaps difficult to recognize (Kostermans and Bompard, 1993). Whether these two species are actually the different varieties within a species or whether these two species were resulted from epigenetic event are still unknown. In which environmental factors are significant to change the morphology of species (Novero *et al.,* 2012).

The possibility of hybrid origin was reported by Yonemori *et al.* (2002) and Teo *et al.* (2002) based on molecular data is still affect the

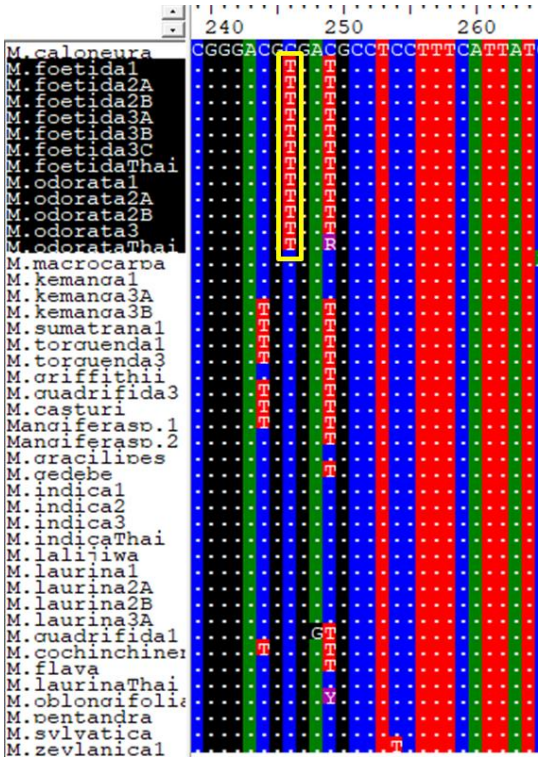

Figure 5. Unique nucleotide sequence variation of ITS region within all of the accession from *M. odorata* and *M. foetida* compared to other accessions (at base number 246, a Thymine not Cytosine).

polytomy of *M. foetida* and *M. odorata*. There were somewhat a relationship among *M. odorata, M. foetida* and *M. indica.* Related to the origin of species, *M. odorata* is only found in Sumatra and has wide distribution throughout the island (Fitmawati and Hayati, 2018). All of the accessions of *M. odorata* and *M. foetida* differ from other species of *Mangifera* at nucleotide position 246 in the nuclear ribosomal DNA of ITS region: Thymine not Cytosine (Figure 5). Giving the fact that all of the *M. foetida* and *M. odorata* accessions were in monophyletic group, means the relationship between this two species is stronger than *M. indica,* which assumed as one of the parental in hybrid origin by Teo *et al.* (2002).

Based on *rbcL* sequence of cladogram reconstruction *M.quadrifida* and *M. sumatrana* formed one group. According Fitmawati *et al.* (2013), this molecular data also supported by morphological data such as the chartaceous leaves texture and *M. quadrifida* has white flower while *M. sumatrana* has red flower. *Mangifera* sp.1 has a close relationship with *M. torquenda. M. torquenda* is placed on subgenus *Mangifera* included in the tetramerous group with morphological characters sepals and petals amounted to 4 (Fitmawati *et al.,* 2017).

Analysis of combination among ITS, *trnL-F* IGS and *rbcL* sequences also could not resolve the division between subgenus *Mangifera* and *Limus* and also among the section of

Table 6. Sources of *Mangifera* ITS sequences for combination analysis.

Species	Acc. No. (ITS)	Acc. No. (*rbcL*)	Acc. No. (*trnL-F*IGS)	Geographic origin	Section
M. kemanga Bl. 3A	MF678503	-	MF919593	Southern Sumatra	*Deciduae*
M. kemanga Bl. 3B	MF990368	-	MF919594	Southern Sumatra	*Deciduae*
M. foetida Lour. 2A	MF678499	-	KY392613	Eastern Sumatra	*Perennes*
M. foetida Lour. 2B	MF678500	-	KY392608	Eastern Sumatra	*Perennes*
M. foetida Lour. 3A	MF678501	-	MF945597	Southern Sumatra	*Perennes*
M. foetida Lour. 3B	MF678505	-	MF997585	Southern Sumatra	*Perennes*
M. foetida Lour. 3C	MF678506	-	MF997584	Southern Sumatra	*Perennes*
M. odorata Griff. 2A	MF678496	-	KY392610	Eastern Sumatra	*Perennes*
M. odorata Griff. 2B	MF678497	-	KY392615	Eastern Sumatra	*Perennes*
M. odorata Griff. 3	MF678507	-	MF945596	Southern Sumatra	*Perennes*
M. laurina Bl. 2A	MF678495	-	KY392612	Eastern Sumatra	*Mangifera*
M. laurina Bl. 2B	MF678498	-	KY392609	Eastern Sumatra	*Mangifera*
M. laurina Bl. 3A	MF678508	-	MF997588	Southern Sumatra	*Mangifera*
M. indica L. 2	MF678502	-	KY392616	Eastern Sumatra	*Mangifera*
M. indica L. 3A	MF678509	-	MF997586	Southern Sumatra	*Mangifera*
M. lalijiwa Kosterm.	MF678504	-	MF997587	Southern Sumatra	*Mangifera*
M. quadrifida Jack. 3	MF678511	-	MF997589	Southern Sumatra	*Mangifera*
Mangifera sp. 2	MF678494	-	KY392607	Eastern Sumatra	Undetermined
M. zeylanica (Bl) Hook. f. 3	MF990364	-	MF997591	Southern Sumatra	*Mangifera*
M. sumatrana Miq. 3	MF990366	-	MF997590	Southern Sumatra	-
M. quadrifida Jack 3	MF678511	-	MF997589	Southern Sumatra	*Mangifera*
M. kemanga Bl. 1*	KX347955	-	MF919592	Central Sumatra	*Deciduae*
M. foetida Lour. 1*	KX347956	-	KY392618	Central Sumatra	*Perennes*
M. odorata Griff. 1*	KX347957	-	KY392623	Central Sumatra	*Perennes*
M. laurina Bl. 1*	KX347963	-	KY392621	Central Sumatra	*Mangifera*
M. indica L. 1*	KX347960	-	KY392619	Central Sumatra	*Mangifera*
Mangifera sp. 1*	KX347964	-	KY392622	Central Sumatra	Undetermined
Bouea macrophylla Griff.*	AB071691	-	AY594500	Thailand	-
Anacardium occidentale L.*	AB071690	-	AY594997	Thailand	-

Note: *Data obtained from GeneBank

genus *Mangifera* proposed by Kostermans based on morphological data only (Table 6). The results of this study suggested that Kostermans' infrageneric classification should be re-considered in order to establish a modern classification which based on monophyly of the genus *Mangifera.* This new modern classification is more robust than previous system, and relationship of some closely related taxa and interspecies can be clearly resolved. To establish a new modern classification of the genus *Mangifera*, molecular characters of *Mangifera* species have to be available, and they represent the species of 6 sections within the Kostermans' infrageneric classification. Preferably, specimens examined in Kostermans and Bompard (1993) are borrowed, however, they are old specimens. It is impossible to amplify the old specimens, otherwise they have to be re-collected. Most of those specimens were collected under 1990, for example *M. pajang* Kosterm. which was collected from North of Sangkulirang, Mapulu in 1957 by Kostermans (Kostermans and Bompard, 1993).

A very limited sequences of ITS regions nrDNA which were applied in this study might be the reason of unresolve phylogenetic relationship of sections within Kostermans' infrageneric classification. Only 44

sequences from 23 species of *Mangifera* was used construct a phylogenetic tree by using MP method and Bayesian Analysis. Twenty sequences from 10 species were generated from this study, and the remaining sequences were obtained from NCBI GenBank. Preferably, the sequences of 46 species were included in the analysis, however, the leaf sample for DNA extraction is difficult to obtain and if it is exist, it is from old specimens. A few new collected specimens are available. Moreover, the availability of sequence data of *Mangifera* in GenBank is very limited, consequently phylogenetic tree based on morphological and sequence ITS nrDNA was unable to be compared. As a result, the phylogenetic of *Mangifera* species was not completely resolved. At present, 44 sequences of M*angifera* are available in GenBank. At present, phylogenetic relationship of *Mangifera* within the genus has not been resolved until more species are added to the analysis.

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