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Short Communication: Using ITS as a molecular marker for *Mangifera* species identification in Central Sumatra

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Abstract. *Fitmawati, Hayati I, Sofiyanti N. 2016. Using ITS as a molecular marker for* Mangifera *species identification in Central Sumatra. Biodiversitas 17: 653-656.* The relationship among *Mangifera* species in Central Sumatra is currently unclear. Previous molecular studies on these taxa using cpDNA were unable to produce well-resolved phylogenetic trees. In this study, we explored the potential of the ITS sequences as molecular markers for *Mangifera* species to better resolve the phylogenetic analysis. Parsimony analysis revealed that the common ancestor *M. quadrifida* as the first species appeared in Central Sumatra. *Mangifera* sp. which assumed as new species had the longest genetic distance among species examined and may assumed as the most primitive species of *Mangifera* in Neighbor-Joining analysis. *M. sumatrana* and *M. torquenda* were closely related as well as *M. foetida* and *M. odorata*. Also, *M. indica* was closely related to *M. kemanga*. This finding and the other marker of cpDNA such as trnL-F and rbcL gene may suggest a possibility to revision in the latest *Mangifera* classification based on morphological character. Our results also revealed and support the genus *Mangifera* is a monophyletic group.

Keywords: Central Sumatra, ITS, *Mangifera*, molecular marker, phylogenetic analyses

INTRODUCTION

The genus *Mangifera* is one of the most important genera from family Anacardiaceae which used for commercial fruit production in the world. The characteristics of *Mangifera* species in Central Sumatra were tolerant to high rainfall, capable of fruiting out of season, high production and flowers resist against wet climate. The species with these traits had a potential as germplasm resources (Fitmawati et al. 2013). Exploration on *Mangifera* species has been done by Fitmawati et al. (2013) in three provinces of Central Sumatra, i.e.: Riau, West Sumatra and Jambi. Ten of *Mangifera* species which typical in Central Sumatra were obtained. *Mangifera* species in Sumatra were divided into three categories such as: wild types, semi cultivated types and cultivated types (Fitmawati et al. 2015). Due to high frequency of forest and land fires in Sumatra, specific types of *Mangifera* Sumatra were threatened in natural habitat, therefore wild germplasm resources must be conserved before it lost in the wild.

The most recent and acceptable classification of *Mangifera* were proposed by Kostermans and Bompard (1993) based on morphological character and divided into two groups based on disc flower characteristic namely sub genus *Limus* and sub genus *Mangifera*. Sub genus *Limus* has narrower disc than the base ovary, stalk-like or even lacking whereas sub genus *Mangifera* has broader disc than the base of the ovary, cushion-like (Kostermans and Bompard 1993). Morphological plasticity and continuity characters were the main problem to define phylogenetic relationship therefore using molecular approach based on DNA sequence which has more informative characters and support morphological characteristics. Molecular Phylogeny of *Mangifera* has been done using nuclear genome marker, ITS region for *Mangifera* in Thailand by Yonemori et al. (2002), as well using chloroplast DNA marker of trnL-F Intergenic spacer on *Mangifera* sp*ecies* in Java and Sulawesi (Fitmawati and Hartana 2010), also matK (Hidayat et al. 2012) and *rbc*L (Suparman et al. 2013) on *Mangifera* mainly in Thailand and a few part of Indonesia.

Internal Transcribed spacer (ITS) of nrDNA has been used for molecular markers at specific level of Angiospermae (Baldwin et al. 1995; Yonemori et al. 2002). Sequences of ITS were also useful because it has conserve region, short size (±700 bp), high evolution rate, informative and universality (Baldwin et al. 1995). Molecular study of specific *Mangifera* in Central Sumatra based on ITS sequences has never been informed, so that tries to reveal relationship among *Mangifera* species in Central Sumatra. Molecular approach has benefit to find the best phylogenetic tree model which useful in conservation and cultivation strategies.

MATERIALS AND METHODS

Plant material and DNA extraction

All samples used in this study (Table 1) were collected in Central Sumatra from exploration in 2012-2013. Two

genera from Anacardiaceae family were used as outgroup obtained from Genbank Data (NCBI) by Yonemori et al. (2002).

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

The genomic DNA was amplified using universal primer ITS4 and ITS5 (White et al. 1990) for the entire ITS regions. 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^{o}C for 5 m, 94^{o}C for 1 m, 47.4^{o}C for 30 s, 72^{o}C for 1 m 30 s, 72^{o}C for 7 m. PCR products were sent to First Base Laboratories, Malaysia. The amplified products were then purified by PCR Clean-Up or Gel Extraction depend 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 (White et al. 1990) (First Base Laboratories).

Phylogenetic analysis

DNA sequences of ITS regions 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). 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.

The data matrix of alligned sequences was analyzed by PAUP 4.0 program (Swofford 2002) for parsimony and neighbor joining method with bootstrap replicate method.

RESULTS AND DISCUSSION

ITS sequence analysis

The length of ITS1 is 264 bp and of ITS2 ranged from 226 to 230 in *Mangifera* species studied. There is not much variation in length for 5.8S gene region having 162 - 163 bp. The G+C content was fairly equivalent between of ITS1 and ITS2, although 5.8S gene has lower content than ITS1 and ITS2 (Table 2).

Allignment of the entire of ITS sequences among *Mangifera* species obtained 660 bp. There were 48 and 98 polymorphic sites in ITS1 and ITS2 respectively, whereas three sites were polymorphic in 5.8S gene region (Table 2). Among these 149 polymorphic sites, 77 sites (33 in ITS1, 42 in ITS2 and two in 5.8S gene region) were supposed to be informative for phylogenetic analysis using parsimony method. However, when the sequences of two outgroup were added to the allignment, it resulted more indels due to short length of outgroup sequences, especially in ITS1. It resulted in 666 bp of the aligned length for the entire sequence in all species including outgroup taxa. The polymorphic sites became 234 in the entire sequence in all species including outgroup taxa, and 90 sites among them were assumed to be informative for parsimony analysis.

Table 1. List of 10 *Mangifera* species collection in 2012-2013 with their distribution and two outgroup taxa used in this study

Species name	Distribution			Accession number
	R	WS	J	
M. kemanga Bl.	√	√	√	KX347955
M. foetida Lour.	√	√	√	KX347956
M. odorata Griff.	√	√	√	KX347957
M. torquenda Kosterm.	√	√	√	KX347958
M. quadrifida Jack.	√	√	√	KX347959
M. indica L.	√	√	√	KX347960
M. sumatrana Miq.	√	√	√	KX347961
M. zeylanica (Bl.) Hooker f.	√	√	√	KX347962
M. laurina Bl.	√	√	√	KX347963
Mangifera sp.	√	-	-	KX347964
Anacardium occidentale L.	√	√	√	AB071690
Bouea macrophylla Griff.	√	√	√	AB071691

Note: R: Riau, WS: West Sumatra, J: Jambi

Table 2. The characteristic features of the ITS region among *Mangifera* species and combination with outgroup taxa

	Length range (nt)	Length mean (nt)	Aligned length (nt)	G+C content (%)	G+C mean (%)	No. of variable sites	No. of informative sites	Tree length	CI	RI
Mangifera spp.										
ITS1	264	264	264	63.6-67.4	65.4	48	33	53	0.92	0.94
5.8s rDNA	162-163	162.9	163	54.6-55.8	55.4	3	2	4	1.00	1.00
ITS2	226-230	228.3	233	55.8-61.4	58.6	98	42	123	0.90	0.80
Entire seq.	652-657	655.2	660	58.9-62.4	60.6	149	77	201	0.81	0.73
Mangifera spp. + 2 outgroup taxa										
ITS1	232-264	261.3	269	62.1-70.6	64.6	90	48	123	0.85	0.83
5.8s rDNA	162-163	162.8	164	54.3-56.7	55.3	14	4	17	0.94	0.80
ITS2	220-230	227.3	233	55.8-69.1	59.9	130	38	200	0.87	0.62
Entire seq.	615-657	651.5	666	58.4-64.4	60.6	234	90	338	0.87	0.76

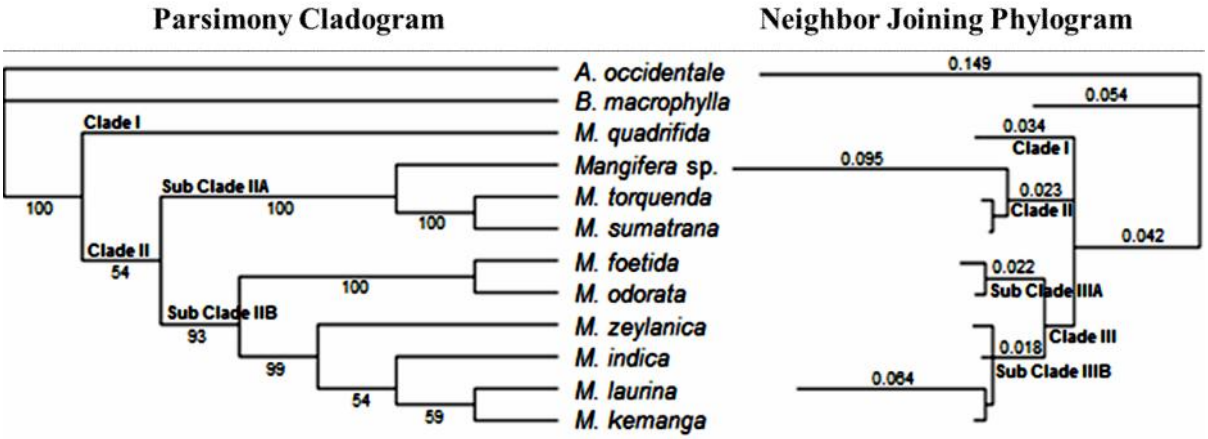

Figure 1. The phylogenetic tree based on the ITS sequences generated from maximum parsimony analysis with bootstrap value below the branch and number on the base of branch showed nodes number (*left side*) and evolution tree from HKY85 model evolution generated from neighbor joining analysis with total branch length 0.55. Branch length (number above line) corresponds to the genetic distance (*right side*).

Phylogenetic analysis of *Mangifera* species in Central Sumatra

The results of parsimony analysis based on the sequence data of ITS region are summarized in Table 2. Based on parsimony criteria, it was obtained a cladogram with CI value 0.87 and RI value 0.76. Monophyletic group of *Mangifera* species were separated from outgroup in the 21st branch with thirty three nucleotide changes (17 different sites in ITS1, 3 base in 5.8S and 13 sites in ITS2). Evolution tree from ten *Mangifera* species formed two clades with bootstrap value 100%. Clade I consists of *M. quadrifida* while Clade II consists of *Mangifera* sp., *M. torquenda, M. sumatrana, M. foetida, M. odorata, M. zeylanica, M. indica, M. laurina* and *M. kemanga.* Clade II evolved and divided into two sub clade. Sub clade IIA consists of *Mangifera* sp., *M. torquenda* and *M. sumatrana* while sub clade IIB consists of two groups were split *M. foetida* and *M. odorata* with *M. zeylanica, M. indica, M. laurina* and *M. kemanga.* (Figure 1 *left side*).

Neighbor joining (NJ) analysis reconstructed three clades. Clade I consisted of *M. quadrifida*, Clade II consists of monophyletic groups of *Mangifera* sp., *M. torquenda* and *M. sumatrana*, and Clade III consists of *M. foetida, M. odorata, M. zeylanica, M. indica, M. laurina* and *M. kemanga* (Figure 1 *right side*). The main contradiction in the NJ tree compared with the parsimonious tree was the place of clade II and clade III. Both clades formed a larger monophyletic group in parsimony analysis whereas in NJ analysis both clades were separate and resulted multifurcating tree.

Discussion

The results of parsimony analysis based on the sequence data of ITS region are noted in Table 2. Based on parsimony analysis *M. quadrifida* became the early wild type founded in lowland rainforest of Central Sumatra (Fitmawati et al. 2015). This finding was supported by fifteen nucleotide base changes in specific sites which separated *M. quadrifida* with the other nine *Mangifera* species. *M. torquenda* was closely related to *M. sumatrana*. They were separated from *Mangifera* sp. with fifty nine different nucleotide base characters. They formed a clade by sharing coriaceous leaf texture.

Mangifera foetida was showed a closely related to *M. odorata*. This theory about *M. odorata* is a hybrid from *M. indica* and *M. foetida* stated by Hou 1978 and also supported by Teo et al. (2002) and Yonemori et al. (2002). This fact does not agree with Kostermans and Bompard (1993) for saying the reticulation of *M. odorata* was definitely different from *M. indica* and *M. foetida* and also the flower was not an intermediate of both *Mangifera*.

Another monophyletic group consisted of *M. zeylanica, M. indica, M. laurina* and *M. kemanga.* The first three species were supported by Kostermans and Bompard (1993) based on morphological character, but we found the contradiction of *M. kemanga* place in this tree (Figure 1 *left side*). It was different with Kostermans and Bompard (1993) which classified *M. kemanga* into sub genus *Limus* but based on this research *M. kemanga* united in one group with *M. zeylanica, M. laurina* and *M. indica,* which is belong to sub genus *Mangifera* according to Kosterman and Bompard 1993. The previous note about relationship among *M. kemanga* and the other three species has never found therefore it became new finding on this study.

Neighbor Joining (NJ) analysis showed that *Mangifera* sp. had the longest evolutionary history from ten *Mangifera* species in this study and it assumed as the most primitive species found in Central Sumatra. *Mangifera* sp. has combination character between sub genus *Limus* and *Mangifera.* This species is included in sub genus *Mangifera* due to cushion-like disc flower while it can be included to sub genus *Limus* by deciduous character. Another important finding is the stomata type of *Mangifera* sp. is cyclocytic whereas the remaining species are anomocytic type (Astuti 2014). The discovery of *Mangifera* sp. in Central Sumatra was assumed as new species by Fitmawati et al. (2013). In this study it was found *Mangifera* sp. has high similarity in morphological

characters with *M. magnifica* Kochummen (Kostermans and Bompard 1993), with slight different found in pear-shape fruit and young bud which deciduous in *Mangifera* sp. but lacking in *M. magnifica*.

Generally, leaf texture is a suitable character to divide genus *Mangifera*. Leaf texture was described by Kostermans and Bompard (1993) where it can be divided in two large groups namely coriaceous type and chartaceous type. Coriaceous type is more primitive in ecological studies (Bews 1927). In case it was synchronize the sequence of ITS, it is assumed that the character of leaf texture was a synapomorph character in *Mangifera* classification. Some species of *Mangifera such as M. quadrifida and M. torquenda* showed transition leaf texture relatively towards coriaceous or chartaceous. It is assumed as biparental inherited from nuclear genome therefore *Mangifera* species which has transition leaf texture is a natural hybrid from different parental such as *M. odorata* hybrid from *M. foetida* and *M. indica*. Hence, ITS marker potentially track origin and evolutionary of polyploidy in plant (Kim and Mabry 1991).

Results of alligned sequence of entire ITS revealed ITS region was flanking conserve 5.8S region (coding region) encoded ribosomal RNA which is important in protein synthesis (add reference). Mutation rate of conserve gene is slower than non coding region. ITS region as non coding region has more variation and higher mutation rate than coding region. Non coding region (intron) has role in gene expression regulation which adaptable with niche/habitat. Most of this non coding region could be observe through phenotypic characters.

Based on this study we found many differentiations between classification based on morphological characteristics by Kostermans and Bompard (1993) and molecular study .This results could be use as strong basis to develop a new system of classification. Classification based on DNA sequence is assumed to produce nature and accurate classification because DNA is a basic unit of information that encode organism.

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