RESEARCH ARTICLE

SABRAO Journal of Breeding and Genetics
55 (1) 175-186, 2023
http://doi.org/10.54910/sabrao2023.55.1.17
http://sabraojournal.org/
pISSN 1029-7073; eISSN 2224-8978

MANGIFERA KEMANGA BLUME (ANACARDIACEAE) TAXONOMIC ASSESSMENT FOR GENETIC DIVERSITY BASED ON MOLECULAR SUBSTANTIATION

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SUMMARY

Mangifera kemanga Blume is a wild relative of mango (*Mangifera indica*) and a local fruit with various potential uses. The community uses its fruit and leaves for food and its wood as a building material. However, the genetic diversity and taxonomic status of *M. kemanga* species still need more exploration and have a dispute with *M. caesia* Jack. The presented study aimed to determine the taxonomic status of *M. kemanga* and its relationship with *M. caesia* through a molecular approach and to recognize the species' delimitation. The recorded data analysis used the *trnL-trnF* intergenic spacer sequence. Carrying out DNA isolation employed the Geneaid Genomic DNA Mini Kit (Plant) protocol, with the DNA sequences analyzed for kinship using Maximum Parsimony and Neighbor-Joining methods and genetic diversity analysis performed using DnaSP 6. A total of 27 *trnL-trnF* intergenic spacer sequence identification and authentication resulted from BLAST on NCBI as sequences derived from the genus *Mangifera*. The phylogenetic tree revealed that the accessions of *M. kemanga* and *M. caesia* are very similar, hence, considered not as independent species. Thus, based on the *trnL-trnF* intergenic spacer sequences, *M. kemanga* is a synonym of *M. caesia* with taxonomic status as a variety of *M. caesia.* This molecular-based taxonomic evidence is significant for determining the valid species status of *M. kemanga* so that it can provide basic information for further studies on biodiversity and germplasm conservation.

Keywords: *Mangifera caesia*, genetic diversity, molecular analysis, morphological traits, phylogenetic, *trnL-trnF* intergenic spacer marker

Key findings: Based on the accessions of *M. kemanga* and *M. caesia* collected from four islands in Indonesia, collecting molecular data used the *trnL-trnF* intergenic spacer marker, which provides several sequences that have yet to be published. The molecular analysis gave new taxonomic substantiation to determine the status of *M. kemanga*. Furthermore, the present data can be a basis for conservation strategies and optimizing its potential use as a genetic resource.

Communicating Editor: Prof. Naqib Ullah Khan

Manuscript received: December 11, 2022; Accepted: February 5, 2023.

INTRODUCTION

Mangifera kemanga Blume is a tropical fruit species and a wild relative of mango belonging to the family Anacardiaceae, which distributes naturally in the Malay Peninsula, Sumatra, Kalimantan (rarely), and West Java (Kostermans and Bompard, 1993; Juliantari *et*

Citation: Resida E, Chikmawati T, Ariyanti N, Fitmawati (2023). *Mangifera kemanga* Blume (Anacardiaceae) taxonomic assessment for genetic diversity based on molecular substantiation. *SABRAO J. Breed. Genet.* 55(1): 175-186. http://doi.org/10.54910/sabrao2023.55.1.17.

al., 2021). This species' common cultivation is in West Java, especially in Bogor (Tapsi *et al.,* 2012). Locally, four cultivars, viz., Sabu, Lokal, Binglu, and Sugar originated from the species *M. kemanga* in Bogor (Mulyaningsih *et al.,* 2022). In Sumatraisland, *M. kemanga* exists along the river basins of Central Sumatra (Riau and Jambi Provinces) and Southern Sumatra (Lampung, Bengkulu, and South Sumatra Provinces) (Resida *et al.,* 2017). Based on the past botanical exploration of wild mangoes in Sumatra, a report stated *M. kemanga* to be one of the rare wild mango species (Fitmawati and Hayati, 2018). The use of *M. kemanga* is quite diverse in Indonesia, with its young leaves usually consumed as fresh vegetables called *'lalapan.'*

In *M. kemanga*, fresh fruits are either consumed directly or processed into juice by adding coffee powder, sugar, and ice (Kostermans and Bompard, 1993). The Sundanese people in West Java processed grated seeds into sauce *'sambal'* by adding chili and salt (Kostermans and Bompard, 1993). In addition, phytochemical screening and antioxidant profile of wild mangoes in Sumatra revealed that *M. kemanga* has the potential as a source of antioxidants with higher quercetin content as compared with other wild species of mangoes (Fitmawati *et al.,* 2018, 2020; Ho and Tu, 2019). Quercetin is a plant flavonol from the flavonoid group of polyphenols, found in fruits, vegetables, leaves, seeds, and grains; red onions and kale are common foods containing appreciable amounts of it. It has a bitter flavor and serves as an ingredient in dietary supplements, beverages, and food. The quercetin found in *M. kemanga* has various pharmacological benefits, including antidiabetic (Gondi and Rao, 2015), anti-influenza (Wu *et al.,* 2016), anticancer (Zhao *et al.,* 2016; Hashemzaei *et al.,* 2017), antioxidant and anti-inflammatory (Lesjak *et al.,* 2018), and antibacterial (Wang *et al*., 2018).

Taxonomically, the problem of the *M. kemanga* species' status and the scientific names still needs resolution since *M. Kemanga's* first publication was a species of the family Anacardiaceae (Blume, 1850). However, disputes on its position as a species have occurred, as some researchers also defined *M. kemanga* as a variety of *M. caesia* Jack (Kostermans, 1965; Hou, 1978). According to Mukherjee (1949), both species have some differences in the leaves, panicles, flowers, and fruits. Hou (1978) also considered Blume's description of *M. kemanga* on the fruit color confusing. Kostermans and Bompard (1993) finally delimited *M. kemanga* as a distinct species from *M. caesia* based on fruit character descriptions. *Mangifera kemanga* fruit is very distinctive with a pear-like shape. At a very early stage of the fruit, some are glossy white with dirty red spots, but when they ripen, the rind becomes brown, dull, and rough. These characteristics differ from *M. caesia*, e.g., the 'Wani' fruit found in Bali, Indonesia, a cultivar of *M. caesia*. The rind of 'wani' showed to be smooth, glossy, yellowish-white, or yellowish-green when ripe (Kostermans and Bompard, 1993).

Classification based on morphological characteristics still causes the taxonomic status of *M. kemanga* to undergo debate. Furthermore, the discovery of a natural hybrid between *M. kemanga* and *M. caesia* also confirmed the taxonomic status of *M. kemanga* as a problematic species (Bompard, 1992). Therefore, the taxonomic status of *M. kemanga* needs reviewing using a molecular approach. The development of molecular data to resolve plant taxonomy problems is reliable by the available higher number of characteristics than morphological characteristics, especially for the lower taxon categories. Molecular characterization can use the chloroplast marker (cpDNA) *trnL-trnF* intergenic spacer. This non-coding regional sequence is easy to amplify and has a short sequence size, producing high variation and highly-frequent mutations (Small *et al.,* 2004). Therefore, using this marker can best review the taxonomic status of *M. kemanga* and its relationship with *M. caesia*, as its close relative. The latest study aimed to assess the taxonomic status of *M. kemanga* and its relationship with *M. caesia* using morphological and molecular approaches.

MATERIALS AND METHODS

Plant material

This study collected 27 samples of *M. kemanga* and *M. caesia* from the various distribution areas in Indonesia, including four islands, i.e., Java (Bogor), Kalimantan (East and South), Bali (Tabanan), and Sumatra (Riau, Jambi, South Sumatra, Aceh, and Bangka Belitung Islands) (Table 1). Seventeen accessions belonged to the *M. kemanga,* while 10 accessions originated from *M. caesia.* The analysis of coordinate data of *M. kemanga* and *M. caesia* used the Quantum Geographic Information System (QGIS 3.20.2) software (Figure 1).

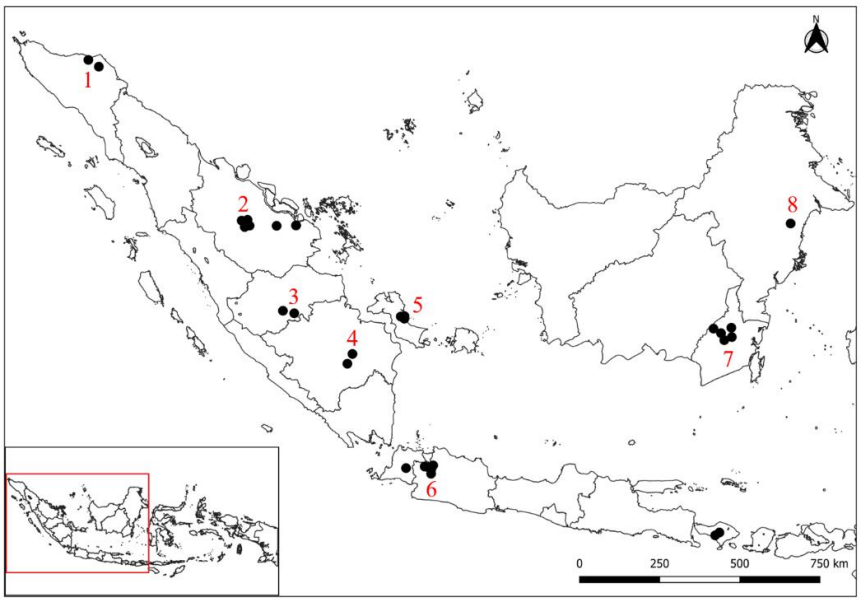

Figure 1. *M. kemanga* and *M. caesia* sampling locations in Indonesia: (1) Aceh, (2) Riau, (3) Jambi, (4) South Sumatra, (5) Bangka Belitung Islands, (6) Bogor, (7) South Kalimantan, and (8) East Kalimantan.

DNA extraction, amplification, and sequencing

DNA extraction employed the Geneaid Genomic DNA Mini Kit (Plant) protocol on *M. kemanga* and *M. caesia* accessions (Table 1). DNA extraction consisted of five steps: tissue dissociation, cell lysis, DNA binding, washing, and DNA elution. DNA amplification used the cpDNA *trnL-trnF* intergenic spacer sequence, as well as, a universal primer of *trnL-trnF* intergenic spacer (Taberlet *et al.,* 1991). The total volume of PCR reactions followed the Thermo Scientific™ protocol. Conducting the PCR reaction according to Fitmawati and Hartana (2010). PCR products examination used the electrophoresis method and documented with a Gel Doc (AlphaDigiDoc™ RT). The PCR product sequencing ensued in the Apical Scientific Laboratory, Malaysia.

Genetic diversity and phylogenetic analysis

The analysis of genetic diversity used DnaSP 6 software (Rozas *et al.,* 2017). Aligning and constructing a phylogenetic tree analyzed the DNA sequences for kinship based on the Maximum Parsimony (MP) and Neighbor-Joining (NJ) methods using the MEGA 11 software: Molecular Evolutionary Genetics Analysis version 11.0.8 (Tamura *et al.,* 2021). Carrying out online data mining BLAST, identified similar sequences based on the National Center for Biotechnology Information (NCBI) database. A total of five accessions of DNA sequences from NCBI served for outgroups, which were *M. indica* L. (two accessions), *Bouea macrophylla* Griffith (two accessions), and *Bouea oppositifolia* (Roxb.) Adelb. The cladogram strength test used bootstrap analysis of 1000 replicates (Felsenstein, 1985).

RESULTS

Sequence profiles of *trnL-trnF* intergenic spacer *M. kemanga* and *M. Caesia*

The DNA amplicons successfully sequenced ranged from 415–459 bp. Similar findings have also resulted in previous studies of *Mangifera* species (Fitmawati *et al.,* 2017b; 2021, Resida *et al.,* 2017). The sequence length used in the analysis of 36 taxa (including outgroups) was 411 bp. A total of 381 (92.70%) characters revealed conservative (constant) sites, 29 (7.05%) were variable sites, 17 (4.14%) were singleton variable sites, and 12 (2.92%) were parsimony-informative sites (Table 2).

Table 1. Accessions of *M. kemanga*, *M. caesia,* and outgroups collected from the field and sequence data from Genbank.

Origin of accession	Collection/ accession number	Species	Geographical Location		
			Latitude	Longitude	Altitude (masl)
Riau (IHR)	ER05 P5	*M. kemanga*	0°22'48.2"S	103°05'01.4"E	7
Riau (IHR)	ER08 P8	*M. kemanga*	0°22'51.0"S	103°05'07.7"E	5
Riau (IHR)	ER09 P9	*M. kemanga*	0°22'56.8"S	103°05'16.8"E	8
Riau (IHR)	ER14 P14	*M. kemanga*	0°23'00.7"S	103°05'17.6"E	7
Riau (IHR)	ER15 P15	*M. kemanga*	0°22'59.5"S	103°05'16.5"E	7
Riau (IHU)	ER18 RGT	*M. kemanga*	0°22'11.4"S	102°31'55.7"E	12
Riau (KSG)	ER38 KG2	*M. kemanga*	0°26'28.0"S	101°40'27.3"E	44
Riau (KSG)	ER39 KG3	*M. kemanga*	0°28'20.3"S	101°37'37.0"E	48
Riau (KSG)	ER40 KG4	*M. kemanga*	0°29'28.4"S	101°39'35.9"E	50
Jambi (SLR)	ER29 JM1	*M. kemanga*	2°07'53.8"S	102°49'30.2"E	33
Jambi (SLR)	ER30 JM2	*M. kemanga*	2°11'58.1"S	102°47'36.7"E	38
Aceh (ACU)	ER23 ACDS	*M. kemanga*	5°05'06.6"N	97°14'59.1"E	9
West Java (BGR)	ER32 BG1	*M. kemanga*	6°34'51.5"S	106°43'19.8"E	191
West Java (BGR)	ER33 BG2	*M. kemanga*	6°33'15.0"S	106°43'47.7"E	165
West Java (BGR)	ER34 BG3	*M. kemanga*	6°33'48.0"S	106°43'21.7"E	171
West Java (BGR)	ER35 BG4	*M. kemanga*	6°34'59.7"S	106°43'21.8"E	192
West Java (BGR)	ER36 BG5	*M. kemanga*	6°34'34.1"S	106°43'40.0"E	185
South Sumatra (OLR)	ER24 PM1	*M. caesia*	3°18'41.5"S	104°43'48.1"E	15
South Sumatra (OLR)	ER25 PM2	*M. caesia*	3°19'07.5"S	104°44'15.7"E	12
Bangka Belitung (PPG)	ER19 PP1	*M. caesia*	-	-	-
Bangka Belitung (PPG)	ER20 PP2	*M. caesia*	-	-	-
East Kalimantan (SMR)	ER01 SMR	*M. caesia*	0°26'06.0"S	117°10'42.1"E	9
South Kalimantan (HST)	ER26 KSL1	*M. caesia*	2°41'55.7"S	115°18'39.6"E	13
South Kalimantan (HSS)	ER43 KSL4	*M. caesia*	2°44'04.2"S	115°18'06.8"E	15
South Kalimantan (HSS)	ER44 KSL5	*M. caesia*	2°44'44.9"S	115°21'17.5"E	109.4
Bali (TBN)	ER27 BL1	*M. caesia*	8°27'56.3"S	115°09'09.3"E	281
Bali (TBN)	ER28 BL2	*M. caesia*	8°27'57.0"S	115°09'09.2"E	280
GenBank (RIA) *	MF919592	*M. kemanga*	00°28'10.1"S	101°37'57.4"E	-
GenBank (STS) *	MF919593	*M. kemanga*	02°52'44.7"S	103°52'07.2"E	-
GenBank (LPG) *	MF919594	*M. kemanga*	05°07'30.0"S	103°59'28.0"E	-
GenBank (SMT)	KY392620	*M. kemanga*	-	-	-
GenBank	MF997586	*M. indica*	-	-	-
GenBank	KY392616	*M. indica*	-	-	-
GenBank	AY594500	*B. macrophylla*	-	-	-
GenBank	KY392617	*B. macrophylla*	-	-	-
GenBank	KP055490	*B. oppositifolia*	-	-	-

IHR=Indragiri Hilir, IHU=Indragiri Hulu, KSG =Kuantan Singingi, SRL=Sarolangun, ACU=Aceh Utara, BGR=Bogor, OLR=Ogan Ilir, PPG=Pangkal Pinang, SMR=Samarinda, HST=Hulu Sungai Tengah, HSS=Hulu Sungai Selatan, TBN=Tabanan, RIA=Riau, STS=South Sumatra, LPG=Lampung dan, SMT= Central Sumatra. *=Resida *et al.,* (2017).

Table 2. Characteristics and statistics of the *trnL-trnF* intergenic spacer *M. kemanga* and *M. caesia* in Indonesia.

Variables	*M. kemanga* + *M. caesia*	*M. kemanga* + *M. caesia* + outgroup
Number of ingroup taxa	31	31
Number of outgroup taxa	-	5
Sequence length (bp)	411	411
Number of constant sites (%)	96,10	92,70
Variable sites (%)	1,94	7,05
Number of parsimony-informative sites (%)	1,70	2,92
Singleton sites (%)	1,94	4,14
Tree length (steps)	21	39
Consistency Index (CI)	0,762	0,821
Retention Index (RI)	0,762	0,816

Based on sequence alignment using BLAST, sequences of *M. kemanga* and *M. caesia* identified as sequences belonging to the genus *Mangifera* and proved by the highest percentage of query cover and percent identity. The range of query cover and percent identity values were 84%–100% and 97.93%–99.26%, respectively. *Mangifera kemanga* sequences also had a high query cover value with *M. foetida*. Morphologically, grouping the two species was into the same subgenus (*Limus*). The *trnL-trnF* intergenic spacer sequences of *M. caesia* were unavailable in the GenBank database. Hence, the *trnL-trnF* intergenic spacer sequences of *M. caesia* resulting in this study are new.

The results of the alignment of *M. kemanga* and *M. caesia* sequences showed that their sequences differed by 1.70% of the total length of the sequence examined (411 bp). Sequence alignment also revealed information on the average nucleotide composition with frequencies of Thymine/T (32.78%), Cytosine/C (22.07%), Adenine/A (28.98%), Guanine/G (16.17%), AT (61.76%), and GC (38.24%). The data confirmed that the most nucleotide compositions were adenine and thymine in the spacer region. Similar findings have also come from other studies using the *trnL-trnF* intergenic spacer sequence in the family Anacardiaceae (Fitmawati and Hartana, 2010; Harsono *et al.,* 2017; Fitmawati *et al.,* 2017a, b). In non-coding region chloroplast DNA, the most abundant nucleotide composition was adenine and thymine (Li, 1997).

Nucleotide diversity analysis

In *M. kemanga* procured from West Java and *M. caesia* obtained from Bali, the analysis results of the haplotype diversity were in the high category (0.9–1). However, in the populations of *M. kemanga* from Sumatra and *M. caesia* from Kalimantan, the haplotype diversity was moderate (0.5–0.73). Furthermore, in *M. caesia* obtained from Sumatra, the haplotype diversity was lowest (0.00) (Table 3). Thus, the genetic variation (level of polymorphism) in the population of *M. caesia* from Sumatra is minimal. According to Nei (1987), the value of haplotype diversity (Hd) ranges from 0.8–1 in the high category, 0.5–0.7 in the moderate, and 0.1–0.4 in the low category. Interestingly, these results showed that the population with the highest genetic diversity comes from areas with high altitude (165–281 masl), namely, *M. kemanga* from West Java and *M. caesia* from Bali, Indonesia compared with other populations generally found in swampy areas (5–50 masl).

Nucleotide diversity plays a vital role in measuring the degree of polymorphism in a population (Nei and Li, 1979). The value of nucleotide diversity (Pi) categorizes as high, with a value of Pi > 0.1 (Jukes and Cantor, 1969). The nucleotide diversity of the entire populations of *M. kemanga* and *M. caesia* was low, with a value of Pi < 0.1 ranging from 0.001–0.009 (Table 3). The low nucleotide diversity was due to the *trnL-trnF* intergenic spacer marker in the chloroplast genome. The population of *M. caesia* from Bali has the highest nucleotide diversity (0.009). The results explain that *M. caesia* obtained from Bali was relatively genetically diverse than *M. caesia* accessions procured from other regions with the same also evidenced by recording the genetic variability in 22 *M. caesia* cultivars in Bali, Indonesia (Rai *et al.,* 2008). Mutation events cause nucleotide diversity in the *trnL-trnF* intergenic spacer sequence.

Table 3. Genetic diversity between species and populations of *M. kemanga* and *M. caesia* using 2-parameter Kimura model analysis.

Species / Populations	Genetic diversity			
	N	Hn	Hd	Pi
M. kemanga	21	9	0.767	0.006
Sumatra	16	6	0.733	0.006
West Java	5	4	0.900	0.005
M. caesia	10	3	0.511	0.003
Sumatra	4	1	0.000	0.000
Kalimantan	4	2	0.500	0.001
Bali	2	2	1.000	0.009
Total	31	10		

N=number of sequences, Hn=number of haplotypes, Hd=haplotype diversity, and Pi=nucleotide diversity.

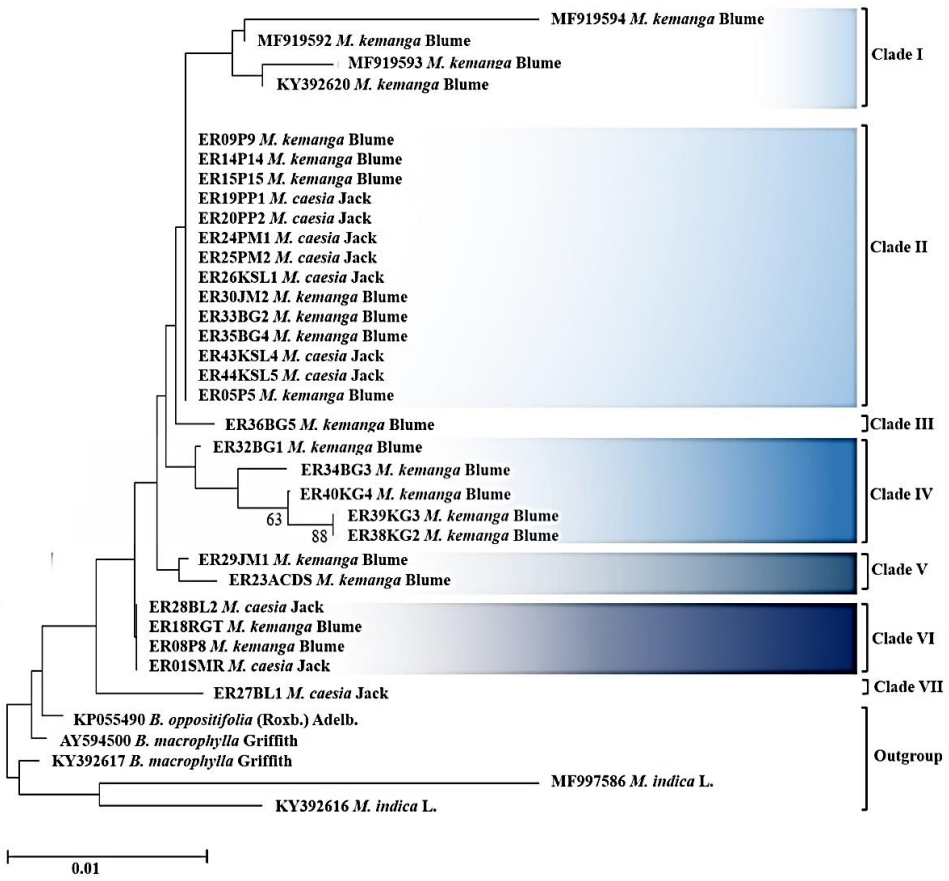

Figure 2. Phylogenetic tree using Neighbor-Joining analysis. Branching was analyzed by bootstrap 1000 replicates. Bootstrap values on nodes that are less than 50% are not shown. ER = accession code (Table 1).

Phylogenetic analysis based on the Neighbor-Joining (NJ) method

Phylogenetic analysis based on the NJ method showed that *M. kemanga* and *M. caesia* form a monophyletic group with seven clades (Figure 2). Clades I, III, IV, and V contain the accessions of *M. kemanga.* However, the clades II and VI were combined clades of *M. kemanga* and *M. caesia*. Clade VII only has accessions of *M. Caesia* occupying it. In clade-I, the accession MF919594 of *M. kemanga* from Lampung, Indonesia evolved the last. The accession experienced the most nucleotide changes in the ingroup because it had the longest evolutionary process compared with other accessions. Therefore, accession from Lampung can be the most advanced accession than other accessions of *M. kemanga* and *M. caesia* found in Indonesia.

Clade II contains the most combination accessions of *M. kemanga* and *M. caesia.* However, occupying clade III was an *M. kemanga* accession from Bogor, Indonesia (ER36BG5) and separated from other Bogor accessions located in clades II and IV. Clade IV grouped three accessions obtained from Riau, Indonesia, and two from Bogor, Indonesia. The accession (ER32BG1) of *M. kemanga* obtained from Bogor evolved earlier than other accessions in clade IV. Furthermore, clade V contained accessions of *M. kemanga* from Jambi (ER29JM1) and Aceh (ER23ACDS), Indonesia. In addition to clade II, the two species also got clustered in clade VI, which consisted of two accessions of *M. kemanga* from Riau (ER18RGT and ER08P8) and two accessions of *M. caesia* obtained from East Kalimantan (ER01SMR) and Bali (ER28BL2). Meanwhile, the accession (ER27BL1) of *M. caesia* procured from Bali appeared to be the earliest in the NJ tree.

Table 4. Genetic distance matrix (pairwise distance) of *trnL-trnF* intergenic spacer in *M. kemanga* and *M. caesia* using NJ analysis with the 2-parameter Kimura evolution model.

	Collection/ Accession Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
1	ER05P5 *M. caesia* Jack																																			
2	ER08P8 *M. caesia* Jack	0,002																																		
3	ER09P9 *M. caesia* Jack	**0,000**	0,002																																	
4	ER14P14 *M. caesia* Jack	0,000	0,002	0,000																																
5	ER15P15 *M. caesia* Jack	0,000	0,002	0,000	0,000																															
6	ER26KSL1 *M. caesia* Jack	0,000	0,002	0,000	0,000	0,000																														
7	ER43KSL4 *M. caesia* Jack	0,000	0,002	0,000	0,000	0,000	0,000																													
8	ER44KSL5 *M. caesia* Jack	0,000	0,002	0,000	0,000	0,000	0,000	0,000																												
9	ER01SMR *M. caesia* Jack	0,002	0,000	0,002	0,002	0,002	0,002	0,002	0,002																											
10	ER27BL1 *M. caesia* Jack	0,010	0,007	0,010	0,010	0,010	0,010	0,010	0,010	0,007																										
11	ER28BL2 *M. caesia* Jack	0,002	0,000	0,002	0,002	0,002	0,002	0,002	0,002	0,000	0,007																									
12	ER19PP1 *M. caesia* Jack	0,000	0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,002	0,010	0,002																								
13	ER20PP2 *M. caesia* Jack	0,000	0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,002	0,010	0,002	0,000																							
14	ER24PM1 *M. kemanga* Blume	0,000	0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,002	0,010	0,002	0,000	0,000																						
15	ER25PM2 *M. kemanga* Blume	0,000	0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,002	0,010	0,002	0,000	0,000	0,000																					
16	ER29JM1 *M. kemanga* Blume	0,002	0,005	0,002	0,002	0,002	0,002	0,002	0,002	0,005	0,012	0,005	0,002	0,002	0,002	0,002																				
17	ER30JM2 *M. kemanga* Blume	0,000	0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,002	0,010	0,002	0,000	0,000	0,000	0,000	0,002																			
18	ER23ACDS *M. kemanga* Blume	0,005	0,002	0,005	0,005	0,005	0,005	0,005	0,005	0,002	0,010	0,002	0,005	0,005	0,005	0,005	0,002	0,005																		
19	ER38KG2 *M. kemanga* Blume	0,010	0,012	0,010	0,010	0,010	0,010	0,010	0,010	0,012	0,017	0,012	0,010	0,010	0,010	0,010	0,007	0,010	0,010																	
20	ER39KG3 *M. kemanga* Blume	0,010	0,012	0,010	0,010	0,010	0,010	0,010	0,010	0,012	0,017	0,012	0,010	0,010	0,010	0,010	0,007	0,010	0,010	0,000																
21	ER40KG4 *M. kemanga* Blume	0,007	0,010	0,007	0,007	0,007	0,007	0,007	0,007	0,010	0,015	0,010	0,007	0,007	0,007	0,007	0,005	0,007	0,007	0,002	0,002															
22	ER32BG1 *M. kemanga* Blume	0,002	0,005	0,002	0,002	0,002	0,002	0,002	0,002	0,005	0,010	0,005	0,002	0,002	0,002	0,002	0,005	0,002	0,007	0,007	0,007	0,005														
23	ER33BG2 *M. kemanga* Blume	0,000	0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,002	0,010	0,002	0,000	0,000	0,000	0,000	0,002	0,000	0,005	0,010	0,010	0,007	0,002													
24	ER34BG3 *M. kemanga* Blume	0,007	0,010	0,007	0,007	0,007	0,007	0,007	0,007	0,010	0,010	0,010	0,007	0,007	0,007	0,007	0,005	0,007	0,007	0,007	0,007	0,005	0,005	0,007												
25	ER35BG4 *M. kemanga* Blume	0,000	0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,002	0,010	0,002	0,000	0,000	0,000	0,000	0,002	0,000	0,005	0,010	0,010	0,007	0,002	0,000	0,007											
26	ER36BG5 *M. kemanga* Blume	0,002	0,005	0,002	0,002	0,002	0,002	0,002	0,002	0,005	0,007	0,005	0,002	0,002	0,002	0,002	0,005	0,002	0,007	0,012	0,012	0,010	0,005	0,002	0,005	0,002										
27	ER18RGT *M. kemanga* Blume	0,002	0,000	0,002	0,002	0,002	0,002	0,002	0,002	0,000	0,007	0,000	0,002	0,002	0,002	0,002	0,005	0,002	0,002	0,012	0,012	0,010	0,005	0,002	0,010	0,002	0,005									
28	MF919594 *M. kemanga* Blume	0,018	0,020	0,018	0,018	0,018	0,018	0,018	0,018	0,020	**0,028**	0,020	0,018	0,018	0,018	0,018	0,020	0,018	0,023	0,020	0,020	0,020	0,020	0,018	0,025	0,018	0,020	0,020								
29	MF919593 *M. kemanga* Blume	0,008	0,010	0,008	0,008	0,008	0,008	0,008	0,008	0,010	0,018	0,010	0,008	0,008	0,008	0,008	0,010	0,008	0,013	0,015	0,015	0,015	0,010	0,008	0,015	0,008	0,010	0,010	0,020							
30	KP055490 *B. oppositifolia* (Roxb.) Adelb.	0,009	0,006	0,009	0,009	0,009	0,009	0,009	0,009	0,006	0,006	0,006	0,009	0,009	0,009	0,009	0,009	0,009	0,006	0,012	0,012	0,012	0,009	0,009	0,009	0,009	0,009	0,006	0,028	0,015						
31	MF919592 *M. kemanga* Blume	0,000	0,003	0,000	0,000	0,000	0,000	0,000	0,000	0,003	0,012	0,003	0,000	0,000	0,000	0,000	0,003	0,000	0,006	0,009	0,009	0,009	0,003	0,000	0,009	0,000	0,003	0,003	0,012	0,000	0,008					
32	KY392620 *M. kemanga* Blume	0,000	0,003	0,000	0,000	0,000	0,000	0,000	0,000	0,003	0,012	0,003	0,000	0,000	0,000	0,000	0,003	0,000	0,006	0,009	0,009	0,009	0,003	0,000	0,009	0,000	0,003	0,003	0,012	0,000	0,008	0,000				
33	MF997586 *M. indica* L.	0,038	0,035	0,038	0,038	0,038	0,038	0,038	0,038	0,035	0,043	0,035	0,038	0,038	0,038	0,038	0,040	0,038	0,038	0,045	0,045	0,045	0,040	0,038	0,045	0,038	0,040	0,035	0,033	0,025	0,033	0,015	0,015			
34	KY392616 *M. indica* L.	0,022	0,019	0,022	0,022	0,022	0,022	0,022	0,022	0,019	0,019	0,019	0,022	0,022	0,022	0,022	0,024	0,022	0,022	0,030	0,030	0,027	0,022	0,022	0,024	0,022	0,022	0,019	0,039	0,031	0,012	0,023	0,023	0,030		
35	AY594500 *B. macrophylla* Griffith	0,008	0,005	0,008	0,008	0,008	0,008	0,008	0,008	0,005	0,013	0,005	0,008	0,008	0,008	0,008	0,010	0,008	0,008	0,018	0,018	0,016	0,010	0,008	0,016	0,008	0,010	0,005	0,027	0,016	0,003	0,009	0,009	0,031	0,014	
36	KY392617 *B. macrophylla* Griffith	0,010	0,008	0,010	0,010	0,010	0,010	0,010	0,010	0,008	0,015	0,008	0,010	0,010	0,010	0,010	0,013	0,010	0,010	0,018	0,018	0,018	0,013	0,010	0,018	0,010	0,013	0,008	0,028	0,018	0,003	0,009	0,009	0,023	0,017	0,003

Table 5. The genetic divergence within the group means distance at the species level using a 2-parameter Kimura model.

Species	D	SE
M. kemanga	0.006	0.002
M.caesia	0.003	0.001
M. caesia and *M. kemanga*	0.005	0.002

D=genetic distance, SE=standard deviation.

Table 6. The genetic divergence between groups means distance at the species level using a 2-parameter Kimura model.

	Population	SE				
		M. kemanga			*M. caesia*	
		Sk	K	B	Sc	J
	M. kemanga (Sumatra/Sk)	**	0.002	0.003	0.001	0.002
	M. caesia (Kalimantan/K)	0.004	**	0.003	0.001	0.002
d	*M. caesia* (Bali/B)	0.009	0.006	**	0.003	0.004
	M. caesia (Sumatra/Sc)	0.004	0.001	0.006	**	0.001
	M. kemanga (West Java/J)	0.006	0.003	0.007	0.002	**

d=genetic distance (below diagonal), SE=standard deviation (above diagonal), Sk=*M. kemanga* population of Sumatra, Sc=*M. caesia* population of Sumatra, J=*M. kemanga* population of West Java, K=*M. caesia* population of Kalimantan, B=*M. caesia* population of Bali.

The genetic distance matrix also revealed the close relationship among the accessions (Table 4). The genetic distance within the *M. kemanga* population was greater than that in the *M. caesia* population, i.e., 0.006 and 0.003, respectively (Table 5). However, the genetic distance between the two species was smaller than the distance within the *M. kemanga* population (0.005). Thus, the distance values revealed that *M. kemanga* and *M. caesia* appeared very closely related because the genetic distance between the species was higher than the distance between the two species. These findings further strengthen the recommendation of the taxonomic status of the two species as synonymous.

Genetic distance between the populations (between group distance) presents that *M. caesia* from Sumatra is very closely related to *M. caesia* from Kalimantan (Table 6). However, the *M. caesia* population from Bali and *M. kemanga* from Sumatra have the most distant relationship (0.009 ± 0.003). Although the collection of both populations was from two different regions. The *M. caesia* population obtained from Bali grows at an altitude ranging 280–281 masl, while the *M. kemanga* population from Sumatra grows at an altitude of 5–50 masl. Moreover, the *M. caesia* has 22 cultivars in Bali, allowing this species to have a higher genetic range (Rai *et al.,* 2008).

Based on the genetic distance between accessions (pairwise distance), *M. kemanga* from Lampung (MF919594) and *M. caesia* from Bali (ER27BL1) were the most distantly related *Mangifera* species, with the highest genetic distance matrix value of 2.8% (0.028) (Table 4). However, several other ingroup accessions had genetic distances ranging from 0.0% to 1.7%. *M. caesia* accession (ER26KSL1) from Kalimantan and *M. kemanga* accessions (ER30JM2, ER33BG2, and ER35BG4) from Sumatra and West Java have the least genetic distance or no genetic distance (0%), thus, considered identical species as were very closely related.

Phylogenetic analysis based on Maximum Parsimony (MP) method

Based on MP analysis, classifying 31 accessions of *M. kemanga* and *M. caesia* into two main clades was successful and separated from five outgroup accessions, the genus *Bouea* and *Mangifera*. The resulting phylogenetic tree had a consistency index (CI) of 0.821, a retention index (RI) of 0.816, and a homoplasy index (HI) of 0.179 (Table 2, Figure 3). The consistency index (CI) and retention index (RI) were close to unity, indicating that the phylogenetic tree was relatively stable (Klingenberg and Gidaszewski, 2010; Fitmawati *et al.,* 2017a).

Clade I contains 26 accessions of *M. kemanga* and *M. Caesia* with a high bootstrap value of 90%. This clade also divided all accessions into two subclades. Subclade IA comprised 19 accessions of *M. kemanga* (procured from Bogor, Jambi, South Sumatra, and Riau, Indonesia) and seven accessions of *M. caesia* (originating from South Sumatra, South Kalimantan, and Bangka Belitung Islands, Indonesia). However, *M. kemanga* accession (ER08P8) from Riau separated from other accessions due to a change in the nucleotide 243 (T-G) site in subclade IB. In the second clade, there were five accessions, three from *M. Caesia,* i.e., ER01SMR obtained from Samarinda (East Kalimantan), ER27BL1 and ER28BL2 from Tabanan (Bali), and two from *M. kemanga* accession ER18RGT from Indragiri Hulu (Riau), and ER23ACDS procured from North Aceh, Indonesia. In all the clades, the accessions of the two species can not form a separate group but instead clustered together.

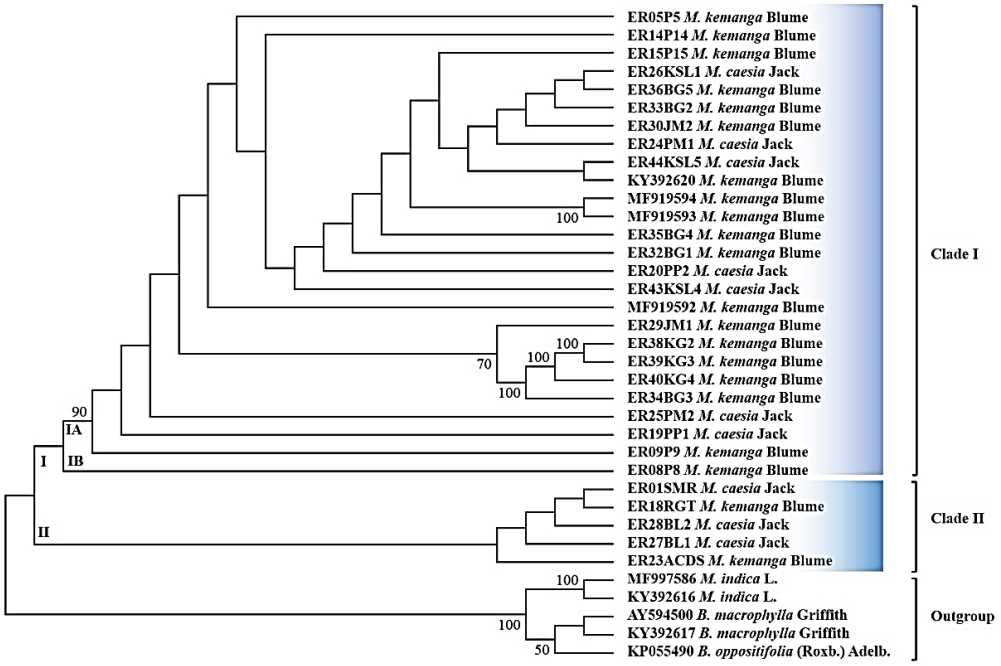

Figure 3. Phylogenetic tree using Maximum Parsimony analysis. Branching was analyzed by bootstrap 1000 replicates. Bootstrap values on nodes that are less than 50% are not shown. ER = accession code (Table 1).

The clustering presented using MP analysis also showed similar results compared with the NJ grouping. Thus, the different accessions of *M. kemanga* and *M. caesia* cannot be strictly delimited as independent species.

DISCUSSION

In the genus *Mangifera,* some species have high plasticity and continuity of morphological characteristics, and it is difficult to identify (Fitmawati and Hartana, 2010). As a result, species complexity and misidentification may occur in this genus (Fitmawati *et al.,* 2021, 2022). The species complexity may occur because the identification of morphological characters has flaws. Environmental factors often influence the morphological appearance and may show inconsistently due to human subjectivity (Bani *et al*., 2017; Elly *et al*., 2018). In addition, morphological characteristics come from both parents, so these characteristics have limitations in tracing intra- and interspecific relationships and natural evolutionary traces through maternal lineage. Therefore, the appropriate molecular evidence will better assist in addressing these limitations.

Employing the *trnL-trnF* intergenic spacer sequence of chloroplasts in the phylogenetic analysis of *M. kemanga* helps to find new evidence. Widely using of chloroplast DNA continues in studying plant phylogenetics. This genome is haploid with a simple and stable genetic structure, generally, uniparental transmission, none or very rare recombination, tracing only the maternal lineage, and using universal primers (Dong *et al*., 2012; Jiang *et al*., 2020; Kim *et al*., 2020). As a non-coding region of cpDNA, the *trnL-trnF* intergenic spacer marker also has a high mutation rate so that this region can change more rapidly than other coding regions in the chloroplast genome (Hocaoglu-Ozyigit *et al*., 2020).

In the *trnL-trnF* intergenic spacer sequence of *M. kemanga* and *M. caesia, the s*equence alignment showed a high percentage of homology (1.70%) (Table 2) and low nucleotide diversity values (Pi < 0.1) (Table 3). These results indicate the close relationship between the two species and confirm that the cpDNA sequence was more conservative than nuclear DNA. Therefore, in the *trnL-trnF* intergenic spacer cpDNA, the base changes occur in a very small number compared with the nuclear genome. However, this data is still important in providing some information to

explain the evolutionary process. The sequence variation in the *trnL-trnF* intergenic spacer sequence was due to a single nucleotide mutation that occurred over a very long time (Fitmawati and Hartana, 2010). The mutation rate of cpDNA loci was very low, ranging from 3.2×10^{-5} to 7.9×10^{-5} (Provan *et al.,*1999).

Furthermore, the differences in the genetic distance describe each accession's evolutionary rate. The rate of evolution can be faster or slower, depending on the adaptation mechanism and the state of the habitat environment (Fitmawati *et al.* 2017b, 2018). Based on genetic distance, accessions of *M. caesia* from Bali and *M. kemanga* from Sumatra were the most distantly related accessions. The populations from Bali also presented a high value of genetic diversity (Hd and Pi values). *Mangifera caesia* is a native species from Sumatra, Indonesia, with a natural habitat in the form of lowlands. This species is usually found on the banks of flooded rivers and swamps periodically, whose altitude is below 400 masl (and rarely up to 800 masl) (Fitmawati and Hayati, 2018). Introducing this species in the Bali region encouraged these plants to adapt to an environment with higher altitudes than their habitat in Sumatra. Thus, accessions from Bali record higher genetic diversity. However, the highest genetic distance was only 2.8% in the *trnL-trnF* intergenic spacer sequence (Table 4). The genetic distance further indicates an increase in the development of adaptation strategies of a species through genetic variations formed to survive in nature. Meanwhile, that genetic variation has implications for creating a diversity of characters that play a vital role in supporting the adaptation process and the sustainability of the existence of a species.

The results of Neighbor-Joining and Maximum Parsimony analyses were congruent with each other. The basis for grouping accessions in both phylogenetic trees was not on geographic distribution and morphological delimitations, as earlier reported (Kostermans, 1965; Kostermans and Bompard, 1993). The *trnL-trnF* intergenic spacer sequence has yet to be able to separate the accessions into two independent species. All the accessions were divided into seven clades in the Neighbor-Joining analysis, while in two clades with Maximum Parsimony analysis. However, all the accessions of M*. kemanga* and *M. caesia* appeared scattered throughout those clades. Using the same marker for *Bouea*, another genus in the family of Anacardiaceae, the marker was able to explicitly separate *Bouea macrophylla* Griffith and *Bouea oppositifolia* (Roxb.) Adelb. (Harsono *et al.,* 2017).

In addition, the proportion of homology between *M. kemanga* and *M. caesia* also authenticated that both populations were closely related. Thus, based on the *trnL-trnF* intergenic spacer marker, presumably, *M. kemanga* and *M. caesia* were identical species. The use of *trnL-trnF* intergenic spacer sequence of chloroplast DNA analyzed the phylogenetic relationships of closely related species, but could not separate infraspecific (within species) groupings (Taberlet *et al.,* 1991). The inability of this sequence to separate the groupings of the two species examined indicates that the two taxa are under species level (infraspecific). *Mangifera kemanga* may also be a variety (intra-species) of *M. Caesia,* the accepted species.

The present findings also got support from Kosterman (1965), who described the status of *M. kemanga* as a variety of *M. caesia* (*Mangifera caesia* var. *kemanga* Blume) based on its morphological characteristics. The present results also recommend reinstating *M. kemanga* as a variety of *M. caesia* Jack. *sensu lato (s.l.).* This species circumscription becomes wider based on molecular data. According to Kosterman's (1965) records, *M. kemanga* in Indonesia only grows in West Java and South Sumatra regions. Furthermore, Kostermans and Bompard (1993) also recorded that the distribution of *M. kemanga* in Indonesia includes West Java (main distribution), Sumatra, and Kalimantan (very rare). In the latest study, *M. kemanga* collections came from West Java and Sumatra, but none from Kalimantan.

In conclusion, the present results provide new evidence for the species limitation of *M. caesia* (*M. caesia* Jack. s.l.), whose current population is decreasing in nature. The provisional IUCN Conservation Status Assessment for *M. caesia* Jack is "Near Threatened (NT)" under criterion B1b (iii). This species has experienced a decline in its natural population due to the losses that occur in its natural habitat caused by plantation development and forest logging (Ganesan, 2021). A decrease in population numbers can have an impact on the genetic diversity of this species. Genetic diversity is the key to sustaining populations in the face of various environmental changes that occur naturally, as well as, human activities. Accurate classification at the molecular level is vital for optimizing the use of species for various

breeding purposes and adopting effective conservation strategies to preserve the genetic diversity of this species.

CONCLUSIONS

The completed research assessed the taxonomic status and relationship of *M. kemanga* and *M. caesia* using molecular markers. Reconstruction of the phylogenetic tree using molecular characteristics showed that separating accessions of *M. kemanga* and *M. caesia* cannot complete on the cladogram. Therefore, based on the *trnL-trnF* intergenic spacer sequences, the study reclassified *M. kemanga* as a variety of *M. caesia* (*Mangifera caesia* var. *kemanga* Blume). The present results provide new evidence for the limitation of the species *M. caesia* (*M. caesia* Jack. *s.l.*).

ACKNOWLEDGMENTS

The authors would like to thank "The Indonesia Endowment Fund for Education (LPDP)" for supporting and funding this research. The authors sincerely acknowledged anonymous reviewers for their critical reading of the manuscript and constructive comments. All the authors also equally contributed to this research.

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