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VARIASI INFRASPESIES MACANG (*Mangifera foetida)* BERDASARKAN SEKUEN GEN *rbc*L

THE INFRASPECIES VARIATIONS OF Mangifera foetida BASED ON THE RBCL GENE SEQUENCES

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Abstrak

Macang (*Mangifera foetida*) adalah jenis mangga kosmopolit dengan vigoritas yang kuat. Macang banyak ditemukan di perkarangan dan kebun dan bersifat semi liar. Penurunan luas lahan seperti deforestasi dapat mengakibatkan keanekaragaman kultivar macang juga mengalami penurunan secara cepat. Penelitian ini bertujuan untuk menganalisis dan merekonstruksi hubungan kekerabatan antar kultivar macang menggunakan sekuen gen *rbc*L. Sampel berasal dari koleksi hasil eksplorasi mangga Sumatera bagian Selatan, yaitu Provinsi Bengkulu, Lampung dan Sumatera Selatan. Ekstraksi DNA dilakukan dengan menggunakan metode CTAB yang dimodifikasi, kemudian DNA diamplifikasi dengan menggunakan primer spesifik *rbc*L *forward* dan *reverse*, dan dilakukan sekuensing serta analisis filogenetik. Rekontruksi pohon filogenetik menggunakan program PAUP* versi 4.0b10 dengan metode *Maximum Parsimony* (MP) dan *Neighbor Joining* (NJ). Kladogram dengan MP diperoleh dua klad, yaitu pada klad I terdiri dari kultivar Macang lonjong dan Macang lado, sedangkan klad II terdiri dari kultivar Macang bulat. Berdasarkan metode NJ, diperoleh Macang bulat memiliki jarak genetik lebih panjang sehingga dianggap sebagai individu yang lebih primitif daripada kultivar yang lain. Dengan demikian, dari penelitian ini diperoleh informasi dan bukti dari status taksonomi kultivar macang.

Kata kunci: Analisis filogenetik; Kultivar; Macang (*Mangifera foetida*); *rbc*L

Abstract

Mangifera foetida is a species of cosmopolitan mango with strong vigor. Some M. foetida are found in some front houses and gardens and has a character of semi-wild in its cultivation. Decreases in land area, such as deforestation, can lead to a rapid decline in their diversity of cultivars. This study aimed to analyze and reconstructs the phylogenetic relationship among M. foetida cultivars using the rbcL gene sequences. Samples were collected from the exploration within the area of South Sumatra, such as Provinces of Bengkulu, Lampung and South Sumatra. The DNA extraction was carried out using the modified CTAB method, followed by DNA amplification using rbcL-specific primers, sequencing, and phylogenetic analysis. The phylogenetic trees were reconstructed using the PAUP version 4.0b10 by using the method of Maximum Parsimony (MP) and Neighbor Joining (NJ). Cladogram of the MP tree showed two clades that the clad I consisted of M. foetida (Macang lonjong) and M. foetida (Macang lado) cultivars, whereas clad II consisted of M. foetida (Macang bulat) cultivar. The NJ tree showed that M. foetida (Macang bulat) has* a *longer genetic distance so it is considered as a more primitive cultivar than others. Therefore, information and evidences from the taxonomic status of the M. foetida cultivars were obtained from this study.*

Keywords*: Cultivars; Macang (Mangifera foetida); Phylogenetic analysis; rbcL*

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INTRODUCTION

Macang (Mangifera foetida) distributes widespread in the mainland of Sumatra, also known as a cosmopolitan species. This mango grows mostly in the primary forest low lands of tropical regions with high rainfall at 0–1200 m asl (Fitmawati *et al*., 2017a). Fitmawati and Hayati (2018) reported that *M. foetida* are spread on dry and mixture land, bushes, primary and secondary dryland forests and open land. *M. foetida* fruits taste range from sour to sweet.

Based on morphological characters, it has been found *M. foetida* cultivars with varying forms of fruit in Bengkulu province, such as *M. foetida (Macang lado)* and small size *M. foetida (Macang lonjong)*, sweet flavor *M. foetida*, the soft meat fruit with orange colored pulp. *Mangifera foetida (Mangifera susu)*, the unique fruit shape like nipple in mammals and *M. foetida (Macang bulat)* that is a large size with yellow pulp (Fitmawati *et al.,* 2017b). This fruit is rarely cultivated and some grow wild in the woods whose existence threatened with extinction.

Expansion of the forest has decreased natural habitats rapidly, this is due to human activities such as deforestation, forest conversion into plantations, such as palm oil and rubber. One of the effort to preserve the diversity of *M. foetida* cultivars is to conduct exploration to collect data base and analyze the relationship morphological and molecular phylogenetics which later can be useful in compiling classifications, determining taxonomic status and as a support in conservation activities.

Diversity analysis by morphological markers is a basic in a grouping. Classification in cultivar level, the morphological approach needs to be supported by a comprehensive approach. one of them is a DNA based molecular approach that much plays a role for high genetic diversity among different species (Wattoo *et al*., 2016). Molecular data is used as a supporter of morphological characteristics in expressing kinship relationships in level of evolution among *M. foetida* cultivars. In this study, the DNA barcoding marker which was used was based on the cpDNA *rbc*L ribulose-1,5-bisphospate carboxylase (RuBisCO) marker. Generally mutations in DNA with morphological characters have different speeds, so to simplify the analysis of phylogenetic relationships, molecular markers are needed which later can support morphological markers in expressing kinship relationships. *rbcL* markers have a fairly strong gene sustainability and slow mutation rate (Li *et al*., 2011). Based on phylogenetic analysis research 9 species mango from Central Sumatra by Fitmawati *et al.* (2017b), stated that nucleotide base variation that obtained from *rbc*L sequences is very high and recommended that these markers can be used for phylogenetic analysis at genus level species and infraspecies.

Research on phylogenetic based on *Mangifera* molecular in Java Island with a marker of *rbc*L has been carried out (Suparman *et al.,* 2013) and Sumatra Island mangoes (Fitmawati *et al*., 2017b). However, the specifications for molecular analysis of the *rbc*L gene at the cultivar level have never been done. Therefore, this study was conducted to analyze the kinship relationship among *M. foetida* cultivars through phylogenetic analysis with *rbc*L markers and as an effort in the conservation of *M. foetida* on Sumatra Island. The purpose of this study is to analyze and reconstruct the kinship relationship among *M. foetida* cultivars from Sumatra Island using *rbc*L genes sequences in chloroplast genome.

MATERIAL AND METHOD

Sampel Collection and DNA Isolation

Three mango samples that were collected from the area of Bengkulu is *M. foetida,* such as *M. foetida (Macang bulat), M. foetida (Macang lonjong), and M. foetida (Macang lado)* Fitmawati *et al.,* 2018) as well as *Bouea macrophylla* Griff as an outgroup (Table 1). The initial procedure was pre-insulation by soaking leaf samples with aqua demineralisation for 48 hours in order to obtain a good quality DNA. After that the leaf samples are dried in the oven and ready to be isolated. DNA isolation used CTAB Doyle and Doyle (1987) method with modification. Furthermore, the isolated DNA was added with TE Buffer and stored at -20 °C. The DNA quality of the DNA isolation was checked using an electrophoresis machine with 1% agarose gel concentration.

Tabel 1. The list of *Mangifera* genus collections at Botanical Laboratory, Faculty of Mathematics and Natural Sciences - University of Riau, was obtained from the studied areas in the Southern Sumatra

Species	Sections	Subgenus	Origin	Sample code
M. foetida Lour.	*Perennis*	*Limus*	Bengkulu	*Macang bulat*
M. foetida Lour.	*Perennis*	*Limus*	Bengkulu	*Macang lonjong*
M. foetida Lour.	*Perennis*	*Limus*	Bengkulu	*Macang lado*

Amplification and Sequencing

The PCR reaction total that was used was as much as 50 µL with solution composition (Thermo Scientific Protocol) that was 5µL *Dream Taq Buffer*; 5µLdNTP mix; 2.5 µL primer F; 2.5 µL primer R; 0.25 µL *Dream Taq DNA Polymerase*; 1 µL DNA prints and 33.75 µL dH_2O (*water, nuclease-free*).

The primers which were used were Forward 5' primer (CTTGGCATTCCGAGTA) 3' and Reverse 5' primer (TCACAAGCAGCCAGTTC) 3' (Suparman, 2013). The temperature and time used for each cycle in the amplification process were; pra-95 °C denaturation for 4 minutes then followed by 35 cycles consisting of denaturation as much as 94 °C for 30 seconds, annealing 53,4 °C for 30 seconds and ekstension 72 °C for 2 minutes and 1 last cycle post-extension 72 °C for 10 minutes. The quality of the isolated DNA was checked by using electrophoresis machine with gel agarose concentration 1.2% in a solution of 1x TAE *(Tris Acetic Acid-EDTA).* Sequencing was carried out at First BASE Laboratories through PT. Genetika Science Indonesia.

Phylogenetic Analysis

Sequence data were aligned using ClustalW and then manually checked with BioEdit. Reconstruction of the phylogenetic tree using the PAUP* version 4.0b10 program with the *Maximum Parsimony* (MP) and *Neighbor Joining* (NJ) methods using 100x bootstrap analysis.

RESULTS

***rbc*L Sequence Analysis**

The results of sequencing or sequencing of the nucleotide base with the length *rbc*L on three *M. foetida* cultivars ranged between 807–1280 bp. The longest nucleotide base is in a *Macang bulat* species that is 1280 bp, while the shortest nucleotide base was found in the *Macang lonjong* cultivar that is 807 bp. The nucleotide base composition is stated as the content of G + C because its character is stable in DNA. The G + C content ranged from 43.62–44.53% with the average G + C obtained is 44%. Alignment results (alignment) of nucleotide base lengths in the sequence of three *M. foetida* cultivars that were used were 829 bp (Table 2). The alignment results of *rbc*L sequence with a total nucleotide base of 829 bp contains 807 bp which characteristically are constant. It means that it has the same nucleotide base in every individuals, 22 bp parsimony-uninformative and the absence of parsimony-informative nucleotide base or not found at least two different characters.

Tabel 2. Alignment sequences of three *Mangifera foetida* cultivars were collected from Bengkulu

Species	DNA Sequences
Macang lado	*TGCT--CTGCTGT-GACAGCAGGCAGCTGCGCGTAGCTGCGGAATCTTCTACTGGTACATGGACAACTGTGTGGACCGAT*
Macang lonjong	*TGCCGCTGCCTGTAGGTAGCAGGC-GCTGCG-GTAGCTGCGGAATCTTCTACTGGTACATGGACAACTGTGTGGACCGAT*
Macang bulat	*TCCCGACTGCTGT-GACAGTAGGCAGCTGCGCGTAGCTGCGGAATCTTCTACTGGTACATGGACAACTGTGTGGACCGAT*
Macang lado	*GGGCTTACCAGCCTTGATCGTTACAAAGGACGATGCTACAACATTGAGCCCGTTGCTGGAGAAGAAAATCAATATATATG*
Macang lonjong	*GGGCTTACCAGCCTTGATCGTTACAAAGGACGATGCTACAACATTGAGCCCGTTGCTGGAGAAGAAAATCAATATATATG*
Macang bulat	*GGGCTTACCAGCCTTGATCGTTACAAAGGACGATGCTACAACATTGAGCCCGTTGCTGGAGAAGAAAATCAATATATATG*
Macang lado	*TTATGTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTG*
Macang lonjong	*TTATGTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTG*

Species	DNA Sequences
Macang bulat	*TTATGTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTG*
Macang lado	*GGTTCAAAGCCCTGCGCGCTCTACGTCTAGAGGATCTACGAATCCCTACCTCGTATATAAAAAGTTTCCAAGGACCACCG*
Macang lonjong	*GGTTCAAAGCCCTGCGCGCTCTACGTCTAGAGGATCTACGAATCCCTACCTCGTATATAAAAAGTTTCCAAGGACCACCG*
Macang bulat	*GGTTCAAAGCCCTGCGCGCTCTACGTCTAGAGGATCTACGAATCCCTACCTCGTATATAAAAAGTTTCCAAGGACCACCGC*
Macang lado	*CATGGGATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGCCGTCCCCTATTGGGATGTACTATTAAACCGAAATTAGGT*
Macang lonjong	*CATGGGATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGCCGTCCCCTATTGGGATGTACTATTAAACCGAAATTAGGT*
Macang bulat	*ATGGGATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGCCGTCCCCTATTGGGATGTACTATTAAACCGAAATTAGGTT*
Macang lado	*TTATCCGCTAAGAACTACGGTAGAGCTGTTTATGAATGTCTACGTGGTGGACTTGACTTTACCAAAGACGATGAGAACGTG*
Macang lonjong	*TTATCCGCTAAGAACTACGGTAGAGCTGTTTATGAATGTCTACGTGGTGGACTTGACTTTACCAAAGACGATGAGAACGTG*
Macang bulat	*TATCCGCTAAGAACTACGGTAGAGCTGTTTATGAATGTCTACGTGGTGGACTTGACTTTACCAAAGACGATGAGAACGTGA*
Macang lado	*AACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCCTATTTTGTACGGAAGCTCTTTTTAAAGCGCAGGCTGAAACAGGT*
Macang lonjong	*AACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCCTATTTTGTACGGAAGCTCTTTTTAAAGCGCAGGCTGAAACAGGT*
Macang bulat	*ACTCCCAACCATTTATGCGTTGGAGAGACCGTTTCCTATTTTGTACGGAAGCTCTTTTTAAAGCGCAGGCTGAAACAGGT*
Macang lado	*GAAATTAAAGGTCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATGAAAAGGGCTATGTGTGCAAGAGAGTT*
Macang lonjong	*GAAATTAAAGGTCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATGAAAAGGGCTATGTGTGCAAGAGAGTT*
Macang bulat	*GAAATTAAAGGTCATTACTTGAATGCTACTGCAGGTACATGCGAAGAAATGATGAAAAGGGCTATGTGTGCAAGAGAGTT*
Macang lado	*GGGAGTTCCTATCGTAATGCATGACTACTT-AACAGGGGG-ATTCACCGCAAA-TACTAGCTTGGCTCATTATTGCCGAG*
Macang lonjong	*GGGAGTTCCTATCGTAATGCATGACTACTT-AACAGGGGG-ATTCACCGCAAA-TACTAGCTTGGCTCATTATTGCCGAG*
Macang bulat	*GGGAGTTCCTATCGTAATGCATGACTACTTTAACAGGGGGGATTCACCGCAAAATACTAGCTTGGCTCATTATTGCCGAG*
Macang lado	*AATAATGGTCTACTTCTTTCACATCC-ATCGTGCCG-TGCATGCAGTTATTGATAGACAGAAGAATCATGGGTATGGCACT*
Macang lonjong	*AATGGTCTACTTCTT-CACATCC-ATCGTGCAA-TGCATGCAGTTATTGATAGACAGAAGAATCATGG-TATG-CACTTTC*
Macang bulat	*ATAATGGTCTACTTCTT-CACATCCCATCGTGCAAATGCATGCAGTTATTGATAGACAAAAAAATCATGG-TATG-CACTT*

Phylogenetic Analysis of *M. foetida* Cultivar

Analysis of *M. foetid*a kinship relationship in reconstructing kinship relations using two methods, namely the method of MP and NJ. MP is a method that allows the formation of phylogeny trees and involves the smallest evolutionary changes (Kannan & Wheeler, 2012). Based on the method of MP it was obtained a cladogram with a tree length was 22 and a value of *C indexency* (CI)= 1. The value of CI= 1 indicates parsimony changes in the character of base while the RI value= 1 indicates the complete character and consistent with phylogeny (Amar *et al*., 2014), so that with high CI also indicates that the cladogram that is produced has a high level of parsimony (Fitmawati *et al*., 2017b).

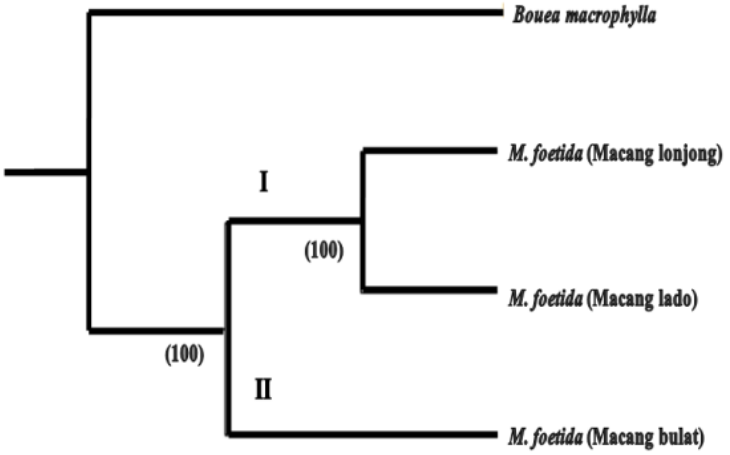

Figure 1. The cladogram of *Mangifera foetida* based on the *Maximum Parsimony* method using the *rbc*L. gene. The number below the branch shows a 100x bootstrap value

The cladogram forms two clad (Figure 1). Clad I consists of *Macang lonjong* cultivar and *Macang lado* cultivar and klad II consists of *Macang bulat* cultivars with 100% bootstrap value. This grouping is divided based on cultivars which experience the smallest evolution distance. *Macang lonjong* and *Macang lado* cultivars grouping to form one clad because they have the smallest genetic distance and experience rapid development, characterized by the distinctive characteristics of each cultivar.

Macang bulat cultivars are separated from clade I because there are differences in the 708 nucleotide base sequence (AC), 709 (AG), 794 (GA), 796 (T-A), and 797 (C-T). The cladogram using the MP method forms a monophyletic clad, that is a clad originating from the same ancestor. The NJ method is a method of reconstructing kinship relationships based on the proximity of evolutionary relationships that occur (Telles *et al*., 2018) and indicated by the existence of different kinship distance matrix coefficients among *M. foetida* cultivars.

Table 3. Kinship distance matrix of *Mangifera foetida* cultivar were collected from Bengkulu using *Neighbor Joining* method

Cultivars	1	2	3	4
Bouea macrophylla	-			
Macang lonjong	0,012	-		
Macang bulat	**0,019**	0,005	-	
Macang lado	0,016	0,002*	0,014	-

Description: The bold number sign indicates the value of the high kinship distance matrix, and asterisk shows a low kinship distance matrix

The NJ method in reconstructing kinship relationships based on the proximity of the evolutionary distance that occurs, indicated by the value of the kinship distance matrix among individuals. The evolutionary distance can be considered as genetic distance which shows the evolutionary rate is different for each cultivar (Maloukh *et al*., 2017). The lower the matrx value the closer kinship relationship and vice versa, the higher kinship matrix value, the farther the kinship distance between the two cultivars in the cladogram.

Figure 2. Cladogram of *Mangifera foetida* based on *Neighbor Joining* method. The number below the branch shows a 100x bootstrap

Distance matrix values obtained from the NJ method, shows the lowest matrix value found on *Macang lonjong* cultivars and *Macang lado* as much as 0.002 (Table 3), so it can be assumed both cultivars have the closest relationship, so that it support analysis of MP where *Macang lonjong* is grouping with *Macang lado*. While matrix value of the highest distance contained in *Macang bulat* cultivar so it can be assumed that this cultivar has the most different relationship. This is due to the MP method grouping one individual with another based on the similarity of nucleotide base and supported by differences in morphological characters. Genetic distance between *M. feotida* cultivars can also be seen from the branch length which shows the rate of evolution among cultivars in cladogram analysis using the NJ method. Based on the phylogenetic tree produced by the NJ method, *M. feotida* cultivars have longer branches than other cultivars (Figure 2).

DISCUSSION

Based on the analysis of *rbc*L sequences with the MP method, it is obtained that *Macang bulat* are very different cultivars compared to the two *M. feotida* cultivars and the are wild species cultivar found in Sumatra. *Macang bulat* have a long evolutionary distance and are assumed to be more primitive individuals. *Macang bulat* cultivar can be indicated as the original parent who has a role in natural hybridization. Among these three *M. feotida* cultivars, *Macang bulat* has a very different character from other cultivars, this *M. feotida* is very big with yellow flesh.

Macang lonjong has the resemblance to the *Macang lado* based on its morphological character by forming a sister group clad. Both of these cultivars are species of *M. feotida* which have a distinct sweet taste from common *M. feotida* with the color of orange flesh. But the *Macang lonjong* has a bigger fruit shape and oval shape, while the *Macang lado* fruit size is smaller than the *M. feotida* species (Fitmawati *et al*., 2018).

Based on the cultivation aspect, *M. feotida* still relatively types spring cultivation that grow wild in some conditions. *M. feotida* status on the Plant list is still unresolve and is supported by a Species List of Threatened (LC) status or a list of species that are endangered on the IUCN Red list that sets criteria for species scarcity status. So by this status, informations are needed regarding taxonomic status and the existence of this type, morphological as well as its molecular information. Based on the phylogenetic analysis, *M. feotida* uses *rbcL* sequence, it is obtained that there are some nucleotide base differences in all three of these *M. feotida* cultivars. Based on the mango phylogenetic study based on the *rbc*L group (Juliantari *et al*., 2018), two *M. feotida* cultivars do not form a sister group and one of the *M. feotida* cultivars forms a sister group with *M. odorata* and show close kinship.

This suggests that, this result can be used as strong basic to develop a new classification system. Classification based on DNA sequence is assumed to produce an accurate classification because DNA is an information basic unit that encodes organisms. Based on the results of this study, the *rbc*L sequence can be used as a molecular marker for analysis of relations to infraspecies (cultivars) level. The results of this classification can be a basis for cultivated species of *M. feotida*.

CONCLUSION

Variations of the three species of *M. feotida* cultivars based on *rbc*L sequences obtain cladogram by using MP method, form a monophyletic clad that grouping. Clad I consists of *Macang lonjong* and *Macang lado* cultivars and Clad II consists of a single *Macang bulat* cultivar clad. Cladogram using NJ method supports MP analysis that shows *Macang bulat* cultivar has a longer genetic distance and assumed as more primitive individuals.

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