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Phylogenetic Analysis of *Baccaurea* Spp. in West Sumatra Using MatK Molecular Markers

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Abstract. *Baccaurea* is a group of fruit-producing plants found in wild forests. Many of these plant species have not yet explored their potential. The high rate of deforestation in West Sumatra poses a threat of extinction to the genetic resources of the genus *Baccaurea* before being explored and identified. The research aims to analyze the sequence characters and phylogenetic of *Baccaurea* found in West Sumatra using the MatK molecular marker. Phylogenetic analysis using the Maximum Likelihood (ML) method in MEGA X application. The results of the analysis of the six species of *Baccaurea* found that the sequence length ranged from 854-1019bp, the percentage of G+C base composition is 33.4%, the percentage of A+T base composition is 66.6%, the genetic distance range is 0-4% with a conservative character of 484bp and informative characters of 4bp. While the phylogenetic analysis using the ML method grouping the six species of *Baccaurea* to form a monophyletic clade with a bootstrap value of 100%, all species collected were in the same clade. These results reveal the first time that the MatK sequences from six species *Baccaurea* native to West Sumatra will be included in NCBI for use by other studies in conducting broader phylogenetic research.

Keywords: *Baccaurea*; MatK gene; Maximum Likelihood (ML); Phylogenetics

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INTRODUCTION

Baccaurea Lour. is a genus of angiosperm plants belongs to the Phyllanthaceae family (Gunawan *et al*., 2016). The number of *Baccaurea* species that have been published is 173 species and 87 species are synonyms (GBIF, POWO, theplantlist.org). The type of bacca fruit (berry) produced from a single ovary is a characteristic that underlies the terminology of the genus *Baccaurea* (Beentje, 2016). Taxonomically, it is important to know detailed information about *Baccaurea*. *Baccaurea* is a fruit-producing wild plant that lives naturally in forests. Based on the results of research conducted by Gunawan *et al.*, (2016) revealed that *Baccaurea* has the potential as a source of natural ingredients for medicine and its distribution area is also widely spread (Gunawan *et al.,* 2018). In addition, its existence is very important for the balance of the ecosystem because of its role as food for many species (Ramayani and Fitmawati, 2020). On the island of Sumatra, more than 25% of *Baccaurea* species are found (GBIF, 2022) and specifically in West Sumatra there are 13 types (GBIF, 2022).

Based on data on forest area, West Sumatra province is experiencing a relatively high rate of forest deforestation. In a period of 25 years, West Sumatra has lost 578,372 ha of forest due to forest conversion, illegal logging and illegal mining in the forest (Vinolia, 2019). This situation will indirectly pose a threat to the existence of genetic resources for wild fruits in tropical forests, including *Baccaurea*. Previous studies show that there are several *Baccaurea* in West Sumatra province with high potential. But it has not been fully explored, especially wild species. The high rate of deforestation can cause the extinction of this plant before its species is explored and identified, which is most likely a new taxon. The

diversity of a taxon is largely determined by the speciation process which can be traced from phylogenetic analysis. Phylogenetic analysis is a method used to evaluate systematics which is expected to be able to explain the diversity of organisms and their kinship relationships depicted in a clade or phylogram (Hidayat and Pancoro, 2016)

In the phylogenetic analysis of the data used is molecular data. MatK (Maturase K) is a gene located in the chloroplast genome of plants which has a low level of genetic recombination and is only inherited maternally, so this gene can be used to view the evolutionary history of an organism (Wang *et al.*, 2013). Maturase K is also a gene that codes for an intron organelle through a maturase protein that breaks group II introns (Barthet *et al.*, 2020). In recent years, the MatK gene has often been used by experts for molecular phylogenetic analysis in plants in order to understand diversity and answer several phylogenetic problems. Examples of research conducted by Manurung *et al.*, (2018) on the genus *Zanthoxylum* and Retnaningati (2017) on the species *Cucumis melo* L. using the MatK marker showed that the MatK marker succeeded in separating the two species at the species level. Therefore, research on the phylogenetic analysis of *Baccaurea* in West Sumatra using the molecular marker MatK is important to do in order to provide convenience in the process of proving and identifying accurately and quickly. The purpose of this study was to analyze the sequence characters and phylogenetic of *Baccaurea* found in West Sumatra using the molecular marker MatK and the benefits of this research, namely to fill the treasures of science, especially in the field of molecular taxonomy of *Baccaurea* found in West Sumatra and the sequence data obtained can be enter into the NCBI database for use by other researchers in conducting broader phylogenetic research.

METHOD

Sample Collection

Six species of *Baccaurea* were obtained from eight districts in West Sumatra, namely Agam, West Pasaman, East Pasaman, Padang-Pariaman, Pesisir Selatan, Solok, Tanah Datar, and 50 kota (Table 1). The samples were stored in a plastic ziplock containing silica gel until the DNA was isolated in the laboratory.

Table 1. Location data and species of the genus *Baccaurea* analyzed

No.	Species	Local Name
1	*Baccaurea deflexa* Müll. Arg	Birah mato
2	*Baccaurea dulcis* (Jack) Müll.Arg.	Kapunduang
3	*Baccaurea lanceolata* (Miq.) Müll.Arg.	Lempaong
4	*Baccaurea motleyana* Müll.Arg.	Rambai
5	*Baccaurea racemosa* (Reinw.) Müll. Arg.	Kisip
6	*Baccaurea sumatrana* (Miq.) Müll.Arg.	Sour

Plant DNA Isolation

DNA isolation from young leaves was carried out using the KIT Bioline Isolate II Plant DNA method. Four stages in the DNA isolation process using the KIT Bioline Isolate II Plant DNA method, namely (1). Lysis: 15 mg of dried young leaf tissue was crushed using a mortar assisted by liquid nitrogen. The powdered grinding results were transferred into a microtube, then 400 µl of PAW I wash buffer solution was added, then vortexed, then 10 µl of Rnase A was added and incubated in a water bath at a 65^0C for 10 minutes. (2). Binding: The incubated sample was transferred into an isolate filter tube (purple), then centrifuged at a 11,000 rpm for 2 minutes. After that, 450µl PB binding buffer solution was added and centrifuged again at 11000 rpm for 1 minute. (3). Washing: the centrifuged sample was transferred into a new tube with a spin column (green).The supernatant will be filtered down the new tube, which will then be discarded. Next, the pellet contained in the spin column tube (green) was added with 400 µl of PAW I wash buffer solution and centrifuged at 11,000 rpm for 1 minute. The supernatant will be filtered down and thrown again. Then 700 µl of PAW II wash buffer solution was added and centrifuged again at 11000 rpm for 1 minute. The supernatant will be filtered down and will be thrown away again. Finally, 200 µl of PAW II wash buffer solution was added and centrifuged again at 11000 rpm for 2 minutes. (4). Elute: the spin column (green) containing DNA is then transferred into a new tube. The PG buffer solution was heated. After that, 50 µl of PG buffer solution was added to the new tube containing the spin column (green). Then the tube was incubated at 65^0C for 5 minutes. After that it was centrifuged at 11000 rpm for 1 minute. Finally, the PG buffer solution was heated again, then 50 µl of PG buffer

solution was put back into a new tube containing the spin column (green) and incubated again at 65^0C for 5 minutes and then centrifuged at 11000 rpm for 1 minute. Then the spin column (green) was removed from the tube, the purified DNA containned in the tube and stored at 20^0C. The quality of the isolated results was examined using an electrophoresis machine with 2% agarose gel concentration.

DNA amplification

The PCR cocktail used consisted of 11µl MyTaq Red HS Mix, 9µl ddH2O, 3µl isolated DNA and 1µl each of forward and reverse primers. The primers used are MatK-F 5' CCCATTTTTGCTAATGATCG'3 and MatK-R 5' GAGGACAAATTTTCGCATTT'3 (Aseny, 2022). Amplification used a SensoQuest PCR machine with 30 programmed cycle. The stages of PCR work procedure used, namely initial denaturation at 95^0C for 5 minutes, followed by denaturation at 95^0C for 30 seconds, annealing at 51.5-53^0C for 30 seconds, elongation at 72^0C for 1 minute and final elongation at 72^0C for 5 minutes.

Electrophoresis

The PCR results were electrophoresed on 2% agarose gel. A total of 2.5 µl of DNA sample was inserted into the agarose gel wells. The electrophoresis machine work with a voltage of 100 volts, 200 A, 20 watts for 45-50 minutes. furthemore, the results electrophoresis were transferred to a UV transilluminator connected to a laptop so that image of DNA band could be observed and documented.

DNA Sequencing and Data Analysis

PCR product were then sent for purification and two-way sequencing analysis at First Base Laboratories Malaysia through PT. Genetics Indonesian Science. The sequences obtained were then contigged with the SegMen program in DNA STAR, then BLASTed through the official NCBI website. Sequence alignment using the Clustal X application, the results obtained were edited using the BioEdit program. Analysis of sequence characters and phylogenetic using the MEGA X program. Analysis of sequence characters to see differences in nucleotide bases and calculate the genetic distance between species. Meanwhile, in the phylogenetic analysis, 15 sequences were added from the gene bank as comparison species (13 in-group sequences and 2 out-group sequences) (Table 2). Phylogenetic tree reconstruction using the Maximum Likelihood (Ml) method with 1000x bootstrap analysis.

Table 2. List of comparative species from the gene bank based on MatK markers

Species	No. Accession	Reference	Information
B. angulata	LC737097.1	Okuno et al., 2022	
B. deflexa	MG838531.1	Amandita et al., 2019	
B. dulcis	MG838533.1	Amandita et al., 2019	
B. dulcis	MG838524.1	Amandita et al., 2019	
B. hookeri	LC737099.1	Okuno et al., 2022	
B. javanica	AY579878.1	Samuel et al., 2016	
B. kunstleri	LC737100.1	Okuno et al., 2022	
B. lanceolata	AY552419.1	Samuel et al., 2016	*In Group*
B. macrocarpa	KJ708847.1	Samuel et al., 2016	
B. minor	LC737098.1	Okuno et al., 2022	
B. mollis	MG838540.1	Amandita et al., 2019	
B. parviflora	MG838543.1	Amandita et al., 2019	
B. pyriformis	LC737101.1	Okuno et al., 2022	
Macaranga praestans	DQ866575.1	Amandita et al., 2019	
Blumeodendron kurzii	DQ866525.1	Amandita et al., 2019	*Out Group*

RESULTS AND DISCUSSION

The results of DNA purification of the six species of *Baccaurea* in the MatK gene showed clear and bright bands (Figure 1). The success of the amplification process is affected by the annealing temperature. Each species of *Baccaurea* has a different annealing temperature. So that several repetitions are done to get the optimum annealing temperature with good band results. The results of modifying the annealing temperature on the MatK marker in obtaining an optimum temperature ranging from 48-51.8^0C with details for each species, namely *B. deflexa*, *B. dulcis*, *B. lanceolata*, and *B. motleyana* (51.5^0C); *B. racemosa* (51.8^0C); *B. sumatrana* (48^{0}C). According to Amanda *et al.,* (2019) determining

the annealing temperature incorrectly will cause the primer attachment to be non-specific.

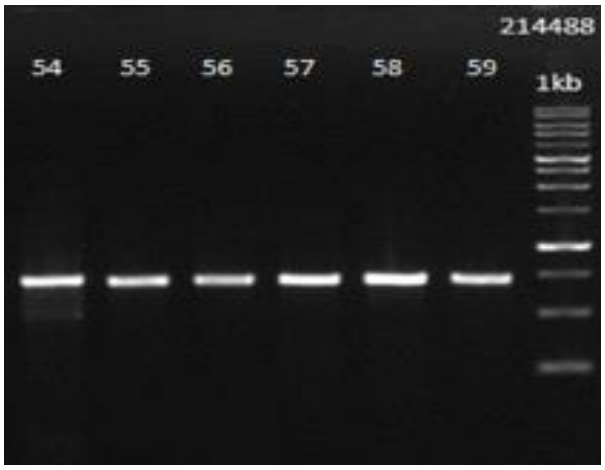

Figure 1. Results of *Baccaurea* DNA purification based on the MatK marker: (59). *B. deflexa*, (58). *B. dulcis*, (57). *B. lanceolata*, (56). *B. motleyana*, (55). *B. racemosa*, (54). *B. Sumatrana*.

Sequence Character Analysis

Sequence character analysis was carried out from sequence analysis using BLAST to verify the sequencing results with existing sequence data on the NCBI website. The results obtained, the six *Baccaurea* sequences were identified as the *Baccaurea* group. The level of similarity (homology) of BLAST results of MatK markers ranges from 99.32-99.86%. This value indicates the similarity range of the samples used based on the MatK marker with the sequences in NCBI. The difference in the species identification results obtained in the field with the sequences in NCBI is strongly suspected to be due to the lack of sequence data in gene banks related to *Baccaurea* species, especially in the Sumatra region. For example, *B. sumatrana* was identified as *Baccaurea* sp. and *B. lanceolata* as *Baccaurea* sp.

The results of the BLAST sequence were then observed using the MEGA program to obtain a comparison of sequence characteristics based on the markers used (Table 3). In determining the sequence characteristics of the DNA sequences of six species of *Baccaurea* from the collection, the addition of *B. lanceolata* sequence from the gene bank as the type species. The length of the DNA sequence obtained based on the MatK marker is 854-1019 bp. The sequence length range obtained is not much different from the results of previous research, namely the length of the MatK marker DNA sequence is 900-1240 bp (Vinitha *et al.*, 2014; Syamsuardi *et al.*, 2018).

Table 3. Characteristics of the Baccaurea sequence based on MatK

No	Sequence Characteristics	MatK
1.	Sequence Length (bp)	854-1019
2.	Sequence Length in Data Analysis (bp)	529
3.	Percentage of G+C Amount (%)	33.4
4.	Percentage of A+T Amount (%)	66.6
5.	Genetic Distance Range (%)	0-4
6.	Conservative character(bp)	484
7.	Informative character (bp)	4

Furthermore, in the analysis of sequence characters, the length of the sequence used is 529 bp. From the sequence analysis, the percentage of G+C base composition in the genus *Baccaurea* based on the MatK marker is 33.4%. While the percentage of A+T base composition is 66.6%. Based on Gunawan's research, (2020) on *B. angulata* using a marker from the chloroplast genome (trnL-FIGS) showed the appropriate results, namely obtaining the percentage results of G + C (38.4%) and A + T (61.7%). According to Manurung *et al.*, (2018) the nucleotide composition in the non-coding areas of chloroplast DNA contains more adenine and thymine bases. Then for the character of the genetic distance range, the MatK marker has a genetic distance range (0-4%). Differences in genetic distance describe the level of evolution of each species of *Baccaurea*. The greater the genetic distance, the higher the genetic adaptation developed by the species to survive in its environment. Finally, for the character of conservative bases and informative bases, Based on 529 bp aligned in data analysis using MatK markers, the number of conservative characters obtained is 480 bp while the informative characters is 4 bp (Table 3). The low number of informative characters on the MatK marker means that the MatK marker has a constant character. The base changes that occur in chloroplast DNA are very small compared to the nuclear genome because it is inherited maternally only, so the information generated from these base changes will provide an overview of the rate of evolution that occurs in the *Baccaurea* genus. In the chloroplast genome, the MatK gene is a highly variable gene that can be used for species identification and verification (Roslim *et al.*, 2016). The informative character are a combination of the parsimony character and the singleton character. The total informative

characters obtained were 4 bp with 1 bp of parsimony and 3 bp of singleton. From the analysis that has been done based on MatK markers, all *Baccaurea* have a base difference of 1 bp with *B. lanceolata* (NCBI), except *B. lanceolata* has no base difference (0 bp) with *B. lanceolata* (NCBI) (Table 4.).

Table 4. Differences in collected *Baccaurea* bases based on MatK markers.

No	Species	1	2	3	4	5	6	7
1.	*B. lanceolata*(NCBI)							
2.	*B. deflexa*	1						
3.	*B. dulcis*	1	2					
4.	*B. lanceolata*	0	1	1				
5.	*B. motleyana*	1	2	2	1			
6.	*B. racemosa*	1	2	0	2	2		
7.	*B. Sumatra*	1	2	2	2	2	2	

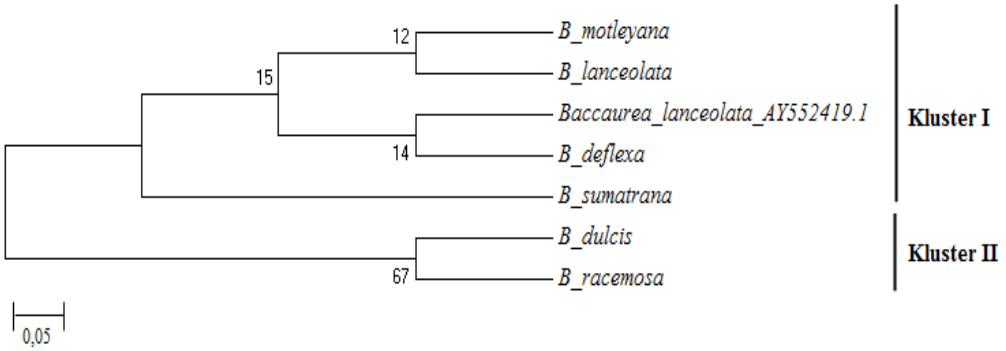

Figure 2. Phylogram of the phylogenetic relationships of the collected Baccaurea based on MatK markers.

Based on the results of the phylogram of the phylogenetic relationship of *Baccaurea* using the MatK marker, the results of grouping are monophyletic, meaning that the seven *Baccaurea* species come from a common ancestor (Figure 2). The grouping formed from the phylogram consists of two clusters, namely cluster I and cluster II. Cluster I consisted of *B. motleyana*, *B. lanceolata*, *B. lanceolata* (NCBI), *B. deflexa*, and *B. sumatrana* while cluster II consisted of *B. dulcis* and *B. racemosa*. In cluster I, *B. motleyana* and *B. lanceolata* are in the same branch with a bootstrap value of 12%. The base difference between the two is 1 bp. then *B. lanceolata* (NCBI) and *B. deflexa* are in the same branch with a bootstrap value of 14%. The base difference between the two, namely 1 bp and finally *B. Sumatrana* is a sister taxa among the four types of *Baccaurea* in cluster I, supported by a bootstrap value of 15% and a base difference of 2 bp.

Next in cluster II, consisting of *B. dulcis* and *B. racemosa*. Both combine with a bootstrap value of 67% and between them there is no difference in base.

The separation of *B. dulcis* and *B. racemosa* from cluster I is due to the large difference in the number of bases and the long genetic distance between *B. dulcis* and *B. racemosa* and the *Baccaurea* species found in cluster I. The *Baccaurea* type in cluster I has the most base differences many with *B. dulcis* are *B. motleyana* and *B. sumatrana* as much as 2 bp. Meanwhile, the species that has the fewest base differences with *B. dulcis* is *B. lanceolata* of 1 bp. Then the species of *Baccaurea* in cluster I which has the most base differences with *B. racemosa* is *B. deflexa*, *B. lanceolata* and *B. motleyana* by 2 bp and the species which has the fewest base differences with *B. racemosa* is *B. lanceolata* (NCBI) as much as 1 bp (Table 4). Morphologically, we can see the differences in fruit shape of each species of *Baccaurea* collected. This difference in fruit shape is one of the key characteristics in distinguinshing each species of *Baccaurea* (Figure 3).

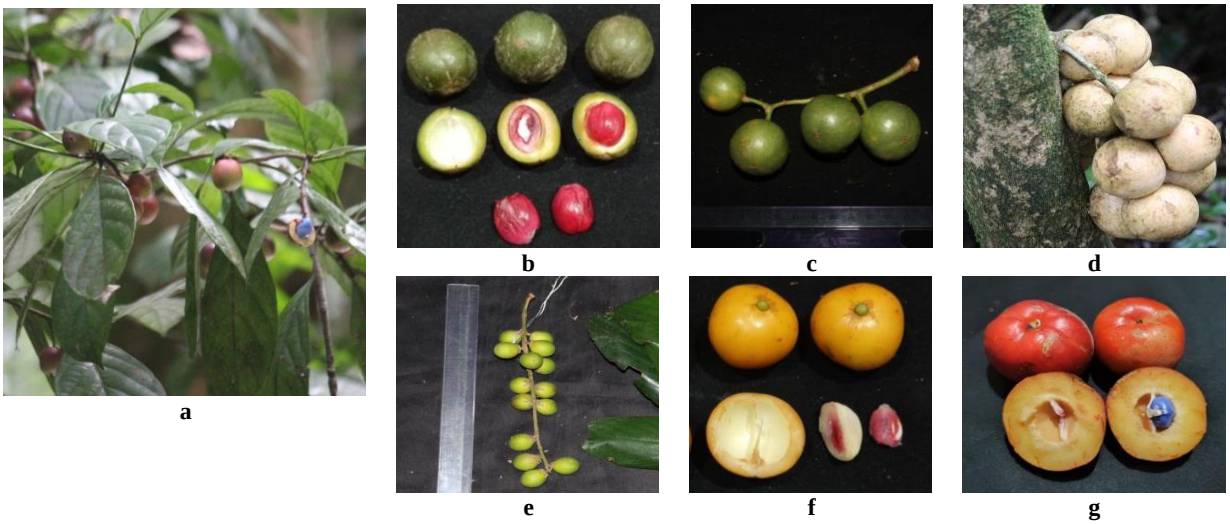

Figure 3. *Baccaurea* fruit shape: a. Examples of appearance of *Baccaurea* twigs, leaves and fruit; b. *B. deflexa*; c. *B. dulcis*; d. *B. lanceolata*; e. *B. motleyana*; f. *B. racemosa*; g. *B. Sumatrana*.

Phylogenetic Analysis

In the phylogenetic analysis, sequence additions were carried out based on the MatK marker from NCBI. The number of sequences added was 15 sequences (Table 2), namely 13 *Baccaurea* sequences as comparison sequences and 2 sequences from different species as outgroups. Two species that became an outgroup, namely *Macaranga praestans* and *Blumeodendron kurzii*. The addition of outgroups in phylogenetic analysis is really needed as a comparison of characters that experience changes, namely apomorphic characters and plesiomorphic characters. Apomorphic characters are derived characters found in ingroups. Meanwhile, plesiomorphic characters are primitive characters that come from one ancestor found in the outgroup (Muzzazinah, 2017).

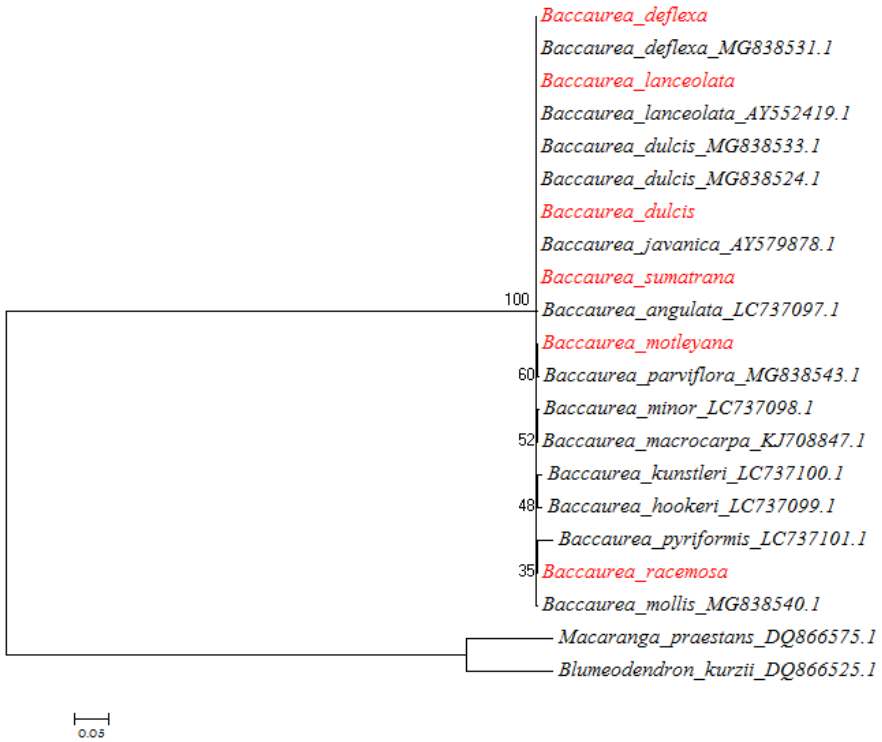

Figure 4. Cladogram of *Baccaurea* kinship relationships collected based on MatK (ML) markers. The species name in red is Baccaurea which was collected in this study.

The results of cladogram reconstruction from *Baccaurea* phylogenetic analysis based on MatK markers analyzed using the Maximum Likelihood (ML) method resulted in groupings originating from the same branch and separated from the outgroup (Figure 4). This grouping is supported by a high bootstrap value, namely 100%. So it can be concluded that the *Baccaurea* grouping formed is monophyletic. The same results also occurred in the studies of *Stelechocarpus* (Tuhardi, 2023), *Opuntia* sp (Aulia, 2022), and *Ixora* sp (Anzani *et al.*, 2021) who used the MatK marker as a DNA barcode. Of the three studies, the ability of the MatK gene to discriminate at the weak species level, the MatK locus has high homogeneity between species, so it cannot be used to differentiate at the species level. MatK is one of the genes originating from the chloroplast genome. DNA from the chloroplast genome is inherited maternally or from the female parent, so that in the chloroplast genome the genetic changes that occur are very few and even take a very long time for a change to occur (Manurung *et al*., 2018). Nevertheless, the data obtained from these few changes can be important information in determining the rate of evolution that occurs in these organisms. The grouping of all *Baccaurea* accessions into one large group and only separated from the outgroup is also supported by small genetic distance values. The range of genetic distance between all *Baccaurea* accessions is 0.00-0.04 (0-4%). Genetic distance is an illustration of the evolutionary rate of each species of *Baccaurea*. The rate of evolution can be faster or slower, depending on the adaptation process and enviromental conditions.

Overall, the relationship between *Baccaurea* in West Sumatra is influenced by differences in the amount of nucleotide base compositions and differences in genetic distance. This difference illustrates the level of evolution of each species of *Baccaurea*. The more different bases and the greater the genetic distance, the more distant the relationship, and vice versa. Differences in the number of bases and genetic distance cause genetic variations in a species, so that the species is more able to survive in its environment and this variation will be passed on to the next generation. The results of this research reveal for the first time MatK sequences from six native *Baccaurea* species in Sumatra which will be included in the NCBI data base for use by other researchers in revealing broader phylogenetic research.

CONCLUSION

Based on the research results, it can be concluded that the sequence characteristics of six *Baccaurea* species in West Sumatra using the MatK marker have a low level of variation between species, namely the genetic distance range of 0-4%, conservative character of 484% and informative character of 4%. While the results of phylogenetic analysis of *Baccaurea* with its relatives illustrate a slow rate of evolutionary, where all *Baccaurea* species collected are in the same clade and are monophyletic with a bootstrap value of 100%. Based on the results of the study, it is recommended to conduct further research using other molecular markers, for phylogenetic analysis of *Baccaurea*.

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