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PHYLOGENETIC ANALYSIS OF POTENTIAL WILD FRUITS OF *BACCAUREA* SPP. (PHYLLANTHACEAE) INDIGENOUS TO WEST SUMATRA, INDONESIA

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SUMMARY

Baccaurea is a group of fruit-bearing potential wild tropical plants. West Sumatra is one of the Indonesian provinces with a sizable forest area. However, the high deforestation rate threatens the germplasm, especially *Baccaurea* wild plants, before well exploring their potential. The study aimed to analyze the sequence characters and phylogenetics of *Baccaurea* species found in West Sumatra using ITS molecular markers. DNA extraction relied on the Kit protocol using ITS molecular markers. The study employed the MEGA X application for sequence analysis and kinship relationships. Results of the analysis based on sequence characters of *Baccaurea*'s six species showed lengths ranging from 696 to 749 bp, the percentage composition of G + C bases (63.8%) and A + T bases (36.2%), the range of genetic distance (1%–5%), the number of conservative characters (549 bp), the number of informative characters (69 bp), and the point mutation in the nucleotide sequence (369 and 489). Meanwhile, the phylogenetic analysis using the ML method grouped the six species of *Baccaurea* to form a monophyletic clade with a bootstrap value of 100%-99%-100%. The results revealed for the first time the inclusion of ITS sequences of six *Baccaurea* species indigenous to West Sumatra in the NCBI database, which can benefit future investigations in identifying *Baccaurea* species and as a valuable reference for plant breeding in *Baccaurea* wild fruit plants' development and conservation.

Keywords: *Baccaurea* wild fruit plants, ITS genes, phylogenetic analysis, sequence characteristics, West Sumatra

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Key findings: The sequence data of six *Baccaurea* wild fruit species based on ITS markers found in West Sumatra are new entries for the NCBI database. The genetic basis identified in *Baccaurea* species will also be a valuable reference for plant breeders to conserve and further develop the *Baccaurea* wild fruit plants.

INTRODUCTION

Baccaurea is a group of angiosperms that belong to the family Phyllanthaceae (Secco *et al.,* 2012). Based on its potential benefits, *Baccaurea* is a genus of fruit-producing plants. Their existence also plays a vital role in balancing the ecosystem because fruit-bearing trees serve as food for many species (Rijksen, 1978). Past studies also enunciated that most *Baccaurea* species have been functioning as a source of food and traditional medicines (Suwardi *et al.,* 2022).

The *Baccaurea* species centers of diversity distribution include the flora Malesiana regions comprising Malaysia, Singapore, Indonesia, Brunei Darussalam, the Philippines, Timor-Leste, and Papua New Guinea (Haegens, 2000). Published reports on the *Baccaurea* species have reached 173 species (Gunawan *et al.,* 2016, 2018; Sivadasan *et al.,* 2020; Charu *et al.,* 2021), with more than 25% of *Baccaurea* species specifically found on the island of Sumatra (GBIF, 2022).

West Sumatra is one of the Indonesian provinces on the west coast of Sumatra islands, with a forest area of 4.2 million hectares, of which around 45.7% are natural forests (BPS, 2022). According to Navia *et al.* (2022), forests provide massive benefits as future sources and reservoirs of native crop plants. More than 80% of wild plants also exist in tropical forests (Suwardi *et al.,* 2022). However, based on the forest area data, West Sumatra is experiencing a relatively high rate of deforestation. Within the last 25 years, West Sumatra has lost 578,372 ha of forest due to the area's conversion and illegal logging and mining activities (Vinolia, 2017). The situation will directly threaten the available genetic resources of wild fruit plants in tropical forests, including *Baccaurea*. Previous studies have also reported several high-potential fruit-bearing *Baccaurea* prevalent in West Sumatra province but require full exploration, especially the wild species (Suwardi *et al.,* 2023). The high deforestation rate could lead to the plants' extinction before even discovering and identifying them, likely to be new taxa.

Phylogenetic analysis is a method that can help determine the diversity and kinship of organisms without the need for complete morphological characters. One of the molecular markers often used in phylogenetic analysis is ITS. ITS is a widely used molecular taxonomy and phylogeny technique because of its fast amplification with a high degree of variation even between closely related species (Hollingsworth*,* 2011). Therefore, research on phylogenetic analysis of potential wild fruits *Baccaurea* spp. (Phyllanthaceae) indigenous to West Sumatra using ITS molecular markers is crucial to provide a convenient, precise, and quick identification processand the latest information on species diversity and kinship relationships in West Sumatra tropical forests. This study sought to analyze the sequence and phylogenetic characters of *Baccaurea* found in West Sumatra using ITS molecular markers. The study will also be beneficial by adding new scientific knowledge, especially in the molecular taxonomy of *Baccaurea* found in West Sumatra, with the obtained sequence data entered into the NCBI database for other researchers' use for broader phylogenetic research.

MATERIALS AND METHODS

Sample collection

The collection of *Baccaurea* six species samples came from eight districts in West Sumatra, namely, Agam, West Pasaman, East Pasaman, Padang-Pariaman, Pesisir Selatan, Solok, Tanah Datar, and 50 Kota. Fresh and young

leaf samples collected from these *Baccaurea* plants underwent genomic DNA isolation (Feng *et al.,* 2014).

DNA extraction

DNA extraction from leaves used the Bioline Isolate II Plant KIT method. Furthermore, the PCR amplification process employed a PCR cocktail consisting of 11 µL My Taq Red HS Mix, 9 µL ddH2O, 3 µL isolated DNA, and 1 µL each of forward and reverse primers. The primers used were ITS5-F 5'GGAAGTAAAAGTCGTAACAAGG'3 and ITS4-R 5'CTCCGCTTATTGATATGC'3 (White *et al.* 1990). Amplification engaged a SensoQuest PCR machine with 30 programmed cycles. The stages of the PCR work procedure comprised initial denaturation at 95 °C for 5 min, followed by denaturation at 95 °C for 30 s, annealing at 51.5 °C–53 °C for 30 s, elongation at 72 °C for 1 min, and final extension at 72 °C for 5 min. PCR results received electrophoresis on a 2% agarose gel. Inserting 2.5 µL of DNA sample into the agarose gel wells transpired. The electrophoresis machine worked with 100 volts, 200 A, and 20 watts for 45–50 min. Transferring the electrophoresis results to a UV transluminator connected to a laptop helped observe and document the images of DNA bands.

Data analysis

PCR product purification and two-way sequencing analysis occurred at the First Base Laboratories, Malaysia, via PT Genetika Science, Indonesia. The obtained sequences reached submission with the SegMen program in DNA STAR (Burland, 2000) and then underwent the BLAST process through the NCBI official website. Sequence alignment used the Clustal X application editing further the results with the BioEdit program (Hall, 1999). Analysis of molecular and phylogenetic characters continued using the MEGA X program (Tamura et al., 2019). Molecular analyses probing nucleotide base differences and calculating genetic distances between species also ensued. In the phylogenetic analysis, an additional 17 sequences from the gene bank served as comparison species (15 in-group sequences and two out-group sequences) (Table 1). Phylogenetic tree reconstruction progressed with the Maximum Likelihood (ML), Neighbor-Joining (NJ), and Maximum Parsimony (MP) methods with the 1000x bootstrap analysis.

Table 1. List of comparison species from the gene bank based on ITS markers.

Species	Accession number	Reference	Description
B. lanceolata	OK052881.1	Tnah *et al.,* 2021	
B. motleyana	OK052882.1	Tnah *et al.,* 2021	
B. parviflora	OK052886.1	Tnah *et al.,* 2021	
B. polyneura	OK052883.1	Tnah *et al.,* 2021	
B. ramiflora	KF186441.1	Fang *et al.,* 2013	
B. ramiflora	ON514069.1	Khang *et al.,* 2022	
B. ramiflora	KR531813.1	Huang *et al.,* 2015	*In Group*
B. ramiflora	KR531812.1	Huang *et al.,* 2015	
B. ramiflora	KR531811.1	Huang *et al.,* 2015	
B. ramiflora	KR531810.1	Huang *et al.,* 2015	
B. ramiflora	KR531809.1	Huang *et al.,* 2015	
B. ramiflora	KF186441.1	Fang *et al.,* 2013	
B. ramiflora	OK052884.1	Tnah *et al*., 2021	
B. ramiflora	KR531808.1	Huang *et al.,* 2015	
B. reticulata	OK052885.1	Tnah *et al*., 2021	
Macaranga praestans	DQ866575.1	Kulju and Van-Welzen, 2006	*Out Group*
Blumeodendron kurzii	DQ866525.1	Kulju and Van- Welzen, 2006	

Table 2. Species and local name from the analyzed genus *Baccaurea*.

No.	Species	Local Name
1	*Baccaurea deflexa* Müll.Arg	Birah mato
2	*Baccaurea lanceolata* (Miq.) Müll.Ar	Lempaong
3	*Baccaurea motleyana* Müll.Arg.	Rambai
4	*Baccaurea polyneura* Hook.f.	Djentikan
5	*Baccaurea racemosa* (Reinw.) Müll.Arg.	Kisip
6	*Baccaurea sumatrana* (Miq.) Müll.Arg.	Semasam

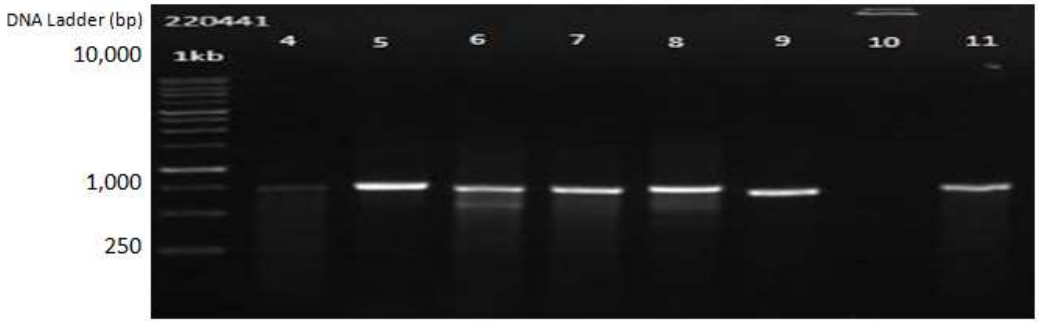

Figure 1. *Baccaurea* DNA purification results based on the ITS marker: (5). *B. deflexa*, (6). *B. lanceolata*, (7). *B. motleyana*, (8). *B. polyneura*, (9). *B. racemosa*, (11). *B. sumatrana*.

RESULTS AND DISCUSSION

The results of DNA purification from six *Baccaurea* species using ITS markers showed transparent and bright bands detailing each type, namely *B. racemosa* (52 °C), *B. deflexa* (52.3 °C), *B. motleyana* (52.3 °C), *B. sumatrana* (52.3 °C), *B. lanceolata* (54.9 °C), and *B. polyneura* (54.3 °C) (Table 2, Figure 1). According to Poulsen *et al*. (2018), the recommended annealing temperature for ITS primers is 55 °C. However, during the PCR process, this temperature did not give good band results; hence, the study modified the annealing temperature to get the optimum temperature, ranging from 52 °C to 54.9 °C.

Molecular character analysis

Molecular character analysis also proceeded from the sequence analysis using BLAST to verify the sequencing results with existing sequence data on the NCBI website. The results revealed that the six *Baccaurea* sequences were distinctly a *Baccaurea* group. The similarity level (homologous) BLAST results of ITS markers ranged from 79.68% to 96.82%. The difference in the results of identified species obtained with the sequences in NCBI was clearly due to the unavailability of sequence data in the gene bank related to *Baccaurea* species, especially in the Sumatra region. For example, species *B. sumatrana* appeared as *B. ramiflora* and *B. racemosa* as *B. ramiflora*.

The results of the BLAST sequences scrutiny used the MEGA program to compare sequence characteristics based on the ITS markers (Table 3). In determining the molecular characteristics of the DNA sequences of six *Baccaurea* species from the collection, adding *B. lanceolata* sequences from the gene bank served as a type species. The DNA sequence length obtained for ITS markers was 696–749 bp. According to past research, the range of DNA sequence length using ITS markers was 500–700 bp (Alvarez and Wendel, 2003; Kress *et al.,* 2005; Theerakulpisut, 2012).

Furthermore, the molecular characters' analysis used a sequence length of 625 bp. In the sequence analysis in the *Baccaurea* genus, the percent of the G + C base composition was 63.8%, while the A + T base composition was

Table 3. Characteristics of *Baccaurea* sequences based on ITS.

No	Sequence Characteristics	ITS
1.	Sequence Length (bp)	696-749
2.	Length of Sequence in Data analysis	625
3.	Percentage of G+C	63.8
4.	Percentage of A+T	36.2
5.	Genetic Distance Range (%)	1-5
6.	Conservative character(bp)	549
7.	Informative characters (bp)	69

36.7%. These results were similar to the findings from Aprilianingsih (2021) on *Homalomena pexa* species, as they recorded the percentage of G + C base composition (69.93%) and A + T (30.05%). According to Arisuryanti *et al.* (2016), the composition of nucleotide bases contains more G + C than in the chloroplast spacer in the ITS region.

For the genetic distance range, ITS markers have a range of genetic distance of 1%–5%. The greater the genetic distance, the higher the genetic adaptation the species develops to survive in the existing environment. However, the differences in genetic distance illustrate the level of evolution of each type of *Baccaurea.* ITS markers have excellent potential for species differentiation in taxonomic classification (Hamid *et al*., 2023).

Finally, for conservative base characters and informative bases, based on 625 bp aligned in the data analysis using ITS markers, the number of conservative characters was 549 bp, while informative characters were 69 bp (Table 3) (Figure 2). Informative characters were the combination of parsimony with singleton characters. Total parsimony characters obtained as much as 19 bp and singleton as much as 49 bp. Informative characters describe the difference in the number of bases owned by the seven *Baccaurea* samples analyzed. Analysis based on ITS markers revealed that the basest difference with *B. lanceolata* was *B. polyneura,* with 16 bases. However, the least base difference with *B. lanceolata* was *B. lanceolata*, with six bases. The base differences with other species, namely, *B. deflexa, B. motleyana, B. racemosa,* and *B. sumatrana,* have differences of 15, 10, 10, and 13 bp, respectively (Table 4). The base difference among the species indicated linkage to the species kinship. The greater the value of the base difference, the more distant the kinship relationship, and the smaller the value of the base difference, the closer the kinship relationship.

Phylogenetic analysis

The phylogenetic analysis added sequences based on ITS markers obtained from the NCBI. The added 17 sequences (Table 1) comprised 15 *Baccaurea* sequences as comparison sequences and two sequences from different species as outgroups. The two outgroup species were *Macaranga praestans* and *Blumeodendron kurzii*. In phylogenetic analysis, adding outgroups was necessary for comparing the changing parameters, i.e., apomorphic and plesiomorphic characters. Apomorphic characters were the derived characters found in the group, while the plesiomorphic characters were the primitive characters from the ancestor in the outgroup (Muzzazinah, 2017).

The cladogram reconstruction of the *Baccaurea* kinship analysis based on ITS markers used a combination of the Maximum Likelihood (ML), Neighbour-Joining (NJ), and Maximum Parsimony (MP) methods. The results obtained through these three analyses were the same, producing groupings from the same branch and separating from outgroups (Figure 3). Therefore, one can conclude that the *Baccaurea* grouping formed was monophyletic. The cladogram of the kinship analysis formed two main groups with a bootstrap value of 100%-99%-100% at ML/NJ/MP, namely, Clade I and Clade II.

Species	1	2	2	4	4	5	5	6	6	6	7	8	8	8	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	2	5	7	6	7	0	7	1	2	3	7	0	2	4	4	3	5	9	4	1	2	6	9	9	6	2	3	7	8	
B_motleyana	C	T	A	C	C	T	G	C	G	G	T	G	T	G	C	A	A	A	C	G	G	C	T	C	G	C	C	A	G	
B_lanceolata	.	.	T	.	T	.	T	.	A	.	.	.	A	T	.	G	.	.	T	.	R	.	.	.	.	.	.	G	.	
Baccaurea_polyneura	.	.	.	.	T	.	.	T	A	A	.	A	.	.	G	G	.	.	.	.	A	G	.	T	C	T	.	G	.	
B_racemosa	.	.	.	T	T	.	.	.	A	C	C	.	C	T	.	G	C	C	.	.	.	.	C	.	.	.	A	G	.	
B_sumatrana	.	C	T	.	T	.	.	.	A	.	.	.	.	T	.	G	.	.	.	A	A	-	.	.	.	.	.	G	.	
B_deflexa	A	.	.	.	.	C	.	.	A	A	C	A	.	.	.	G	.	.	.	.	.	G	.	T	.	.	.	G	A	
Baccaurea_lanceolata_OK052881.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Species	2	2	2	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5
	0	0	2	4	6	1	1	1	2	2	2	3	3	4	6	6	7	7	0	0	0	1	3	3	4	6	7	7
	0	9	1	9	0	0	5	8	3	4	5	6	0	4	4	4	8	0	4	5	6	9	6	1	9	8	3	4	7	
B_motleyana	C	T	C	C	C	T	C	A	A	T	T	-	G	A	A	T	G	A	C	A	A	C	G	G	C	C	T	A	A	
B_lanceolata	.	.	.	.	T	.	.	.	.	.	.	-	.	C	.	.	A	.	.	.	.	.	A	A	T	T	C	.	.	
Baccaurea_polyneura	T	.	.	.	.	.	T	C	-	.	-	C	.	-	.	.	A	G	T	G	.	.	.	A	T	.	.	.	.	
B_racemosa	.	.	.	A	.	.	.	.	.	.	.	T	.	C	.	C	A	.	.	.	G	.	.	A	T	.	.	.	.	
B_sumatrana	.	C	T	.	T	A	.	.	.	.	C	-	T	C	G	.	A	.	.	G	.	.	.	A	T	.	.	.	.	
B_deflexa	T	C	.	T	.	.	.	C	T	C	.	-	.	C	.	.	A	G	.	.	.	A	.	A	T	.	.	G	.	
Baccaurea_lanceolata_OK052881.1	-	-	-	.	.	.	.	.	.	.	.	-	.	C	.	.	A	.	.	.	.	.	A	A	T	T	.	.	G	

Species	5	5	5	5	5	6	6	6	6	6	6
	8	8	9	9	9	0	0	0	0	1	1
	1	2	4	5	6	0	1	4	8	0	9
B_motleyana	T	G	C	G	T	A	C	C	T	C	A
B_lanceolata	.	.	.	T	.	.	.	.	.	.	.
Baccaurea_polyneura	C	.	G	.	C	.	.	G	C	T	.
B_racemosa	.	A	.	.	C	.	.	.	.	.	.
B_sumatrana	.	.	.	.	.	.	T	.	.	.	.
B_deflexa	.	.	G	.	C	.	.	.	.	.	C
Baccaurea_lanceolata_OK052881.1	.	.	.	A	A	G	.	.	.	.	.

Figure 2. Sequence of different base positions in *Baccaurea* collected based on ITS markers.

Table 4. Differences in *Baccaurea* bases collected based on the ITS marker.

No.	Species	1	2	3	4	5	6	7
1.	*B. lanceolata* (NCBI)							
2.	*B. deflexa*	15						
3.	*B. lanceolata*	6	31					
4.	*B. motleyana*	10	27	18				
5.	*B. polyneura*	16	23	30	28			
6.	*B. racemosa*	10	29	21	22	33		
7.	*B. sumatrana*	13	32	17	22	29	26	

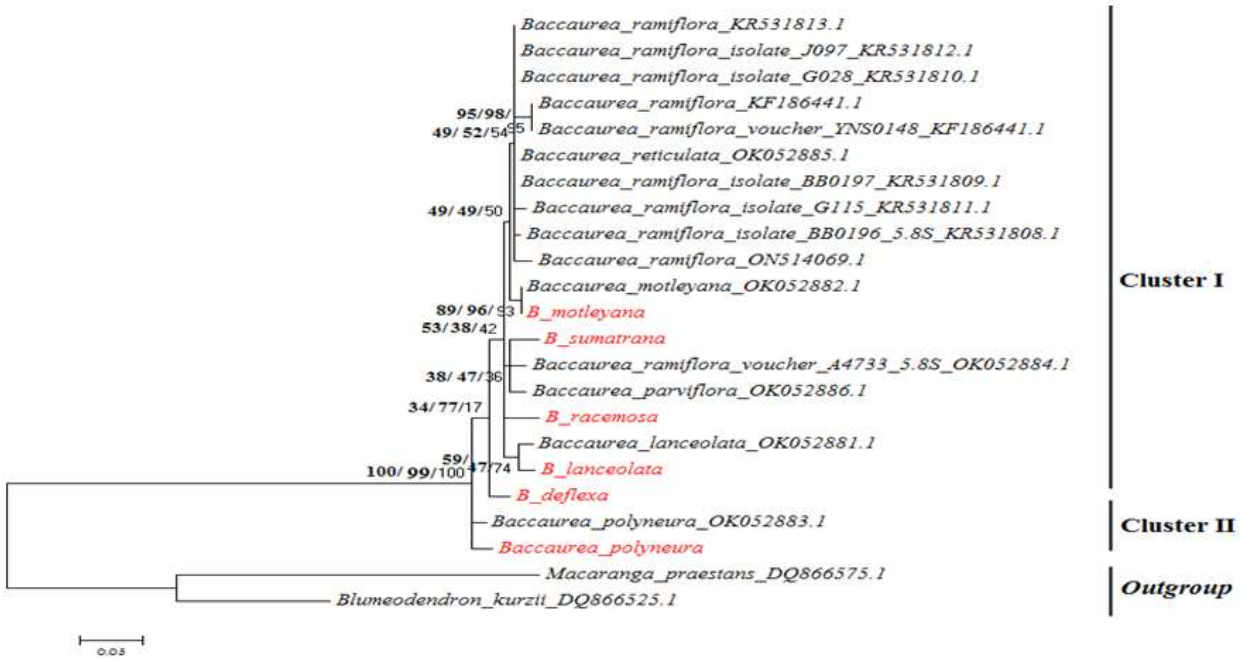

Figure 3. Cladogram of *Baccaurea* kinship relationships collected based on ITS markers (NJ/MP/ML). The species name in red is *Baccaurea* that was collected in this study.

Clade-I consists of 19 species, while Clade II consists of only two species, *B. polyneura* (NCBI) and *B. polyneura*.

The grouping formation of these two clades was due to differences in bases and genetic distance from the entire sequence. The nature of the phylogenetic tree based on molecular markers can provide more accurate classification results than using morphological character analysis, although the bootstrap value obtained was low (Chatrou *et al.,* 2012). Clade I included the samples of *B. motleyana*, *B. sumatrana, B. racemosa, B. lanceolata,* and *B. deflexa. B. motleyana* and *B. lanceolata* have sister taxa with sequences from the gene bank. Their branching also attained support from high bootstrap values (89%-96%-93% and 59%-47%-74%). The genetic distance between the two was 0.03 (3%). For *B. sumatrana*, it formed a subclade with *B. ramiflora* and *B. parviflora* with a bootstrap value of 38%-47%-36% and a genetic distance range that separates the three at 0.03 (3%). Meanwhile, *B. deflexa* formed its branch in Clade I, supported by a bootstrap value of 53%-38%-42%. The difference in the genetic distance range of *B. deflexa* with the four *Baccaurea* species in the collection contained in Clade I ranges from 0.04 to 0.05 (4%–5%).

Furthermore, in Clade II, viz., *B. polyneura* (NCBI) and *B. polyneura*, separated from Clade I with a bootstrap value of 100%-99%-100%, these two species formed a single branch. The genetic distance between *B. polyneura* (NCBI) and *B. polyneura* was 0.02 (2%). Separating these Clade-II species from Clade I referred to numerous nucleotide base differences and a long genetic distance. In the number of bases, the range of differences between *B. polyneura* and the type of sample collection in Clade I was 23–33. The range of difference in genetic distance between *B. polyneura* from the type of sample collection in Clade I was 0.04–0.07 (4%–7%). In addition to the difference in the number of bases and the long genetic distance of the separation of *B. polyneura* with the type of sample collection in Clade I, different morphological appearances supported these. For example, *B. polyneura* is unique, and the fruit skin is hollow and easy to open.

Moreover, the morphological character distinguishing the species *B. polyneura* and *Baccaurea* in Clade I depended on the upper and lower leaf blade surfaces. *B. polyneura* has a sparse, hairy to densely hairy upper leaf blade surface, while the *Baccaurea* in Clade I have a bare upper leaf blade surface. For the character of the lower leaf blade surface, *B. polyneura* has a sparsely hairy leaf surface, while the *Baccaurea* in Clade I have a bare to hairy leaf blade surface. Overall, the results of the species *Baccaurea* cladogram formed based on ITS markers have a high ability to distinguish up to the species level. Genetic variation produced by ITS markers has low homology, and it was good to use in phylogenetic analysis up to the species level. According to Seprianto (2017), the ITS region was easy to change or mutate; hence, the species have different characteristics. Sources of genetic variation include mutation, migration, and recombination (Doyle and Griffiths, 2000; Asra *et al*., 2018). The results obtained through the phylogenetic tree were similar to past research of phylogeny analysis of 43 *Baccaurea* based on morphological markers, forming a monophyletic phylogenetic tree (Haegens, 2000). The members of the monophyletic group reached a closely related classification because the members inherit the same genetic and biochemical attributes; therefore, the identification process and species differences were accurate (Rahayu and Nugroho, 2015).

CONCLUSIONS

The sequence characteristics of six *Baccaurea* species using ITS markers have a low level of genetic variation among the species, such as the range of genetic distances (1%–5%), conservative characters (549 bp), and informative characters (69 bp) in West Sumatra, Indonesia. The phylogenetic analysis of *Baccaurea* with its relatives illustrated the rapid rate of evolution, with the collected *Baccaurea* forming two clades, namely, Clade - I and Clade II, however, separating from the outgroup. The clades formed were monophyletic with bootstrap values of 100%-99%-100%. Based on the results, a recommendation to conduct further research is necessary to explore *Baccaurea* in the coastal areas of West Sumatra Island, Indonesia.

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