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Research Article

Assessment of Durian Diversity and Its Wild Relatives (*Durio* spp.) Based on Leaf Morphology and Molecular Marker

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Abstract: Durian (*Durio* spp.) is native to Southeast Asia and has potential for development. However, some species are threatened due to deforestation and extensive land conversion. This study aimed to determine the genetic diversity and relationships of durian and wild relatives (*Durio* spp.) on the Indonesia Island Borneo using a leaf morphology and DNA barcoding (*mat*K) marker. In this study, 15 durian samples from this region were used, excluding 'Monthong' (*Durio zibethinus*) and 'Bengang' (*Neesia strigosa*) as the outgroups from the GenBank database. The leaf morphology was analyzed descriptively, whereas the genetic diversity was by the nucleotide diversity index (π%). The relationship of durians was revealed by the maximum likelihood (ML) method and examined with the bootstrap statistics for 1000 replicates, also confirmed by the PCA (principal component analysis). Based on the leaf morphology, the durians are divided into five forms, i.e., obovate-lanceolate, elliptic, ovate, oblong, and linear-oblong. 'Pampaken' and 'Pampaken Burung Kecil' indicated the earliest form (obovate-lanceolate), whereas the linear-oblong was by 'Kamundai.' Following the molecular marker, it was seen that the durians have low genetic diversity (π%) with only 0.015. However, phylogenetically, the durians were separated into four similar clades or groups for ML and PCA. In this instance, it has appeared that most of the durians evaluated in the current study have close relationships, except for the taxa with the farthest relationship. The results provide valuable information for the local and global durian conservation mission, including future breeding programs.

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1. Introduction

Durian, belonging to the genus *Durio*, is essential for economic and ecological purposes (Aziz and Jalil, 2019). For example, nine durian species, e.g., *Durio lowianus*, *D. graveolens*, *D. kutejensis*, *D. oxleyanus*, *D. testudinarum*, *D. grandiflorus*, *D. dulcis*, *D. excelcus*, and *D. zibethinus*, are edible

fruits with various tastes (Aziz and Jalil, 2019). The *D. zibethinus* is an essential commodity for export demands today (Cheon et al., 2017). In 2020, Indonesia, for example, one of the highest world durian producers, was prosperous in exporting this commodity to many other nations, in Asia (China, Hongkong, Singapore, and Malaysia), Middle Eastern (Saudi Arabia and Qatar) and Europe (Netherlands, Portugal, and Russia) with a total transaction of US$ 232,000 (Rizaty, 2021). In contrast to edible fruit, 14 species of durian also generate wood that can be useful as interior materials. It can also be used for medicinal and pharmaceutical purposes, particularly in malaria treatment (Feng et al., 2016).

Ecologically, this germplasm is native to Southeast Asia, particularly Indonesia and Malaysia (Mursyidin and Daryono, 2016). In Indonesia, 18 of 27 durian species worldwide have been reported, and most are recognized as endemic. Importantly, Borneo Island is part of the Indonesian region, which has a tremendous genetic diversity of durian (Uji, 2005). Indonesia is also known as the center of the world's durian diversity. However, most of them are threatened. Deforestation and extensive land conversion are the main factors causing the problem (Wilcove et al., 2013). The International Union for Conservation of Nature or IUCN (2023) reported several threatened durians, e.g., *D. acutifolius*, *D. dulcis*, *D. grandiflorus*, *D. kutejensis*, *D. testudinarium*, and *D. lanceolatus*.

Consequently, preservation or conservation, including cultivation and breeding tasks, is necessary. In general, conservation is an activity directed at saving and preserving the existence of threatened species (Wintle et al., 2019). Meanwhile, cultivation and breeding activities aim to explore and utilize functional genes to develop new superior cultivars in the future (van Huylenbroeck, 2018). According to Mursyidin and Daryono (2016), most germplasm (wild durian relatives) has beneficial traits or genes that aid in the preservation and breeding efforts, such as a high tolerance to environmental stresses and specific (patch canker) diseases; their existence has been disturbed. In other words, the collection and identification of germplasm are critical for facilitating future durian preservation and breeding tasks (Acquaah, 2015).

So far, genetic characterization of durian germplasm is commonly done using morphological markers (Mursyidin and Daryono, 2016; Mursyidin, 2023). While this marker has many shortcomings, such as being time-consuming and strongly impacted by environmental influences, including multiple gene inheritance (Wu et al., 2021), it is still commonly used to evaluate germplasm because it is strongly related to gene expression results (Mursyidin and Daryono, 2016). Recently, the genetic diversity and relations of durians have been studied using sequencing-based DNA barcoding markers (Mursyidin and Daryono, 2016; Santoso et al., 2017). According to Lee et al. (2017), these markers often exhibit great accuracy and repeatability. Hence, its application is faster, more effective, and more efficient, which can complement the available morphological data.

Our study is focused on determining the genetic diversity and relationship of durian and wild relatives (*Durio* spp.) from Borneo Island, Indonesia, by the leaf morphology and a DNA barcoding (*mat*K) marker. According to Barthet et al. (2020), this molecular marker has a high phylogenetic signature and a moderately rapid mutation rate, which is usable in determining the evolutionary relationships between different plant taxa. Thus, the results of this study will help assist durian preservation and breeding efforts in the future, both locally and globally.

2. Material and Methods

2.1. Plant materials

For the study, fifteen durian samples (*Durio* spp.) were collected from Borneo Island, Indonesia, using the purposive sampling method (Figure 1). The two others, ‘Monthong’ (*Durio zibethinus*) and ‘Bengang’ (*Neesia strigosa*), were collected from the GenBank and used as outgroups (Table 1). All were prepared for morphological and molecular assays.

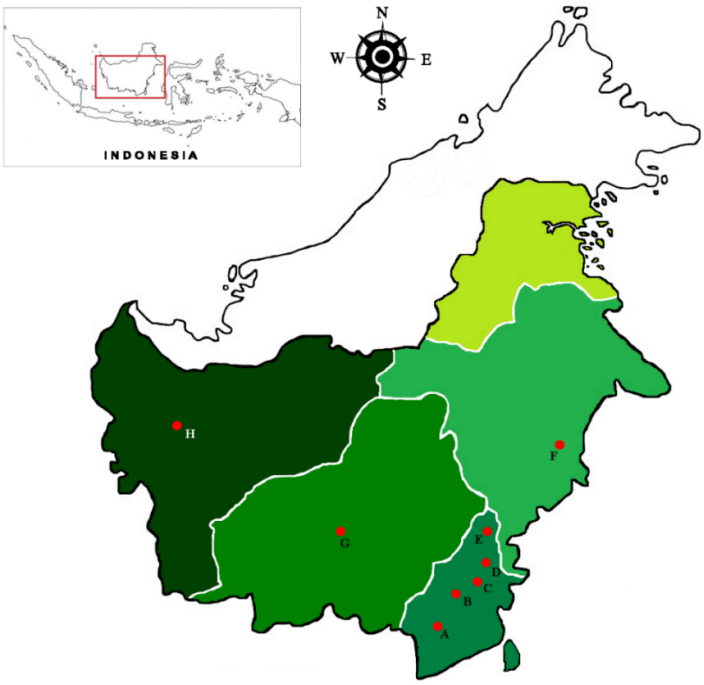

Figure 1. Sampling locations on the Borneo Island of Indonesia (red marks), where fifteen durians (*Durio* spp.) samples were collected and used in this study, i.e., A=Banjar, South Kalimantan; B = South Hulu Sungai, South Kalimantan; C = Central Hulu Sungai, South Kalimantan; D = Balangan, South Kalimantan; E = Tabalong, South Kalimantan; F = Kutai, East Kalimantan; G = Katingan, Central Kalimantan; H = Sekadau, West Kalimantan (see Table 1 for details).

Table 1. Fifteen samples of durian (*Durio* spp.) were used in the study, including their province origin

Name of species	Name of accession	Code	Province origin
D. kutejensis	'Kalih Haliyang'	4	Balangan, South Kalimantan
	'Pampaken Burung Kecil'	1	South Hulu Sungai, South Kalimantan
	'Pampaken'	8	Tabalong, South Kalimantan
	'Kamundai'	14	Tabalong, South Kalimantan
	'Lai Lidung'	2	Kutai, East Kalimantan
D. lowianus	'Malutu'	6	South Hulu Sungai, South Kalimantan
	'Lahung Alang'	10	Balangan, South Kalimantan
D. oxleyanus	'Maharawin Hamak'	9	Banjar, South Kalimantan
	'Karantungan Besar'	12	Katingan, Central Kalimantan
D. excelsus	'Burung Besar'	7	Balangan, South Kalimantan
	'Mantuala Batu Hayam'	13	Central Hulu Sungai, South Kalimantan
D. testudinarium	'Kura-Kura'	15	Sekadau, West Kalimantan
D. zibethinus	'Likol'	5	Tabalong, South Kalimantan
	'Sahang'	3	Tabalong, South Kalimantan
	'Si Japang'	11	Banjar, South Kalimantan
	'Monthong*'	-	Thailand
Neesia strigosa	Bengang*	-	USA

Note: *outgroup, obtained from the GenBank database with the accession numbers MT321069.1 and AY321189.1, respectively.

2.2. Morphological assay

Morphological characteristics of leaves were observed according to durian (*Durio zibethinus* Murr.) descriptors (Bioversity International, 2007).

2.3. Molecular assay

A DNA isolation kit (GP100, Geneaid Biotech Ltd.) was used to prepare the young leaf samples of durian. The quantification of DNAs was employed using the UV-VIS spectrophotometry method (GE Healthcare, UK). Amplification of DNA was employed using a Multigene Optimax PCR (Labnet International Inc., USA) and the universal primers of *mat*K: *mat*K-F (5'-CGTACAGTACTTTTGTGTTTACGAG-3'); *mat*K-R (5'-ACCCAGTCCATCTGGAAATCTTGG TTC-3') (Le et al., 2020). Amplification (PCR) was done with 25 µL of a total volume reaction, consisting of MyTaq HS Red PCR Mix (22 µL), ten µM primers (2.0 µL), ten ng DNA template (1 µL). The reaction was programmed by initial denaturation (94 ºC, 5 min); denaturation (94 ºC, 30 sec), annealing (48 ºC, 30 sec), extension (72 ºC, 45 sec) for 35 cycles; and final extension (72 ºC, 7 min) (Mursyidin et al., 2021). The DNA targets were examined using a UV transilluminator after being separated using 2% agarose gel electrophoresis in a 1X TBE buffer solution and GelRed staining (SMOBiO, Taiwan). At 1st Base Ltd. in Malaysia, it was subsequently purified and bidirectionally sequenced using the Sanger method.

2.4. Data analysis

The forward and reverse sequence of *mat*K of durians were assembled and analyzed manually using the MEGA11 to generate consensus (Tamura et al., 2021). All were then aligned using ClustalW (Thompson et al., 2002). The genetic diversity was measured by the nucleotide diversity index (π%), using the criteria of low (0.1 - 0.4), moderate (0.5 - 0.7), and high (0.8 - 2) (Nei and Li, 1979). The genetic relationship was employed by ML (maximum likelihood), using MEGA11 (Tamura et al., 2021), and the PCA (principal component analysis), with the assistance of MVSP ver. 3.1 (Kovach, 1999). The bootstrap methodology with 1000 replicates was used to examine the phylogenetic trees (Lemey et al., 2009).

3. Results and Discussion

3.1. Leaf diversity

The durians show different characteristics of leaf morphology, both in shape and size (Figure 2). The complete traits of the leaves can be seen in Table 3, whereas their length and width are presented in Table 2. Based on its size (Table 2), 'Kura-Kura' (*D. testudinarum*) is a durian sample that has the lengthiest leaf size (23.3 cm), whereas the 'Pampaken Burung Kecil' was the shortest with 9.3 cm. In this study, 'Pampaken' (*D. kutejensis*) has a leaf length of 16.0 cm. In contrast, the shortest width (2.6 cm) was pointed out by 'Pampaken Burung Kecil,' and the most comprehensive (8,0 cm) was by 'Mantuala Batu Hayam' and 'Kamundai.'

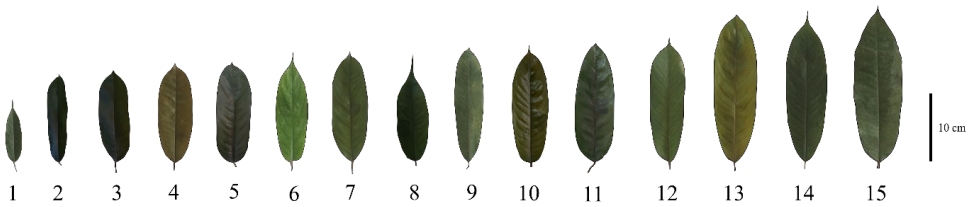

Figure 2. The leaf morphology of durian and its wild relatives (*Durio* spp.) samples used in this study shows differences in shape and size. The name of each sample is provided in Table 2.

Based on their shape (see Table 3), the durians are divided into five forms, i.e., obovate-lanceolate, elliptic, ovate, oblong, and linear-oblong. 'Pampaken Burung Kecil' and 'Pampaken' indicated the obovate-lanceolate form, whereas the linear-oblong was by 'Kamundai.' Based on the shape of the leaf apex, the acuminate, long-acuminate, and cuspidate were presented. In this case, the latest (cuspidate) by 'Pampaken.' Following the leaf base trait, round and cuneate are the dominant forms, whereas the two others (acute and obtuse) are not, as shown by 'Kalih Haliyang,' 'Kamundai,' and 'Si Japang.' Concerning the adaxial (upper) and abaxial (lower) leaf surfaces, durian was divided

into smooth-shiny, slippery, slightly rough, and rough. For the last character (leaf margin), most durian leaves are entire, except 'Kamundai', which is undulate.

Table 2. The leaf length and width of fifteen durian (*Durio* spp.) samples used in the study

Local name	Code	Species	leaf length (cm)	leaf width (cm)
'Pampaken Burung Kecil'	1	*D. kutejensis*	9.3	2.6
'Lai Lidung'	2	*D. kutejensis*	13.0	4.1
'Sahang'	3	*D. zibethinus*	13.4	3.5
'Kalih Haliyang'	4	*D. kutejensis*	14.3	4.2
'Likol'	5	*D. zibethinus*	14.8	4.4
'Malutu'	6	*D. lowianus*	15.7	4.1
'Burung Besar'	7	*D. excelsus*	16.0	5.0
'Pampaken'	8	*D. kutejensis*	16.0	4.7
'Maharawin Hamak'	9	*D. oxleyanus*	16.5	4.5
'Lahung Alang'	10	*D. lowianus*	17.2	5.6
'Si Japang'	11	*D. zibethinus*	17.4	6.2
'Karantungan Besar'	12	*D. oxleyanus*	18.5	5.6
'Mantuala Batu Hayam'	13	*D. excelsus*	21.8	8.0
'Kamundai'	14	*D. kutejensis*	22.1	8.0
'Kura-Kura'	15	*D. testudinarium*	23.3	7.0

Table 3. Morphological characteristics of durian (*Durio* spp.) leaves

Local name	Code	Leaf blade shape	Leaf apex shape	Leaf base shape	Adaxial (upper) leaf surface	Abaxial (lower) leaf surface	Leaf margin
'Pampaken Burung Kecil'	1.	Obovate-lanceolate	Acuminate	Cuneate	Smoot shiny	Slightly rough	Entire
'Lai Lidung'	2.	Elliptic	Long acuminate	Cuneate	Smoot shiny	Slippery	Entire
'Sahang'	3.	Ovate	Acuminate	Round	Smoot shiny	Rough	Entire
'Kalih Haliyang'	4.	Oblong	Acuminate	Acute	Slippery	Rough	Entire
'Likol'	5.	Ovate	Acuminate	Round	Smoot shiny	Smoot shiny	Entire
'Malutu'	6.	Oblong	Long acuminate	Round	Smoot shiny	Rough	Entire
'Burung Besar'	7.	Elliptic	Long acuminate	Round	Smoot shiny	Smoot shiny	Entire
'Pampaken'	8.	Obovate-lanceolate	Cuspidate	Cuneate	Slightly rough	Slippery	Entire
'Maharawin Hamak'	9.	Oblong	Long acuminate	Cuneate	Smoot shiny	Smoot shiny	Entire
'Lahung Alang'	10.	Ovate	Acuminate	Round	Smoot shiny	Smoot shiny	Entire
'Si Japang'	11.	Ovate	Acuminate	Obtuse	Smoot shiny	Smoot shiny	Entire
'Karantungan Besar'	12.	Oblong	Long acuminate	Round	Smoot shiny	Rough	Entire
'Mantuala Batu Hayam'	13.	Oblong	Acuminate	Cuneate	Smoot shiny	Slightly rough	Entire
'Kamundai'	14.	Linear-oblong	Acuminate	Acute	Smoot shiny	Rough	Undulate
'Kura-Kura'	15.	Ovate	Acuminate	Round	Smoot shiny	Slightly rough	Entire

According to Dkhar and Pareek (2014), the emergence of leaf diversity in plants may be caused by genetic and environmental factors. For example, leaf size will shrink as altitude decreases, rainfall increases, and soil nutrient content increases (Ke et al., 2022). Light and temperature also sometimes affect the shape and size of the leaves, although this requires further explanation (Dkhar and Pareek, 2014). Genetically, gene regulatory networks (GRNs) and signaling pathways play an essential role in bringing out the diversity of leaf shapes (Dkhar and Pareek, 2014), for example, the *KANADI* gene family (Zumajo-Cardona et al., 2019).

Referring to Tsukaya (2017), the diversity of leaves in shape and size is closely related to their role as the location of photosynthesis. Photosynthesis requires efficient absorption of light energy and the exchange of CO_2 for O_2, as well as water content and temperature. Consequently, the most high-yielding form can differ according to environmental conditions, and leaf shape also varies (Tsukaya, 2017).

3.2. Sequence characteristics, genetic diversity, and phylogenetic relationship

In this study, we used the *maturase* K (*mat*K) gene, a part of the chloroplast genome (cpDNA), to determine the genetic identity, including diversity and relationship, of this germplasm from Borneo Island, Indonesia. Conceptually, *mat*K is an intron-encoded gene in chloroplast with unique features. It has a variety of lengths, both partial and complete regions. According to Mustafa et al. (2018), the total length of this sequence is 1,536 bp. Based on Table 4, the part of *mat*K of durian germplasm ranged from 829 to 865 bp.

Table 4. Genetic information for the *mat*K sequence of durian (*Durio* spp.) germplasm

Parameter	***mat*K**
Range of sequence (bp)	829 to 865
Total length sequence observed (bp)	810
Parsimony informative sites (Pi)	18
Singleton sites (S)	61
Variable sites (V)	80
Insertion-deletion (indels) sites	20
Transition/transversion (Ti/Tv) bias value (*R*)	0.94
Nucleotide diversity (π%)	0.015
Guanine-cytosine/GC content (%)	33.18
Maximum likelihood value (ln*L*)	-1736.270
Akaike information criterion (AICc)	3532.677
Bayesian information criterion (BIC)	3758.150

However, it is different with a similar gene from several other plants, mainly Angiosperms, e.g., *Ficus* (Li et al., 2012), *Lycopersicum* (Căprar et al., 2017), *Tetrastigma* (Habib et al., 2017), *Theobroma* (Immanissa et al., 2020), and *Zanthoxylum* (Suriani et al., 2021), with a range of 830 to 857 bp (Tosh et al., 2016). The different lengths of *mat*K in germplasm are related to substitution and single indels (insertions-deletions) mutations (Chen and Shiau, 2015). In this study, 20 indels are present in the *mat*K sequence of durians, including transversion and transition (Table 4). In this case, the last (12) is higher than the transversion (6.5) (Table 5).

Table 5. The nucleotide substitution pattern on the *mat*K sequence of durians (*Durio* spp.)

Nucleotide	Code	A	T	C	G
Adenine	A	-	6.50[a]	6.50[a]	12.00[b]
Thymine	T	6.50[a]	-	12.00[b]	6.50[a]
Cytosine	C	6.50[a]	12.00[b]	-	6.50[a]
Guanine	G	12.00[b]	6.50[a]	6.50[a]	-

Note: a = transversions; b = transition

Following Aloqalaa et al. (2019), transversion occurs in this sequence more frequently than transversions. Therefore, it is typical in the evolution of molecules (Stoltzfus and Norris, 2016). As a part of the cpDNA genome, *mat*K has a relatively high mutation rate (Kar et al., 2015; Barthet et al.,

2020). However, the rhythm and type of its evolution differ from one another. According to Kar et al. (2015), the mutation rate of *mat*K is three times higher than the *rbc*L, so it is called a fast- or rapidly-evolving gene.

Referring to Suriani et al. (2021), a long-lasting single nucleotide polymorphism causes genetic variation in cpDNA, including *mat*K. As a result, polymorphism has generated a strong phylogenetic signal that may be used to resolve evolutionary connections among plants at all taxonomic levels (Kar et al., 2015). Further, phylogenetically informative features are changeable and not the result of homoplasy in phylogenetic analysis (parallel evolution). These phylogenetically informative features are not so variable that they can not be aligned across taxonomic levels. According to Kar et al.(2015), in locations with minimal variability and conserved sequence, *mat*K possesses many critical features that can be aligned to demonstrate evolutionary links from species to divisional or even higher taxonomic levels.

The *mat*K sequence of durians has a low level of polymorphism, with only 80 variable sites of 810 bp (see Table 4). As a result, this germplasm has a low-level nucleotide diversity ($\pi\% = 0.015$). Natural selection and founder effects may affect this genetic diversity, including genetic isolation and inbreeding (Gao et al., 2017). In this case, inbreeding is the most probable because it may reduce genetic diversity (Mursyidin et al., 2017) and decrease disease resistance and resilience to extreme conditions or environmental depression (Lloyd et al., 2016). In addition, a few samples used in this study may also generate a low level of genetic diversity. Then, for further studies, it is suggested that we use a larger sample size to confirm our results.

Moreover, natural selection and evolutionary processes require genetic variation to produce a core population (Govindaraj et al., 2015). It is, therefore, genetic variation is a crucial factor in the evolution or necessity of upcoming adaptive modifications. Consequently, genetic variation significantly affects conservation-related tasks (Lloyd et al., 2016). To increase the efficacy and efficiency of this attempt, it is imperative to comprehend genetic variation in this context. Since large-scale population genetic studies are the only way to address certain conservation elements, like the loss of gene diversity (Luan et al., 2006).

Plant breeding can benefit from genetic diversity information as well. To develop new, superior cultivars with desired traits or associated with a variety of abiotic and biotic stress tolerance, breeders, in this case, use all available plant genetic resources or genetic diversity (Swarup et al., 2021). Furthermore, it increases the genetic diversity of the population that will follow to adapt to future changes. Stated differently, only the present population needs a significant degree of gene variety to react rapidly to environmental changes (Lloyd et al., 2016).

Apart from the genetic diversity, the threatened durians of Borneo Island, Indonesia, showed unique phylogenetic relationships. This germplasm was grouped into four clades following ML (Figure 3) and PCA (Figure 4). Further, the genetic divergence revealed that 20 pairs of durian have the closest relation. At the same time, the farthest is shown by four durians, e.g., 'Kura-Kura' (*D. testudinarium*) and 'Burung Besar' (*D. excelsus*) (Figure 5).

In this instance, the phylogenetic trees showed a monophyletic divergence of germplasm. Slobodan and Pastana (2020) define it as a group of taxa descended from a single taxon or a common ancestor. It is noteworthy that two unique durian germplasms from Borneo Island, Indonesia, named 'Likol' (*D. zibethinus*) and 'Lai Lidung' (*D. kutejensis*), are closely linked to 'Monthong' as an outgroup (Figure 5). Because the outgroup can affect ingroup relationships and polarizing characteristics when determining the root's location, the outgroup is crucial in phylogenetic analyses (Wilberg, 2015).

However, future conservation efforts will benefit from this phylogenetic information (Flint-Garcia, 2013), mainly when calculating the genetic diversity of the progeny (Acquaah, 2015). Turner-Hissong et al. (2020) state that a wide range of genetic variants may be present in the offspring of individuals with distant ties who cross. In contrast, when individuals have a closely related cross, their progeny may be homozygous genetically. Once more, this knowledge is helpful for future durian management, preservation, and breeding efforts (Fernández-García, 2017).

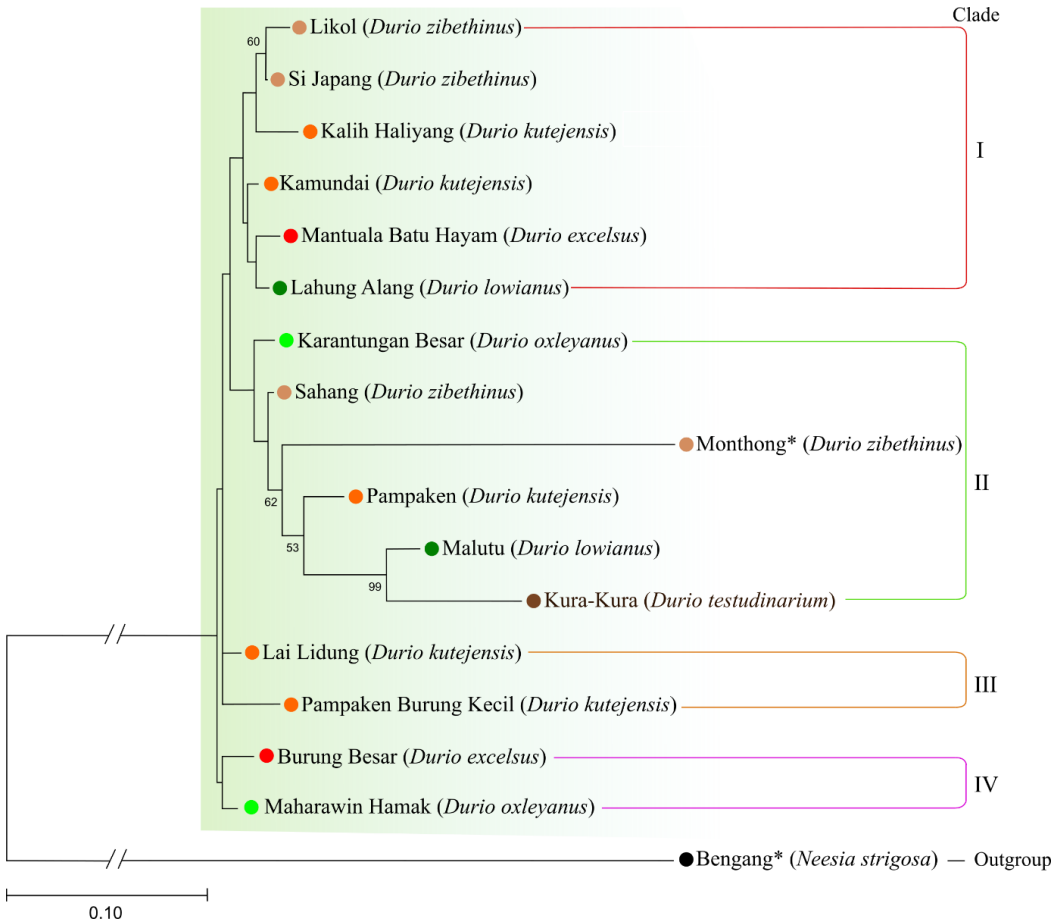

Figure 3. The genetic relationship of durian and its wild relatives (*Durio* spp.) from Borneo Island, Indonesia, were grouped into four clades based on the ML (maximum likelihood) with a bootstrap of 1,000 replicates.

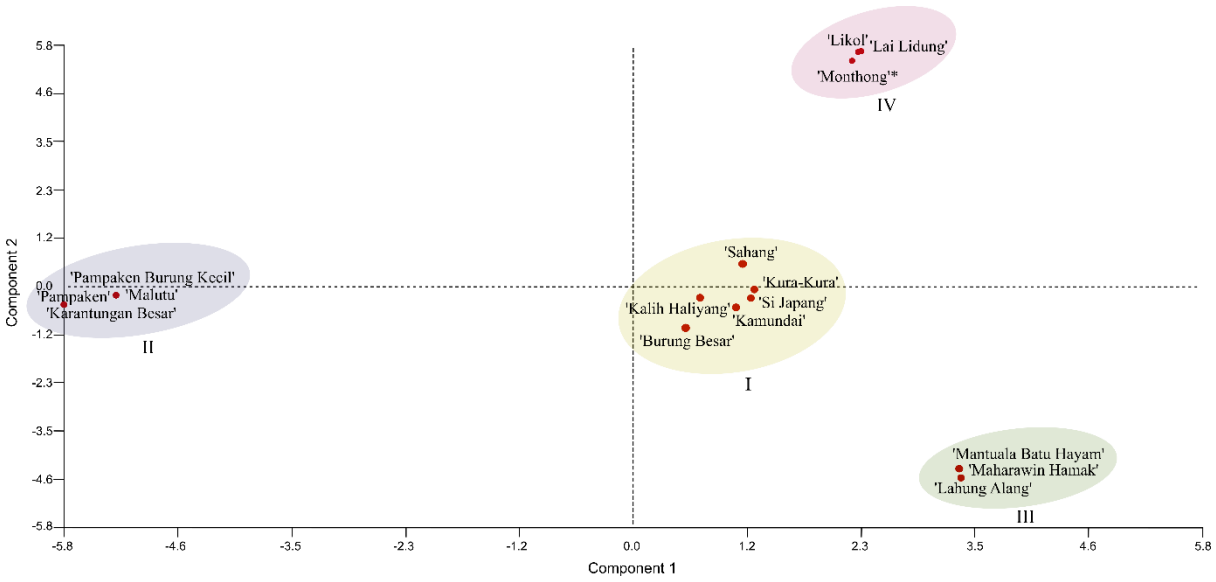

Figure 4. The durians (*Durio* spp.) from Borneo Island of Indonesia were grouped into four clusters based on the principal component analysis (PCA).

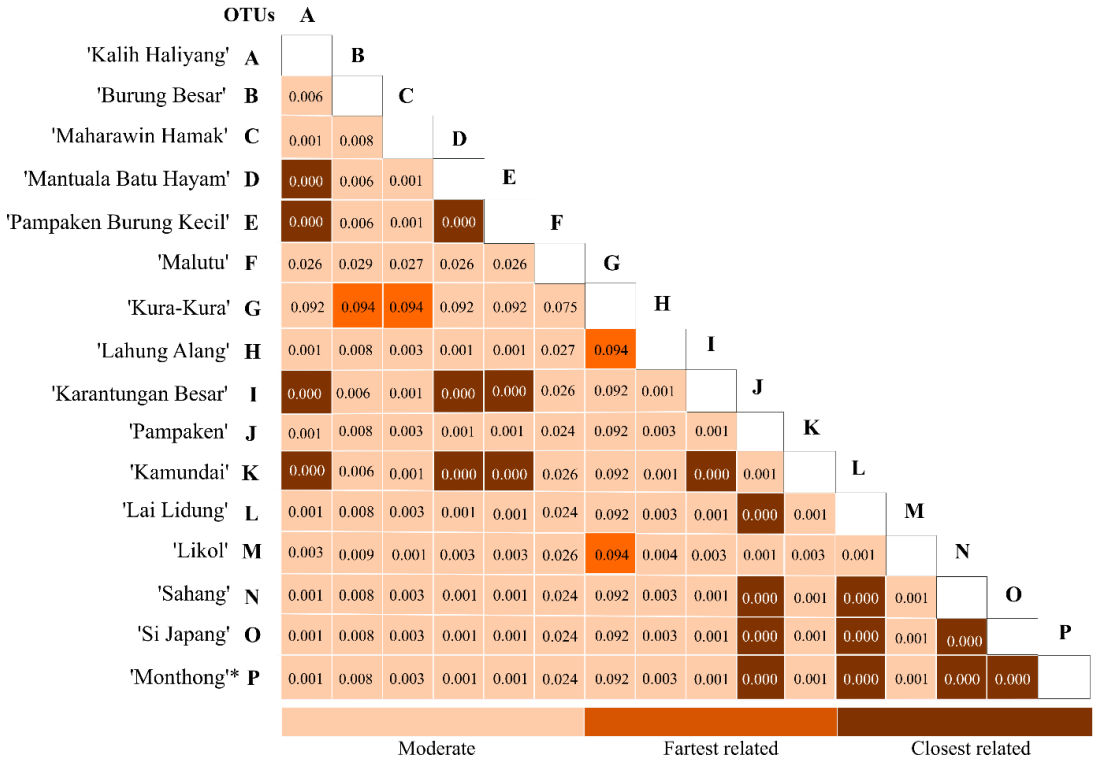

Figure 5. Genetic differentiation among durian (*Durio* spp.) accessions, revealed by maximum likelihood model. OTUs= Operational taxonomic units; * = Outgroup.

Conclusion

Based on the leaf morphology, the durians are divided into five forms, i.e., obovate-lanceolate, elliptic, ovate, oblong, and linear-oblong. 'Pampaken Burung Kecil' and 'Pampaken' indicate the obovate-lanceolate, while the linear-oblong is by 'Kamundai.' Molecularly, the durian germplasm of Borneo Island, Indonesia, has a low-level nucleotide diversity ($\pi\% = 0.015$). The phylogenetic analysis revealed that the durians are grouped into four clades, ML and PCA. In this case, most durian has the closest relationship, whereas the farthest by four durians, e.g., 'Kura-Kura' (*D. testudinarium*) with 'Burung Besar' (*D. excelsus*). Thus, this information is helpful for future durian preservation and breeding efforts.

Ethical Statement

Ethical approval is not required for this study because plant samples are collected freely and are not included as protected ones.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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Author Contributions

DHM and YAN conceptualized and designed the study. YAN is responsible for the sample collection and laboratory analysis conducted by MRF. DHM and MRF are involved in data analysis. DHM drafted the initial article. All authors finalized the article.

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