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GENETIC DIVERSITY AND PHYLOGENETIC RELATIONSHIP IN DURIAN KEBAU (*DURIO* SP.) USING THE ITS REGION ANALYSIS

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SUMMARY

Lahat Regency, South Sumatra, Indonesia, is home to diverse *Durio* populations, including the durian Kebau, which has received acclaim as a superior local cultivar. However, molecular data on this cultivar remain limited. This study aimed to assess the genetic diversity and phylogenetic relationships of durian Kebau compared with other Indonesian durian cultivars using the internal transcribed spacer (ITS) region analysis. The analyzed ITS sequences totaled 13, comprising newly generated sequences from Kebau and sequences retrieved from the NCBI database, including accessions of *Durio zibethinus* and *Durio* sp. Kura-Kura from West Kalimantan. Conducting multiple sequence alignment used ClustalW, with genetic diversity parameters calculated using DnaSP. A haplotype network construction resulted in using PopART, while phylogenetic relationships inferred utilized the maximum likelihood (ML) and neighbor-joining (NJ) methods implemented in MEGA X with 1,000 bootstrap replicates. The analysis revealed high genetic diversity, with pairwise distances ranging from 0.000 to 0.227, yielding 11 distinct haplotypes and 49 parsimony-informative sites. Phylogenetic trees showed the durian Kebau exhibited a close genetic relationship with the *D. zibethinus* cultivar Monthong and clustered with durians from West Kalimantan. These findings highlight the genetic richness of local durian germplasm and provide data for breeding programs and genetic resource conservation

Keywords: Durian germplasm, internal transcribed spacer (ITS), genetic diversity, genetic relationship, quantitative traits

Key findings: The study revealed the highest genetic diversity in Indonesian durian germplasm. The research also established that durian Kebau showed considerable genetic relationship with *Durio zibethinus* and durian Kura-Kura.

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INTRODUCTION

Durian (*Durio* spp.), a member of the family Malvaceae, is a higher plant known for its considerable species diversity, comprising approximately 27 species (Ketsa *et al.*, 2020). Durian is a tropical fruit known for its distinctive strong odor and spiky outer husk. Often called the 'king of fruits,' it is very popular in Southeast Asia and has a wide distribution across Asia (Thorogood *et al.*, 2022). Indonesia, recognized as the major center for durian diversity, hosts 20 species (Surya *et al.*, 2023). Despite its cultural and economic importance, limited molecular data exist for many local cultivars, including the certified superior Kebau cultivar from Lahat, South Sumatra, Indonesia—hindering targeted conservation and breeding efforts. However, molecular data on certified superior durian varieties like Kebau remain particularly scarce, especially those based on the internal transcribed spacer (ITS) region. Hence, the presented study aimed to assess the genetic variation and phylogenetic relationship of the Kebau cultivar using the ITS region in comparison with other Indonesian regional cultivars to address such a gap.

Durian holds considerable ecological and economic value (Khoo, 2024). Indonesia, being one of the world's leading producers, has been actively exporting durian to various countries (Ketsa *et al.*, 2020), with an export volume of 87.2 tons in 2024 (www.cnbcindonesia.com). Within Indonesia, South Sumatra Province is popular for its rich genetic diversity of local durian germplasm (Anggadhania *et al.*, 2023). Among these, Lahat Regency is also the prominent area known for durian production through a wide range of local cultivars. However, presently only five durian cultivars, viz., Bantal Mas, Burung Api, Linsing, Kebau, and Linar, have been certified by the Agriculture Department of the Indonesian Government (Herawati *et al.*, 2024).

Furthermore, several wild durian species are facing conservation threats, with some classified as vulnerable or near-threatened by the International Union for Conservation of Nature, or IUCN (Rahman,

2021; Sudarmono *et al.*, 2023). This emphasizes the urgency of conservation efforts through breeding and cultivation programs, which require genetically diverse germplasm (Swarup *et al.*, 2021; Salgotra and Chauhan, 2023).

In further supporting these breeding efforts, molecular markers provide rapid and reliable tools for characterization of the germplasm (Bunjkar *et al.*, 2024). Among them, the internal transcribed spacer (ITS) region has proven particularly effective for assessing genetic diversity in crop plants, including durian (Magandhi *et al.*, 2024). Located between ribosomal RNA genes within a precursor transcript, the ITS region evolves rapidly, making it suitable for evaluating genetic relationships at the genus, species, and subspecies levels (Letsiou *et al.*, 2024). With these characteristics, ITS proved to be widely utilized for assessing genetic relationships at the genus, species, and subspecies levels (Ahmed *et al.*, 2022).

Previous studies have utilized various molecular markers, with a notable focus on the ITS region, for assessing genetic diversity and phylogenetic relationships in Indonesian durian germplasm (Mursyidin *et al.*, 2023; Magandhi *et al.*, 2024). Therefore, based on the above discussion, the latest study aimed to carry out the ITS-based assessment of genetic variation and phylogenetic relationships of durian Kebau from Lahat, South Sumatra, in comparison with other regional cultivars in Indonesia. These findings will contribute to the expansion of the durian DNA database, support breeding initiatives, and strengthen the efforts in genetic resource conservation.

MATERIALS AND METHODS

Plant material

This study utilized leaf samples from the seed tree of the durian Kebau cultivar. It currently serves as the sole documented genetic source of this officially registered variety under the decree of the Indonesian Minister of Agriculture, Keputusan Menteri Pertanian Republik Indonesia No.

440/Kpts/PV.240/D/XII/2022, known as superior variety (Herawati *et al.*, 2024). The promising study aimed to analyze the genetic variation and phylogenetic relationships of durian Kebau using the internal transcribed spacer 1 (ITS1) and ITS4 regions, with primers 5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3', respectively. These primers have already been beneficial in phylogenetic and taxonomic studies due to their high effectiveness in species identification (Letsiou *et al.*, 2024). The ITS region has also had extensive applications in previous studies on the DNA barcoding of Indonesian durian germplasm (Mursyidin *et al.*, 2023; Magandhi *et al.*, 2024).

DNA isolation

Genomic DNA extraction from approximately 100 mg of fresh leaf tissue used a modified protocol of the Wizard® Genomic DNA Purification Kit (Promega). The DNA concentration, as measured, utilized a NanoDrop Lite Plus Spectrophotometer (Thermo Fisher). Following extraction, the DNA pellet reached resuspension in 50 µL of TE (Tris-EDTA) buffer. The DNA attained an initial storing at 4 °C for short-term use before subsequently preserving at -30°C for long-term storage.

PCR condition

The PCR reaction, as performed in a 25 µL volume, included 12.5 µL of MyTaq™ HS Red Mix (Bioline), 1 µM of each primer, 1 µL of DNA template (<250 ng), and 9.5 µL of nuclease-free water. Thermal cycling included an initial denaturation at 95 °C for 3 min, followed by 34 cycles of 95 °C for 30 s, 50 °C for 45 s, and 72 °C for 1 min, with a final extension at 72 °C for 6 min. Amplicons underwent analysis by electrophoresis on a 1% GelRed-stained agarose gel in 1X TAE buffer at 100 volts for 30 min. The gel visualization used the Gel Doc System (Bio-Rad), afterward sending the purified amplicons for Sanger sequencing to 1st Base, Singapore.

Data analysis

The durian Kebau sequence analysis was successful using 12 related DNA barcoding sequences from species (Table 1) obtained from the NCBI Gene Bank (<http://www.ncbi.nlm.nih.gov>) (Sayers *et al.*, 2020). The sequences' processing used BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and aligning with MultAlin (<http://multalin.toulouse.inra.fr/multalin/>). Genetic parameters, including polymorphic sites (S), haplotypes (H), haplotype diversity

Table 1. Durian specimens and accession numbers downloaded from the NCBI GenBank used in this study.

No.	Specimens	NCBI GenBank ITS region accession numbers
1	<i>Durio testudinarius</i>	OR593301.1
2	<i>Durio testudinarius</i>	OR593305.1
3	<i>Durio testudinarius</i>	OR593302.1
4	<i>Durio acutifolius</i>	OR631856.1
5	<i>Durio lanceolatus</i>	OR631859.1
6	<i>Durio zibethinus</i>	MW316599.1
7	<i>Durio zibethinus</i>	XM 022990025.1
8	<i>Durio zibethinus</i>	MF629776.1
9	<i>Durio oxyleyanus</i>	OR631860.1
10	<i>Durio graveolens</i>	OR631857.1
11	<i>Durio kutejensis</i>	OR631858.1
12	<i>Cavanillesia chicamochae</i>	KM245249.1

(Hd), nucleotide diversity (π), recombination rate (R), and Tajima's D, received calculations with DnaSP software. Phylogenetic trees generated employed the maximum likelihood (ML) and neighbor joining (NJ) methods (Zou *et al.*, 2024) in MEGA-XI software (Tamura *et al.*, 2021) with bootstrap support from 1,000 replicates (Goloboff *et al.*, 2022). The haplotype network analysis utilized POPART version 1.7. The heatmap generation was in RStudio using the pegas package (Paradis, 2023).

RESULTS AND DISCUSSION

Durian DNA barcoding profile

The nuclear internal transcribed spacer (ITS) region has a wide employment in phylogenetic and genetic diversity analyses due to the highest sequence variability and capacity to distinguish various species (Zeng *et al.*, 2024). The ITS region, recognized as one of the most informative genomic markers for assessing genetic relationships within plant genera, has offered valuable insights, particularly in taxonomic studies of complex groups such as *Durio* (Santoso and Pancoro, 2020).

In the presented study, the ITS region amplification in durian Kebau produced a DNA fragment measuring 561 base pairs (bp), consistent with the expected size range for ITS sequences in angiosperms and suitable for subsequent genetic analysis. Similarly, Mursyidin *et al.* (2023) also reported an average ITS region length of approximately 684–747 bp across various *Durio* species. These results were also greatly analogous to past findings, which revealed ITS sequence lengths ranged from 827 to 863 bp in durian Kura-Kura from South Kalimantan, Indonesia (Magandhi *et al.*, 2024). These comparative studies and observed data highlighted the inherent length variation within the ITS region among *Durio* taxa, reflecting the genetic diversity within the genus.

In the durian Kebau ITS sequence, the nucleotide composition determination was as follows: thymine—T/U (12.2%), cytosine—C (35.9%), adenine—A (19.8%), and guanine—G

(32.1%). For comparative purposes, the sequence analysis was alongside eight durian Kura-Kura accessions from West Kalimantan and one outgroup, *Cavanillesia chicamochae* (Magandhi *et al.*, 2024) (Table 1). Multiple sequence alignment, being a standard approach in molecular phylogenetic studies, continued using this dataset (Shi *et al.*, 2021). The said analysis enabled the identification of genetic variation critical for constructing evolutionary relationships, elucidating population structure, and formulating the conservation and breeding strategies for *Durio* species (Lin *et al.*, 2022).

Haplotypes diversity

Among the analyzed specimens, distinct haplotypes identified totaled 11 (Figure 1), yielding a haplotype diversity value of 0.962. Hap_1 was notable as a central and ancestral haplotype from which several other haplotypes derived, such as Hap_2, Hap_3, Hap_4, Hap_6, and Hap_5, radiate. Haplotypes, like Hap_8, Hap_9, and Hap_7, showed close associations to these, indicating recent divergence. Hap_10 and Hap_11 appeared as more distant, suggesting higher divergence with less frequent and uniform gene flow. This haplotype pattern reflects a genetic structure characterized by a predominant ancestral haplotype with subsequent diversification, likely driven by geographical and evolutionary factors.

The sequence alignment revealed 49 parsimony-informative sites, reflecting multiple mutation events and the presence of insertions and deletions (InDels) (Table 2). By comparing durian Kebau from Lahat, South Sumatra, to durian Kura-Kura from West Kalimantan, the results displayed a substantial genetic variation within the ITS region. While reports of similar levels of ITS variability came from Magandhi *et al.* (2024) in different *Durio* accessions, our study observed a distinct haplotype pattern and higher pairwise genetic distances, particularly between geographically distant cultivars. These differences suggest possible regional differentiation and support the role of ITS as a useful marker not only for species identification but also for detecting intraspecific

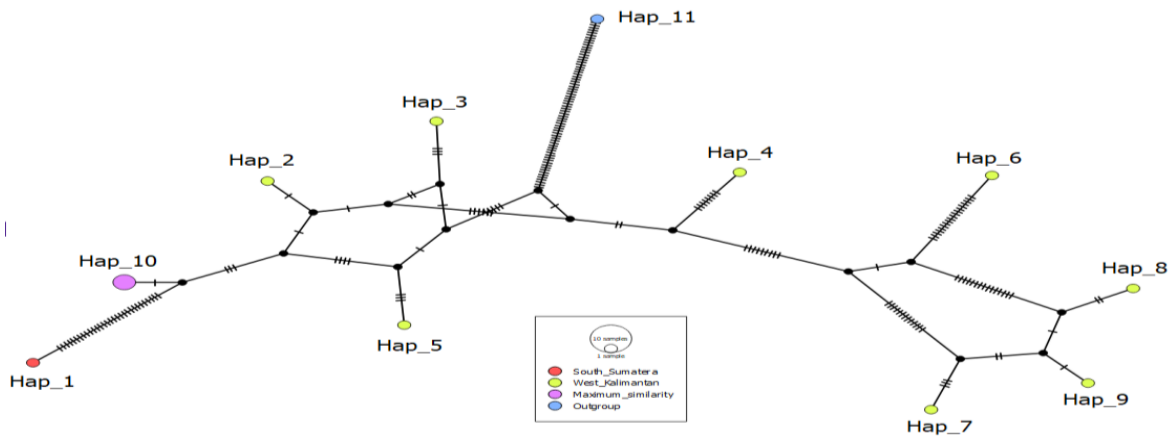


Figure 1. Analysis of genetic relationships and distribution of all the durian haplotypes based on ITS region variations using a haplotype network. The network illustrates genetic relationships among the haplotypes from different geographic regions, including the outgroup and the sequence with maximum similarity, with branch lengths representing genetic distances. Each dash on the connecting lines indicates a single nucleotide difference between haplotypes. Haplotype network of durian samples based on ITS region sequences. Each circle represents a unique haplotype, with size proportional to the number of sequences and colors indicating geographic origin. Each dash represents a single nucleotide substitution between adjacent haplotypes. Branch lengths reflect genetic distances, with dashes on connecting lines denoting single nucleotide differences. The network reveals clear genetic clustering by region, notably showing Kebau, suggesting shared ancestry or recent divergence among these cultivars.

Table 2. Genetic information about various parameters in durian genotypes.

No.	Parameters	ITS
1	Range of sequence length (bp)	561
2	Monomorphic sites	386
4	Polymorphic sites (S)	143
5.	Singleton variable sites	94
6	Parsimony informative sites	49
7	Number of haplotypes (h)	11
8	Haplotype diversity (Hd)	0.962
9	Nucleotide diversity (Pi)	0.071
10	InDel sites	19

diversity across local *Durio* varieties. The observed variations underscore the genetic differentiation among these geographically separated populations and offer insights into their distinct evolutionary histories. This level of genetic variability confirms the effectiveness of the ITS region as a molecular marker for assessing genetic diversity and phylogenetic relationships among the *Durio* populations across different Indonesian regions. The significant divergence proposed intraspecific differentiation potentially driven by geographic

isolation, ecological factors, and evolutionary processes (Sánchez *et al.*, 2023).

Moreover, the highest haplotype diversity highlighted notable genetic differentiation among the populations, providing valuable data for conservation of the plant genetic resources (PGR) and plant breeding programs. Haplotype information is essential for elucidating population structure, tracing lineage-specific traits, and identifying the rare and unique alleles crucial for preserving genetic integrity (Bhat *et al.*, 2021;

Table 3. Pairwise distance of all the durian genotypes used in this study.

Genotypes	A	B	B	C	D	E	F	G	H	I	J	K	L
A Durian Kebau, Lahat													
B <i>Durio graveolens</i> OR631857.1	0.068												
B <i>Durio kutejensis</i> OR631858.1	0.077	0.013											
C <i>Durio lanceolatus</i> OR631859.1	0.090	0.034	0.038										
D <i>Durio oxleyanus</i> OR631860.1	0.077	0.019	0.024	0.048									
E <i>Durio acutifolius</i> OR631856.1	0.138	0.077	0.079	0.073	0.088								
F <i>Durio testudinarius</i> OR593302.1	0.138	0.074	0.080	0.070	0.086	0.073							
G <i>Durio testudinarius</i> OR593301.1	0.142	0.078	0.080	0.074	0.088	0.073	0.015						
H <i>Durio testudinarius</i> OR593305.1	0.140	0.076	0.080	0.072	0.086	0.075	0.013	0.009					
I <i>Durio zibethinus</i> Monthong MW316599.1	0.060	0.011	0.020	0.042	0.022	0.086	0.082	0.087	0.084				
J <i>Durio zibethinus</i> XM 022880025.1	0.060	0.011	0.020	0.042	0.022	0.086	0.082	0.087	0.084	0.000			
K <i>Durio zibethinus</i> MF629776.1	0.060	0.011	0.020	0.042	0.022	0.086	0.082	0.087	0.084	0.000	0.000		
L <i>Cavanillesia chicamochae</i> KM245249.1	0.227	0.166	0.169	0.166	0.166	0.181	0.185	0.185	0.183	0.176	0.176	0.176	-

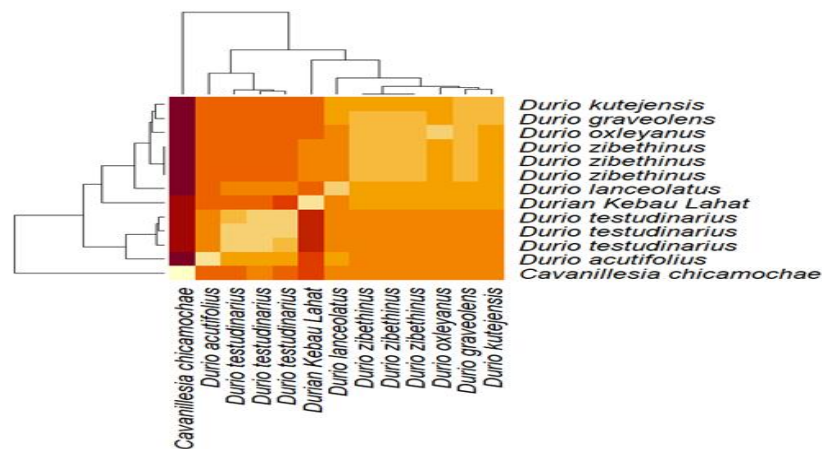


Figure 2. A heatmap of the distance matrix generated to visualize the genetic relationship among all the durian samples based on pairwise nucleotide differences. This visualization offers an intuitive representation of the sequence divergence between the individuals, highlighting the degree of genetic variation across the samples.

Zhang *et al.*, 2021). For conserving threatened and endemic species, this type of information is particularly vital for guiding targeted strategies to maintain genetic diversity.

Moreover, haplotype-based analysis plays a strategic role in crop improvement by enabling the selection of parental lines with complementary genetic profiles, thereby enhancing breeding efficiency and resilience against environmental stress factors (Sivabharathi *et al.*, 2024). The haplotype data integration with breeding programs contributes to the development of superior cultivars and sustainable management of genetic resources. These results emerged as crucial for understanding the genetic structure and

evolutionary history of durian populations. Likewise, such findings will help formulate strategies for conservation and breeding programs aimed at preserving genetic diversity and enhancing cultivar development.

Genetic diversity

Based on the ITS region, the genetic diversity analysis revealed values ranging from 0.000 to 0.227 (Table 3). An intuitive visualization of this genetic relationship appears in Figure 2, illustrating the degree of sequence divergence among the individual durian samples. The observed highest level of diversity was likely because of the mutation and insertions and

deletions (InDels), supported by the considerable number of InDel sites identified in the presented study (Table 2). Teixeira and Huber (2021) also reported that mutations were the primary drivers of genetic diversity. This process plays a crucial role in elucidating species differentiation and the evolution mechanism (Pathirana, 2021).

According to Paloi *et al.* (2022), mutation within the ITS region is a common occurrence, contributing genetic variations across the species. Comparative genetic distance analysis showed the durian Kebau from Lahat, South Sumatra, exhibited the lesser genetic distance (0.068) to *Durio graveolens* (OR631857.1) from West Kalimantan, while the considerable distance (0.142) was evident with *Durio testudinarius* (OR593301.1) (Magandhi *et al.*, 2024). These findings further highlighted and confirmed the substantial genetic diversity observed within *Durio* species in Indonesia.

BLAST analysis confirmed that durian Kebau shares the highest sequence similarity with *Durio zibethinus* (MF629776), *Durio zibethinus* (XM 022880025.1), and *Durio zibethinus* Monthong (MW316599.1). This type of genetic diversity is essential for conservation and breeding programs, providing valuable

genetic material for cultivar development with resilience to environmental conditions and future adaptation (Mishra *et al.*, 2024). The genetic information generated in this study will support ongoing efforts in *Durio* conservation and crop improvement initiatives.

Phylogenetic tree analysis

Phylogenetic analysis is essential for understanding genetic relationships among the various species and advancing breeding programs (Ahmad *et al.*, 2020). Therefore, it is particularly useful for identifying beneficial hybrid compositions and comparing them with their parental lines for economic traits, offering insights into genetic inheritance and aiding in the selection of desirable breeding traits (Adriansyah *et al.*, 2021). Phylogenetic analyses based on the maximum likelihood (ML) tree (Figure 3) and neighbor-joining (NJ) tree (Figure 4) revealed the grouping of *Durio* samples into eight major clades. Durian Kebau showed a close genetic relationship with *Durio zibethinus* Monthong (MW316599.1), highlighting their phylogenetic proximity and reinforcing the genetic similarities between these two species, offering insights into their evolutionary relationship.

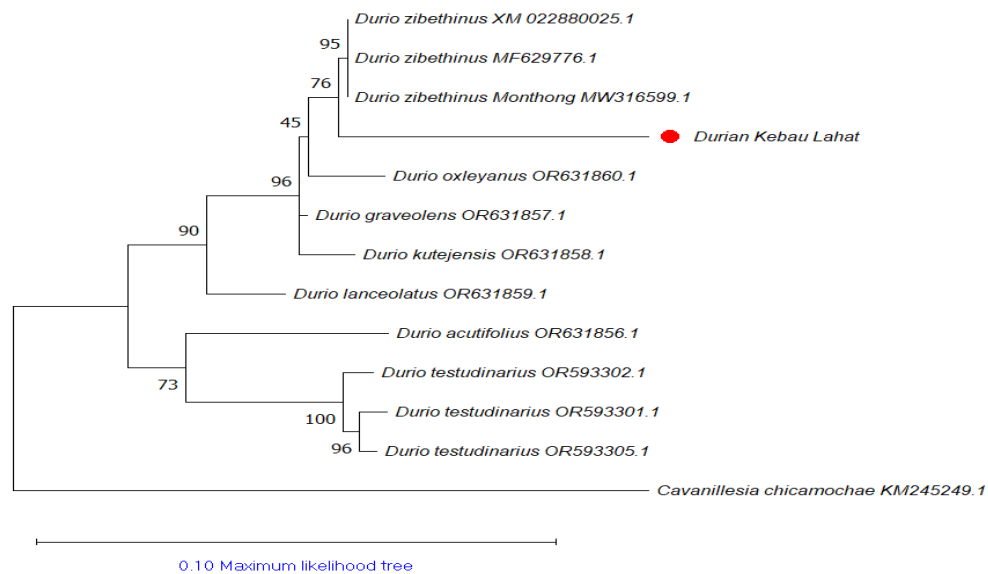


Figure 3. Genetic relationship of all the durian genotypes based on maximum likelihood (ML) analysis. The value on the internal branch shows the results of the bootstrap analysis of 1000 replicates.

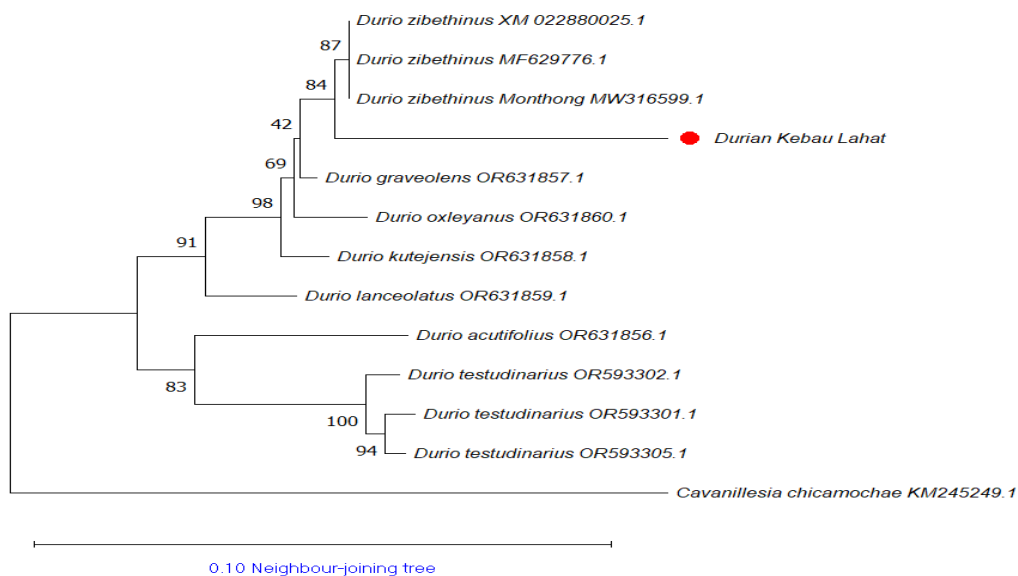


Figure 4. Genetic relationship of all the durian genotypes based on neighbor-joining (NJ) analysis. The value on the internal branch shows the results of the bootstrap analysis of 1000 replicates.

Additionally, the three accessions most closely associated with durian Kura-Kura from West Kalimantan were *Durio graveolens* (OR631857.1), *Durio kutejensis* (OR631858.1), and *Durio oxleyanus* (OR631860), with *Cavanillesia chicamochae* (KM245249.1) identified as the nearest outgroup (Magandhi *et al.*, 2024). These findings will surely provide valuable reference data for *Durio* conservation and breeding programs, particularly in Indonesia. However, the analysis focused only on the ITS region, and it may not capture the complete genomic and functional diversity. Hence, recommending more studies employing additional molecular markers and whole-genome sequencing to validate the said relationship and further improve breeding strategies.

The highest genetic diversity and close genetic relationship identified among the durian Kebau, *Durio zibethinus* Monthong (MW316599.1), and durian Kura-Kura revealed significant implications for conservation and breeding strategies. This genetic variability underscores the importance of implementing both in situ and ex situ conservation efforts to preserve valuable genetic traits. Additionally, molecular insights facilitate the targeted breeding programs by enabling the selection of

parent lines with desirable traits, thereby improving cultivar development efficiency. Furthermore, the genetic markers identified can serve as reliable tools for varietal authentication and certification, supporting market integrity and protecting intellectual property. Overall, these results highlighted the potential for utilizing genetic diversity to ensure sustainable utilization and conservation of durian germplasm in Indonesia. In addition to analyzing more accessions of durian and other local cultivars, we suggest expanding molecular scrutiny using additional chloroplast markers, such as *matK* and *rbcl*. These could provide complementary insights into phylogenetic relationships and enhance the resolution of species differentiation within the *Durio* genus.

CONCLUSIONS

Based on the ITS region analysis, durian Kebau enunciated close genetic similarity to *Durio zibethinus* Monthong (MW316599.1) and to three durian Kura-Kura accessions from West Kalimantan, viz., *Durio graveolens* (OR631857.1), *Durio kutejensis* (OR631858.1), and *Durio oxleyanus* (OR631860). The results

further suggested that durian Kebau shares a common genetic lineage with these taxa. This genetic proximity offered opportunities for targeted breeding and conservation initiatives to enhance durian diversity and resilience to diverse environmental conditions in Indonesia.

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