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DNA BARCODING OF ENDEMIC DURIAN KURA-KURA IN WEST KALIMANTAN, INDONESIA

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SUMMARY

Durio testudinarius is an indigenous durian species classified under the Malvaceae family, primarily distributed within the Borneo region. It has a specific feature of bearing fruit from the main trunk. Understanding its genetic diversity is crucial for its utilization. This study aimed to analyze the genetic diversity and phylogenetic relationships of five *D. testudinarius* accessions from West Kalimantan. A sample of *D. testudinarius* from five West Kalimantan populations, six *Durio* species (*D. graveolens*, *D. acutifolius*, *D. lanceolatus*, *D. dulcis*, *D. kutejensis*, and *D. oxleyanus)* and 17 *Durio* species NCBI dataset underwent genetic diversity and phylogenetic analysis using DNA barcoding markers from three chloroplast regions (*mat*K, *rbc*L, and *trn*L-*trn*F intergenic spacer) and a nuclear DNA Internal Transcribed Spacer (ITS) region. The ClustalW alignment of the five *D. testudinarius* accessions revealed limited nucleotide variation in the chloroplast regions but significant variation in the ITS region, relating to genetic variation in the nuclear genome. Phylogenetic analysis showed that *D. testudinarius* is genetically more similar to *D. beccarianus*, which confirms that they flower on the trunk. This DNA barcoding data improves genetic libraries and assists conservation and breeding programs by revealing *D. testudinarius* and related species' genetic variation.

Keywords: Chloroplast marker, ITS spacer, nucleotide variation, species phylogenetic

Key findings: This research offers valuable insights into genetic variation by providing DNA barcoding sequence data on four barcodes for *D. testudinarius* from West Kalimantan. This contribution enriches existing DNA libraries, supporting conservation efforts and plant breeding programs.

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INTRODUCTION

Durian Kura-Kura (*Durio testudinarius* Becc.) is a species native to Borneo and under the *Durio* genus of the Malvaceae family (POWO, 2023). This species in Indonesia is specifically prevalent in Central Kalimantan (East Kotawaringin, Seruyan, and Pangkalan Bun Regencies) and West Kalimantan (Sintang, Sekadau, Sambas, Landak, Kapuas Hulu, and Ketapang Regencies) (GBIF secretariat, 2019). This species possesses a distinctive characteristic that sets it apart from most of its *Durio* relatives, specifically in its flowers and fruit arrangements, which grow at the base of the trunk (Figure 1) (Kostermans, 1958). Although edible, it is a widely unfavored fruit due to its excessively soft flesh, somewhat moist texture, and intense aroma (Aprilianti, 2019). Nevertheless, this unique characteristic served as a genetic resource for harnessing in developing and enhancing superior durian varieties through targeted plant breeding programs for high production and easy-to-harvest varieties (Uji, 2005).

Durio testudinarius received a rare species classification and a designation as vulnerable on the IUCN red list in 1989, although its conservation status changed subsequently to Least Concern in 2020 (Rahman, 2021). Recognizing the genetic diversity within rare and endangered plant species is crucial for formulating effective management strategies for both in-situ and ex-situ conservation efforts (Ma *et al*., 2012). Genetic diversity analysis can proceed through morphological and molecular approaches. Environmental factors can strongly influence morphological or phenotypic traits and are subject to instability, especially when assessing species that have not yet produced flowers or fruit, such as in the juvenile phase. Hence, an alternative approach less susceptible to environmental influence, namely, molecular markers (genomics), is necessary to corroborate morphological characteristics.

One of the genomic markers uses a DNA (deoxyribonucleic acid) barcoding approach. DNA barcoding entails a standardized sequence of short DNA segments (400–800 bp) designed to facilitate rapid and accurate species identification (Vijayan and Tsou, 2010). This method is particularly useful in biodiversity research, especially in highly diverse regions like tropical areas with high morphological similarity among species (Hebert *et al*., 2004). Apart from aiding taxonomists in identifying biological organisms and conducting phylogenetic analysis, DNA barcoding is a valuable tool for biodiversity experts to unveil potential new species (Kress and Erickson, 2012).

Figure 1. The fruit arrangement in the Sekadau accession of *D. testudinarius* appears at the base of the main trunk.

The utilization of DNA barcoding in investigating genetic diversity and kinship relations within the *Durio* genus is widespread, particularly in cultivated durian (*D. zibethinus* L.) (Sundari *et al*., 2019, 2022; Mursyidin, 2022) and various well-known wild relatives, such as Lai (*D. kutejensis* [Hassk] Becc.) and red durian (*D. graveolens* Becc.) (Santoso *et al*., 2017). However, applying DNA barcoding to underutilized *Durio* species, including *D. testudinarius*, remains limited. This study aimed to analyze the genetic variation and phylogenetic relationship of five *D. testudinarius* accessions from West Kalimantan based on three chloroplast regions (*mat*K, *rbc*L, and *trn*L-*trn*F intergenic spacer) and the nuclear region of the Internal Transcribed Spacer (ITS). This research seeks to provide information regarding the genetic diversity of *D. testudinarius* in five populations in West Kalimantan and contribute to enriching the DNA library to support plant breeding programs and genetic resource conservation efforts.

MATERIALS AND METHODS

Genetic material

Variation nucleotide analysis ensued on five samples of *D. testudinarius* collected from five populations in West Kalimantan of Sambas, Landak, Sanggau, Sekadau, and Ketapang districts (Table 1). Phylogenetic analysis continued on five *D. testudinarius* samples and six variants of *Durio* species (*D. graveolens*, *D. acutifolius* [Mast.] Kosterm. and *D. lanceolatus* Mast., collected from the Balikpapan Botanic Gardens in East Kalimantan, and *D. dulcis* Becc., *D. kutejensis,* and *D. oxleyanus* Griff., collected from the Bogor Botanical Gardens in West Java). The analysis also used 17 DNA barcoding sequence datasets from other *Durio* relative species along with one outgroup dataset of *Theobroma cacao* L., accessed from the Gene Bank of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm .nih.gov) (Sayers *et al*., 2022).

Isolation of genomic DNA

Genomic DNA extraction commenced from about 100 mg of desiccated leaf tissue using a cetyltrimethylammonium bromide (CTAB) buffer solution with a pH of 8.0 (Doyle and Doyle, 1987). The dried DNA pellet reached resuspension in 50 µl of TE (Tris-EDTA) buffer and initially stored at 4 °C, followed by long-term storage at -30 °C. The isolated DNA quantification used the Multiskan SkyHigh Microplate spectrophotometer.

Marker amplification and sequencing

The PCR experiment had a final volume of 50 µL, utilizing MyTaq™ Master Mix 2X (Bioline), with 1 µM for each forward and reverse primer and approximately 100 ng of genomic DNA. The amplification process adhered to the following thermal profile: pre-denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 52 °C, 54 °C, or 58 °C for 45 s as determined by appropriate markers (Table 2), and 72 °C for 45 s, with a final extension at 72 °C for 5 min. The analysis of amplicons used a 1% GelRed-stained agarose gel in 1X TAE (Tris-Acetate-EDTA) buffer via electrophoresis for 30 min at 100 volts. Visualizing the resulting amplicons from electrophoresis assessment employed the Bio-Rad Gel Doc EZ Imager. The sequencing procedure transpired at 1st Base, Singapore, through the service provided by PT. Genetika Science Indonesia.

Data analysis

Performing the multiple sequence alignment utilized the ClustalW algorithm (Thompson *et al*., 1994). The alignment results incurred meticulous examination for any potential nucleotide ambiguities stemming from insertions, deletions, or substitutions, with sequence editing executed when necessary. The edited sequences underwent further processing for dendrogram construction to establish the phylogenetic tree. Determining the optimal model for reconstructing each marker's phylogenetic tree engaged the Maximum Likelihood method (Nei and Kumar,

Table 1. The information on five population accessions of *D. testudinarius* in West Kalimantan.

Population	Accession code	Coordinates	Elevation (masl)
Sambas Botanic Gardens, Sambas District	*D. testudinarius* 1 (Dt1)	01°16′21.9″N 109°29′12.5″E	34
Mandor, Landak District	*D. testudinarius* 2 (Dt2)	00°14′54.4″N 109°18′59.4″E	28
Tayan Hulu, Sanggau District	*D. testudinarius* 3 (Dt3)	00°15′16.8″N 110°17′22.5″E	29
Nanga Taman, Sekadau District	*D. testudinarius* 4 (Dt4)	00°22′28.2″S 110°51′03.2″E	166
Gunung Palung National Park, Ketapang District	*D. testudinarius* 5 (Dt5)	01°13′S 110°7′E	200

Table 2. The information on primers used in the DNA barcoding analysis.

No.	Primer		Sequence (5′-3′)	Ta (°C)	References
1.	*rbc*L	*rbc*La-*F*	ATGTCACCACAAACAGAGACTAAAGC	52	Levin *et al.*, 2003
		*rbc*La-*R*	GTAAAATCAAGTCCACCRCG		Kress and Erickson, 2012
2.	*mat*K	3F-KIM	CGTACAGTACTTTTGTGTTTACGAG	54	Cuénoud *et al*., 2002
		1R-KIM	ACCCAGTCCATCTGGAAATCTTGGTTC		Cuénoud *et al*., 2002
3.	*trn*L-*trn*F intergenic spacer	*eForward*	GGTTCAAGTCCCTCTATCCC	52	Sang *et al*., 1997
		fReverse	ATTTGAACTGGTGACACGAG		Tate and Simpson, 2003
4.	ITS	AB101F	ACGAATTCATGGTCCGGTGGAGTGTTCG	58	Cheng *et al*., 2016
		AB102R	TAGAATTCCCCGGTTCGCTCGCCGTTAC		Cheng *et al*., 2016

2000; Tamura *et al*., 2021). Dendrogram construction progressed using the Maximum Likelihood method (Kumar *et al*., 2018), incurring rigorous testing using 1,000 bootstrap replications (Felsenstein, 1985). All parameters and analyses used the MEGA X software (Kumar *et al*., 2018).

RESULTS

DNA barcoding profile of five accessions of *D. Testudinarius*

Sequence analysis of five *D. testudinarius* accessions revealed specific genetic characteristics: the length of the *rbc*L gene ranged from 564 to 566 base pairs (bp), with a guanine (G)-cytosine (C) content of 44.5%–44.6%; the *mat*K sequence spanned 793–796 bp, with a GC content of 32.7%–33.0%; the *trn*L-*trn*F intergenic spacer ranged from 419 to 437 bp, with a GC content of 30.8%–32.3%; and the ITS sequence was 827–863 bp long, with a GC content of 57.7%–66.2% (Table 3). These findings demonstrated that the tested *D. testudinarius* accessions exhibited a relatively lower proportion of guanine (G)-cytosine (C) in their chloroplast genes than the proportion of adenine (A)-thymine (T). Conversely, the proportion of GC in the ITS region was higher than that of AT.

Nucleotide variations within the five *D. testudinarius* accessions unveiled differences among the four DNA barcoding markers employed. Generally, nucleotide variations in chloroplast genes exhibited relatively lower variations (Table 4) than in the ITS region (Table 5). Specifically, the *mat*K gene displayed only one variation, characterized by a G to A substitution mutation at nucleotide site number 645 in accession Dt5. Meanwhile, the *trn*L-*trn*F intergenic spacer exhibited three nucleotide variations: a T to G substitution at nucleotide site 411, an A to G at nucleotide site 420 (in accession number Dt5), and a C to T substitution at nucleotide site 438 (in accessions Dt3 and Dt5). Notably, the *rbc*L

Table 3. Nucleotide composition of four barcoding DNA sequences of five *D. testudinarius* accessions.

Accessions	*rbc*L (%)					Total	*mat*K (%)					Total
	T(U)	C	A	G	G+C		T(U)	C	A	G	G+C	
Dt1	27,6	21,8	27,8	22,8	44,6	565	37,2	16,6	29,9	16,2	32,8	795
Dt2	27,7	21,6	27,8	22,9	44,5	564	37,1	16,7	29,9	16,3	33,0	790
Dt3	27,6	21,7	27,9	22,8	44,5	566	37,2	16,8	29,9	16,1	32,9	793
Dt4	27,6	21,6	27,9	23,0	44,6	566	37,1	16,7	30,0	16,2	32,9	796
Dt5	27,6	21,8	27,8	22,8	44,6	565	37,2	16,6	30,2	16,1	32,7	796
Accessions	*trn*L-*trn*F (%)					Total	ITS (%)					Total
	T(U)	C	A	G	G+C		T(U)	C	A	G	G+C	
Dt1	39,6	18,9	28,2	13,2	32,1	439	14.9	33.5	19.5	32.1	65.6	863
Dt2	39,4	19,0	28,4	13,3	32,3	437	14.7	34.0	19.3	31.9	66.0	858
Dt3	39,6	18,8	28,6	13,0	31,8	437	18.9	30.6	23.5	27.1	57.7	827
Dt4	41,1	17,2	28,2	13,6	30,8	419	15.8	32.0	21.2	31.0	63.0	835
Dt5	39,2	18,8	28,4	13,5	32,3	436	15.1	33.5	18.7	32.7	66.2	856

Table 4. Nucleotide variations of three chloroplast gene sequences in five *D. testudinarius* accessions. The number above the nucleotide code is the nucleotide site number in the sequence.

Accessions	*rbc*L	*mat*K	*trn*L-*trn*F		
		645	411	420	438
Dt1	-	G	T	A	C
Dt2	-	.	.	.	.
Dt3	-	.	.	.	T
Dt4	-	.	.	.	.
Dt5	-	A	G	G	T

Table 5. Nucleotide variations of the nuclear DNA of ITS in five *D. testudinarius* accessions. The number above the nucleotide code is the nucleotide site number in the sequence. Red boxes indicate insertion-deletion mutations.

Accessions	92	94	112	119	120	123	133	136	139	145	159	183	200	201	205	207	218	224	235	236	240	241	242	248	249	251	255
Dt1	C	G	C	G	A	G	G	C	G	G	T	G	A	C	C	G	C	G	C	G	G	G	A	C	G	C	G
Dt2	.	.	.	.	.	.	.	.	.	.	C	.	G	.	.	C	T	A	.	.	.	.	.	.	.	.	A
Dt3	T	A	A	A	G	A	A	.	T	A	C	.	G	.	.	C	T	A	.	.	A	A	C	G	C	T	C
Dt4	.	.	.	.	.	.	.	T	.	.	.	C	G	G	G	.	T	.	G	T	A	.	.	.	.	.	T
Dt5	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	T	.	.	.	.	.	.	.	.	.	.
Accessions	260	261	263	265	267	268	269	270	273	276	277	278	279	282	286	287	289	290	291	297	298	304	309	315	317	320	322
Dt1	G	C	C	C	G	T	C	G	C	C	G	A	A	C	G	C	C	G	-	C	G	G	G	G	A	C	G
Dt2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.
Dt3	C	.	.	T	A	A	T	A	A	A	A	C	C	A	T	.	A	A	T	.	-	A	T	A	G	T	T
Dt4	.	.	.	.	.	.	.	A	.	.	A	.	.	.	.	G	.	A	-	G	.	.	A	.	.	.	.
Dt5	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.
Accessions	324	328	333	335	337	344	346	349	354	366	367	371	372	373	375	380	384	385	387	389	390	397	400	403	404	410	412
Dt1	G	G	C	G	C	C	G	C	T	C	G	T	C	G	T	C	C	G	C	C	G	T	A	C	G	C	G
Dt2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
Dt3	.	A	T	A	T	T	A	T	.	G	A	C	T	A	G	T	T	T	T	T	.	C	T	A	A	T	A
Dt4	C	.	.	.	.	.	T	.	C	.	.	.	.	.	G	.	.	.	.	.	A	.	.	A	.	.	.
Dt5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
Accessions	416	421	426	427	432	433	441	444	448	452	458	459	464	480	485	486	488	489	493	496	497	505	512	518	520	522	530
Dt1	C	A	G	G	C	G	G	G	G	C	G	C	C	G	C	C	T	G	C	C	G	G	A	G	C	C	C
Dt2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Dt3	T	T	C	A	T	A	.	.	A	T	A	A	A	T	T	T	.	.	A	.	A	.	.	A	.	T	T
Dt4	.	.	.	.	.	.	A	A	.	.	.	A	.	A	.	.	A	A	.	A	.	A	T	.	A	.	.
Dt5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Accessions	534	535	536	550	552	558	564	565	569	577	637	667	727	730	733	734	738	739	740	745	746	778	799	813	849	851	852
Dt1	C	C	G	C	G	C	T	C	G	C	G	G	T	C	G	T	G	C	G	C	G	A	T	C	A	G	C
Dt2	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
Dt3	.	T	A	.	A	.	C	T	C	.	.	.	.	T	A	C	A	G	A	T	A	.	.	.	T	A	A
Dt4	G	.	.	G	.	T	.	.	.	-	C	T	.	.	.	.	.	.	.	.	.	G	.	T	.	.	.
Dt5	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table 6. Specimens and accession numbers deposited in the NCBI Gene Bank used in this study.

No.	Specimens	Accession numbers in NCBI Gene Bank			
		*rbc*L	*mat*K	*trn*L-*trn*F intergenic spacer	ITS
1.	*D. testudinarius 1*	OR601135.1	OR601140.1	OR601145.1	OR593301.1
2.	*D. testudinarius 2*	OR601136.1	OR601141.1	OR601146.1	OR593302.1
3.	*D. testudinarius 3*	OR601137.1	OR601142.1	OR601147.1	OR593303.1
4.	*D. testudinarius 4*	OR601138.1	OR601143.1	OR601148.1	OR593304.1
5.	*D. testudinarius 5*	OR601139.1	OR601144.1	OR601149.1	OR593305.1
6.	*D. acutifolius*	OR601150.1	OR601156.1	OR601162.1	OR631856.1
7.	*D. dulcis*	OR601151.1	OR601157.1	OR601163.1	OR631920.1
8.	*D. graveolens*	OR601152.1	OR601158.1	OR601164.1	OR631857.1
9.	*D. kutejensis*	OR601153.1	OR601159.1	OR601165.1	OR631858.1
10.	*D. lanceolatus*	OR601154.1	OR601160.1	OR601166.1	OR631859.1
11.	*D. oxleyanus*	OR601155.1	OR601161.1	OR601167.1	OR631860.1

Table 7. Specimens and accession numbers downloaded from the NCBI Gene Bank used in this study.

No.	Specimens	Accession numbers in NCBI Gene Bank			
		*rbc*L	*mat*K	*trn*L-*trn*F intergenic spacer	ITS
1.	*D. acutifolius*	MZ479678.1	-	-	AF287700.1
2.	*D. affinis*	-	-	-	AF287705.1
3.	*D. beccarianus*	MH332492.1	-	-	AF287707.1
4.	*D. carinatus*	-	-	-	AF287708.1
5.	*D. dulcis*	MZ479687.1	-	-	AF287715.1
6.	*D. excelsus*	MZ479682.1	MH332624.1	-	-
7.	*D. grandiflorus*	-	-	-	AF233320.1
8.	*D. graveolens*	OK052759.1	-	-	AF287720.1
9.	*D. griffithii*	LC736242.1	KJ708909.1	-	AF233310.1
10	*D. kutejensis*	MZ479692.1	-	-	AF287717.1
11	*D. lanceolatus*	-	-	-	AF287709.1
12	*D. lowianus*	MZ479684.1	-	-	AF287711.1
13	*D. oblongus*	-	LC737192.1		AF287703.1
14	*D. oxleyanus*	MZ479689.1	NC064728.1	NC064728.1	AF233306.1
15	*D. singaporensis*	KJ594689.1	KJ708910.1	-	AF287702.1
16	*D. testudinarius*	MZ479685.1	-	-	AF287704.1
17	*D. zibethinus*	MG895967.1	KY860084.1	NC036829.1	AF233305.1
18	*T. cacao*	NC014676.2	NC014676.2	NC014676.2	JQ228377.1

gene provided no nucleotide variations among the tested accessions. In contrast to chloroplast genes, the ITS region showed a significant nucleotide variation, amounting to 135 disparity points encompassing substitution, insertion, and deletion mutations (detailed in Table 5).

Phylogenetic tree analysis of five accessions of *D. Testudinarius*

A phylogenetic tree using sequence data from five *D. testudinarius* accessions and comparable data from other *Durio* relatives, including an outgroup dataset available in the NCBI Gene Bank, succeeded in construction (Tables 6 and 7), based on three chloroplast genes and one DNA nuclear region of ITS (Figure 2). The number of DNA barcoding sequence datasets available in the NCBI Gene Bank from *Durio* species varies, depending on the respective marker. Twelve *rbc*L gene sequence datasets, seven *mat*K gene sequence datasets, two *trn*L-*trn*F intergenic spacer sequence datasets, and 16 ITS region sequence datasets achieved successful downloading (Table 7).

Overall, the resulting bootstrap frequencies on many branches in all constructed phylogenetic trees were relatively

(A) (B) (C) (D)

Figure 2. Phylogenetic trees construction using the Maximum Likelihood method with a bootstrap of 1,000 replications. A. *rbc*L; B. *mat*K; C. *trn*L-*trn*F intergenic spacer; D. ITS region. The numbers above the branches represent the bootstrap frequencies. The code corresponding to the species name is the accession number in the NCBI Gene Bank. The red box indicates the grouping of the five *D. testudinarius* accessions. Scale bars denote measures of genetic distance.

low, less than 50%. However, the branches forming a cluster of the five *D. testudinarius* accessions (highlighted in the red box on the phylogenetic tree) produced relatively high-frequency values (>50%), except for the tree constructed based on the *rbc*L gene. The

phylogenetic tree revealed that the five accessions (*D. testudinarius* 1-5) clustered together in the same clade/group with close branch distances and low genetic divergence across the chloroplast *rbc*L, *mat*K, *trn*L-*trn*F intergenic spacer, and ITS region. The findings of phylogenetic analysis suggested a stronger genetic relationship between *D. testudinarius* accessions and *D. beccarianus* than the other species studied, as determined using chloroplast genes and the ITS region.

The analysis of the *rbc*L gene signified the formation of two main groups among the test accessions. Specifically, the identified five *D. testudinarius* accessions in the same clade received designations as clade I.a.2, along with *D. beccarianus* (accession number MH332492.1) and *D. singaporensis* (accession number KJ594689.1). In the case of the *mat*K gene, the five *D. testudinarius* accessions grouping within the same clade acquired the clade I.a label, along with *D. beccarianus* (accession number MH332635.1), *D.* cf. *oblongus* (accession number LC737192.1), and *D. singaporensis* (accession number KJ708910.1).

Furthermore, the *trn*L-*trn*F intergenic spacer examination demonstrated that the five *D. testudinarius* accessions were also in the equal clade location, specifically clade I.a, along with the *D. acutifolius* accession. Similarly, the analysis of the ITS region markers indicated that the five *D. testudinarius* accessions had the same clade grouping, referred to as clade II, together with *D. testudinarius* (accession number AF287704) and *D. beccarianus* (accession number AF287707). This clade appeared as a sister group with *D. affinis* (accession number AF287705.1), D. *singaporensis* (accession number AF287702.1), and *D. oblongus* (accession number AF287703.1).

DISCUSSION

The evaluated accessions of *D. testudinarius* demonstrated a lower ratio of guanine (G) and cytosine (C) in the chloroplast genes when compared with the ratio of adenine (A) and thymine (T). In contrast, the relative abundance of guanine-cytosine (GC) content in the internal transcribed spacer (ITS) region emerged higher than the relative abundance of adenine-thymine (AT) content. Previous studies have documented comparable patterns in various *Durio* species (Nyffeler and Baum, 2001) and *Acacia* species (Ismail *et al*., 2020), although the extent of these tendencies may differ across different species. Generally, Kwon *et al*. (2020) stated both gymnosperms and angiosperms in seed plants showed similar GC contents, about 38% in the chloroplast genome. The analysis of GC content in plant barcoding DNA sequences can provide significant insights into genomic variation and genetic inequalities among the different plant species (Layton *et al*., 2014; Mohosina *et al.,* 2020; Setiawan *et al*., 2022).

The analysis of the genetic diversity of *D. testudinarius* from five populations using DNA barcoding markers based on the nuclear DNA ITS region showed much higher variation than *rbc*L, *mat*K, and the *trn*L-*trn*F intergenic spacer. It confirmed that the nuclear genome has a faster evolutionary rate than the chloroplast genome (Smith, 2015). The rates of evolution of chloroplast and nuclear genomes differ significantly due to several internal and external factors, including mode of inheritance, mutation rate, and evolutionary pressures (Petit *et al*., 2005). High nucleotide variation in the ITS region may result from the high crossing-over frequency during cell division and the recombination process (Cheng *et al.,* 2016). In contrast, an inheritance of the chloroplast genome in many plants is often through maternal mechanisms, in addition to its vital functions related to photosynthesis and adaptation to environmental changes (Camus *et al*., 2022). DNA barcoding derived from chloroplast genome sources is widely used in studying plant evolutionary processes and phylogenetic relationships between plant taxa (Chen *et al*., 2022; Yan *et al*., 2023). Meanwhile, DNA barcoding originating from the nuclear genome, especially the ITS region, is applicable for species-level identification, studying genetic diversity, and conducting phylogenetic analysis in unique taxa, both at the interspecies and intraspecies (cultivar) levels (Ningrum *et al*., 2020; Matra *et al*.,

2021). Some researchers combined chloroplast and nuclear genomes to identify and analyze the phylogenetic relationships of taxa for comprehensive and robust results (Hashim *et al*., 2021; Cahyaningsih *et al*., 2022).

In addition to the study utilizing own samples, including *D. testudinarius*, and six specimens of other durian relatives (*D. acutifolius*, *D. dulcis*, *D. graveolens*, *D. kutejensis*, *D. lanceolatus*, and *D. Oxleyanus)*, it incorporated sequence datasets already available in the NCBI Gene Bank. The aim was to offer further insights into the positioning of *D. testudinarius* from various locations concerning its relatives in the taxonomic tree. Nevertheless, the analyzed data remains relatively limited, considering the significant number of available *Durio* species worldwide. As of 2023, there are 28 recognized *Durio* species (POWO, 2023), with a massive distribution in Kalimantan, including *D. testudinarius* (Kostermans, 1958). Among the four DNA barcoding markers, the greater availability of ITS region marker sequence data in the NCBI Gene Bank suggests a more prevalent use of ITS markers than other markers.

The relative arrangement of the five *D. testudinarius* accessions on the phylogenetic tree revealed a notable degree of proximity. The cluster bore support from reasonably high bootstrap frequencies observed on the branches connecting the five accessions. In the context of phylogenetic tree analysis, bootstrap frequencies served as indicators of the robustness or reliability of the branches delineated within the tree. According to Felsenstein (1985), higher bootstrap values indicate increasing confidence in the positioning of branches within a phylogenetic tree. Conversely, lower values suggest a higher level of doubt regarding the location of these branches. Regarding the phylogenetic tree derived from the *rbc*L gene, it is evident that the corresponding bootstrap frequency is below 50%. It suggests an uncertain ordering of the taxa within the branch (Berry and Gascuel, 1996).

The five *D. testudinarius* accessions exhibited a remarkably close relationship with *D. beccarianus.* The genetic proximity between the two accessions can be visible from the close genetic distance in the phylogenetic tree based on the *rbc*L, *mat*K, and ITS regions. The closeness also reflected morphological similarities, as *D. beccarianus* shares several traits with *D. testudinarius*, including white flowers in clusters and fruit emerging at the base of the stem (Kostermans, 1958). Based on the ITS region markers, Nyffeler and Baum (2001), with an update from Mursyidin *et al*. (2023), reported a close relationship between *D. testudinarius* and *D. beccarianus*. Notably, among the five accessions, the *D. testudinarius* fifth accession was the closest to the *D. beccarianus* accession. *Durio beccarianus,* with accession numbers MH332492.1 and MH332635.1 (Dean *et al*., 2018), originates from the exact location as the *D. testudinarius* fifth accession, namely, the Gunung Palung National Park, Ketapang. Furthermore, *D. beccarianus,* with accession number AF287707.1 (Nyffeler and Baum, 2001), originates from Serawai, Sintang Regency, which aligns with the distribution location of *D. testudinarius*. These observations suggest a potential identity between the *D. testudinarius* fifth accession and the *D. beccarianus* accessions MH332492.1, MH332635.1, and AF287707.1, possibly belonging to the same species. However, further investigation is necessary to establish this conclusively.

CONCLUSIONS

Five accessions of *D. testudinarius* exhibited relatively low nucleotide variation in the chloroplast *rbc*L, *mat*K, and *trn*L-*trn*F intergenic spacer. However, it demonstrated substantial variation in the ITS region. Phylogenetic analysis indicated a closer relationship between *D. testudinarius* accessions were more to *D. beccarianus* than the other evaluated *Durio* species, as determined based on the chloroplast and ITS region.

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