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Discovery of Simple Sequence Repeat Markers in the Endemic Durian Kura-Kura (*Durio testudinarius* Becc.)

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ABSTRACT

Durio testudinarius, known as Durian Kura-Kura, is a native Bornean durian species in the Malvaceae family with fruit at the base of the main stem. Recent advances in genomic-based next-generation sequencing are being used to conserve germplasm and enhance plant breeding. This method rapidly and cost-effectively sequences plant genomes, allowing for simple sequence repeat markers to be constructed *in silico*. This study aimed to design and evaluate novel, simple sequence repeat markers utilizing next-generation sequencing microsatellite data of *D. testudinarius*. A total of 20 sequences containing di-, tri-, tetra-, and penta-nucleotide motif repeats were selected and designed to generate primers using Primer3 online software. The PCR results of the twenty primers tested on *D. testudinarius* and 19 genomic DNA samples of species from the Malvales order showed successful amplification. These newly developed simple sequence repeat markers are crucial for genetic population analyses, determining population structure, and enhancing plant breeding programs and conservation strategies for *D. testudinarius* and its relative's germplasm resources.

1. Introduction

Durio testudinarius, also referred to locally name as Durian Kura-Kura, is a native durian species (*Durio*) belonging to the Malvaceae family, specifically found exclusively in the region of Borneo (POWO 2023). The IUCN Red List classified *D. testudinarius* as a rare species and listed it as vulnerable in 1989, although its status to Least Concern in 2020 (Rahman 2021). This species exhibits unique characteristics that distinguish it from other durian relatives, particularly in the inflorescences and fruits that emerge at the base of the stem (Kostermans 1958). These distinctive traits make it a valuable genetic resource with the potential for enhancing and developing superior durian characteristics (Uji 2005).

It is critical to evaluate a species' genetic diversity and population structure when managing genetic resources. Various tools can be employed for these purposes, including morphological and molecular markers. People frequently prefer molecular markers due to their speed, applicability at any growth stage, and insensitivity to environmental influences. One relatively popular PCR-based molecular marker in many research studies is the microsatellite. Microsatellites occasionally referred to as simple sequence repeats (SSRs), are short DNA sequences characterized by the repetition of a core motif in a tandem arrangement, high polymorphism, and abundance throughout the genomes of organisms (Ellegran 2004). SSRs have been the preferred marker for more than two decades due to their ability to exist in multiple alleles, exhibit codominance, transfer effectively across species, offer high levels of information, and demonstrate excellent reproducibility (Vieira *et al.* 2016). Scientific investigations widely utilize SSR markers, including

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studies on genetic diversity, paternity examination, population analysis, identification of genetic features, and forensic research (Kumar 2022).

The utility of SSR markers in genetic studies is undeniable, yet their reliance on pre-existing sequence information has been a significant limitation. The advent of next-generation sequencing (NGS) has transformed this landscape by enabling cost-effective and swift genome sequencing. This technological leap allows researchers to harness a wealth of data for the development of SSR markers through computational methodologies. This study aimed to develop and validate new SSR markers through in silico analysis using microsatellite data of *D. testudinarius* derived from NGS technology. This study also evaluated the transferability of the developed marker to other species in the Malvales order. This paper provides novel SSR markers that can assist in the study of genetic diversity and population structure to enhance plant traits through plant breeding programs. It is also valuable for making recommendations in the conservation strategy for the germplasm resources of *D. testudinarius* and its relatives.

2. Materials and Methods

2.1. Filtering and Selecting Microsatellite Sequences

The microsatellite data of *D. testudinarius*, as documented in the research conducted by Magandhi *et al.* (2021), was employed as a data source for the development of new microsatellite markers. The whole genome sequencing data of *D. testudinarius* was provided by the National Center for Biotechnology Information (NCBI) Gene Bank with accession ID DRX242869. A total of 4,315 sequences containing microsatellites underwent a filtering process, retaining only the single for each repeat. From these, 20 contig datasets were selected, encompassing di-, tri-, tetra-, and penta-nucleotide patterns. Selected sequences were then deposited in the DNA Data Bank of Japan (DDBJ) repository.

2.2. Extracting Genomic DNA

The genomic DNA of *D. testudinarius* utilized for the validation primer was sourced from a living collection preserved at Sambas Botanic Garden, West Kalimantan. Transferability of SSR markers was tested on 19 other species in the Malvales order, including 18 species from the Malvaceae (*D. dulcis, D. kutejensis, D. macrantha, Durio* sp.1, *D. acutifolius, D. graveolens, D. lowianus, D. oxleyanus, D. lanceolatus, Neesia sinandra, Durio* sp.2, *Durio* sp.3, *Theobroma cacao, D. connatus, Kleinhovia hospita, Hibiscus macrophyllus, Ceiba pentandra*, and *Thespesia populnea*), and one species from the Thymelaeaceae family, namely *Aquilaria malaccensis*, collected from the living collection of Bogor Botanic Gardens. Genomic DNA extraction from about 100 mg of desiccated leaf tissue was carried out using a cetyltrimethylammonium bromide (CTAB) buffer solution with a pH of 8.0 (Doyle and Doyle 1987). The dried DNA pellet was dissolved in 50 µL of TE (Tris-EDTA) buffer and stored at 4°C overnight before being transferred for long-term storage at -30°C.

2.3. Designing and Validating SSR Primers

The selected microsatellite sequences were then designed to obtain SSR primer candidates, utilizing the online iteration of Primer3 Web version 4.1.0 (https://primer3.ut.ee/) (Koressaar *et al.* 2018). Primer picking was arranged on these conditions: primer size 18–23 base pairs (bp) (optimum on 20 bp), primer Tm 59.0–62.0°C (optimum on 60.0°C), primer GC% 40–80% (optimum on 60%), and product size range 150-250 and 100-300 bp. The other parameters were set following the default program. The pre-designed primer candidates were then ordered through oligonucleotide provider Macrogen Singapore.

All selected primers were verified in two genomic DNA samples from two individuals of *D. testudinarius* through the polymerase chain reaction (PCR) method. The PCR amplification was conducted using an Agilent SureCycler 8800, and the Type-IT PCR kit (Qiagen) was used in a 10 µL reaction mixture. The reaction mixture consisted of approximately 20 ng of genomic DNA, 5 µL of 2x Type-IT Multiplex PCR Master Mix, 0.2 µM of each reverse primer, 0.1 µM of each forward primer, and 0.1 µM of each fluorescence-labeled primer (FAM, VIC, NED, and PET). The PCR protocol consisted of an initial denaturation step at 95°C for 3 minutes, followed by 32 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute 30 seconds, and extension at 72°C for 30 seconds. The reaction was concluded with a final extension step at 72°C for 30 minutes. Detailed information on the grouping and fluorescent labeling of the twenty pairs of primers is provided in Table 1.

Following that, amplicons underwent electrophoresis for 65 minutes at 70 volts using 1.5% GelRed-stained agarose gel in 1X TAE buffer. The electrophoretic band was visually examined using the Axygen® Gel

Documentation System instrument, and visual images were documented for evaluation. The determination of the allele number and size was conducted through DNA fragment analysis using the capillary electrophoresis method, which a service provider facilitated. Subsequently, the obtained data were manually processed using GeneMapper Software 5 (Applied Biosystems).

2.4. Marker Transferability

The PCR was conducted in a 10-µL MyTaq™ Master Mix 2X (Bioline) containing 0.1 µM each of forward and reverse primers and approximately 20 ng of template DNA. The thermocycler was programmed to denature DNA at 94°C for 3 min, followed by 35 cycles of 94°C for 60 s, annealing temperature at 60°C for 60 s, 72°C for 60 s, and a final 5-min extension step at 72°C. Amplicons were separated by electrophoresis through 3% GelRed-stained agarose gel in 1X TAE (Tris-Acetate-EDTA) buffer for 180 minutes at 70 volts. Visualization of the resulting amplicons from electrophoresis was assessed using the Axygen® Gel Documentation System instrument. Amplification was indicated by visible bands of the expected size (intense or faint) on the agarose gels.

Table 1. Information on grouping 20 pairs of primers under multiplex PCR conditions

Grup multipleks	ID primer	Label fluoresen	Ta (°C)
M1	P4	6-FAM	60
	P10	VIC	60
	P2	NED	60
	P20	PET	60
M2	P16	6-FAM	60
	P1	VIC	60
	P9	NED	60
	P14	PET	60
M3	P17	6-FAM	60
	P5	VIC	60
	P3	NED	60
	P18	PET	60
M4	P15	6-FAM	60
	P6	VIC	60
	P8	NED	60
	P12	PET	60
M5	P7	6-FAM	60
	P11	VIC	60
	P13	NED	60
	P19	PET	60

Ta was annealing temperature;
6-FAM: 5'-GCCTCCCTCGCGCCA-3',
VIC: 5'-GCCTTGCCAGCCCGC-3',
NED: 5'-CAGGACCAGGCTACCGTG-3',
PET: 5'-CGGAGAGCCGAGAGGTG-3'

3. Results

3.1. Selected Microsatellite Sequence Data and Primer Design

A total of 20 sequences were selected from di-, tri-, tetra-, and penta-nucleotide motifs of the *D. testudinarius* microsatellite data, ranging in length from 227 to 650 bp (Table 2). Among these, there were five sequences in dinucleotide repeats, six sequences in each of the tri- and tetra-nucleotide repeats, and three sequences in pentanucleotide repeats. These twenty sequences have been deposited in the DDBJ repository as publicly accessible data (https://ddbj.nig.ac.jp/arsa/). They are also available in the National Centre for Biotechnology Information (NCBI) gene bank (https://ncbi.nlm.nih.gov) with accession numbers LC767465-LC767484.

A set of 20 SSR primers was successfully generated from the selected microsatellite sequences data using the Primer3 software. The software generated primers that produced products spanning a size range of 163 to 299 bp. These new primers have a melting temperature (Tm) ranging from 59.02 to 60.61°C and a guanine and cytosine percentage (primer GC%) ranging from 40.91 to 63.16%. Comprehensive information regarding the new 20 primers is provided in Table 3.

Table 2. Selected microsatellite sequences of *D. testudinarius* deposited in DDBJ repository

Accession number in DDBJ	Locus ID	SSR motif	SSR site	Sequence length (bp)
LC767465	dt24564	(TC)11	197-218	333
LC767466	dt18725	(AG)10	266-285	391
LC767467	dt2717	(CT)10	164-183	477
LC767468	dt37193	(TA)10	121-140	421
LC767469	dt41585	(AG)10	119-138	362
LC767470	dt2294	(AAT)8	310-333	473
LC767471	dt33464	(ATA)8	150-173	331
LC767472	dt33709	(GAC)8	145-168	396
LC767473	dt17599	(AAG)7	253-273	536
LC767474	dt19746	(TTA)7	157-177	401
LC767475	dt24697	(TTA)7	287-307	466
LC767476	dt88788	(AAAT)6	148-171	300
LC767477	dt12934	(TGAT)6	188-211	445
LC767478	dt55948	(CATA)6	192-215	329
LC767479	dt30243	(TTAA)6	143-166	345
LC767480	dt5254	(TTTCA)5	171-195	650
LC767481	dt17930	(ACAG)6	227-250	530
LC767482	dt17247	(TCTTT)5	248-272	413
LC767483	dt18696	(ATAAA)5	207-231	404
LC767484	dt126809	(ATTT)5	101-120	227

Table 3. Presents the newly designed SSR primers for *D. testudinarius*, which were generated using the Primer3 online software

Primer number	Accession number		Primer sequence (5'-3')	Tm (°C)	Primer GC (%)	Product Size (bp)
P1	LC767465	F	GTGGTCCTTCTCTGCTGCC	60.38	63.16	164
		R	TGATCGGGCTTTTCAGGTTCT	59.65	47.62	
P2	LC767466	F	TCGACAACCTGGAGCTTTACA	59.31	47.62	239
		R	TGTGGGCGATGATGAGAGTC	59.54	55.00	
P3	LC767467	F	AGCCTTTTCTTACCTCCGCT	59.02	50.00	168
		R	CTAAACACCCTCCACTCCGC	60.39	60.00	
P4	LC767468	F	TCCTTGAAGCTTCGGATGGT	59.02	50.00	163
		R	TGGAGAGAGGTAGGCTGACA	59.30	55.00	
P5	LC767469	F	TTCCGCCATATCTCCATCATCA	59.09	45.45	218
		R	ACGATAAACTTGGAGCGTGTTC	59.26	45.45	
P6	LC767470	F	AATCTGCCGTTTGTCCAGCA	60.54	50.00	250
		R	GCCCAGATGCGATTTAAGGC	59.69	55.00	
P7	LC767471	F	GCCTTGGATTATGCAACGTCA	59.26	47.62	215
		R	TGGAACCCAAACTACACTGACT	59.22	45.45	
P8	LC767472	F	TGGTGATGGACGGAGAGGAT	60.03	55.00	218
		R	GCAAGGCTTTTCTTCCGCAT	59.76	50.00	
P9	LC767473	F	ATCCGCTGCTGTTCATCCAA	60.04	50.00	202
		R	TAAGAGGGTGTCGAGGGTGG	60.61	60.00	
P10	LC767474	F	AGGAAGTTCTCGCCGGATTG	60.11	55.00	201
		R	ACCCAACCACACCATCCATC	59.96	55.00	
P11	LC767475	F	ATGTGCCAGCTAAGCCTGTT	59.96	50.00	230
		R	TCCCCAAAGTTGCATCATACAGT	60.25	43.48	
P12	LC767476	F	TCTGCGACCAAAACAAAGTGAC	59.90	45.45	170
		R	TGTAGCATCCAATGAGAAAGCAG	59.06	43.48	
P13	LC767477	F	CGTTCTCGCTTCTCATTTCAGC	60.22	50.00	280
		R	AACCCATCACCCATGCCTTT	59.88	50.00	
P14	LC767478	F	TGACCAACCATGTGATATGCTCA	60.05	43.48	299
		R	GCACGCATACACAAAGATCCA	59.26	47.62	
P15	LC767479	F	TGTGGAGGAAAGGACAACGT	59.17	50.00	228
		R	GAGAGCAAAGACCCAACCCA	59.89	55.00	
P16	LC767480	F	AGGTGTGGAAGTTTGGACCG	60.18	55.00	244
		R	CCAGATACCAAGACCAGCGG	60.18	60.00	
P17	LC767481	F	ACAAGCATGCATGGACCACA	60.54	50.00	200
		R	ACAGCTGTGCCTCTCAACAG	60.25	55.00	
P18	LC767482	F	CACCAAAGGCCTACTCTTGCT	60.27	52.38	220
		R	TGCGCCATGAACCACCTATT	60.03	50.00	
P19	LC767483	F	GGAGCAGATGACGAGTAACCC	60.20	57.14	240
		R	ACCGAACTGTATGCTTTGCTTT	59.12	40.91	
P20	LC767484	F	TGGTGGAATTTGTCATGGGATCA	60.25	43.48	213
		R	AGGATGCCAGTCAACACCTG	59.96	55.00	

3.2. SSR Primers Evaluation

The gel electrophoresis test revealed successful amplification of all twenty primers in samples 1 and 2 of *D. testudinarius* genomic DNA (Figure 1). Based on the capillary electrophoresis method, one to two alleles were observed for each of the twenty primers on each sample using GeneMapper Software 5 (Table 4). Notably, among these loci, seven were observed that exhibited the detection of two alleles, while the remaining 13 loci indicated the presence of only one allele. The deviation between the expected and observed allele sizes varied from 0 bp (detected in P17) to 23 bp (detected in P4 and P7).

3.3. Transferability of SSR Markers

The results of gel electrophoresis separation of 20 SSR primers tested on 19 genomic DNA samples from species belonging to the Malvaceae and Thymelaceae families showed visible bands of the expected size for each primer with varying levels of amplification (Figure 2). Specifically, across all tested samples, 10 primers were amplified at 100%, 4 primers were amplified at

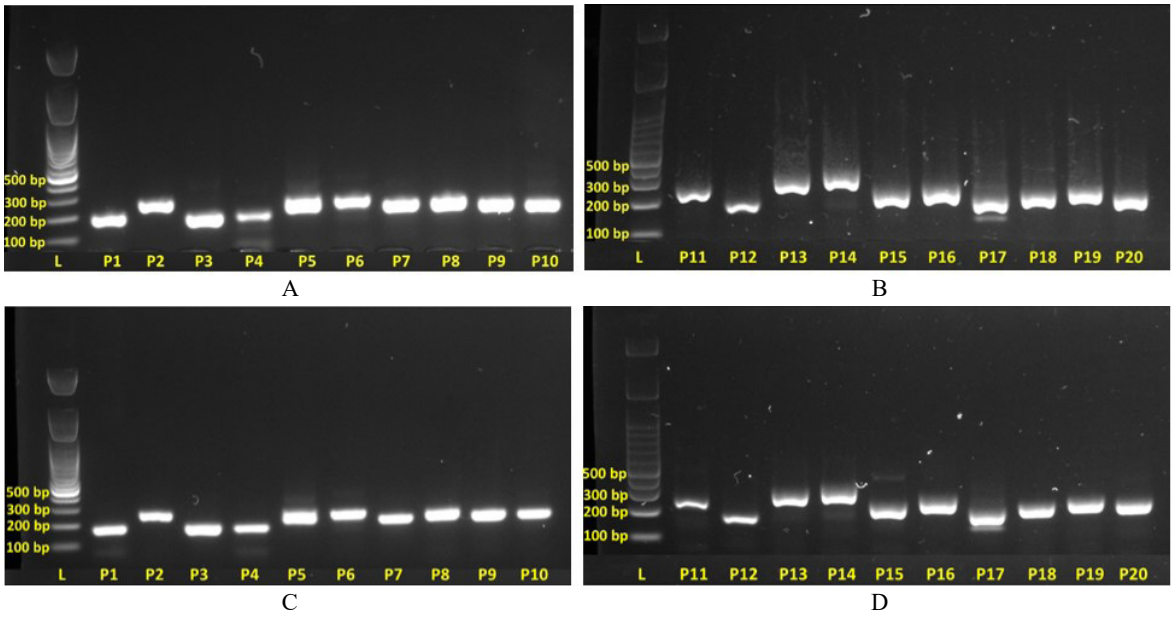

Figure 1. Visualization of amplicons on 1.5% GelRed-stained agarose gel using the Axygen® gel documentation system instrument on validation of twenty primers. (A) and (B) were samples 1, (C) and (D) were samples 2 of genomic DNA from two individuals of *D. testudinarius*. P1 to P20 were primer numbers 1 to 20. L was a ladder with 100 bp

Table 4. Presents the allele number and size of the twenty selected primers, as determined using the capillary electrophoresis method and evaluated by GeneMapper Software 5, for samples 1 and 2 of the genomic DNA of *D. testudinarius*

Primer number	Expected allele size (bp)	Observed allele number		Observed allele size (bp)	
		Sample 1	Sample 2	Sample 1	Sample 2
P1	164	1	1	177	181
P2	239	2	1	250; 252	254
P3	168	1	1	186	188
P4	163	2	1	182; 186	178
P5	218	1	1	231	231
P6	250	1	1	263	263
P7	215	2	2	220; 235	229; 238
P8	218	2	1	228; 231	237
P9	202	1	1	219	219
P10	201	1	1	216	219
P11	230	1	1	246	246
P12	170	1	1	188	188
P13	280	1	1	294	299
P14	299	2	1	315; 317	317
P15	228	1	1	241	241
P16	244	1	2	259	259; 265
P17	200	2	2	200; 211	197; 215
P18	220	1	1	241	241
P19	240	1	1	256	256
P20	213	1	1	233	233

95%, 4 primers were amplified at 90%, and 1 primer each was amplified at 85 and 70% (Table 5).

4. Discussion

Researchers in the past have typically identified SSRs by creating genomic libraries that focus on specific SSR patterns and using recombinant DNA techniques to isolate and sequence clones containing these SSRs (Zalapa *et al.* 2008). Nevertheless, the utilization of library-based methodologies has been demonstrated to be both laborious and expensive, as they heavily depend on low-throughput sequencing techniques and are limited to isolating specific enriched

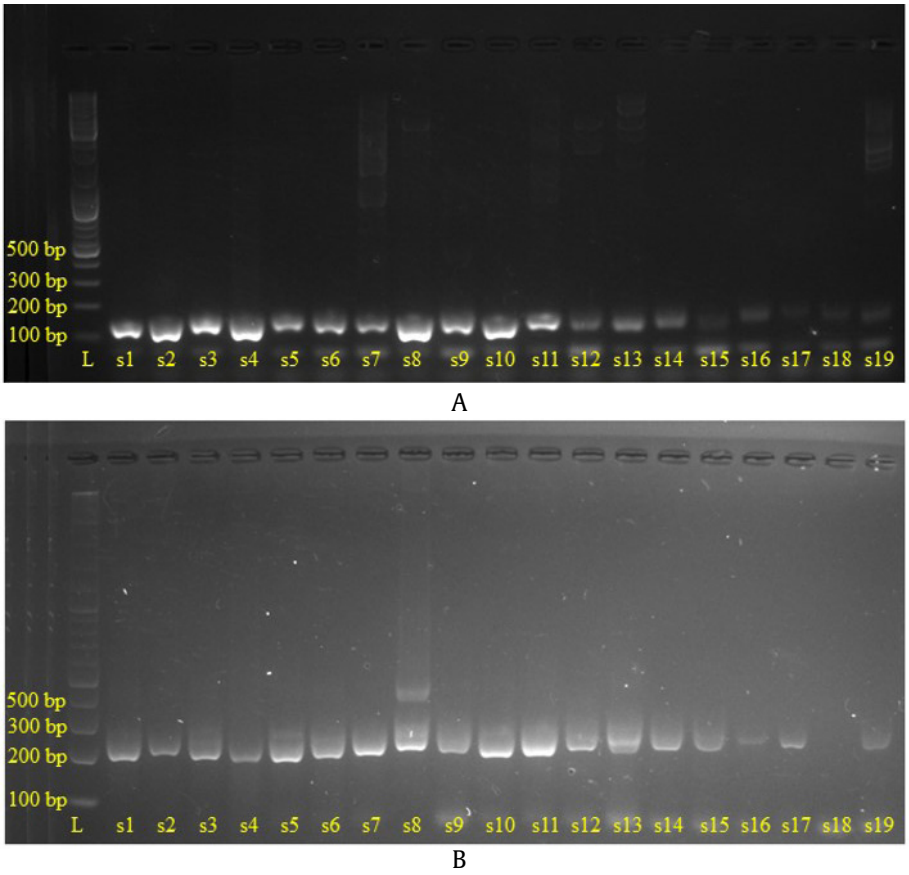

Figure 2. Visualization of amplicons on 3% GelRed-stained agarose gel using the Axygen® gel documentation system instrument on the transferability assay of P1(A) and P5 (B). s1 to s19 were samples of genomic DNA of *Durio dulcis, Durio kutejensis, Durio macrantha, Durio* sp.1, *Durio acutifolius, Durio graveolens, Durio lowianus, Durio oxleyanus, Durio lanceolatus, Neesia sinandra, Durio* sp.2, *Durio* sp.3, *Theobroma cacao, Durio connatus, Kleinhovia hospita, Hibiscus macrophyllus, Ceiba pentandra, Thespesia populnea*, and *Aquilaria malaccensis* L. was a ladder with 100 bp

SSR motifs (Zhu *et al.* 2012). Recent advancements in the field of molecular biology have facilitated the emergence of DNA sequencing technology with high throughput, effectively surmounting these limitations.

PCR primers can be effectively designed using bioinformatics tools and software. There is a diverse range of freely accessible software and tools on the internet for PCR primer design, such as Primer3, Web Primer, Gene Fisher2, and Gene Runner. The selection of the Primer3 software for this research was based on its comprehensive features for PCR primer design, user-friendly interface, and convenience of default options (Kumar and Chordia 2015). Utilizing Primer3, a set of 20 SSR primers was successfully generated from the selected sequences containing microsatellites. A wet laboratory phase subsequently complemented this computational stage to validate the functionality of the designed primers on actual genomic DNA samples. In simpler terms, this confirmation involved conducting PCR, and the product was evaluated through gel electrophoresis. The results demonstrated successful amplification of the tested primers in both genomic DNA samples of *D. testudinarius* and its nineteen relatives. These twenty confirmed SSR markers might then be applied to larger samples from various populations to determine the level of marker polymorphism as well as the genetic diversity and population structure of *D. testudinarius* and its relatives.

The capillary electrophoresis method was employed in this study to evaluate the existence of alleles, as well as their number and size, for each locus in two genomic DNA samples of *D. testudinarius*. The use of capillary electrophoresis has been extensively employed in determining the number and size of alleles at a microsatellite locus in various studies, including those on the genetic diversity of medicinal plum varieties (Wang *et al.* 2021), the *Saccharum* and *Erianthus* genera (Ali *et al.* 2019), as well as upland and sea island cotton genotypes (Aydin 2023). In the results of this study, among the 20 loci evaluated in the

Tabel 5. Transferability of SSR markers on Malvales order

Spesies	Family	Primer number																			
		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20
Durio dulcis	Malvaceae	+	+	+	+	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+	+
Durio kutejensis	Malvaceae	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+
Durio macrantha	Malvaceae	+	+	+	+	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+	+
Durio sp.1	Malvaceae	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	++	+	+	+	++
Durio acutifolius	Malvaceae	+	+	+	+	++	++	++	+	+	+	+	+	+	++	+	+	+	+	+	+
Durio graveolens	Malvaceae	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Durio lowianus	Malvaceae	+	+	+	+	+	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+
Durio oxleyanus	Malvaceae	+	+	+	+	++	+	++	+	+	+	+	++	+	+	+	+	++	+	+	+
Durio lanceolatus	Malvaceae	+	+	+	+	+	+	++	+	+	+	+	++	+	+	+	+	+	+	+	+
Neesia sinandra	Malvaceae	+	+	+	+	+	+	++	+	+	+	+	+	+	+	+	+	++	+	+	+
Durio sp. 2	Malvaceae	+	+	+	+	+	+	++	+	+	+	+	++	+	+	+	+	+	+	+	+
Durio sp. 3	Malvaceae	+	+	+	++	+	+	++	++	+	+	+	+	+	+	+	+	+	+	+	+
Theobroma cacao	Malvaceae	+	++	+	+	++	+	++	++	++	+	+	-	+	+	+	+	+	+	+	+
Durio connatus	Malvaceae	+	++	+	+	+	+	++	++	+	+	+	+	+	+	+	+	+	+	+	+
Kleinhovia hospita	Malvaceae	+	++	+	+	+	+	++	++	+	+	+	+	+	+	+	+	+	+	+	+
Hibiscus macrophyllus	Malvaceae	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++
Ceiba pentandra	Malvaceae	+	++	+	+	+	+	-	-	+	-	-	-	+	+	+	+	+	+	+	++
Thespesia populnea	Malvaceae	+	-	-	+	-	+	+	-	+	-	-	-	-	+	+	+	+	+	+	++
Aquilaria malaccensis	Thymelaeaceae	+	++	+	+	+	+	++	++	++	-	+	+	+	+	+	+	+	+	+	+
% Amplified primer		100	95	95	100	90	100	90	90	100	85	90	70	90	95	100	100	100	100	100	100

A letter (-) indicates not amplified, (+) indicates amplified with only one band detected, and (++) indicates amplified with multiple band detected

genomic DNA samples, seven exhibited the detection of two alleles. In comparison, the remaining 13 loci indicated the presence of only one allele.

Microsatellite or SSR markers have been extensively employed in various studies, including the analysis of genetic diversity and population structure in underutilized and wild species, such as jackfruit (*Artocarpus heterophyllus*), Himalayan raspberry (*Rubus ellipticus*), rambai (*Baccaurea motleyana*), guava (*Psidium guajava*), and wild passiflora (*Passiflora* spp.) (Vianna *et al.* 2019; Nasir *et al.* 2020; Sing *et al.* 2021; Sharma *et al.* 2021; Lin *et al.* 2022; Ojwang *et al.* 2022). In the case of *Durio* species, SSR markers have been predominantly employed in the well-known durian (*Durio zibethinus*) (Siew *et al.* 2018; Hannum *et al.* 2019; Kumar et al. 2023), with comparatively limited use in its wild relative species, including *D. testudinarius*. The results of this study are poised to make a substantial contribution to the application of SSR markers, specifically in *D. testudinarius* and broadly in its relatives. These markers can play a crucial role in studying genetic dynamics and population structure, particularly within the realms of plant breeding and the conservation of genetic resources.

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