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Assessment of the Genetic Variation of Malaysian Durian Varieties using Inter-Simple Sequence Repeat Markers and Chloroplast DNA Sequences

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ABSTRACT

To date, 124 durian varieties have been registered with the Malaysian Department of Agriculture based on phenotypic characteristics. However, the levels and patterns of genetic variation among the varieties are still unknown. In this study, the leaves of 27 durian varieties were sampled from four durian orchards in Universiti Putra Malaysia, namely Bukit Ekspo, Putra Mart, Ladang Puchong and Ladang 5. Twenty five inter-simple sequence repeat (ISSR) primers were tested for PCR amplification on DNA samples. Twelve ISSR primers amplified 133 clear and reproducible DNA fragments and 122 (91.73%) were polymorphic, indicating a high level of genetic variation among these durian varieties. Primers flanking four chloroplast DNA (cpDNA) regions (*trn*L-*trn*F, *atp*B-*rbc*L and *trn*H-*psb*A intergenic spacers as well as the partial *mat*K gene) were tested for PCR amplification. Two cpDNA regions (*trn*L-*trn*F and *mat*K) were successfully amplified, but showed no variation in their DNA sequences, even when additional samples from Vietnam were included. The findings in this preliminary study lay a foundation for more comprehensive future studies on the genetic variation among durian varieties.

Keywords: Chloroplast DNA sequence, DNA barcoding, *Durio zibethinus*, genetic diversity, inter-simple sequence repeat

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INTRODUCTION

Durio is one of the genera in the family Malvaceae and is characterised by its most striking feature i.e. spiny fruit containing large seeds covered with fleshy or leathery arils (Nyffeler & Baum, 2001). A total of 34 species of *Durio* have been recorded (Idris, 2011; "The Plant List", 2013), and at least nine species of these produce edible fruit (Idris, 2011). Of the nine species, durian (*D. zibethinus*) is the most common and most widely cultivated. It is also one of the most popular tropical fruit in Southeast Asia.

In Malaysia, 124 durian varieties are registered with the Malaysian Department of Agriculture ("Varieties Registered for National Crop List", http://pvpbkkt.doa.gov.my/NationalList/Search.php) as of February 2017. It is noteworthy that the different 'types' of durian have always been termed differently; by the Malaysian Department of Agriculture as "varieties," and by the Malaysian Agricultural Research and Development Institute (MARDI) and Universiti Putra Malaysia (UPM) as "clones" (e.g. Abidin, 1991; Jawahir & Kasiran, 2008). For convenience, in this paper we shall use the terminology used by the Malaysian Department of Agriculture i.e. durian varieties. These varieties are registered solely based on their morphological character such as fruit shape, thorn size, aroma of the fruit and seed shape (Department of Agriculture, 2010). Morphological character in plants is easy to observe, but plants are subject to phenotypic plasticity as a direct result of environmental factors (e.g. climate, nutrient and moisture content, soil type etc.) and age, which may contribute to morphological variation (Ruwaida, Supriyadi, & Parjanto, 2009). To overcome the limitation of phenotypic plasticity, there is a need to carry out genetic characterisation on the registered durian varieties. Such data on genetic variation are important not only for the management of durian genetic resources, but also for exploring the possibility of developing genetic markers for future identification of durian varieties.

Inter-simple sequence repeats (ISSRs) and chloroplast DNA (cpDNA) sequences are two useful markers to study genetic variation in plants. ISSR is a PCR-based method which uses microsatellite sequences as primers to amplify regions in the genome that fall between two similar microsatellite sequences. The result is a series of amplified DNA fragments for each sample that can then be scored and compared to other samples to evaluate the amount of genetic variation present in the samples (Ng & Tan, 2015). CpDNA is maternally inherited, has a lower mutation rate compared to nuclear DNA and is widely used in genetic variation studies of plants at various taxonomic levels (Dong, Liu, Yu, Wang, & Zhou, 2012; Gielly & Taberlet, 1994).

ISSR markers do not require prior knowledge of genomic sequences and a high number of loci across genomes can be easily screened, while universal PCR primers have been developed for several cpDNA loci, making them suitable markers for genetic variation studies on durian, for which we have very little genetic information. Also, although all commercial durian types are identified morphologically as *D. zibethinus*, it is unsure if all current varieties belong to the same species, as there has been no study done at the genetic level addressing this question. There is always a possibility of cross-breeding between different *Durio* species (i.e. interspecific hybridisation) to produce edible fruit leading to the array of varieties we see today. One way to determine if the durian types were mothered by *D. zibethinus* is through cpDNA sequencing.

In this study, we used ISSR and cpDNA markers to evaluate the levels and patterns of genetic variation present in a subset of Malaysian durian varieties. Specifically, we asked the questions: (1) What is the level of genetic variation present among Malaysian durian varieties? (2) What are the genetic relationships among the different durian varieties, and did they arise naturally in their assumed places of origin? (3) Are the commercial durian varieties we have today derived solely from *D. zibethinus*? Are there interspecific hybrids?

MATERIALS AND METHOD

Sampling of Durian Varieties, DNA Extraction, and Purification

Leaf samples of 27 durian varieties were collected for this study (Table 1). They were sampled from four orchards in UPM, namely Bukit Ekspo, Putra Mart, Ladang Puchong and Ladang 5. For DNA extraction, 100mg of fresh leaf material was ground in liquid nitrogen, and the total genomic DNA was extracted using the CTAB method (Doyle & Doyle, 1990). The crude DNA extract was further purified using the GF-1 Plant DNA Extraction Kit (Vivantis).

Table 1
Details of durian samples used in this study

No.	Variety	Common Name	Location of Sampling	Place of Origin*
1	D2	Dato' Nina	Putra Mart	Melaka
2	D7	-	Ladang Puchong	Selangor
3	D8	-	Ladang Puchong	Kuala Lumpur
4	D10	Durian Hijau	Putra Mart	Selangor
5	D16	-	Bukit Ekspo	-
6	D24	-	Putra Mart	Perak
7	D84	-	Ladang 5	Perak
8	D88	Bangkok 8	Ladang 5	Selangor
9	D96	Bangkok A	Ladang 5	Selangor
10	D99	Kop Kecil	Putra Mart	Thailand
11	D125	Kop Jantung	Ladang 5	Kedah
12	D145	Tuan Mek Hijau/Beserah	Ladang Puchong	Pahang
13	D148	Paduka	Ladang Puchong	Perak
14	D158	Kan Yau/Tangkai Panjang	Ladang Puchong	Kedah

Table 1 *(continue)*

No.	Variety	Common Name	Location of Sampling	Place of Origin*
15	D159	Mon Thong/Bantal Mas	Ladang Puchong	Kedah
16	D160	Buluh Bawah	Ladang Puchong	Selangor
17	D162	Tawa	Ladang Puchong	Selangor
18	D168	Durian Mas Hjh. Hasmah	Putra Mart	Johor
19	D169	Tok LiTok	Ladang Puchong	Kelantan
20	D172	Durian Botak	Ladang Puchong	Johor
21	D175	Udang Merah	Ladang Puchong	Pulau Pinang
22	D188	MDUR 78	Ladang Puchong	Terengganu
23	D189	MDUR 79	Ladang Puchong	Terengganu
24	D190	MDUR 88	Putra Mart	Terengganu
25	D197	Raja Kunyit/Musang King	Putra Mart	Kelantan
26	Durian Gergasi (DG)	-	Ladang Puchong	-
27	Durian Siam (DS)	-	Bukit Ekspo	-
28	Chanee	-	Vietnam	Thailand
29	Kanyao	-	Vietnam	Thailand
30	B31	-	Vietnam	Vietnam
31	Bi	-	Vietnam	Vietnam
32	Chuong Bo	-	Vietnam	Vietnam
33	Chin Hoa	-	Vietnam	Vietnam
34	HB11	-	Vietnam	Vietnam
35	Kho Qua	-	Vietnam	Vietnam
36	La Queo	-	Vietnam	Vietnam
37	Ri 6	-	Vietnam	Vietnam
38	Sau Huu	-	Vietnam	Vietnam
39	Tam Son	-	Vietnam	Vietnam

Place of origin of Malaysia samples is according to Department of Agriculture ('Recommended plant varieties in Malaysia'*, n. d.)

ISSR Genotyping

Twenty-five ISSR primers were initially tested on a subset of two durian DNA samples in two replicates, and only those that generated multiple, clear and reproducible bands were subsequently used to genotype all 27 durian samples featured in this study. The details of the ISSR primers are listed in Table 2. Single-primer PCR reactions were performed in 10 µL reaction mixtures, each containing 1× NEXpro™ e PCR Master Mix (Genes Laboratories, Korea), 1 µM ISSR primer and approximately 10 ng genomic DNA. A touch-down PCR profile was used, which comprised an initial denaturation of 3 min at 95°C, followed by 13 cycles of 30 s at 95°C, 30 s at 58-46°C (-1°C/cycle) and 1.5 min at 72°C, 25 cycles of 30 s at 95°C, 30 s at 45°C and 1.5 min at 72°C, and finally an extension step at 72°C for 7 min. The PCR amplicons were analysed by electrophoresis on 2% agarose gel, stained with ethidium bromide and viewed under UV illumination. DNA bands between the range of 100 bp and 1500 bp were scored as 'present' (1) or 'absent' (0) for each individual to generate a binary ISSR data matrix before estimation of the basic parameters, including total number of bands, number of polymorphic bands and the percentage of polymorphic bands. To visualise the genetic relationship among the durian varieties, a Neighbour-Joining (NJ) tree was constructed based on the Dice similarity coefficient, using DARwin 6.0 (Perrier & Jacquemoud-Collet, 2006). The degree of confidence at each node of the NJ tree was evaluated through 1,000 bootstrap replicates.

Sequencing of cpDNA Loci

Table 2
ISSR Primers used in this study

No.	Primer name	Primer sequence (5'–3')	No. of bands	No. of polymorphic bands
1.	UBC 834	$(AG)_8$YT	-	-
2.	UBC 841	$(GA)_8$YC	9	9
3.	UBC 848	$(CA)_8$RG	12	10
4.	UBC 855	$(AC)_8$YT	12	10
5.	UBC 856	$(AC)_8$YA	-	-
6.	Ng2.01	$(AC)_8$B	7	7
7.	Ng2.02	$(AG)_8$B	-	-
8.	Ng2.03	$(TC)_8$V	-	-
9.	Ng2.04	$(TG)_8$V	-	-
10.	Ng2.05	$(CA)_8$D	-	-
11.	Ng2.06	$(CT)_8$D	-	-
12.	Ng2.07	$(GA)_8$H	-	-
13.	Ng2.08	$(GT)_8$H	-	-
14.	Ng2.09	$(AC)_8$SS	-	-
15.	Ng2.10	$(AG)_8$SS	13	13
16.	Ng3.01	$(ACA)_5$SS	13	11
17.	Ng3.02	$(AGA)_5$SS	12	10
18.	Ng3.03	$(TCA)_5$SS	16	15
19.	Ng3.04	$(TGA)_5$SS	-	-
20.	Ng3.05	$(ACT)_5$SS	-	-
21.	Ng3.06	$(AGT)_5$SS	11	11
22.	Ng3.07	$(TCT)_5$SS	-	-
23.	Ng3.08	$(TGT)_5$SS	4	3
24.	Ng3.09	$(ATC)_5$SS	9	9
25.	Ng3.10	$(ATG)_5$SS	15	14
		Total	133	122 (91.73%)

Note: Degenerate bases Y=C/T; R=A/G; B=C/G/T; V=A/C/G; D=A/G/T; H=A/C/T; S=C/G

Four sets of published primers (Table 3) were tested to amplify the partial matK gene, as well as the *trn*L-*trn*F, *atp*B-*rbc*L and *trn*H-*psb*A intergenic spacers. Primers that resulted in positive amplification were used to genotype all 27 samples from UPM. PCR amplicons were analysed by electrophoresis on 1% agarose gel, stained with ethidium bromide and viewed under UV illumination. PCR amplicons were then purified and sequenced on an ABI 3730 platform, through services provided by First Base Laboratories Sdn. Bhd. The nucleotide sequences were edited and assembled using the ATGC version 6.0 (Genetyx Corporation) software and finally aligned using Clustal W embedded in MEGA 7.0 (Kumar, Stecher, & Tamura, 2016). Sequences of both cpDNA loci were deposited in GenBank with the accession numbers KY860031–860084.

Table 3
cpDNA loci used in this study. Only the trnL-trnF and matK loci were successfully amplified in this study

Locus	Primer Name	Primer sequence (5'-3')	Amplification (+/-)	Approximate amplicon size (bp)	Source
*trn*L-*trn*F	*trn*L-c	CGAAATCGGTTAGACGTACG	+	1000	Taberlet et al., 1991
	*trn*L-f	ATTTGAACTGGTGACACGAG			
*atp*B-*rbc*L	*atp*B-1	ACATCKARTACKGGACCAATAA	-	-	Chiang, Schaal, & Peng, 1998
	*rbc*L-1	AACACCAGCTTTRAATCCAA			
*mat*K	*mat*K472F	CCCRTYCATCTGGAAATCTTGGTTC	+	800	Yu, Xue, & Zhou, 2011
	*mat*K1248R	GCTRTRATAATGAGAAAGATTTCTGC			
*trn*H-*psb*A	*trn*H-1	CGCGCATGGTGGATTCACAATCC	-	-	Kress, Wurdack, Zimmer, Weigt, & Janzen, 2005

Note: Degenerate bases: Y=C/T; R=A/G; K=G/T

RESULTS

Analysis of ISSR Data

Of the 25 ISSR primers tested (Table 2), only 12 primers produced clear and reproducible bands, and these were subsequently used to genotype all the samples. The 12 primers generated a total of 133 bands that fell within the range of 100-1500 bp in molecular weight. An example of the generated banding pattern is shown in Figure 1. The number of bands amplified per primer ranged from 4 to 16 with an average of 11.08 bands per primer. Of the 133 amplified bands scored, 122 (91.73%) were polymorphic.

An NJ tree (Figure 2) was constructed to visualise the relationship among the different durian varieties sampled in this study. While general clustering of varieties was observed in the tree, support for the tree was low; only three nodes showed ≥50% bootstrap support.

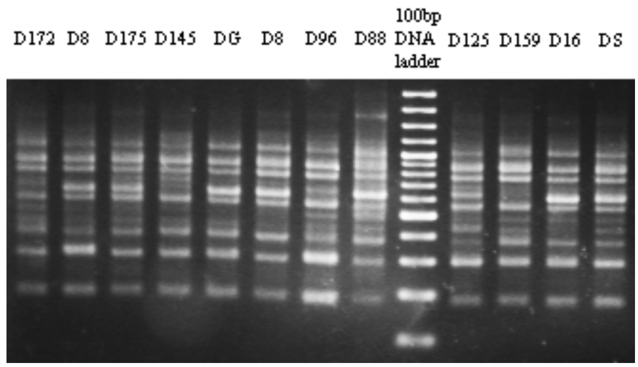

Figure 1. Example of ISSR amplification products of 12 varieties using primer Ng3.01, electrophoresed through 2% agarose gel

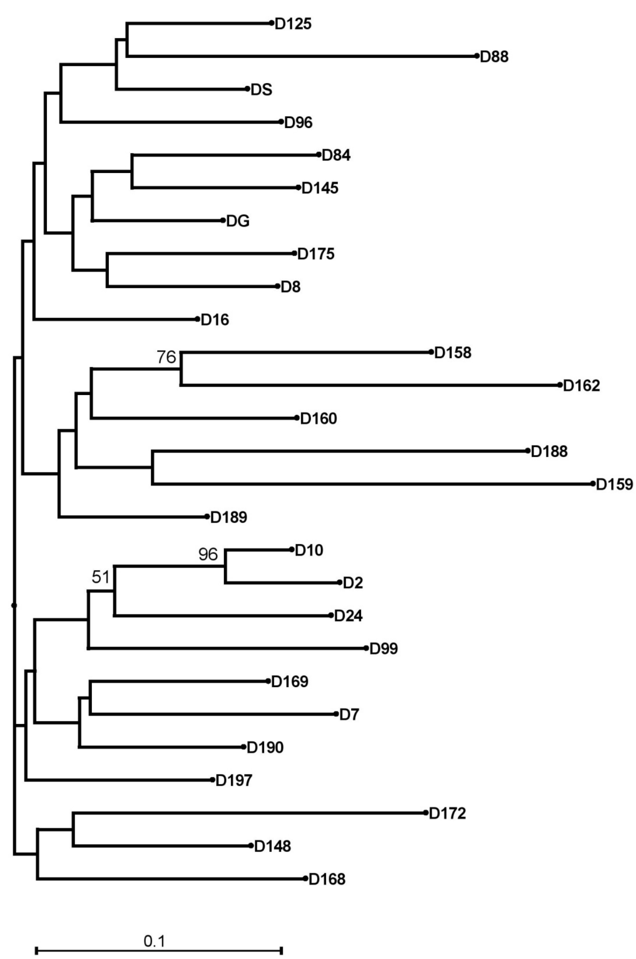

Figure 2. NJ tree of 27 durian varieties constructed on DARwin, with 1,000 bootstrap replicates. Only bootstrap values ≥50% are labelled on the nodes

Analysis of cpDNA Data

Two out of four cpDNA loci, *mat*K and *trn*L-*trn*F, were successfully PCR-amplified and sequenced. Sequencing of the cpDNA sequences across the 27 samples at the *mat*K and *trn*L-*trn*F loci revealed identical sequence lengths within each locus. The aligned lengths were 732 bp for matK and 870 bp for *trn*L-*trn*F. No variation was observed at both cpDNA loci.

DISCUSSION

Levels and Patterns of Genetic Variation

The level of genetic variation found in the durian varieties sampled in our study using ISSR markers was higher than what was found in the studies carried out by Vanijajiva (2012) using ISSR markers and Vanijajiva (2011) using RAPD markers, both on durian varieties from Thailand. The higher number of samples and loci used in this study could be the reason for the higher genetic diversity observed. Our results were comparable to the study done by Ruwaida et al. (2009), who used six RAPD markers to evaluate the genetic diversity in Indonesian durian varieties, which showed an average of 81.89% polymorphic bands. This shows that there is considerably high genetic variation among the different varieties of Malaysian durian.

CpDNA is known to be less polymorphic compared to nuclear DNA, within species (Banks & Birky, 1985). However, the observation of some degree of intraspecific cpDNA variation was not unexpected, as observed in some other cultivated species such as *Pisum sativum, Nicotiana debneyi, Quercus* and *Liriodendron* (Neale, Saghai-Maroof, Allard, Zhang, & Jorgensen, 1988; Okaura & Harada, 2002). The absence of genetic variation detected at the cpDNA loci may be due to the small sample size in this study. To further explore this possibility, we compared our cpDNA data to DNA sequences at the same loci across 12 Vietnamese commercial durian varieties (Giang, Tri, Ky, Muoi, & Hien, unpublished data; see Table 1). However, no variation was observed at both loci across the Malaysian and Vietnamese durian varieties. As cpDNA is maternally inherited, this raises the possibility that the various commercial durian varieties could have been derived from a small group of related mother trees through asexual propagation, among which cpDNA variation would have been very low.

From the aspect of biogeography, taxa that originated from geographically nearer areas would be more genetically related, assuming that these taxa were of natural origin (i.e. not human-mediated). The NJ tree that was constructed to shed light on the relationship among the various durian varieties used in this study however, did not display significant levels of confidence. This means that the varieties were essentially genetically closely related, and no significant relationship between a variety and its corresponding place/region of origin (see Table 1) was found. As durian has become a popular fruit crop in most of Southeast Asia, human activity (e.g. transplant of a variety from a source location to another location followed by crossing with local varieties) seems to be the most possible cause for the non-conformity to biogeographical expectation in the derivation of these durian varieties.

Hybridisation in the Evolution of Malaysian Durian

Hybridisation between different durian varieties (intraspecific hybridisation) has been utilised to come up with superior varieties (e.g. D24 × D10 = D190, D10 × D24 = D188; (Sani, Abbas, Buniamin, Nordin, & Rashed, 2015). However, hybridisation between different species of *Durio* (interspecific hybridisation), to increase genetic diversity in cultivated durian, is not unheard of (e.g. *D. kutejensis* × *D. zibethinus* in Indonesia; [Hariyati, Kusnadi, & Arumingtyas, 2013]). While this study could not confirm if hybridisation between *D. zibethinus* and other *Durio* species took place in the evolution of popular Malaysian durian varieties, the durian varieties sampled in this study were most probably mothered only by *D. zibethinus*, as only a single genotype at each cpDNA locus was found. Future studies incorporating nuclear DNA loci would be useful to further explore the possibility of gene flow from other *Durio* species to the cultivated *D. zibethinus*.

CONCLUSION

Our results demonstrated the potential of using genetic markers to assess the genetic variability of durian varieties. The high level of genetic variation found in a subset of Malaysian durian varieties using ISSR markers provided a preliminary view for the potential development of strategies for germplasm conservation and genetic improvement of existing local durian varieties. However, such a result was not reflected in the cpDNA sequences used in this study. A higher number of cpDNA loci, as well as other genetic markers, should be included in future studies.

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