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PHYLOGENETIC RELATIONSHIPS AMONGST 10 *Durio* SPECIES BASED ON PCR-RFLP ANALYSIS OF TWO CHLOROPLAST GENES

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ABSTRACT

Twenty seven species of *Durio* have been identified in Sabah and Sarawak, Malaysia, but their relationships have not been studied. This study was conducted to analyse phylogenetic relationships amongst 10 *Durio* species in Malaysia using PCR-RFLP on two chloroplast DNA genes, i.e. *ndh*C-*trn*V and *rbc*L. DNAs were extracted from young leaves of 11 accessions from 10 *Durio* species collected from the Tenom Agriculture Research Station, Sabah, and University Agriculture Park, Universiti Putra Malaysia. Two pairs of oligonucleotide primers, N_1-N_2 and $rbcL_1$-$rbcL_2$, were used to flank the target regions *ndh*C-*trn*V and *rbc*L. Eight restriction enzymes, *Hind*III, *Bsu*RI, *Pst*I, *Taq*I, *Msp*I, *Sma*I, *Bsh*NI, and *Eco*R130I, were used to digest the amplicons. Based on the results of PCR-RFLP on *ndh*C-*trn*V gene, the 10 *Durio* species were grouped into five distinct clusters, and the accessions generally showed high variations. However, based on the results of PCR-RFLP on the *rbc*L gene, the species were grouped into three distinct clusters, and generally showed low variations. This means that *ndh*C-*trn*V gene is more reliable for phylogenetic analysis in lower taxonomic level of *Durio* species or for diversity analysis, while r*bc*L gene is reliable marker for phylogenetic analysis at higher taxonomic level. PCR-RFLP on the *ndh*C-*trn*V and *rbc*L genes could therefore be considered as useful markers to phylogenetic analysis amongst *Durio* species. These finding might be used for further molecular marker assisted in *Durio* breeding program.

[*Keywords*: *Durio*, phylogeny, genetic markers, PCR-RFLP]

INTRODUCTION

Malaysia is one of the centers of origin of durian (*Durio* spp.). There are 27 species of the genus *Durio* in the country, mainly grow in Malaysian Borneo (Sabah and Sarawak) (Popenoe 1974; Soon and Lum 1984; Nanthachai 1994). Twenty-three species of *Durio* have been recorded, while 10 species had already been characterized (Idris 2000). In Sarawak, 16 species have been recorded, which seven of those are considered as bearing edible fruits (Gadug and Voon 2000). The most economically important species is *Durio zibethinus* Murr. (Tinggal *et al*. 1994).

Studies on population structure and genetic diversity are essential for the conservation and management strategies of the genetic resources (Karp *et al*. 1997). Phylogenetic analysis is a population genetic study attempting to define the evolutionary history of plants. Current plant populations are grouped to explain their development from their ancestor (Henry 1997). According Bretting and Widrlechner (1995), plant genetic resource management comprises of germplasm conservation and encouraging utilization. Effective conservation is required to maximize and ensure that its genetic potential will be fully available for use in the future (Esquinas-Alcazar 1993).

The analysis of genetic relationships among samples is started with the construction of a matrix specifying the characters-state of each marker for each sample (Karp *et al*. 1997). The result is often presented in the form of a matrix of similarities or dissimilarities (Henry 1997) and illustrated by means of a phylogenetic tree (Li and Graur 1991) or dendrogram tree (Karp *et al*. 1997), where aggregation of samples linking together in clusters are more genetically similar each other than to samples in other clusters.

Polymerase chain reaction-based restriction fragment length polymorphism (PCR-based RFLP) or shortly called PCR-RFLP combines both the techniques of PCR, which has inherent ability to utilize small amount of relatively crude genomic DNA, and RFLP, which has the ability to discriminate between genotypes based upon the presence or absence of restriction sites within the amplified DNA (Karp *et al*. 1997). Genetic and breeding efforts have applied this technique in various plant groups such as *Imperata cylindrica* (Chou and Tsai 1999), Dipterocarpaceae (Tsumura *et al*. 1996),

conifers (Tsumura *et al.* 1995), maize (Kaufman *et al.* 1996), rice (Cordesse *et al.* 1990), *Brassica* (Bhatia *et al.* 1996), and pepper (Livneh *et al.* 1992). The advantages of PCR-RFLP approach are in the quality of the data and the information obtained from it. The polymorphisms studied are of known identity and reveal information on phylogenetic relationships. This technique is also called cleaved amplified polymorphic sequence (CAPS) (Karp *et al.* 1997).

Chloroplast DNA (cpDNA) is a genome frequently used for population structure studies (Comes and Abbott 1999), and has been concentrated on relatively conservative genes such as *rbc*L (Doyle and Doyle 1999). This method has successfully analysed the population structure and phylogeographic studies of several species (Ziegenhagen and Fladung 1997). Beside *rbc*L, *ndh*C-*trn*V is also the type of cpDNA that has successfully been used in genetic studies of *Coffea* spp. (Lashermes *et al*. 1996). The objective of this study was to analyse phylogenetic relationships amongst 10 *Durio* species in Malaysia using PCR-RFLP on two cpDNA genes, *ndh*C-*trn*V and *rbc*L.

MATERIALS AND METHODS

The study was conducted at the Genetics and Plant Breeding Laboratory, Department of Crops Science, Faculty of Agriculture, Universiti Putra Malaysia from December 2002 to May 2003. Plant samples were 11 accessions from 10 *Durio* species collected from the Tenom Agriculture Research Station and Sabah Agriculture Park, Sabah, and University Agriculture Park, Universiti Putra Malaysia (Table 1).

DNAs were extracted from 300 mg of young leaves of durian accessions using modified CTAB-based protocol of Mathius and Hutabarat (1997). Leaf samples taken from Sabah were preserved in plastic bag containing 1% mercaptoethanol-enriched CTAB buffer (Santoso *et al.* 2003), while leaf samples from University Agriculture Park were used in fresh. Two pairs of oligonucleotides N_1-N_2 and $rbcL_1$-$rbcL_2$ primers (Table 2) obtained from Research Instrument were used to flank the target regions. Eight restriction enzymes (*Hind*III, *Bsu*RI, *Pst*I, *Taq*I, *Msp*I, *Sma*I, *Bsh*NI, and *Eco*R130I) were used to digest the amplicons.

Amplification of *ndh*C-*trn*V and *rbc*L regions used the modified method of Edwards (1998). Amplicon was performed in a volume of 50 µl containing 27.2 µl distilled water, 1x PCR buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 0.6 mM of each primer, 60 ng genomic DNA, and 1 unit *Taq* polymerase. The PCR mixture was put into the 0.5 ml thin-walled microcentrifuge tube, placed in the thermal-cycler model PTC-200 (MJ Research), and amplified for 35 cycles of denaturation process at 95°C for 20 seconds, annealing process at 55°C for 60 seconds, extension process at 72°C for 60 seconds, and final extension at 72°C for 10 minutes.

First gel electrophoresis was performed in a volume of 12 µl containing 8 µl of distilled water, 2 µl of PCR products, and 2 µl of gel loading buffer. The samples were loaded onto a 2.0% agarose gel in 1x TAE along with DNA marker. The gel was run in 1x TAE buffer at 80 volts for 1 hour. After that, the gel was stained in the ethidium bromide solution for 15 minutes and then destained in distilled water for 30 minutes. The image of the stained gel was captured using the photo document system (model Chemilmager 5500-Alpha Innotech, USA).

Digestion of the PCR products was performed in a volume of 25 µl containing 15 µl distilled water, 2.5 µl 10x RE buffer, 5.0 µl amplicon, and 2.5 µl restriction enzyme. The reaction mixture was placed into the 0.5 µl thin-walled microcentrifuge tube. The mixture was mixed by pipetting up and down several times, and incubated at 37° C for 1.5-3.0 hours. The second electrophoresis

Table 1. Eleven accessions from 10 *Durio* species used in this study.

Species	Origin
D. kinabaluensis	Tenom, Sabah
D. oxleyanus	Tenom, Sabah
D. dulcis	Tenom, Sabah
D. testudinarum	Tenom, Sabah
D. kotejensis	Tenom, Sabah
D. graveolens (orange fruit)	Tenom, Sabah
D. graveolens (green fruit)	Tenom, Sabah
D. grandiflorus	Tenom, Sabah
D. purureus	Tenom, Sabah
D. sabahensis	Tenom, Sabah
D. zibethinus (clone D24)	UPM Serdang, Selangor

Table 2. Targeted DNA regions, primers and their sequences, Tm values and GC contents.

Region	Primer	Sequence	Tm value	GC content (%)
*ndh*C-*trn*V	N_1	5'-ACGGTTCGAAT CCGTATAGC-3'	57.3	50.0
		5'-CATATTCGTGA AGCAGAAAC-3'	53.2	40.0
*rbc*L	$rbcL_1$	5'-TGTCACCAAAA ACAGAGACT-3'	53.2	40.0
	$rbcL_2$	5'-TTCCATACTT CACAAGCAGC-3'	55.3	45.0

and capturing image were then conducted following incubation.

Variations of the restricted PCR products were scored based on the presence and absence of bands on the images taken from the gels. The NTSys-PC software (Rohlf 2000) was used to compute Jacquard's coefficients of similarity, and the dendrogram was then constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

RESULTS AND DISCUSSION

Phylogeny of Ten *Durio* Species based on PCR-RFLP of *ndh*C-*trn*V Gene

Primers N_1-N_2 were able to amplify DNA sequences of 10 *Durio* species that ranged in size of approximately 900-1100 bp (Fig. 1). Following digestion with eight restriction enzymes, PCR-RFLP products of the *ndh*C-*trn*V gene produced restricted fragments with sizes ranging from 250 to 1100 bp (Fig. 2). Number of fragments produced from DNA of each accession ranged from 1 to 6; average number was 3.25. A total of 286 fragments were produced, of which 275 (96.15%) were polymorphic, while 11 (3.85%) were monomorphic. The monomorphic bands were observed by digestion using *Sma*I enzyme. Generally, the highest number of fragments was produced using *Sma*I (48 fragments), and the lowest was using *Taq*I (16 fragments).

Genetic distance matrices constructed based on restriction products of all fragments analysed are presented in Table 3. These matrices show the genetic distances ranging from 2.2% to 96.3%. The widest was between *D. sabahensis* and *D. testudinarum,* having similarity value of 2.2%. Two accesions of *D. graveolens* (orange fruit and green fruit) are closely related, having similarity value of 96.3%. This means both *D. graveolens* are identic. The wide range in genetic distances (2.2% to 96.3%) shows that there were high variations amongst the 11 accessions of *Durio* species.

The dendrogram tree indicating the phylogenetic relationships amongst species based on RFLPs on

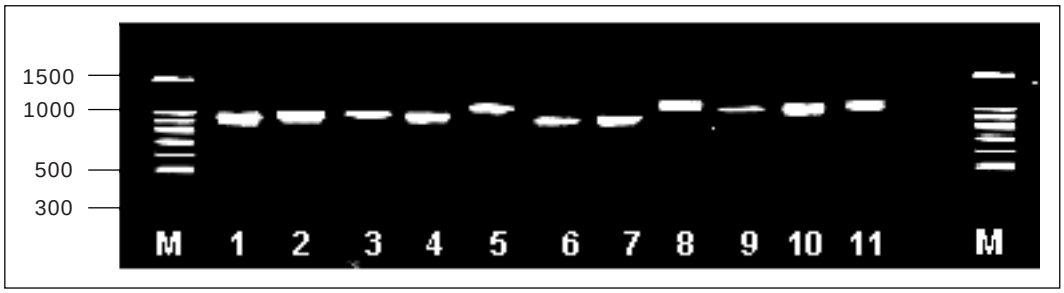

Fig. 1. Banding patterns of the amplified *ndh*C-*trn*V region of DNA from 10 *Durio* species; M = 100 bp DNA ladder, 1 = *D. kinabaluensis,* 2 = *D. oxleyanus*, 3 = *D. dulcis,* 4 = *D. testudinarum,* 5 = *D. kotejensis,* 6 and 7 = *D. graveolens*, 8 = *D. grandiflorus*, 9 = *D. purureus,* 10 = *D. sabahensis,* and 11 = *D. zibethinus* (clone D24).

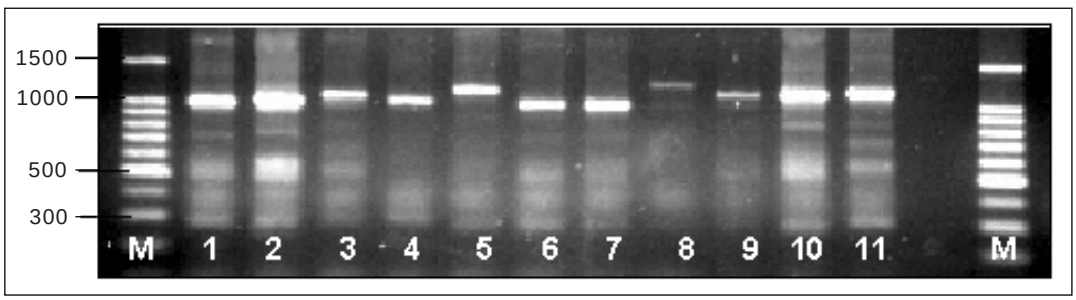

Fig. 2. Banding patterns of RFLPs from *Sma*I digestion of the amplified *ndh*C-*trn*V region of DNA from 10 *Durio* species; M = 100 bp DNA ladder, 1 = *D. kinabaluensis,* 2 = *D. oxleyanus*, 3 = *D. dulcis,* 4 = *D. testudinarum,* 5 = *D. kotejensis,* 6 and 7 = *D. graveolens*, 8 = *D. grandiflorus*, 9 = *D. purureus,* 10 = *D. sabahensis,* and 11 = *D. zibethinus* (clone D24).

Table 3. Genetic distance matrices involving 10 *Durio* species, using Jacquard's coefficients of similarity, based on PCR-RFLP on *ndh*C-*trn*V region.

Species	*D. knb.*	*D. oxl.*	*D. dlc.*	*D. tsd.*	*D. ktj.*	*D. gr.* (orange fruit)	*D. gr.* (green fruit)	*D.grd.*	*D. prr.*	*D. sbh.*
D. oxleyanus	0.231									
D. dulcis	0.176	0.231								
D. testudinarum	0.095	0.087	0.070							
D. kotejensis	0.133	0.222	0.159	0.057						
D. graveolens (orange fruit)	0.163	0.452	0.267	0.132	0.333					
D. graveolens (green fruit)	0.143	0.429	0.273	0.135	0.343	0.963				
D. grandiflorus	0.125	0.167	0.125	0.069	0.161	0.167	0.171			
D. purureus	0.122	0.163	0.179	0.067	0.423	0.194	0.200	0.240		
D. sabahensis	0.220	0.226	0.271	0.022	0.576	0.318	0.326	0.150	0.382	
D. zibethinus (D24)	0.175	0.092	0.196	0.152	0.318	0.143	0.145	0.040	0.233	0.417

D. knb. = *D. kinabulensis*, *D. oxl.* = *D. oxleyanus*, *D. dlc.* = *D. dulcis*,
D. tsd. = *D. testudinarum*, *D. ktj.* = *D. kotejensis*, *D. gr.* = *D. graveolens*,
D. grd. = *D. grandiflorus*, *D. prr.* = *D. purureus*, *D. sbh.* = *D. sabahensis*

*ndh*C-*trn*V region is presented in Fig. 3. The dendrogram reveals the formation of five clusters. Three of those, the first, fourth and fifth clusters, were scattered off and occupied by single species of *D. kinabaluensis*, *D. grandiflorus* and *D. testudinarum* in each cluster, respectively. This reveals their low genetic similarities to other species, indicating that independent evolutionary processes involving *ndh*C-*trn*V region might have occurred on those three species. The second cluster consists of four accessions belonging to three species, i.e. *D. oxleyanus*, two accesions of *D. graveolens* (orange fruit and green fruit), and *D. dulcis*. This reveals high genetic similarities amongst the three species, indicating the presence of some associations in

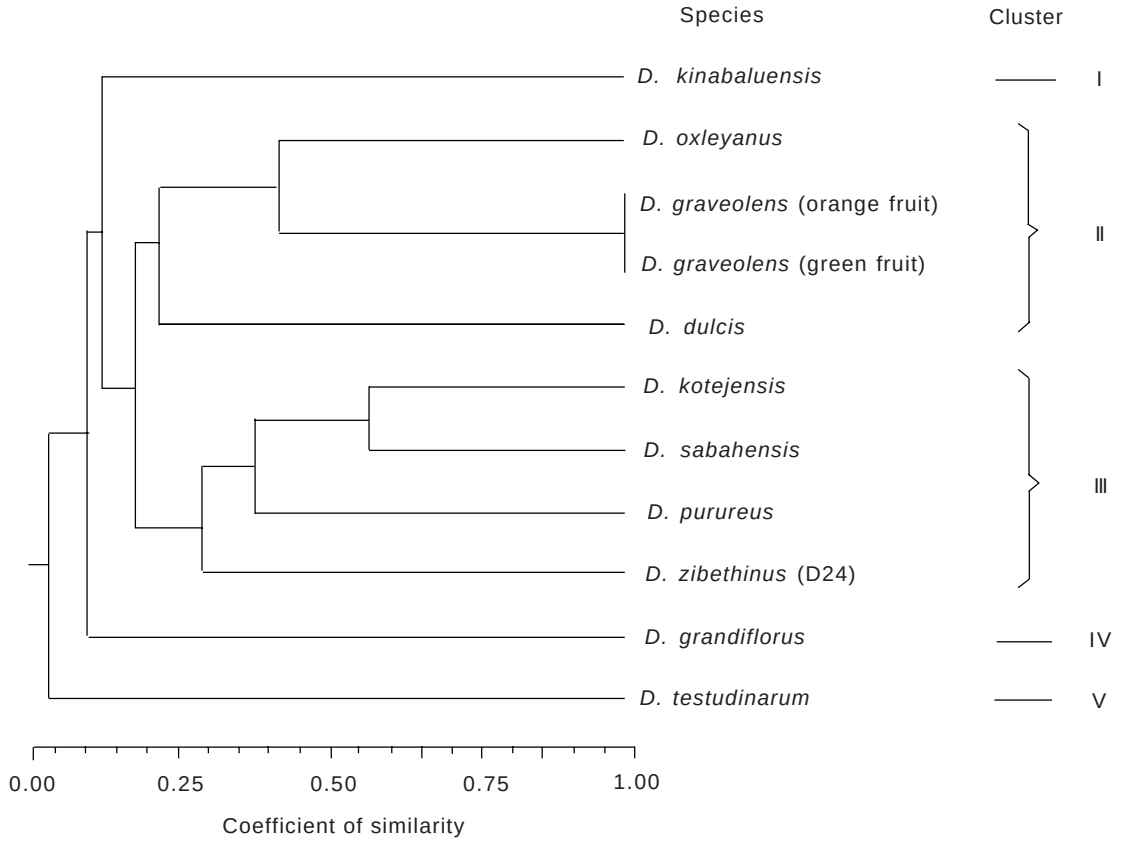

Fig. 3. Dendrogram showing the phylogenetic relationships amongst 10 *Durio* species based on PCR-RFLP on *ndh*C-*trn*V region.

the evolutionary processes involving *ndh*C-*trn*V region found in the three species within the cluster.

A similar trend was observed in the third cluster, which consisted of four species, i.e. *D. kotejensis*, *D. sabahensis*, *D. pururеus* and *D. zibethinus.* This cluster reveals high genetic similarities amongst the four species, indicating the presence of some associations in the evolutionary processes involving *ndh*C-*trn*V region in the four species.

Phylogeny of Ten *Durio* Species based on PCR-RFLP of *rbc*L Gene

Primers *rbc*L_1-*rbc*L_2 were able to amplify targeted DNA sequences of 10 *Durio* species using and produced an aligning band approximately 1400 bp (Fig. 4). Digestion of the PCR product using eight restriction enzymes revealed restricted fragments sized between 150 and 1400 bp (Fig. 5). Number of fragments produced from DNA of each accession ranged from 1 to 4; average number was 2.13.

A total of 188 fragments were produced, of which, 34 (18.09%) were polymorphic and 154 (81.91%) were monomorphic. Generally, the highest number of fragments was produced using *Taq*I (41 fragments), and the lowest were using *Pst*I, *Msp*I and *Sma*I (each with 11 fragments).

Genetic distance matrices constructed based on restriction products of all fragments analysed are presented in Table 4. These matrices show that the genetic distances ranged from 76.1% to 100%. The widest distance was between *D. sabahensis* and *D. testudinarum*, having similarity value of 76.1%, while the closest distances were between the two accessions of *D. graveolens*, and amongst *D. oxleyanus*, *D. dulcis* and *D pururеus*, having similarity value of 100%. This result also confirmed that two accession of *D. graveolens* are identic (Fig. 6).

The dendrogram reveals the formation of three clusters. The first cluster consists of three accessions belonging to *D. kinabaluensis* and the two accessions of *D. graveolens*. The second cluster

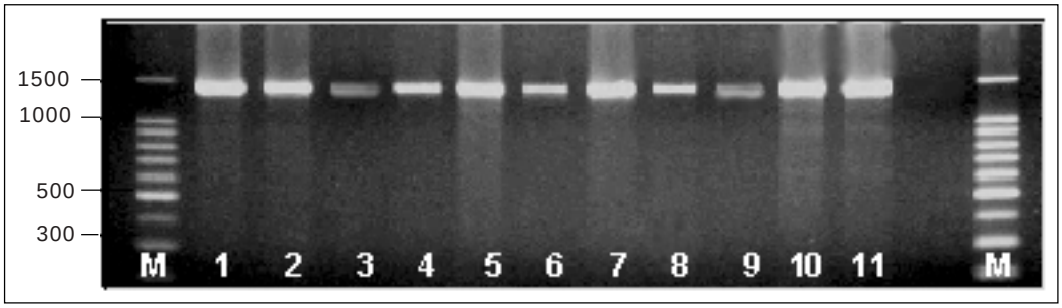

Fig. 4. Banding patterns of the amplified *rbc*L region of DNA from 10 *Durio* species; M = 100 bp DNA ladder, 1 = *D. kinabaluensis,* 2 = *D. oxleyanus*, 3 = *D. dulcis,* 4 = *D. testudinarum,* 5 = *D. kotejensis,* 6 and 7 = *D. graveolens*, 8 = *D. grandiflorus*, 9 = *D. pururеus,* 10 = *D. sabahensis,* and 11 = *D. zibethinus* (clone D24).

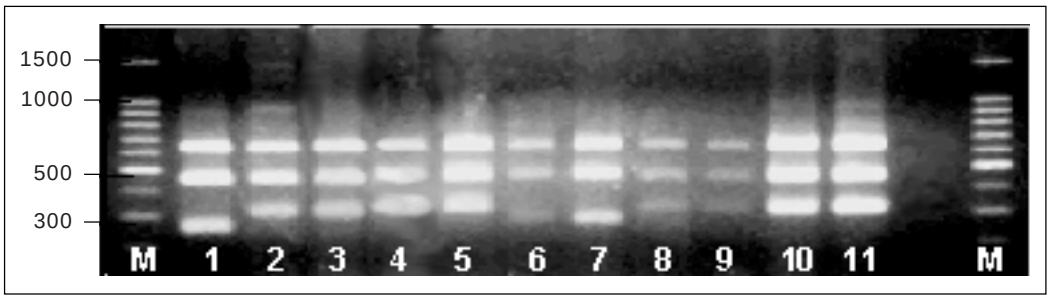

Fig. 5. Banding patterns of RFLPs from *Bsu* RI digestion of the amplified *rbc*L region of DNA from 10 *Durio* species; M = 100 bp DNA ladder, 1 = *D. kinabaluensis,* 2 = *D. oxleyanus*, 3 = *D. dulcis,* 4 = *D. testudinarum,* 5 = *D. kotejensis,* 6 and 7 = *D. graveolens*, 8 = *D. grandiflorus*, 9 = *D. pururеus,* 10 = *D. sabahensis,* and 11 = *D. zibethinus* (clone D24).

Table 4. Genetic distance matrices involving 10 *Durio* species, using Jacquard's coefficients of similarity, based on PCR-RFLP on *rbc*L region.

Species	*D.knb.*	*D.axl.*	*D. dlc.*	*d.tsd*	*d.ktj*	*D.gr. (orange fruit)*	*D.gr. (green fruit)*	*D.grd.*	*D.prr.*	*D.sbh.*
D. oxleyanus	0.842									
D. dulcis	0.842	1.000								
D. testudinarum	0.789	0.941	0.941							
D. kotejensis	0.894	0.944	0.944	0.888						
D. graveolens (orange fruit)	0.894	0.842	0.842	0.789	0.894					
D. graveolens (green fruit)	0.894	0.842	0.842	0.789	0.894	1.000				
D. grandiflorus	0.850	0.894	0.894	0.842	0.947	0.850	0.850			
D. pururеus	0.842	1.000	1.000	0.941	0.944	0.842	0.842	0.894		
D. sabahensis	0.772	0.809	0.809	0.761	0.857	0.772	0.772	0.818	0.809	
D. zibethinus (D24)	0.809	0.850	0.850	0.800	0.900	0.809	0.809	0.857	0.850	0.952

D. knb. = D. kinabulensis, D. oxl. = D. oxleyanus, D. dlc. = D. dulcis,
D. tsd. = D. testudinarum, D. ktj. = D. kotejensis, D. gr. = D. graveolens,
D. grd. = D. grandiflorus, D. prr. = D. pururеus, D. sbh. = D. sabahensis

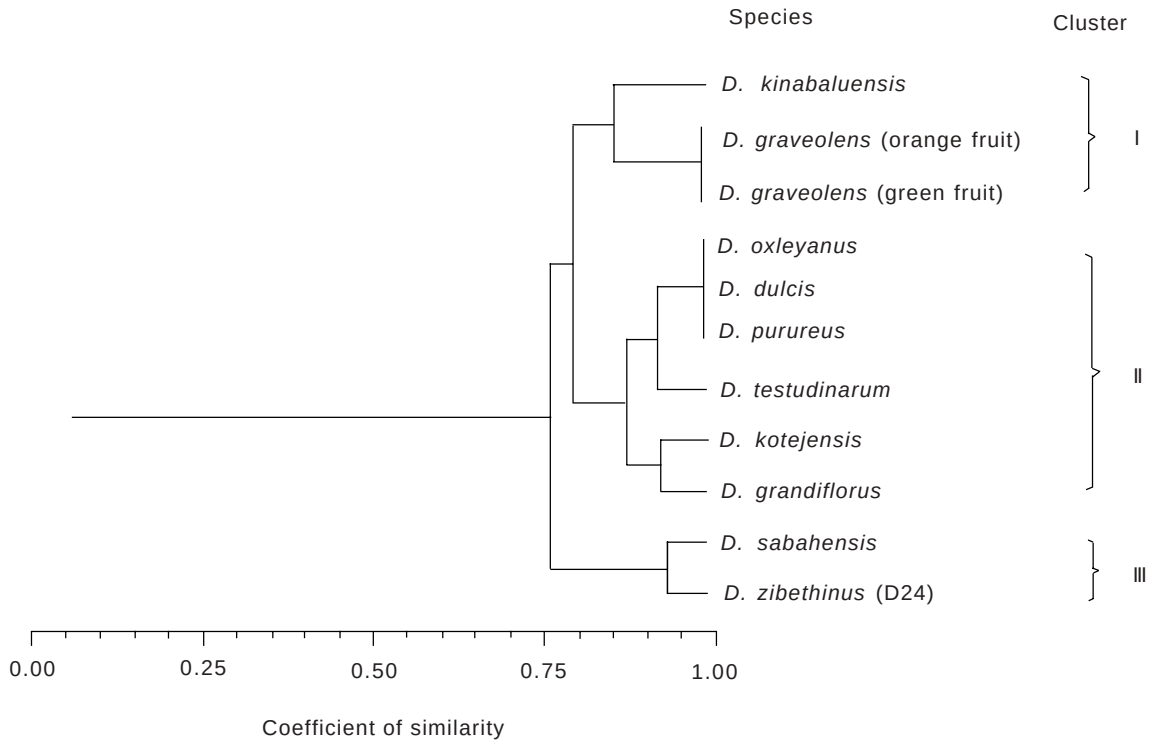

Fig. 6. Dendrogram showing the phylogenetic relationships amongst 10 *Durio* species based on PCR-RFLP on *rbc*L region.

consists of six species, i.e. *D. oxleyanus, D. dulcis, D. pururеus, D. testudinarum, D. kotejensis* and *D. grandiflorus*, and the third consists of two species, *D. sabahensis* and *D. zibethinus*.

Generally, the variation amongst 10 species revealed by the PCR-RFLPs on the *rbc*L region was lower than those on the *ndh*C-*trn*V region. Coefficients of similarities ranged from 76.1% to 100%, showing that there were close genetic distances amongst the accessions. Low variation was also shown by two identical groups involving five accessions belonging to four species, which constitutes 45.45% of the total samples.

These results indicated the difference in the level of alteration in the DNA sequences of the two genes, where the sequences in the *ndh*C-*trn*V gene have altered more intensively than those in the *rbc*L gene. The *ndh*C-*trn*V region is a noncoding region of

chloroplast DNA, which corresponds to intergenic spacer between *ndh*C and *trn*V genes (Lashermes *et al*. 1996). Noncoding region tends to rapidly in sequence alteration (Clegg and Zurawski 1992), makes it more reliable to be used in plant diversity analysis, and might be used to analyse the phylogenetic relationship in lower taxa. The evidence shown by the two accessions of *D. graviolens*, both have little mutation from the same length gene, differed from other species. Meanwhile, the *rbc*L region is a coding region of chloroplast DNA, which corresponds to *RuBisCo* large subunit (Petit *et al*. 1998). The gene is highly conserved among various plants and slow in sequence evolution, hence *rbc*L is a powerful marker to be used for phylogenetic analysis (Ziegenhagen and Fladung 1997), mainly at higher taxonomic level (Doyle and Doyle 1999).

Although there was a different level of variations between the two genes studied, the technique involving PCR-RFLP on the *ndh*C-*trn*V and *rbc*L genes, have succeeded distinguish the 10 *Durio* species into distinct clusters. This technique can therefore be considered as useful markers for phylogenetic analysis of *Durio* species.

The results also showed that two cpDNA primers could be used in molecular analysis of *Durio* species, besides the intergenic spacer of *rbc*L-ORF106 gene (Kanzaki *et al.* 1997) and *ndh*F gene (Nyffeler and Baum 2000). This finding might be useful for further molecular marker assisted selection in *Durio* breeding program in both Malaysia and Indonesia, since these countries have the shared area of *Durio* species origin, and so the diversity. Discovering any other markers are, however, required to maximize recovery of *Durio* genome map.

CONCLUSION

Based on PCR-RFLP on the *ndh*C-*trn*V gene, the 10 *Durio* species were grouped into five distinct clusters, where the accessions generally showed high variations. In contrast, based on PCR-RFLP on the *rbc*L gene, the accessions were grouped into three distinct clusters, and generally showed low variations.

*Ndh*C-*trn*V gene is more reliable for phylogeny analysis in lower taxonomic level of *Durio* or to be used for diversity analysis, while *rbc*L gene is reliable marker to be used for phylogeny analysis at higher taxonomic level.

The technique, PCR-RFLP on the *ndh*C-*trn*V and *rbc*L genes therefore, could be considered as useful markers for phylogeny analysis amongst *Durio* species. The finding might be useful for further molecular marker assisted selection in *Durio* breeding program.

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