
Genetic variability among durian (*Durio zibethinus* Murr.) cultivars in the Nonthaburi province, Thailand detected by RAPD analysis

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The genetic variability among cultivars of Durian (*Durio zibethinus* Murr.) from Nonthaburi province, Thailand was examined using the random amplified polymorphic DNA (RAPD) technique. Genomic DNA was extracted from fresh leaf samples of 14 accessions collected from the Nonthaburi province. Nine primers (OPAM-03, OPAM-12, OPAM18, OPB-01, OPB-14, OPC-01, OPC-05, OPK-05, and OPZ-03) were selected for analysis. A total of 90 DNA fragments, varying from 100-3000 bp, were amplified, of which 34 (37.77%) were polymorphic. Based on the results from the dendrogram analysis, two clusters could be separated with similarity coefficients ranging from 0.235-0.956. RAPD analysis showed promise as an effective tool in estimating genetic polymorphism in different accessions of cultivars Durian in Nonthaburi.

Key words: *Durio zibethinus*, Nonthaburi, RAPD, genetic diversity

Introduction

Durian (*Durio zibethinus* Murr.), “King of Fruits”, is one of the most important agricultural economic fruits in Thailand. The country is the world’s largest producer and exporter of Durian, followed by Malaysia and Indonesia (Somsri, 2007). Durian belongs to the family Malvaceae (APG II, 2003) and distinctive for its large size, unique odour, and thorn-covered husk (Brown, 1997). Over the centuries, numerous Durian cultivars propagated by vegetative clones have arisen in Thailand. They used to be grown with mixed results from seeds of trees bearing superior quality fruit, but are now propagated by layering, or more commonly, by grafting, including bud, veneer, wedge, whip or grafting onto seedlings of randomly selected rootstocks (Somsri, 2007).

Approximately 200 Durian cultivars have been named in Thailand (Somsri, 2008). However, the difference between its cultivars is practically not

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studied. There is not much information available on the genetic relationship between cultivated Durian varieties in Thailand (Somsri, 2007), particularly in the Nonthaburi province, where it has been cultivated for hundreds of years. Earlier classification and evaluations of Durian were done primarily based on phenotypic expression of the plants such as shape of fruit, size of thorns on the skin and other morphological characters (Somsri, 2007). Unfortunately, morphological variation has limited ability to distinguish genetically similar individuals. For this reason, the use of molecular markers has become a standard method to study variability among closely related taxa (Weising *et al.*, 1995).

Genetic markers, such as isozymes (Crawford, 1990; Vanijajiva *et al.*, 2003) and polymerase chain reaction (PCR) based methods, are more reliable for identification of genetic diversity than morphological markers, although each technique has advantages and limitations (Weising *et al.*, 1995). Polymorphisms detected by randomly amplified polymorphic DNA (RAPD) markers have been used for numerous applications in genetics research despite having the disadvantage of poor reproducibility and not generally being associated with distinct gene regions (Vanijajiva *et al.*, 2005). But the cost advantage of RAPD to other molecular techniques tends to favor it, especially for initial genetic studies. Another advantage is that it is a multi loci marker with the simplest and fastest detection technology (Williams *et al.*, 1990). The technique has been successfully employed for determination of genetic diversity in several agricultural plants such as Jackfruit (Pushpakumara and Harris, 2007) and Cotton (Chaudhary *et al.*, 2010). It has been used successfully in the assessment of genetic diversity of Durian cultivars in Philippines (Tacca *et al.*, 2005).

The objective of this study was to use the RAPD technique to evaluate the genetic diversity and relatedness of 14 cultivars of Durian in the province of Nonthaburi using nine RAPD primers. To date, there is no published literature on the assessment of genetic diversity in Durian cultivars using molecular techniques in this province of Thailand.

Materials and methods

Plant materials

DNA isolation and RAPD analysis were carried out using fresh leaf samples from 14 accessions collected from the Nonthaburi province (Table 1). All cultivars are cultivated in a greenhouse at the Faculty of Science and Technology, Phranakhon Rajabhat University. Voucher specimens of all accessions are deposited in the Phranakhon Rajabhat University Herbarium.

Table 1. Samples of Durian used in this study.

Durian Cultivars	Collection site in Nonthaburi	Vouchers	Sample number
Kop Chainam	Bang Kruai	OV012-10	D1
Kop Saonoi	Bang Kruai	OV015-10	D2
Kop Watklaul	Bang Kruai	OV011-10	D3
Kop Maethao	Bang Kruai	OV007-10	D4
Kop Tatao	Bang Kruai	OV002-10	D5
Kop Takum	Bang Kruai	OV019-10	D6
Kanyao	Bang Bua Thong	OV020-10	D7
Yummawad	Bang Kruai	OV004-10	D8
Luang	Bang Kruai	OV022-10	D9
Thongyoichat	Bang Kruai	OV026-10	D10
Kampan	Bang Kruai	OV027-10	D11
Chatsrithong	Bang Kruai	OV032-10	D12
Monthong	Pak Kret	OV003-10	D13
Chanee	Pak Kret	OV005-10	D14

Genomic DNA isolation

Genomic DNA was extracted from the leaves of 14 accessions using the CTAB method following the procedure of Doyle and Doyle (1987) with minor modifications. The leaves (0.05 g) were ground in a mortar with a pestle. Extraction buffer [(1% (w/v) CTAB, 50 mM Tris-HCl (pH 8), 0.7 M NaCl, 0.1% β -mercaptoethanol)] 500 μ l was added and the solution was incubated at 60 C^o for 30 min. The homogenate was mixed with 25:24:1 phenol:chloroform: isoamyl alcohol (v/v/v) by gentle inversion. After centrifugation at 13,000 rpm for 15 min, the upper aqueous layer was transferred to a fresh tube. RNA was removed by treating with 2.5 μ l of the RNase (10 mg/ml) for 30 min at 37 C^o. The extraction of DNA with phenol/chloroform/isoamyl alcohol was repeated one more time. DNA in the solution was precipitated with 0.6 volume of ice-cold isopropanol and washed with 70% ethanol. Following this, the DNA was extracted using CTAB DNA extraction protocol without RNase. The process was repeated until the DNA pellet was free of color (two to three times) and the final pellet was dissolved in sterile deionized water. DNA quality and quantity were determined on 0.8% agarose gel. The DNA was stored at 20 C^o, for further use as templates for PCR amplification.

RAPD-PCR analysis

To optimize the PCR amplification condition, experiments were carried out with varying concentrations of MgCl₂ and DNA template. Three different concentrations of MgCl₂ (3, 4, 5 mM) were used. PCR mixture (25 ml) contained: 10 Promega reaction buffer (100 mM Tris-HCl pH 9, 500 mM KCl, 1% Triton X-100), 0.4 mM of each dNTP, 0.6 mM of primer, 0.5 unit of Taq polymerase (Promega), 5 mM MgCl₂ and 100 ng template DNA. Ten decanucleotide primers (Operon Technologies, California, USA) of random sequences were used (Table 2). PCR was performed using a Thermohybaid PX2, programmed for an initial melting step at 94 C° for 4 min, followed by 45 cycles, each cycle consisting of three steps of 94 C° for 1 min, 36 C° for 1 min and 72 C° for 2 min. A final extension step at 72 C° for 4 min was performed after the 45 cycles. A negative control reaction in which DNA was omitted was included in every run in order to verify the absence of contamination. The RAPD products were separated by agarose (1.8% w/v) gel electrophoresis at 100 A for 30 minutes in 0.04 M TAE (Tris-acetate 0.001 M-EDTA) buffer pH 8. The gels were stained with ethidium bromide (10 mg/ml), and photographed on a UV transluminator. To determine RAPD profiles, the size of each DNA band was inferred by comparison with a 100 bp DNA ladder (Promega), used as a molecular weight marker (M). Polymorphisms at all loci were confirmed by three repeating tests for each primer at different times.

Table 2. Sequences of arbitrary primers, sizes and number of amplified bands, and the percentage of polymorphic bands resulting from the RAPD analysis.

Primer	Sequence	Size range (bp)	No. of RAPD bands	Polymorphic bands (%)
OPAM-03	5'- CTCCCTGTG -3'	100-2000	12	5 (41.6%)
OPAM-12	5'- TCTCACCGTC -3'	600-1800	5	2 (40%)
OPAM-18	5'- ACGGGACTCT -3'	380-3000	7	3(42.8%)
OPB-01	5'- GTTTCGCTCC-3'	180-1500	13	5(38.41%)
OPB-14	5'- TCCGCTCTGG -3'	100-2200	13	3(23.07%)
OPC-01	5'- TTCGAGCCAT -3'	100-2000	14	4(28.57%)
OPC-05	5'- GATGACCGCC -3'	300-2500	13	4(30.76%)
OPK-05	5'- TCTGTTCGAGG -3'	420-2500	8	4 (50%)
OPZ-03	5'- CAGCACCGCA -3'	200-3000	17	4(23.52%)

Gel scoring and data analysis

Only strong and reproducible RAPD bands were scored. Different patterns observed were scored as discrete variables, using 1 to indicate the

presence and 0 to indicate the absence of a unique pattern. The SPSS (version 18) data analysis package was used for the statistical analyses (Backeljau *et al.*, 1996; Norusis, 2010). Relationships among individuals were determined by the distance matrix method. Nei and Li's Dice similarity coefficients were calculated for all pair-wise comparisons between individual samples to provide a distance matrix (Nei and Li, 1979). A dendrogram was constructed from this matrix on the basis of the hierarchical cluster analysis, which is based on the average linkage between group, i.e. the unweighed pair-group method algorithm (UPGMA) as described by Sneath and Sokal (1973). A principal component analysis (PCA) was also conducted using a genetic distance matrix obtained from the binary data set. It was negated and rescaled (0–1), using the Euclidean distance between pair-wise comparison of individuals (Ludwig and Reynolds, 1988). The PCR amplicons were analyzed on 1.8% agarose gels and detected by staining with ethidium bromide. UV trans-illuminated gels were photographed in gel documentation and image analysis system (Syngene, Synoptics Group, Cambridge, UK).

Results and discussion

DNA extraction and optimum PCR conditions

DNA extracted from Durian using a modified Doyle and Doyle (1987) method gave green or brown-colored DNA. Initial RAPD-PCR amplification using this DNA extract did not produce any band for visualization, possibly because the presence of the polysaccharide and phenolic compound that intercalates irreversibly with the DNA helices as reported in the case of DNA from another group of tropical plants (Rajaseger *et al.*, 1997; Rath *et al.*, 1998). The extraction of high quality DNA was optimized by re-extracting the DNA using CTAB DNA extraction protocol and phenol:chloroform:isoamyl alcohol extraction instead of chloroform:isoamyl alcohol extraction. The phenolic compound and pigment co-precipitating with the DNA were easily removed and good RAPD profiles were obtained with all samples. The reproducibility of RAPD results may be overcome by optimizing experimental conditions and following precisely a chosen experimental protocol. Therefore, a necessary precondition for RAPD analysis is the establishment of PCR conditions that ensure reliable and reproducible results (Ramser *et al.*, 1996). In our PCR reactions, 5 mM MgCl₂ gave the best result.

RAPD profiles

All nine random primers used for initial screening (OPAM-03, OPAM-12, OPAM18, OPB-01, OPB-14, OPC-01, OPC-05, OPK-05, and OPZ-03) gave optimum RAPD profiles with all the plants studied. Ninety bands were generated using the nine primers. The amplified product varied between 100 and 3000 bp. The number of fragments produced by a primer ranged from 5 to 17 (Table 2). Examples of the RAPD polymorphisms produced by the random primer OPAM-03, OPB-14, OPC-05 and OPZ-03 are shown in Fig. 1. The maximum numbers of bands were observed in OPZ-03 primer (17), while minimum number of bands was recorded with OPAM-12 primer (5) (Table 2). It has been suggested that the sequence of OPZ-03 primer may occur frequently in all Durian cultivars and scored maximum number of bands, whereas primer OPAM-12 was found less polymorphic within and between the Durian cultivars. Quite considerable genetic variability does exist among different varieties of Durian cultivated in Nonthaburi province.

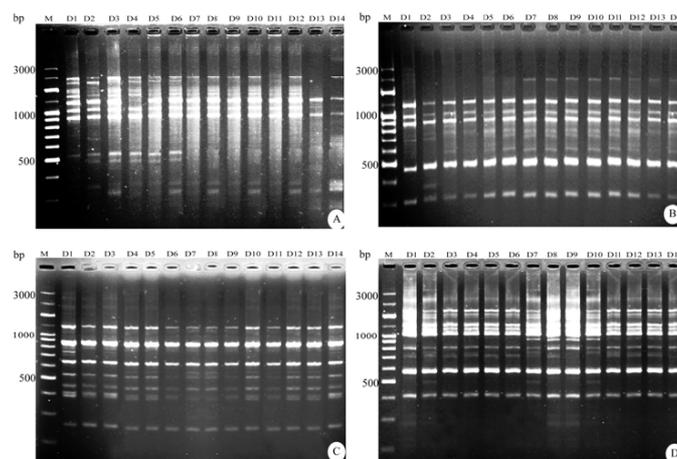


Fig. 1. Examples of the RAPD polymorphisms from 14 Durian cultivars revealed by decanucleotide primers (A) OPAM-03 (B) OPB-14 (C) OPC-05 and (D) OPZ-03 (left to right: lane M, molecular weight marker 1 kb ladder DNA; lanes D1–D14 stand for individual species of plants).

Relationships between cultivars

In order to estimate genetic variability among Durian cultivars in Nonthaburi, genetic similarity coefficients were calculated. The similarity matrix obtained using Nei and Li's coefficient (Nei and Li, 1979) is shown in Table 3. Similarity coefficients ranged from 0.235-0.956 in 14 Durian cultivars

tested in the present experiment, with the lowest value obtained for D5-D8, whereas D4-D6 show the highest similarity values.

Based on RAPD bands amplified by nine primers, genetic distances among the 14 accessions were calculated and a dendrogram was constructed by UPGMA method (Fig. 2). The dendrogram consisted of two major clusters. The first cluster contained a group of the six Durian cultivars of Kob varieties (D1-D6) and was divided into two sub-groups. The first subgroup is made up of two cultivars: D4 and D6. Inside the second subgroup of the first main cluster are four cultivars: D3 and D5, related to D1 and D2. The remaining cultivars were divided into three sub-groups. Cultivars belonging to the second main cluster are grouped in three sub-clusters. The first subgroup is made up of three cultivars: D9, D14 and D8, with close relationship to D10 and D12. D11 and D13 form the third subcluster with a distantly related D7.

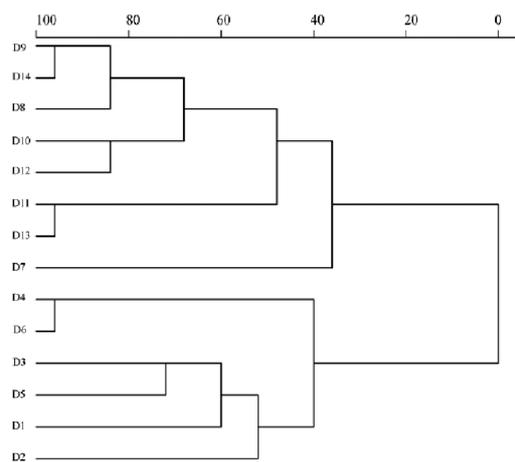


Fig. 2. Dendrogram based on UPGMA analysis of genetic similarity of 14 Durian cultivars obtained from RAPD, showing relationships among individual plants.

The intraspecific relationship among the 14 Durian cultivars by means of the first and second component of the principal components analysis shows two major groups (Fig. 4) which is in good agreement with the dendrogram analysis (Fig. 3). The members in the first major group are dispersed. They split into two distinct subgroups. The first subgroup includes D2, D3, D5, and D1, the second includes D4 and D9. Another major group is formed by eight accessions and split into two distinct subgroups. The first subgroup includes D8, D9, D12, and D14, the second includes D7, D10, D11 and D13. The result agrees very well with the taxonomic characters, for example, shape of fruit, size of thorns on the skin and leaf characters (Somsri, 2007).

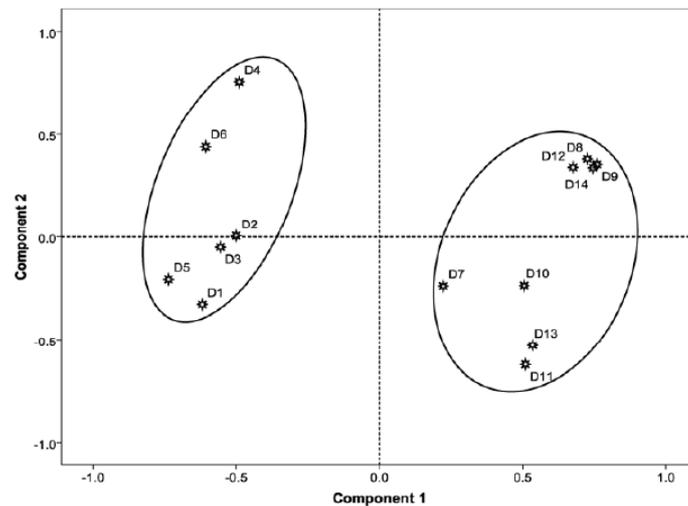


Fig. 3. Plot of PCA analysis of genetic distance of 14 Durian cultivars from RAPD, showing groups of individual plants.

Table 3. Pair-wise genetic similarity of 14 cultivars Durian according to the index of Nei and Li (1979).

Taxa	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14
D1	1.000													
D2	.800	1.000												
D3	.846	.696	1.000											
D4	.750	.696	.750	1.000										
D5	.750	.800	.846	.636	1.000									
D6	.750	.800	.636	.956	.750	1.000								
D7	.696	.636	.696	.571	.571	.421	1.000							
D8	.421	.333	.571	.696	.235	.571	.636	1.000						
D9	.500	.571	.333	.636	.333	.636	.696	.889	1.000					
D10	.636	.571	.636	.500	.500	.500	.696	.800	.846	1.000				
D11	.696	.500	.421	.421	.421	.571	.750	.750	.800	.800	1.000			
D12	.421	.636	.571	.571	.421	.421	.750	.846	.889	.889	.636	1.000		
D13	.571	.500	.421	.421	.421	.571	.636	.846	.800	.800	.929	.636	1.000	
D14	.500	.421	.500	.636	.333	.500	.800	.889	.929	.750	.800	.889	.696	1.000

In conclusion, RAPD markers provide basic genetic knowledge among the cultivars of Durian. This research supports the current varietal classification of Durian. This is a preliminary study, and that a more detailed molecular study such as SSR and AFLP could help solve this: the existence of homonyms and synonyms, particularly with regard to varieties that have been cultivated for centuries and are widely distributed in Nonthaburi.

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