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Differentiation of Three Varieties of *Zingiber officinale* Rosc. by RAPD Fingerprinting

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ABSTRACT The genetic variation between three varieties of *Zingiber officinale* Rosc. was investigated using Random Amplified Polymorphic DNA (RAPD) analysis. The varieties are reported to be useful in traditional medicine and food flavours in Peninsular Malaysia. Analysis was carried out on the RAPD profiles generated by 16 arbitrary primers to determine genetic differences between the varieties. The clearest polymorphic bands were obtained from OPA1, OPA8, OPA9, OPA10, OPA13, OPA16 and OPA20 primers. A total of 104 bands were scored and analysed.

ABSTRAK Perbezaan genetik di antara tiga varieti *Zingiber officinale* Rosc. dikaji dengan menggunakan teknik 'Random Amplified Polymorphic DNA' (RAPD). Varieti-varieti tersebut merupakan tumbuhan yang banyak digunakan di dalam perubatan tradisional dan sebagai perasa masakan di Semenanjung Malaysia. Analisis dilakukan berdasarkan profil-profil RAPD yang dihasilkan dengan menggunakan 16 jenis primer rambang untuk menentukan perbezaan genetik di antara varieti tersebut. Jalur-jalur polimorfik yang jelas telah diperolehi daripada primer-primer OPA1, OPA8, OPA9, OPA10, OPA13, OPA16 dan OPA20. Sejumlah 104 jaluran telah diskor dan dianalisa.

(*Zingiber officinale*, RAPD, primer, ginger, fingerprint)

INTRODUCTION

Holttum [1] recorded 13 species of *Zingiber* for Peninsular Malaysia and of these approximately 5 to 6 species are widely cultivated. In Malaysia, the cultivated species of *Zingiber* such as *Z. officinale*, *Z. montamum* provide an important source for spices, flavour and traditional medicine.

Holttum [1] also reported on three local races for *Zingiber officinale* Rosc., but only two races were discovered and these are not common. These races differ in size and colour of their rhizomes. Mature plants of *Z. officinale* Rosc. var. *officinale* (local name - halia), *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) and *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) may be confused in their general appearance in the absence of their inflorescences or flowers. Theilade [2] reported that *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) can only be differentiated from *Z. officinale* Rosc. var. *officinale* by its smaller, red coloured rhizomes which have stronger and more pungent smell.

At present, there is limited information on the RAPD markers of Zingiberaceae species. RAPD markers have been found to be useful in characterizing and studying the diversity of crop plants [3, 4]. RAPD markers have been found to be useful in differentiating species, varieties, cultivars and races especially in the absence of flower parts. The aim of this study is to analyse the genetic variation between the varieties investigated and to evaluate the significance of these molecular fingerprint.

MATERIALS AND METHODS

Plant Material and DNA extraction

Leaves of *Z. officinale* Rosc. var. *officinale* (halia) and *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) were collected from plants that were grown at Rimba Ilmu, University

var. *rubrum* Theilade (halia padi) which were collected at Muar, Johor and were planted at Rimba Ilmu. DNA was extracted from very young leaves using modified CTAB method [5] with additional RNase treatment.

RAPD Amplification Condition

The RAPD reaction mixture consisted of the following ingredients in a final volume of 25µl, autoclaved water, 12.5µl RAPD 'Master Mix' (Fermentas) containing Taq DNA polymerase (0.05units/µl), $MgCl_2$ (4mM) and dNTPs (0.4mM), 50 picomoles primer and 50 ng template DNA. Random primers (10-mer kits) (Operon Technologies,Inc., CA.) were used as sequences of Kit labeled OPA1 to OPA20. All the primer sequences given are in the 5' to 3' direction. RAPD was carried out using PCR machine (Eppendorf-Mastercycler gradient). The amplification conditions were as follows: 94°C for 90 sec and 45 sec (DNA denaturation), 40°C for 30 sec (DNA annealing), and 72°C for 120 seconds (DNA synthesis) for 40 cycles. After the completion of 40 cycles, the reactions were kept at 4°C. The RAPD products were separated by agarose (1.7%) gel electrophoresis in 0.5X TAE buffer at 80 volts for about two hours. The gels were viewed and photographed under UV light. The bands were visually scored as present (1) or absent (0). The band sizes were estimated by comparing them to bands of GeneRuler 100bp DNA Ladder Plus (Fermentas). To compare and calculate genetic distance between pairs of variety studied, a formula called similarity index (S) was used. S was calculated from band sharing data for each pair of bands according to the formula:

$$S_{xy} = 2n_{xy}/n_x + n_y$$

Where n_x and n_y represent the number of scorable fragments from individual x and y, respectively. The number of fragments shared by both individuals is represented by n_{xy}. S will give the value of similarity index between 0 and 1, where values near to 0, signify less similarity or not similar and values near to 1, indicate high similarity.

RESULTS AND DISCUSSION

RAPD technique is often described as not reliable because of the inability to reproduce the results. Hence in this work, RAPD analysis was repeated to ensure the reproducibility of the banding pattern and concomitantly the validity of the results.

Out of 20 primers examined, only 16 primers produced scorable bands. The remaining four primers either did not produce any bands or the bands were not clear enough to be evaluated. In evaluating the number of bands, only those bands with clear intensities were used. The size of RAPD bands produced was between 2333 bp to about 135 bp (Table 1). The clearest banding patterns differentiating the three varieties studied were produced by seven primers (43.8% of all primers tested) i.e. OPA1, OPA8, OPA9, OPA10, OPA13, OPA16 and OPA20 where a total of 62 scorable bands were evaluated (Table 1). No polymorphic bands were observed from primers OPA2, OPA3, OPA4, OPA5, OPA6, OPA7, OPA11, OPA15 and OPA18. Bands of equal sizes (monomorphic patterns) obtained in the three varieties studied are 27 (65.9% genetic similarity) out of a total of 41 bands produced. The highest number of RAPD bands observed for *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) was 6 (OPA20) and 5 for *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) (OPA20).

Figure 1 shows polymorphic patterns in primers OPA1, OPA8 and OPA20. In primer OPA1 and OPA8, one specific band was obtained at 1500 bp for *Z. officinale* Rosc. var. *officinale* and 2000 bp for *Z. officinale* Rosc. var. *rubrum* Thelaide (halia padi) respectively. Monomorphic bands were visualised at 1031 bp and 500 bp (OPA1), 1500 bp and 550 bp (OPA8) and 1750 bp, 1200 bp and 700 bp (OPA20).

The binary matrix of polymorphic primers is presented in Table 2. The number of rare RAPD markers was very low that is only five variety specific markers were found (OPA1 – 1500 bp marker and OPA20 – 1350 bp marker, specific for *Z. officinale* Rosc. var. *officinale*; OPA20 – 800 bp marker, specific for *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara); OPA8 – 2000 bp marker and OPA16 – 1031 bp marker, specific for *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi). Table 2 showed that all 41 patterns were identified by as few as seven of the 16 informative primers and from a total of 14 bands (OPA1 – 1500 bp; OPA8 – 2000 bp; OPA9 – 400 bp; OPA10 – 1200 bp, 600 bp, 150 bp; OPA13 – 200 bp, 1200 bp, 900 bp; OPA16 – 1031 bp; OPA20 – 1350 bp, 900 bp, 800 bp, 550 bp). The results also showed that *Z . officinale* Rosc. var.

officinale (halia) shared 32 bands (82.1%) with *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) higher than 27 bands (67.5%) shared with *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi). Whilst, *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) shared 31 bands (77.5%) with *Z . officinale* Rosc. var. *rubrum* Theilade (halia padi).

From the values of the similarity index calculated, the three varieties of the *Z. officinale* were shown to be very similar (Table 3). However, in comparison, *Z. officinale* Rosc. var. *officinale* (halia) and *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) showed the highest S value (0.94), followed by the latter and *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) (0.91) and the most dissimilar being, Z. *officinale* Rosc. var. *officinale* (halia) and *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) at 0.84.

These results implicate that there is low genetic variation within *Z. officinale* varieties, which is in agreement with their close morphological affinities. With the observed similarities and dissimilarities in RAPD profiles and morphology, we may conclude that *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) and *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) should not be treated as synonyms. Earlier report [6] has regarded 'halia bara' as synonymous to 'halia padi'. This result may not be conclusive but a very good indicator of such differentiation among varieties and other DNA finger printing methods could be employed to further strengthen these results.

Table 1. RAPD bands of *Z. officinale* Rosc. var. *officinale*, *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara and *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) for selected OPA primers and estimated size of the bands (bp) distinguishing the varieties.

Primers	H	HB	HP	No. of shared bands by 3 varieties	Estimated size (bp) of the most distinguishable bands for the 3 varieties studied		
					H	HB	HP
OPA1	3	2	2	2	1436,1039,491	1019,485	1012,479
OPA2	1	1	1	1	312	310	310
OPA3	2	2	2	2	911,782	890,782	886,777
OPA4	2	2	2	2	2278,575	2333,571	2333,571
OPA5	2	2	2	2	1519,668	1519,668	1556,664
OPA6	1	1	1	1	975	984	966
OPA7	1	1	1	1	1750	1788	1750
OPA8	2	2	3	2	1586,548	1586,548	1931,1500,544
OPA9	2	3	3	2	1200,857	1162,850,448	1125,864,439
OPA10	2	3	1	0	1192,613	1175,608,135	135
OPA11	1	1	1	1	753	753	753
OPA13	4	4	1	1	1963,1137,955,632	1925,1119,960,626	639
OPA15	2	2	2	2	1893,733	1929,740	1893,727
OPA16	3	3	4	3	1330,727,486	1322,727,486	1314,1056,736,497
OPA18	2	2	2	2	1183,717	1183,706	1183,711
OPA20	4	6	5	3	1740,1325,1130,671	1720,1120,939,763,676,548	1720,1130,939,676,548

*The most prominent bands are underlined
H = *Z .officinale* Rosc. var. *officinale* (halia), HB = *Z .officinale* Rosc. var. *rubrum* Theilade (halia bara), HP = *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi).

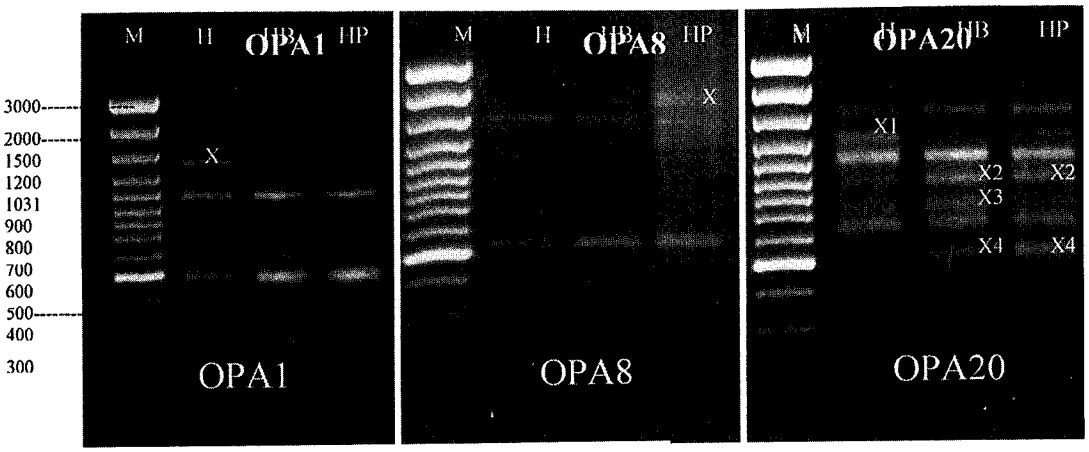

Figure 1. RAPD markers produced by primers OPA1, OPA8 and OPA20.

'X' shows the polymorphic banding patterns distinguishing all varieties studied;
(OPA1; X=1500bp, OPA8; X=2000bp, OPA20; X1=1350bp, X2=900bp, X3=800bp, X4=550bp)
H = *Z. officinale* Rosc var.*officinale* (halia), HB = *Z. officinale* Rosc var. *rubrum* Theilade (halia bara),
HP = *Z. officinale* Rosc var. *rubrum* Theilade (halia padi), M = Marker 100bp Ladder Plus.

Table 2. RAPD markers of three varieties of *Z. officinale*

Primer	bp	*Z. officinale* var. *officinale*	*Z. officinale* var. *rubrum*	*Z. officinale* var. *officinale* (halia padi).
OPA1	1500	1*	0	0
	1031	1	1	1
	500	1	1	1
OPA2	300	1	1	1
OPA3	900	1	1	1
	800	1	1	1
OPA4	2000	1	1	1
	600	1	1	1
OPA5	1500	1	1	1
	700	1	1	1
OPA6	1031	1	1	1
OPA7	1750	1	1	1
OPA8	2000	0	0	1 *
	1500	1	1	1
	550	1	1	1
OPA9	1200	1	1	1
	900	1	1	1
	400	0	1	1
OPA10	1200	1	1	0
	600	1	1	0
	150	0	1	1
OPA11	800	1	1	1
OPA13	2000	1	1	0
	1200	1	1	0
	900	1	1	0
	600	1	1	1
OPA15	2000	1	1	1
	700	1	1	1
OPA16	1300	1	1	1
	1031	0	0	1*
	700	1	1	1
	500	1	1	1
OPA18	1200	1	1	1
	700	1	1	1
OPA20	1750	1	1	1
	1350	1*	0	0
	1200	1	1	1
	900	0	1	1
	800	0	1 *	0
	700	1	1	1
	550	0	1	1

1- bands present, 0- bands absent, * - variety specific band

Table 3. Values of Similarity Index between varieties of *Zingiber officinale*.

Primer	H + HB	H + HP	HB + HP
OPA1	0.8	0.8	1.0
OPA2	1.0	1.0	1.0
OPA3	1.0	1.0	1.0
OPA4	1.0	1.0	1.0
OPA5	1.0	1.0	1.0
OPA6	1.0	1.0	1.0
OPA7	1.0	1.0	1.0
OPA8	1.0	0.8	0.8
OPA9	0.8	0.8	1.0
OPA10	0.8	0.0	0.5
OPA11	1.0	1.0	1.0
OPA13	1.0	0.4	0.4
OPA15	1.0	1.0	1.0
OPA16	1.0	0.9	0.9
OPA18	1.0	1.0	1.0
OPA20	0.6	0.7	0.9
	0.94	**0.84**	**0.91**
	82.1%	**67.5%**	**77.5%**

H = *Z. officinale* Rosc. var. *officinale* (halia), HB = *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara), HP = *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi).

CONCLUSION

Polymorphic RAPD patterns distinguishing *Z. officinale* Rosc. var. *officinale*, *Z. officinale* Rosc. var. *rubrum* Theilade (halia bara) and *Z. officinale* Rosc. var. *rubrum* Theilade (halia padi) were generated using 16 primers. Based on these results, it can be concluded that RAPD technique is useful for the differentiation of varieties of *Z. officinale* using the primers used in this work.

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