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Sex-specific Random Amplified Polymorphic DNA (RAPD) Markers in *Carica papaya*

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ABSTRACT. *Carica papaya is valued as an important fruit crop in tropical and warmer subtropical regions of the world. It exhibits polygamous as hermaphrodite, male and female that usually takes 5–8 months for phenotypic expression. Nursery culling aided by sex specific DNA markers was envisaged to alleviate the unnecessary cost farmers have to incur by maintaining unproductive males that make up 40–50% of the population. The mechanism of sex determination in Carica papaya was described as a tri-allelic single gene with alleles, M_1, dominant for maleness, M_2, dominant for hermaphrodism and m recessive for femaleness dual dominant zygotes (M_1M_1, M_2M_2 and M_1M_2) being inviable.*

Bulked DNA samples of male, female and hermaphrodite Carica papaya were screened using 100 random decanucleotide primers in the RAPD-PCR and selected 20 promising primers for further assaying of individual DNA samples with repeated PCR runs for accurate detection of sex-specific RAPDs.

Initially, a total of 971 amplification products and 89 polymorphisms were obtained by screening bulked samples with 75 primers of which 89 products were polymorphic. The selected twenty primers yielded 927 bands with 375 being polymorphic among 12 individuals. The RAPD based genetic distances clearly indicated genetic differentiation of the population on individual's sex. Three well-amplified, highly reproducible sex-specific fragments, OPC 9–1.7 kb and OPE 3–400 bp (maleness and hermaphrodism) and OPE 19–2.18 kb (femaleness) were detected possibly with tight linkage. Converting these to more authentic SCAR markers by cloning, sequencing the fragments and designing more appropriate primers for developing a single PCR diagnostic assay was anticipated for accurate determination of Carica papaya sex in the nursery.

INTRODUCTION

Carica papaya L. (Papaya or Papaw), native of tropical America spread to most of the Caribbean and Asia, is a widely distributed fruit crop throughout the tropical and warmer subtropical regions of the world. The consumption of *Carica papaya* is growing steadily in parallel with the increase in health conscious food consumers, as the fruit is low in calories and sodium but high in dietary fiber, calcium, potassium and vitamins A and C. The *Carica papaya* enzyme, papain is used in meat tenderizes, face and hair care products

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and various manufacturing applications such as leather, wool, rayon and beer (Wysiwyg://304/http://www.hort.purdue.edu/newcrop/morton/papaya-ars.html).

Carica papaya has good agronomic features such as easy cultivation, rapid growth, minimum-growing space, early production, high yields, multiple uses, prompt returns and adaptation to diverse climatic and soil conditions. Breeding research conducted so far have shown immense promise in development of characters such as tree dwarfing, uniform fruiting, fruit size and quality, more flesh, cold tolerance, fruiting precocity, and tolerance to various stress conditions (Medagoda, 1998). *Carica papaya* trees exhibit sexual dimorphism as hermaphrodite, male and female plants although it can take between 5 – 8 months to discriminate between the sexes. The striking feature is the close association of sex to desirable agronomic traits. Hermaphrodite plants produce rich fleshed fruits of attractive shape while female plants produce fruits rich in papain with a larger number of seeds (Parasnis *et al.*, 1999). The males are unproductive and the growers incur a high cost by maintaining them for a substantial period of time with all inputs. Identification of sex specific DNA markers would alleviate this problem as these markers are reliable that they are unaltered by the environment.

The sex of papaya is determined by five closely linked genes on the sixth chromosome whose phenotypic effects can be described as a tri-allelic single gene with alleles, M_1, dominant for maleness, M_2, dominant for hermaphrodism and *m* recessive for femaleness (Hofmeyr, 1939). The model fitted well with expected Mendelian ratios upon assumption that diploid zygotes; M_1M_1, M_2M_2 and M_1M_2 are inviable. Parasnis *et al.* (1999) provided molecular evidence for a putative Y chromosome in *Carica papaya* diverging in a sex-specific manner. Niroshini *et al.*, 1998 reported preliminary data supporting the possibility of detecting RAPD markers linked to sex expression of *Carica papaya*. Similar studies have also been reported by Somsri *et al.* (1998) and Sondur *et al.* (1996). This study was initiated to identify suitable RAPD markers with tight linkage to sex that could potentially be used to determine the sex of *Carica papaya* seedlings.

MATERIALS AND METHODS

Tender *Carica papaya* leaves from 10 individuals each of male, female and hermaphrodite plants were collected from gardens around Kadawatha. Total genomic DNA was isolated using the simplified CTAB protocol described by Dassanayake *et al.* (1999) and the concentration of DNA was determined spectrophotometrically at 260 nm. DNA amplification for RAPD analysis was carried out using 100 arbitrary decamer primers of the series OPA 01–20, OPB 01–20, OPC 01–20, OPD 01–20 and OPE 01–20 (Operon Technologies, USA) in accordance with Williams *et al.* (1990). Initial amplification was carried out using bulked DNA samples from individual male, female and hermaphrodite plants, separately.

Polymerase chain reactions (PCR) were performed in 25 µl volumes comprising 1×PCR buffer (Promega), 1.8 mM $MgCl_2$ (Promega), 0.16 mM each dNTPs (Pharmacia), 10 pmol primer, one unit of *Taq* polymerase (Promega) and 75 ng of template DNA (2.5 µl, 10×PCR buffer, 2.0 µl, 2 mM dNTPs, 1.8 µl, 25 mM, $MgCl_2$, 0.2 µl, 5 unit/µl *Taq* polymerase, 1.0 µl primer, 15.5 µl PCR water). Amplification was performed in a

programmable thermocycler (PTC–100) with 45 cycles of 94°C for one minute, 36°C for one minute and 72°C for two minutes. Amplification products were separated on 1.2% agarose gel containing ethidium bromide 0.5 µg per ml. Electrophoresis was carried out at 5 v/cm for two and a half hours and the gels were scanned under UV by Photo-Print Gel Documentation system. The images were captured and scored visually as presence or absence of bands and the accurate size of bands were estimated with the help of Photo-Capt software.

Polymerase chain reactions were repeated with twenty selected primers, based on well-resolved amplification profiles and presence of polymorphisms with individual DNA, four from each sex. The amplification products were scored as presence of band, 1 and absence of band 0 and based on these scores, pair-wise similarity in the banding patterns in plants within and between the sexes were computed by Nei and Li's formula (Similarity index, $SI = 2N_{ab} / (N_a + N_b)$ where, N_a and N_b refer to the total number of bands present respectively, in individual 'a' and 'b' and N_{ab} refers to the total number of bands commonly shared by the two individuals N_a and N_b). Polymerase chain reactions were repeated several times with primers, OPC9, OPE3 and OPE19 with newly isolated DNA and also in another thermocycler, Perkin Elmer 4800.

RESULTS

Among 100 primers, 75 generated clear DNA profiles yielding a total of 971 amplification products (4–5 bands per primer) for the bulked sample of male, female and hermaphrodite plants with 64% being very strong amplifications. A total of 89 amplified fragments exhibited polymorphism of which 55% were very strong amplifications (Table 1). The best twenty primers yielded 927 bands (strongly amplified) of which 357 were polymorphic (Table 2).

The genetic relationship of the 12 individuals, constructed by Nei and Li genetic similarity index clearly indicates a clear separation of female group from other sexes. This implies a genetic differentiation of the population based on the sex of the individuals (Fig. 1).

The most striking result was the detection of three characteristic amplification fragments (bands), OPC9–1.7 kb, OPE3–400 bp and OPE19–2.18 kb (named according to the primer number and the size of the band) with very tight linkage to sex. OPC9–1.7 kb and OPE3–400 bp (Figs. 2 and 3) were characteristic for maleness and hermaphrodism while OPE3–400 bp was characteristic for femaleness (Fig. 4).

DISCUSSION

The results elucidate important features of the sex expression in *Carica papaya* and provoke developing a molecular-marker-based method to identify sex at seedling stage. A considerable number of agriculturally important plants including buffalograss, nutmeg, date palm, hop, hemp, pistachio, kiwi fruit, asparagus and papaya are dioecious. In all of these crops, the farmers can be greatly benefited by methods for detection of sex at an early

Table 1. Summary of DNA profiles obtained by RAPD-PCR amplification of bulked DNA samples of female, male and hermaphrodite *Carica papaya*.

Primer	Fragment Size Range (kb)	Number of amplified fragments						Number of polymorphic fragments					
		F		M		H		F		M		H	
		S	W	S	W	S	W	S	W	S	W	S	W
OPA 1-20	<0.5-2.9	39	25	41	24	49	24	1	1	2	1	5	3
OPB 1-20	0.5-2.5	27	25	30	19	31	22	3	5	5	1	5	3
OPC 1-20	<0.5-2.4	53	34	55	35	56	32	1	4	3	3	4	1
OPD 1-20	<0.5-2.3	45	21	34	22	36	22	9	4	1	2	2	3
OPE 1-20	<0.5-2.3	41	13	41	16	41	18	2	1	3	3	3	5
Total		205	118	201	116	213	118	16	15	14	10	19	15

F - Female M- Male H-Hermaphrodite
Strongly (S) and weakly (W) amplified bands are given in separate columns

Table 2. The twenty best primers selected and their DNA profiles obtained by RAPD-PCR amplification of individual DNA.

Primer	Primer sequence	Number of Amplified Fragments			Number of Polymorphic Fragments		
		F	M	H	F	M	H
OPA 11	CAATCGCCGT	11	14	15	6	10	11
OPA 12	TCGGCGATAG	20	22	16	16	17	12
OPA 15	TTCCGAACCC	11	11	10	3	3	2
OPB 12	CCTTGACGCA	26	26	28	6	6	8
OPB 13	TTCCCCCGCT	18	18	19	6	6	7
OPB 15	GGAGGGTGTT	23	23	23	7	7	7
OPC 03	GGGGGTCTTT	9	7	9	5	3	5
OPC 05	GATGACCGCC	20	20	22	8	6	8
OPC 06	GAACGGACTC	24	20	25	17	14	20
OPC 09	CTCACCGTCC	6	18	20	6	10	12
OPC 17	TTCCCCCCAG	16	16	17	1	1	1
OPD 07	TTGGCACGGG	30	26	33	9	6	13
OPD 18	GAGAGCCAAC	25	26	25	1	3	1
OPD 20	ACCCGGTCAC	25	23	24	5	3	4
OPE 03	CCAGATGCAC	12	16	16	0	4	4
OPE 08	TCACCACGGT	13	12	11	6	7	6
OPE 19	ACGGCGTATG	21	15	16	13	7	8

F - Female M - Male H - Hermaphrodite

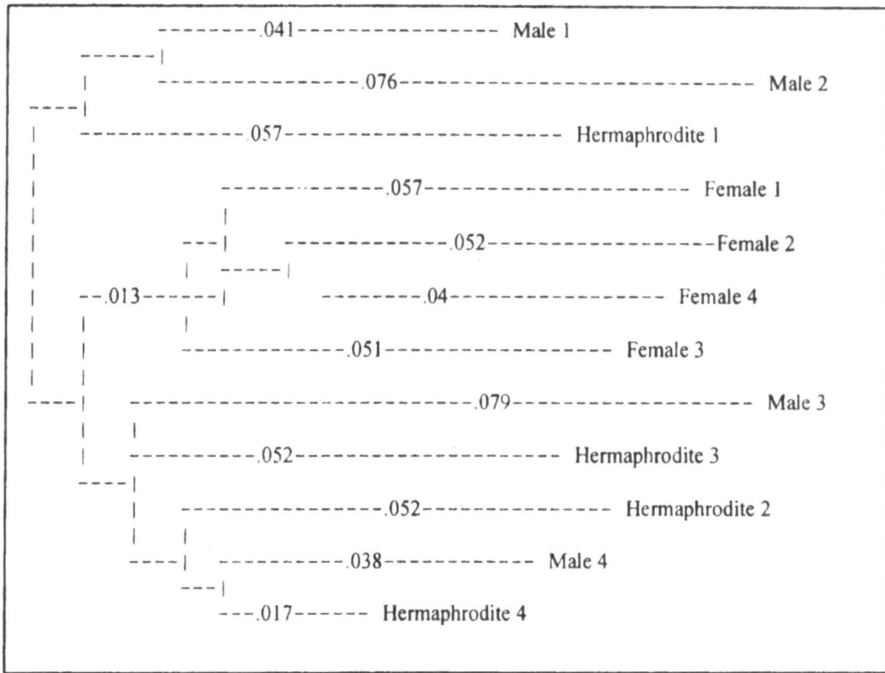

Fig. 1. **Genetic relationship of male, female and hermaphrodite *Carica papaya* as revealed by 20 Operon primers in the RAPD-PCR.**

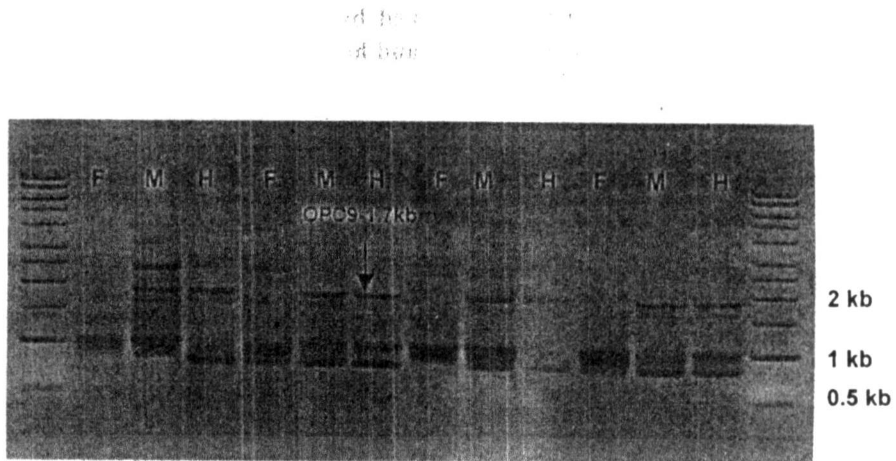

Fig. 2. **RAPD-PCR DNA profiles obtained by amplification of individual DNA samples of female (F), male (M) and hermaphrodite (H) *Carica papaya* by Operon primer OPC9.**

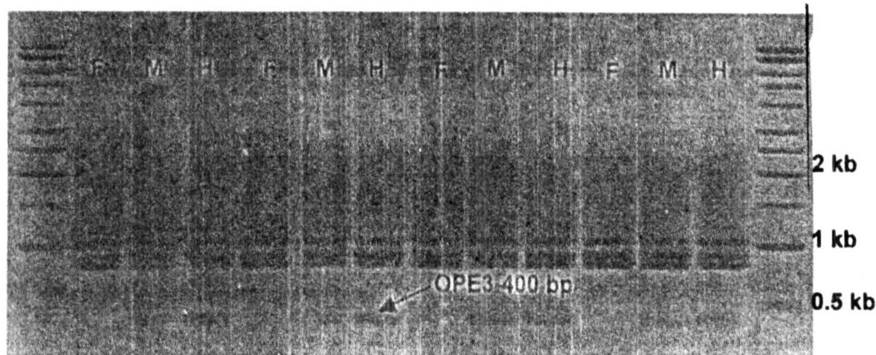

Fig. 3. RAPD-PCR DNA profiles obtained by amplification of individual DNA samples of female (F), male (M) and hermaphrodite (H) *Carica papaya* by Operon primer OPE3.

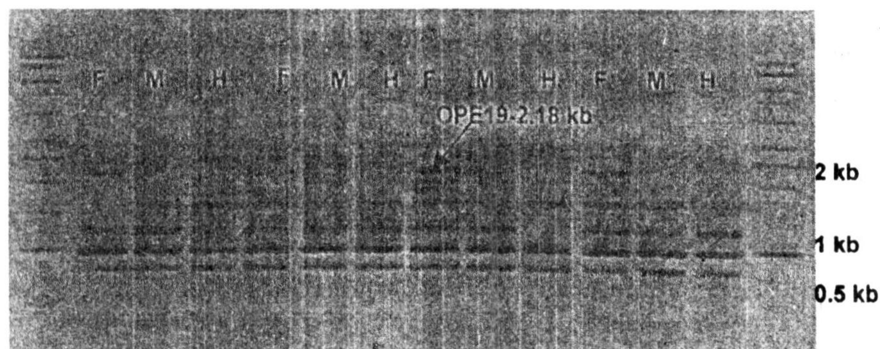

Fig. 4. RAPD-PCR DNA profiles obtained by amplification of individual DNA samples of female (F), male (M) and hermaphrodite (H) *Carica papaya* by Operon primer OPE19.

stage as they can cultivate sufficiently larger number of productive hermaphrodite or female (depending on the market preference) plants with only a minimal number of unproductive male trees.

But *C. papaya* is polygamous, wherein the male and female flowers are borne on separate plants that promote cross-pollination, similar to plant features such as self-compatibility, male sterility and floral devices. This is a most striking example of evolutionary speciation where extreme specialization of sexes into separate plants has

evolved as a mechanism to ensure outbreeding or for optimal allocation of resources between the sexes (Shibu *et al.*, 2000; Delph *et al.*, 1993). The genetic mechanisms involved in dioecy vary in different plants. For *Carica papaya* Hofmeyr (1939) and Storey (1941) provided evidence to suggest that male and hermaphrodite plants are heterogamous (XY) while female is homogamous (XX). The clustering of males and hermaphrodites together separating females of *Carica papaya* by the dominant marker, RAPD too support this mode of inheritance that the genetic factors for sex are active in males and hermaphrodites and not in females.

The three polymorphic bands, OPC9–1.7 kb, OPE3–400 bp and OPE19–2.18 kb generated by Operon primers assure great promise in developing a molecular marker based technique for early identification of sex expression in *C. papaya*. Similarly, Somsri *et al.* (1998) attempted to identify molecular markers for sex determination in *Carica papaya* using two similar PCR based methods RAPD and DAF. As expected of the smaller size of the primers used in DAF a five-fold increase was observed in the number of bands amplified than in RAPD and a large number of DAF markers specific to male or hermaphrodite pooled DNA products were detected. However, they had meager success in converting DAF markers to SCAR (sequence characterized amplified region). The potential of detecting sex-specific RAPD markers have also been established using bulk segregant analysis with several other dioecious plants. Hormaza *et al.* (1994) screened 700 random primers in the RAPD-PCR and detected a band (OPO08–945 bp) tightly linked to female sex of the nut crop, *Pistacia vera*. Polley *et al.* (1997) obtained 32 male specific RAPD bands in hop (unfertilized female flowers are as a beer ingredient) by screening 900 primers while Shibu *et al.* (2000) reported female specific band in nutmeg by screening 60 Operon primers. Jiang *et al.*, 1997 detected two RAPD markers linked to M locus (maleness) in Asparagus and successfully converted one to a SCAR (sequence characterized amplified region) marker.

In addition to RAPD markers, the diagnostic potential of micro-satellite markers was also, exploited to identify sex expression of *C. papaya* by Parasnis *et al.* (1999) using $(GATA)_4$ and $(GAA)_6$ as probes on restriction digested *C. papaya*. This study has also generated useful information to support, the heterogamous male/hermaphrodite and homogamous female, sex determination mechanism in *C. papaya*. More interestingly the same group reported to have developed a method based on restriction digestion and PCR for early detection of *C. papaya* males which has been filed for patent rights in India and USA.

The diagnostic potential of the three polymorphic bands, OPC9–1.7 kb, OPE3–400 bp and OPE19–2.18 kb has to be addressed with due consideration to problems associated with reproducibility of RAPD profiles. A study carried out by a network of European laboratories have confirmed that RAPD profiles were not found to be sufficiently reproducible despite efforts to maintain constant reaction conditions (Jones *et al.*, 1998). However, we have found that the amplification of above three fragments were highly reproducible with several repeated runs of PCR and a large number of DNA samples extracted on many occasions and with two different thermocyclers. This need to be further tested with PCR reagents of different origin and laboratories elsewhere. Nevertheless these three sex specific fragments have been cloned for converting them to more authentic SCAR

markers towards developing a single PCR diagnostic assay for sex determination in *Carica papaya.*

CONCLUSIONS

Genetic differentiation of *Carica papaya* by sex was revealed by RAPD screening, indicating great opportunities for detection of sex-specific molecular markers in *Carica papaya.* The three RAPD markers, OPC9–1.7 kb, OPE3–400 bp and OPE19–2.18 kb detected are highly promising in that they themselves as RAPDs have shown very high level of reproducibility. Cloning and sequencing of these fragments are very likely to give useful information in developing a very accurate molecular marker screening tool for detecting sex expression of *Carica papaya*, so that the farmers can cultivate sufficiently larger number of plants with a marginal number of unproductive male individuals.

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