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VALIDATION AND USE OF DNA MARKERS FOR SEX DETERMINATION IN PAPAYA (*CARICA PAPYA*)

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Abstract

Profitable papaya production requires female and hermaphrodite plants in higher number than male plants. This is only possible if sex of plants is determined at an early growth stage. The present study was conducted to validate sex-linked DNA markers using plants from two Pakistani papaya varieties and subsequently utilize them for determination of sex in juvenile papaya plants. One hundred and five plants (including 49 male and 56 female) of two Pakistani Papaya varieties at flowering stage were screened with six DNA markers viz., W-11, T12, SDP, Napf-76Napf-76, PKBT4 and PKBT5. All male plants exhibited amplification of sex-linked alleles with markers T12 and W11, whereas, 96% and 95% of female plants showed the absence of sex-linked allele with these markers, respectively. Markers SDP, PKBT5 and Napf-76 showed the presence of sex-linked alleles in 98%, 96% and 93% of male plants, respectively, whereas the same markers showed the absence of sex-linked alleles in 100%, 96% and 94% of female plants. One marker, PKBT4 could not produce expected PCR amplification reported previously. The five DNA markers were further used to screen 171 papaya seedlings. These markers clearly differentiated male and female sex types in the studied papaya plants. Results of our study are likely to facilitate Pakistani papaya breeders and growers to incorporate DNA based screening at juvenile stage to determine sex at early stage and to ensure profitable papaya production.

Key words: Papaya; Sex determination; DNA markers.

Introduction

Papaya (*Carica papaya*) is widely grown fruit tree of tropical and subtropical regions (Wu *et al*., 2012). Although papaya has been originated from tropical America but its cultivation is steadily increasing throughout the world due to its nutritional value as well as industrial use.

Tariq, (2014) reported the origin and evolution of male and female plant from hermaphrodite plant experimentally in Pakistan. Papaya fruit is a low caloric diet, rich in dietary fiber and vitamin A and C, whereas, it contains less quantity of sodium thus beneficial for health conscious people (Parasnis *et al*., 1999; Gamage *et al*., 2003). In Pakistan, climate of Punjab and Sind are suitable for papaya production. However, Malir in Karachi and coastal areas of Sind are well known for commercial scale papaya production.

Papaya cultivation is liked by farmers due to its fast growth, short duration and early maturity. Moreover, papaya is a high yielding crop and has a variety of uses but adaptability to diverse range of environments is the salient feature for increased papaya cultivation in various regions in the world (Evans *et al*., 1999). Various breeding efforts have been carried out for short stature, large fruit size, resistance to cold stress and quality.

Papaya plants are divided into three types based on sex-types i.e., male, female and hermaphrodite (both sex-types). Papaya is sexually dimorphic flowering plant, constituting a population of male and female plants in almost equal ratio. Female plants are more valued commercially compared to male papaya plants due to their fruit production. Female plants, however, contain less flesh and produce large number of seed compared to hermaphrodite plants. Cultivation of hermaphrodite and female plants vary depending upon the utilization of its fruits. For instance, pyriform papaya fruit is preferred in foods like desserts, whereas, female papaya fruits are used for industrial purpose. Papaya is the source of a proteolytic enzyme known as papain, which is used in pharmaceutical, leather and food processing industries. Enzyme activity has been reported superior in female papaya fruits compared to hermaphrodite.

Sex determination in Papaya is delayed until flowering which takes six to eight months. Male plants are only needed for pollination as these do not bear fruit. Only 5% male plants are needed for pollination in a papaya population, so most male papaya plants are discarded for not bearing fruit. Therefore, cultivation of female and hermaphrodite plants is preferred to ensure profitable papaya cultivation. For this reason, male plants are chopped down after flowering stage which is less economical to the farmers. Some other dioecious species exhibiting delayed sex identification until flowering like Silene, Rumex, Humulus and Coccinia have a Y chromosome, thus sex can be determined cytologically. However, other species lacking this chromosome cannot be detected through cytology. This constraint has been overcome by the development of sex-linked DNA based markers which allow sex determination at juvenile stage through polymerase chain reaction. Phenotypic sex types of papaya are linked with three alleles (M1 allele for male, M2 for hermaphrodite and m for female) at the same locus SEX1 (Storey, 1938). They hypothesized that plants having homozygous dominant alleles such as M_1M_1, M_2M_2 and M_1M_2 are lethal phenotypes. Later on,

Storey (1953) revised the model, stating that the sex in papaya is controlled by a gene complex rather than single gene and that the gene complex is confined to a small region on sex chromosome. Sondur *et al*. (1996) constructed a model by developing genetic linkage map using RAPD (Random Amplified Polymorphic DNA) marker suggesting that the *Sex1-M* allele for maleness codes a trans-acting element involved in induction of male floral organs and in reduction of carpal development. According to the model, *SEX1-H* allele for hermaphrodite plays an intermediate role in induction of male floral parts but is capable of inhibiting carpal development. On the other hand, *SEX1-F* allele that is responsible for female phenotype is incapable to develop male reproductive parts in flowers. As a result of interspecific hybridization in *Circaceae* family, XX-XY type sex determination model has been proposed where XX is female genotype, XY male genotype and XY2 is hermaphrodite genotype.

In papaya, RAPD and microsatellite markers linked to sex have been reported (Sondur *et al*., 1996; Parasnis *et al*., 2000; Lemos *et al*., 2002; Urasaki *et al*., 2002), and their conversion to SCAR (sequence characterized amplified region) (Urasaki *et al*., 2002; Deputy *et al*., 2002). In present study, we report validation of these markers in 105 papaya plants belonging to two varieties grown in Pakistan. Furthermore, we also report sex determination in 171 papaya seedlings using these DNA markers.

Materials and Methods

Plant material: The plant material for this study comprised of 105 papaya plants, belonging to two Pakistani Papaya varieties, at flowering stage grown at National Agricultural Research Centre, Islamabad and 171 papaya plants at seedling stage that were grown in plastic bags in the glass house of IABGR, National Agricultural Research Center (NARC), Islamabad, Pakistan. The plants were tagged and numbered. Two to three leaf tissues from two weeks old seedlings were obtained for genomic DNA extraction and plants were transplanted in the field for phenotypic sex determination at the flowering stage.

Genomic DNA extraction: Genomic DNA was extracted from fresh leaves using CTAB method (Doyle & Doyle, 1987) with slight modification previously described (Qamar *et al*., 2014). DNA was quantified on 1% agarose gel and diluted to a 50 ng/ul working concentration.

Polymerase chain reaction: Polymerase chain reaction (PCR) was performed using six SCAR markers T12, W11, SDP, PKBT4, PKBT5 and Napf-76 (Table 1). PCR reaction was performed in 20 µl volume, containing 10 mM Tris-HCl (pH 8.5), 50 mM $(NH_4)_2$ SO_4, 1.5 mM $MgCl_2$, 50 ng genomic DNA, 10 pmol of each primer, 2.5 mM of each dNTPs, and 1 U of *Taq* polymerase. The reaction mixture was pre-incubated at 94˚C for 4 minutes and then PCR was repeated for 35 cycles of 94˚C for 45 seconds, 58˚C for 45 seconds, and 72˚C for 1 minute, followed by final extension at 72˚C for 10 minutes. PCR products were resolved on 1.5% agarose gel in TBE buffer. Bands associated with male or female plants were visualized under Gel Documentation system and subsequently scored. Flowering data of 105 plants were recorded by observing flower type i.e male, female and hermaphrodite (having both floral parts). However, among 171 plants subjected to screening, flowering data was available only for 70 plants.

Results

Validation of markers: Six DNA markers were used to determine sex-types in 105 Papaya plants belonging to two Pakistani Papaya varieties, dwarf papaya (Karachi) and local papaya (unknown variety) (Table 2). DNA markers viz; W-11, T12, SDP, Napf-76, and PKBT5 yielded reproducible results, whereas, marker PKBT4 could not produce expected PCR amplification reported previously (Sobir and Pandia, 2008). SCAR markers W11 and T12 were preliminarily tested on few male and female papaya plants. Both markers amplified 800-bp fragments in male plants, whereas, no band was detected in female plants (Fig. 1). Results of these two markers were similar in terms of amplification of sex-linked allele in male plants and non-amplification of this allele in female plants. Among 105 papaya plants, marker T12 produced 800-bp fragment in 48 of 49 male plants (one sample missing), whereas, 54 of 56 female plants did not show any amplification. However, two plants having female floral morphology amplified 800-bp band size, indicating male sex type. Likewise, marker W11 also yielded 800-bp fragment linked to male sex types in all 48 male plants (one missing), showing brighter bands except one sample with very faint amplification (Fig. 1). Similarly, no 800-bp band was observed in 54 of 56 female plants with marker W11, suggesting the absence of sex-linked allele (Table 2).

We used SCAR primers SDP-2 and SDP-3 to detect the presence or absence of *SEX1* gene. This marker resulted in a DNA fragment of 221-bp in 47 plants, indicating the presence of *SEX1* gene, whereas, no amplification of the 221-bp fragment was observed in the remaining 57 plants (Fig. 1). Similarly, PKBT5 was used to detect the presence or absence of *SEX1* in 105 papaya plants. All the male plants showed an expected 320-bp fragment while no band was observed in female plants except 320-bp amplification in two plants. We also used primer pairs Napf-76 and Napf-77, which amplified a brighter band of 831-bp in all the male plants; 3 male plants did not show any fragment, whereas, no amplification was observed in female plants except three plants showing male sex type (17 samples were missing) (Fig. 1). Marker PKBT4 did not produce the expected fragment size previously reported with sex type in papaya.

Results for marker SDP were 99% consistent with flower morphology of papaya plants followed by T12 (98%), W11 (97%), and PKBT5 (96%). Marker Napf76 also diagnosed sex type in accordance with morphological data in case of male plants, whereas, some of the female plants showed faint bands. Two plants (No. 72 and 87) exhibited male sex type based on marker data, but they were female based on flower morphology.

Table 1. Markers used for sex determination in papaya with primer names and sequences.

No.	Primer name	Primer sequence	Reference
1.	T12-F	GGGTGTGTAGGCACTCTCCTT	Deputy *et al*. (2002)
	T12-R	GGGTGTGTAGCATGCATGATA	
2.	W11-F	CTGATGCGTGTGTGGCTCTA	Deputy *et al*. (2002)
	W11-R	CTGATGCGTGATCATCTACT	
3.	SDP-1	GCAGGATTTAGATTAGATGT	Urasaki *et al*. (2002)
	SDP-2	GGATAGCTTGCCCAGGTCAC	
4.	PKBT4-F	GAGGGCGAGGTTTGAATTTGG	Sobir & Pandia (2008)
	PKBT4-R	TTTGGTGTCCTGGTTACCCTC	
5.	PKBT5-F	AGCCAGGGTCGTGGTAAGAG	Sobir & Pandia (2008)
	PKBT5-R	TCCCATGGCGTGTCGCGCTG	
6.	Napf-76-F	GAGGATCCCTATTAGTGTAAG	Parasnis *et al*. (2000)
	Napf-77-R	GAGGATCCCTTTTGCACTCTG	

Screening of Papaya plants for sex-type at juvenile stage: One hundred seventy one papaya plants of unknown sex were screened with marker T12, W11, SDP and PKBT-5 (Table 3). PCR was performed along with one male (M1) and one female (F1) genotypes with known sex as positive and negative control to avoid false positive and negatives. Marker SDP produced a band size of 221-bp in 76 plants and positive control, indicating the presence of male/hermaphrodite locus, whereas, remaining 95 genotypes and negative control did not show any fragment, suggesting absence of sex-linked marker.

Marker T12 produced 800-bp fragment size in 74 samples and positive control, indicating these samples to be male plants, whereas remaining 96 plants and negative control showed absence of the 800-bp PCR amplicon, indicating the female sex type (one sample missing). Out of 171 papaya seedlings, 74 were predicted as male by marker W11 yielding 800 bp fragments in these samples and positive control. On the other hand, this marker did not amplify 800-bp PCR amplicon in rest of the 92 samples and negative control, suggesting them as female plants. Five plants did not amplify any fragment. Marker PKBT-5 was used to determine the sex of 84 papaya plants. Among these, 30 plants and positive control produced a 320-bp band linked to male sex-type (MM), whereas 54 plants did not amplify any band, suggesting female sex type (mm).

Overall, among 171 papaya plants, the frequencies of male and female plants were 70% and 90%, respectively. Inconsistent results were observed for 11 plants with the different markers used. However, 94% plants showed consistency in results for sex types with the different DNA markers used.

Discussion

Identification and validation of DNA markers linked to sex-specific locus has been a robust and cost effective diagnostic assay in various animals including cattle, pig, poultry and fish (Bredbacka, 1995; McGraw, 1993; Hew, 1996). However, such diagnostic assays are not very common in plants due to the differences in plant genetic programs and animal or forensics. To establish such type of diagnostic procedure in plants, efficiency and cost-effectiveness are pre-requisites. This is because we need to perform the assay on a large number of plants compared to forensic subjects (Rafalsky, 1993). DNA markers linked to sex-linked alleles in papaya provide an accurate and cost effective way for sex determination in papaya. In present study, we report the validation of already developed sex- linked DNA markers in papaya and screening of papaya varieties for reliable sex determination.

Sequence characterized amplified regions (SCARs) markers W11 and T12 synthesized by cloning RAPD fragments, have been reported to detect male and hermaphrodite papaya plants (Deputy *et al*., 2002). Our results are comparable with Deputy *et al*. (2002), indicating reliable sex diagnosis by marker T12 and W11. They mapped both markers 0.3cM apart from sex-linked gene *Sex1* showing no recombination. This distance is closer than 7cM reported previously by Sondur *et al*. (1996). Although, no crossovers were observed by both markers, but marker W11 has been reported more reliable compared to marker T12 (Deputy *et al*., 2002). However, in our study both markers were equally reliable for sex determination in papaya.

Another set of SCAR primers has been developed from RAPD markers named as PSDM (Urasaki *et al*., 2002). These markers were designated as SCARps and are present in male and hermaphrodite papaya genotypes but absent in female genotypes. Urasaki *et al*. (2002) reported that these markers sometimes may amplify false negatives due to the low quality or quantity of DNA. To overcome this problem, they developed multiplex PCR of SCARps and papain gene, involved in the production of thiol protease in papaya, as an endogenous control. However, we did not use this internal control. Instead, we compared marker data with flower types to validate the markers. It was observed that marker SDP showed maximum similarity (99%) with flower type making it the most reliable marker for sex detection in papaya. Our results for marker PKBT5 are in agreement with Sobir *et al*. (2008) as they reported a smaller band size compared to the band size of 450 bp, originally reported by Lemos *et al*. (2002). Despite the difference in band size, these markers can successfully differentiate male and hermaphrodite plants from the female papaya plants at very early stage.

Table 2. Flowering and marker data for 105 plants of two Pakistani papaya varieties.

Sr. No.	Sex	T12	W11	SDP	PKBT5	Napf-76	Sr. No.	Sex	T12	W11	SDP	PKBT5	Napf-76
1	male	+	+	+	+	+	54	female	-	-	-	-	-
2	male	+	+	+	+	+	55	female	-	-	-	-	-
3	male	+	+	+	+	+	56	female	-	-	-	-	-
4	male	+	+	+	+	-	57	female	-	-	-	-	-
5	male	+	+	+	+	+	58	female	-	-	-	-	-
6	male	+	+	+	+	+	59	female	-	-	-	-	-
7	male	+	+	+	+	+	60	female	-	-	-	-	-
8	male	+	+	+	+	+	61	female	-	-	-	-	-
9	male	+	+	+	+	+	62	female	-	-	-	-	-
10	male	+	+	+	+	+	63	female	-	-	-	-	-
11	male	+	+	+	+	+	64	female	-	-	-	-	-
12	male	+	+	+	+	+	65	female	-	-	-	-	-
13	male	+	+	+	+	+	66	female	-	-	-	-	-
14	male	+	+	+	+	+	67	female	-	-	-	-	-
15	male	+	+	+	+	+	68	female	-	-	-	-	-
16	male	+	+	+	+	+	69	female	-	-	-	-	-
17	male	+	+	-	-	+	70	female	-	-	-	-	-
18	male	+	+	+	-	+	71	female	-	-	-	-	-
19	male	+	+	+	+	+	72	female	-	+	-	+	+
20	male	+	+	+	+	+	73	female	-	-	-	-	-
21	male	+	+	+	+	+	74	female	-	-	-	-	-
22	male	+	+	+	+	+	75	female	-	-	-	-	-
23	male	+	+	+	+	+	76	female	-	-	-	-	-
24	male	+	+	+	+	+	77	female	-	-	-	-	-
25	male	+	+	+	+	+	78	female	-	-	-	-	-
26	male	+	+	+	+	+	79	female	-	-	-	-	-
27	male	+	+	+	+	+	80	female	-	-	-	-	-

Table 2. (Cont'd.).

Sr. No.	Sex	T12	W11	SDP	PKBT5	Napf-76	Sr. No.	Sex	T12	W11	SDP	PKBT5	Napf-76
28	male	+	+	+	+	-	81	female	-	-	-	-	-
29	male	+	+	+	+	+	82	female	-	-	-	-	-
30	male	+	+	+	+	+	83	female	-	-	-	-	-
31	male	+	+	+	+	+	84	female	-	-	-	-	-
32	male	+	+	+	+	+	85	female	-	-	-	-	-
33	male	+	+	+	+	+	86	female	-	-	-	-	-
34	male	+	+	+	+	+	87	female	+	+	-	+	+
35	male	+	+	+	+	+	88	female	-	-	-	-	+
36	male	+	+	+	+	+	89	female	+	+	-	-	-
37	male	+	+	+	+	+	90	female	-	-	-	-	-
38	male	+	+	+	+	+	91	female	-	-	-	-	-
39	male	+	+	+	+	-	92	female	-	-	-	-	-
40	male	+	+	+	+	+	93	female	-	-	-	-	-
41	male	+	+	+	+	+	94	female	-	-	-	-	-
42	male	missing	missing	missing	missing	+	95	female	-	-	-	-	-
43	male	+	+	+	+	missing	96	female	-	-	-	-	missing
44	male	+	+	+	+	missing	97	female	-	-	-	-	missing
45	male	+	+	+	+	missing	98	female	-	-	-	-	missing
46	male	+	+	+	+	missing	99	female	-	-	-	-	missing
47	male	+	+	+	+	missing	100	female	-	-	-	-	missing
48	male	+	+	+	+	missing	101	female	-	-	-	-	missing
49	male	+	+	+	+	missing	102	female	-	-	-	-	missing
50	female	-	-	-	-	-	103	female	-	-	-	-	missing
51	female	-	-	-	-	-	104	female	-	-	-	-	missing
52	female	-	-	-	-	-	105	female	-	-	-	-	missing
53	female	-	-	-	-	-							

+ indicates presence of band associated with maleness
- indicates absence of band associated with maleness

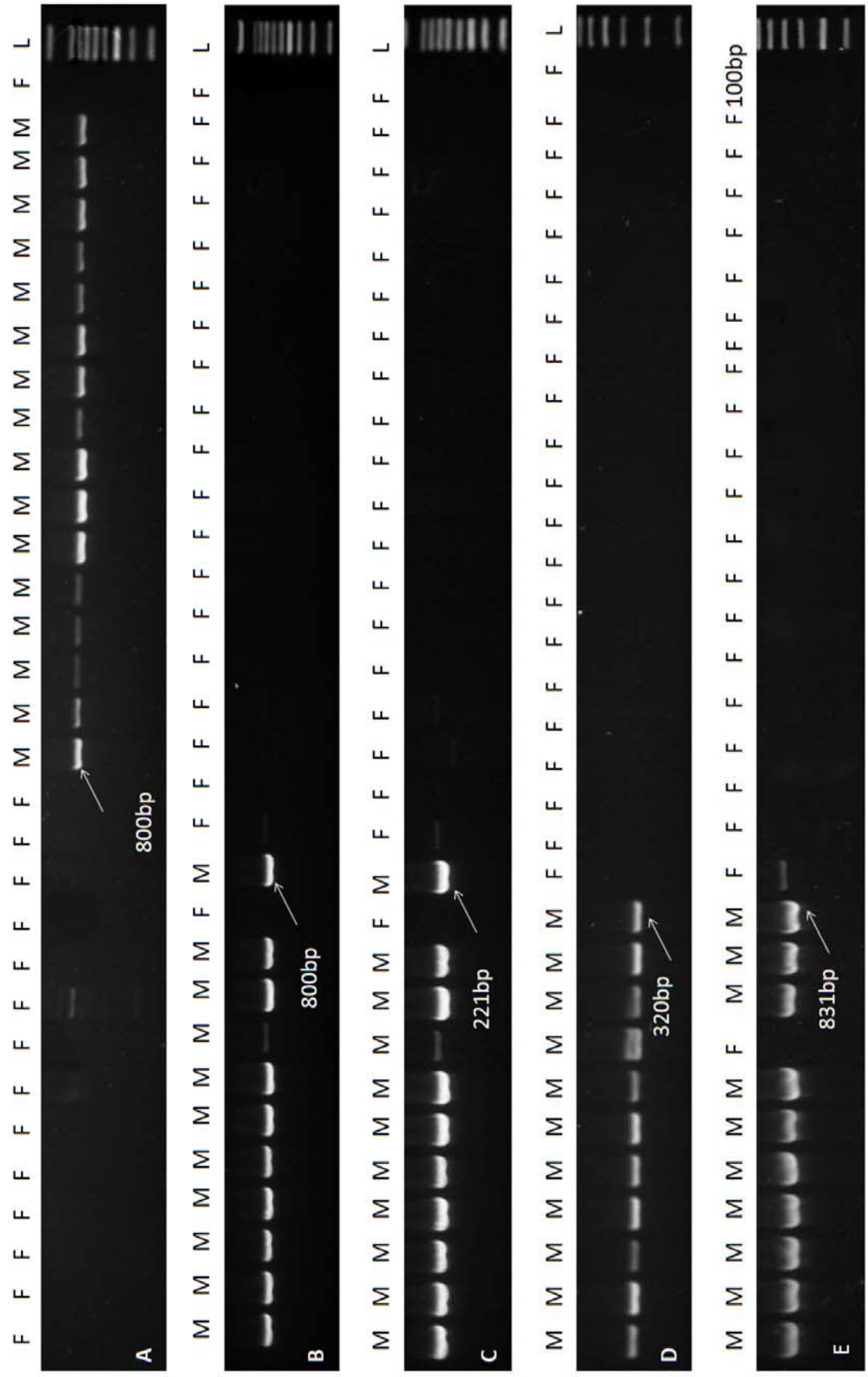

Fig. 1. Banding pattern of marker A) T12 in papaya plants electrophoresed on 1.5% agarose gel; B) W11 in papaya plants using 1.5% agarose; C) SDP in papaya plants resolved on 2% agarose; D) PKBT5 in papaya plants separated on 2% agarose, and E) Napi-76 electrophoresed on 1.5% agarose gel. M= Male sex type: F= Female sex type: L= 100bp ladder.

Table 3. Determination of sex in 171 papaya plants at seedling stage with markers SDP, T12, W11 and PKBT5.

S. No.	SDP	T12 R1	W11	T12 R2	PKBT5	S. No.	SDP	T12 R1	W11	T12 R2
1.	+	+	+	+	+	87.	+	+	+	-
2.	+	+	+	+	+	88.	-	-	-	-
3.	-	-	-	-	-	89.	+	+	+	+
4.	+	+	+	+	+	90.	+	+	+	+
5.	+	+	+	+	+	91.	-	-	-	-
6.	-	-	-	-	-	92.	+	+	+	+
7.	+	missing	missing	+	+	93.	+	+	missing	+
8.	+	+	+	+	+	94.	-	+	missing	-
9.	+	+	+	+	+	95.	-	+	missing	-
10.	+	+	+	+	+	96.	-	-	missing	-
11.	-	-	-	?	-	97.	+	+	+	+
12.	-	-	-	-	-	98.	-	-	-	-
13.	-	-	-	-	-	99.	-	-	-	-
14.	-	-	-	-	-	100.	-	-	-	-
15.	+	+	+	+	+	101.	-	-	-	-
16.	+	-	+	+	+	102.	-	-	-	-
17.	-	-	-	-	-	103.	+	+	+	+
18.	-	-	-	-	-	104.	+	+	+	+
19.	+	+	+	+	+	105.	-	-	-	-
20.	-	-	-	-	-	106.	-	-	-	-
21.	-	-	-	-	-	107.	-	-	-	-
22.	-	-	-	-	-	108.	-	-	-	-
23.	+	+	-	+	+	109.	-	-	-	-
24.	+	+	+	+	+	110.	-	-	-	-
25.	-	-	-	-	-	111.	-	-	-	-
26.	-	-	-	-	-	112.	+	+	+	+
27.	-	-	-	-	-	113.	-	-	-	-
28.	+	+	+	+	+	114.	+	+	+	+
29.	+	+	+	+	+	115.	-	-	-	-
30.	-	-	-	-	-	116.	+	+	+	+
31.	+	+	+	+	+	117.	-	-	-	-
32.	-	-	-	-	-	118.	+	+	+	+
33.	-	-	-	-	-	119.	-	-	-	-
34.	+	+	+	+	-	120.	-	-	-	-
35.	+	+	+	+	-	121.	-	-	-	-
36.	+	+	+	+	-	122.	-	-	-	-
37.	-	-	-	-	-	123.	-	-	-	-
38.	-	+	+	+	-	124.	+	-	+	-
39.	+	-	+	+	+	125.	+	+	+	+
40.	-	-	-	-	-	126.	-	-	-	-
41.	-	-	-	-	-	127.	+	-	+	+
42.	+	-	+	+	-	128.	+	+	+	+
43.	+	+	-	+	+	129.	+	-	+	+
44.	+	+	+	+	+	130.	-	-	-	-

Table 3. (Cont'd.).

S. No.	SDP	T12 R1	W11	T12 R2	PKBT5	S. No.	SDP	T12 R1	W11	T12 R2
45.	-	-	-	-	-	131.	+	-	+	-
46.	+	+	+	+	+	132.	+	+	+	+
47.	-	-	-	-	-	133.	+	+	+	+
48.	-	-	-	-	-	134.	+	+	+	+
49.	-	-	-	-	-	135.	-	-	-	-
50.	-	-	-	-	-	136.	+	+	+	+
51.	-	+	-	-	-	137.	+	+	+	+
52.	-	-	-	-	-	138.	-	-	-	-
53.	-	-	-	-	-	139.	+	+	+	+
54.	+	+	+	+	+	140.	+	+	+	+
55.	-	-	-	-	-	141.	+	+	+	+
56.	+	+	+	+	+	142.	+	+	+	+
57.	-	-	-	-	-	143.	+	+	+	+
58.	+	+	+	?	+	144.	-	-	-	-
59.	+	+	+	+	+	145.	-	-	-	-
60.	-	-	-	-	-	146.	-	-	-	-
61.	-	-	-	-	-	147.	-	-	-	-
62.	-	-	-	-	-	148.	-	-	-	-
63.	+	+	+	+	+	149.	-	-	-	-
64.	+	+	+	-	+	150.	+	+	+	+
65.	+	+	+	-	+	151.	+	+	+	+
66.	+	+	+	+	-	152.	-	-	-	-
67.	-	+	-	-	?	153.	+	+	+	+
68.	+	+	+	+	-	154.	+	+	+	+
69.	-	-	-	-	-	155.	-	-	-	-
70.	-	-	-	-	-	156.	-	-	-	-
71.	-	-	-	-	-	157.	-	-	-	-
72.	-	-	-	-	-	158.	-	-	-	-
73.	+	+	+	+	+	159.	+	+	+	+
74.	-	-	-	-	-	160.	+	+	+	+
75.	+	+	+	+	+	161.	-	+	+	+
76.	+	+	+	+	+	162.	+	+	+	+
77.	-	-	-	-	-	163.	+	+	+	+
78.	-	-	-	-	-	164.	+	+	+	+
79.	-	-	-	-	-	165.	-	-	-	-
80.	-	-	-	-	-	166.	-	-	-	-
81.	-	-	-	-	-	167.	-	-	-	-
82.	-	-	-	-	-	168.	+	+	+	+
83.	+	+	+	+	-	169.	-	-	-	-
84.	+	+	+	-	-	170.	-	-	-	-
85.	-	-	-	-		171.	-	-	-	-
86.	-	-	-	-						

'+' indicates presence of band associated with maleness, whereas '-' indicates absence of band associated with maleness; R1 and R2 refer to two replications for marker T12

Male-specific SCAR marker Napf developed from RAPD markers OPF2 (Parasnis *et al*., 2000) have been reported reliable marker for PCR-based seedling sex diagnostic assay (SSDA). In our study, this marker showed 92% reliable sex diagnosis in male plants as indicated by the amplification of 831-bp fragment. Nevertheless, some discrepancies were observed in case of female sex-types where seven genotypes showed very faint amplicons of 831-bp in several plants. Although, we tried different annealing temperatures for this marker but this problem could not be fixed. Therefore, this marker needs to be tested again on some other genotypes to determine its reliability. Based on the results of these markers, we were able to differentiate male and female sex types, but hermaphrodite sex-types could not be detected. In future, hermaphrodite sex diagnostic assays should be performed to establish routine screening of female as well as hermaphrodite papaya plants. This approach will benefit growers in planting male and female papaya plants in appropriate ratio.

Conclusion

In conclusion, early stage sex diagnosis in papaya through DNA markers is an accurate assay to save time and cost. It would greatly facilitate papaya growers to plant more female plants for increased fruit production. Therefore, a targeted male: female ratio (45:5) can be achieved during transplanting inside the field. In this way, immense capital input could potentially be exploited for female papaya cultivation. Female papaya plants can produce an average of 100 fruits in its life cycle and 250gm of dry papain/year. Increasing number of fruiting trees per hectare will automatically boost up papaya fruits and papain production directing towards more profitable papaya cultivation. In this regard, the present study will facilitate Pakistani papaya breeders and growers to determine sex-type at early growth stage which is likely to boost up papaya production.

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