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# MOLECULAR DIVERSITY ANALYSIS AND SEX DETERMINATION IN PAPAYA (*CARICA PAPAYA* L.) USING MOLECULAR MARKERS

**P. SHIVKUMAR, P. SHAMPRASAD*, B. ROHINI AND A. PETER**
Department of Plant Biotechnology,
University of Agricultural Sciences, GKVK, Bangalore - 560 065, INDIA
e-mail: shamphadnis08@gmail.com

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***Corresponding author**

**ABSTRACT**
A total of twenty simple sequence repeat (SSR) primers were used to understand the genetic diversity between seven Indian papaya (*Carica papaya* L.) cultivars. The SSRs produced amplicons in all the genotypes. A total of 42 alleles were detected from these primers. Sixteen primers were polymorphic with average polymorphic percentage of eighty.The similarity coefficient ranged from 0.33 to 1.00 and was minimum (33 %) between Arka Prabhat and Co-5 and divided the genotypes into two broad clusters in a dendrogram. ArkaPrabhat, Honey Dew, Red Lady grouped into subcluster I and Solo and Arka Surya in subcluster II in main cluster I while Co-2 and Co-5 varieties were in main cluster II.The RAPD-SCAR marker OP-Y7 developed for the sex determination of Colombian papaya varieties amplified a 369 bp fragment in male plants of all the Indian cultivars except female plants of Co-2. Diversity in papaya cultivars needs to be exploited for developing superior cultivars for characteristics like better fruit quality and disease resistance. SCAR marker for sex determination will probably aid papaya cultivation and breeding by saving time, space and labor cost otherwise which is required to grow plants of undesirable sex and will be useful to the farmer and is of commercial value.

## INTRODUCTION

Papaya (*Carica papaya* L.) is one of the most important fruit crops of tropical and subtropical regions of the world. It is one of the easily available, affordable, fruit grown extensively all over the world, rich in vitamins A and C, a good source of the minerals like magnesium (Mg), potassium (K), boron (B), and copper (Cu), and flavonoids like β-carotene, lutin, zeaxanthin and cryptoxanthins. These compounds are known to have antioxidant properties that play a role in delaying ageing and various disease healing processes (Starley *et al.*, 1999; Neha and Dharmashila, 2013). It is also important for its papain content which is of commercial value.

Wide range of germplasm variability and production of seeds have made papaya a potentially valuable fruit tree model crop for genomic and genetic diversity analysis (Liu *et al.*, 2004; Yu *et al.*, 2008).

Molecular markers are widely used in practical plant breeding to access the genetic variability available in germplasm banks, manage and develop core collections, target crosses, classify germplasm into interest groups and identify duplicate accessions (Martins-Lopes *et al.*, 2007). Without determining the diversity reliably, it would not be possible to identify molecular markers or qualitative trait associations (Kumar *et al.*, 2013). A large number of different molecular techniques are at present available and each of them differ in their informational content. Although in principle all types of markers are suitable for genetic diversity analysis, microsatellites *i.e.* simple sequence repeats (SSR) are especially useful for diversity studies (Baumung *et al.*, 2004).

The genetic analysis based on molecular markers has made it possible to investigate the occurrence and variability of SSRs at the whole genome level in germplasm accessions of papaya using different markers like SSRs (Oliveira *et al.* 2010, Asudi *et al.*, 2013 and Sengupta *et al.*, 2013), inter simple sequence repeats (ISSR) (Sudha *et al.*, 2013), random amplified polymorphic DNA (RAPD) (Van Droogenbroeck *et al.*, 2002) polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) (Van Droogenbroeck *et al.*, 2004) and amplified fragment length polymorphism (AFLP) (Van Droogenbroeck *et al.*, 2002).

Papaya is a polygamous species with three basic sex types: female, male, and hermaphrodite. The ratio of female to male plants in the field is around fifty per cent and the increment in female papaya plants per hectare increases the fruit and papain production making the plantation much more profitable. In general, male plants are not useful commercially and hence the farmers must eliminate a considerable number of male plants which increases production cost (Ma *et al.*, 2004). If the sex type of dioecious papaya can be identified at plantlet stage before transfer to field it is possible to produce a plantation with a desirable ratio of 5 % males to 95 % females and subsequently, farmers can save resources such as land, fertilizers and water.

The segregation of male and female plants at the plantlet level is possible with modern biotechnological approaches using molecular markers. Cloning of the sex linked genes and understanding the sex determination process could have profound application in papaya production (Ming *et al.*, 2007). Random amplified polymorphic DNA - sequence

characterized amplified regions (RAPD-SCAR) marker for sex determination in papaya has been reported by several workers (Deputy *et al.*, 2002, Urasaki *et al.*, 2002 and Chaves-Bedoya and Nunez, 2007). Parasnis *et al*. (1999) has found a microsatellite for identifying male plants in papaya.

The present investigation was hence designed with the objectives of studying the genetic diversity in a small collection of Indian papaya cultivars from the germplasm resources of the National Bureau of Plant Genetic Resources (NBPGR), Government of India and other institutes using SSR markers and validating a RAPD-SCAR marker for sex determination (Chaves-Bedoya and Nunez, 2007) in some Indian papaya cultivars with the hypothesis that a specific marker exists at the genomic level for segregating the male and female plants at an early stage of plant development. This can be commercially utilized for separating plants of the two sexes at an early stage which will help the farmer profitably.

## MATERIALS AND METHODS

### Collection of papaya cultivars

The seed and plant material of papaya cultivars in the present study was collected from the Indian Institute of Horticultural Research (IIHR), Hessaraghatta, Bangalore where the NBPGR germplasm is maintained, Tamil Nadu Agricultural University (TNAU) Coimbatore, Tamil Nadu and University of Agricultural Sciences (UAS), Bangalore. Solo, Red Lady, Honey Dew, Arka Surya, Arka Prabhat, Co2 and Co5, were the seven cultivars that were available and used (Table 1). All cultivars were used in the genetic diversity studies and Co5 female parent was not used in the sex determination study using RAPD-SCAR marker. The seeds were sown in pots with a mixture of peat soil and vermiculite in the at a temperature of 25°C for 40-45 days and the first two expanded leaves were used for DNA isolation.

### DNA isolation from papaya leaves

DNA was isolated from fresh 40-45 day old papaya leaves using modified CTAB (2%) method [Doyle and Doyle (1990)], checked for purity on 0.8 % agarose gel electrophoresis stained with ethidium bromide (0.5 mg/mL) and quantified using Biospectrometer (Eppendorf).

### Genetic diversity studies in papaya using SSR markers:

In this study 20 SSR markers (Table 2) developed by Oliveira *et al*. (2008) were used and checked for amplification and polymorphism between the papaya cultivars. The PCR reaction was carried out in a total volume of 20 $\mu$L using 10x PCR buffer with 15 mM $MgCl_2$, 5 $\mu$M to each of the forward and reverse primer, 2.5 mM dNTPs, 3U/$\mu$L of Taq polymerase, 20 ng of template DNA and distilled water (Oliveria *et al.*, 2010). The amplification was carried out in a Biorad MyCycler thermal cycler with the following conditions-Initial denaturation 94ºC for 4 min, 35 cycles of 94ºC for 40 sec, 55ºC – 60ºC for 40 sec annealing temp and 72ºC for 1 min with final extension of 72ºC for 8 min and stored at 4ºC.

### Data analysis

The genotype profiles produced by SSR markers were scored visually for the presence (1) or absence (0) to form binary matrix for each of the SSR loci. The reproducible and polymorphic bands were considered for the analysis. Data sets were analyzed using SIMQUAL option to generate pair-wise Jaccard's similarity coefficient (1908) using NTSYS-pc version 2.11 (Rohlf, 2000). The similarity matrices thus generated was utilized for construction of dendrogram based on SSR marker data using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm and SAHN (Sequential, Agglomerative, Hierarchic and Non-overlapping) clustering. Polymorphism information content (PIC) was calculated using POWERMARKER program version, 3.0 (Liu and Muse, 2005).

### Sex determination in papaya using RAPD-SCAR marker:

RAPD-SCAR marker developed by Chaves-Bedoya and Nunez, (2007) for differentiating male, female and hermaphrodite plants in Colombian papaya cultivars was validated in the Indian cultivars used in the present study. The forward and reverse SCAR marker sequence is mentioned below

The PCR reaction was carried out in a total volume of 20 $\mu$L using 10x PCR buffer with 15 mM $MgCl_2$, 5 $\mu$M of each the forward and reverse primer, 2.5 mM dNTPs, 3U/ $\mu$L of Taq polymerase, 20 ng of template DNA and distilled water (Chaves-Bedoya and Nunez, 2007).

| Primer name | Forward sequence (5'-3') | Reverse sequence (5'-3') | Annealing Temp. |
|---|---|---|---|
| OP-Y7 | AAACTACCGTG CCATTATCA | AGAGATGGGTT GTGTCACTG | 55ºC |

The amplification was carried out in a Biorad MyCycler thermal cycler with the following conditions-Initial Denaturation 94ºC for 5 min, 35 cycles of 94ºC for 1 min, annealing temperature of 55ºC for 1 min and 72ºC for 1 min, final extension of 7 min and stored at 4ºC.

## RESULTS AND DISCUSSION

DNA was isolated from the 40-45 day old seedlings with the first two expanded leaves of *Carica papaya* cultivars. The purity of DNA was 1.8 to 2.15 at absorbance 260 nm and 280 nm and the DNA concentration ranged from 250.4 ng/$\mu$L to 1121.8 ng/$\mu$L.

### Genetic diversity analysis of seven papaya cultivars

Twenty SSR primers were used for genetic diversity analysis of seven papaya varieties. The primers used produced amplicons in all the genotypes. The SSR primers CP 18, CP 20, CP 22 and CP 23 (four of twenty) showed no polymorphism, but the other 16 SSRs showed polymorphism giving average polymorphic percentage of eighty.The number of alleles ranged from 1 - 4 with the maximum number of alleles (4) in

**Table 1: List of some Papaya accessions**

| Sl. No. | Accession | Sex type | Source |
|---|---|---|---|
| 1 | Arka Prabhat | Hermophrodite | IIHR,Hessaraghatta |
| 2 | Honey Dew | Gynodioecious | UAS, Bangalore |
| 3 | Red Lady | Hermophrodite | UAS, Bangalore |
| 4 | Solo | Hermophrodite | UAS, Bangalore |
| 5 | Arka Surya | Hermophrodite | IIHR, Hessaraghatta |
| 6 | Co-2 | Dioecious | TNAU, Coimbatore |
| 7 | Co-5 | Dioecious | TNAU, Coimbatore |

**Table 2: List of SSR primers used in the study**

| Sl. No | Primer name | Forward sequence (5′ – 3′) | Reverse sequence (5′-3′) | Ta (°C) |
|---|---|---|---|---|
| 1 | CP02 | aggcgaaatcggaagagag | ctggtaaaacgacgatgacg | 59°C |
| 2 | CP03 | gaaggcccgtgtaagtgc | tggtgaaaattggaaaggag | 58°C |
| 3 | CP04 | aagggagaagagaagcagagt | ctccagtttgcctccaaag | 57°C |
| 4 | CP05 | gtcctcaatccgaagcat | catacacccttgtggcttct | 57°C |
| 5 | CP06 | ttgcccaccaggcttaat | tgacgttacggtttcatctg | 58°C |
| 6 | CP07 | cctagcattgccttgaggtc | gcccactattcacattcacacc | 60°C |
| 7 | CP09 | cccaattcatgtccaaatcc | atgttgaccaaaggaagcaa | 59°C |
| 8 | CP10 | aaaaatcacagcacgtatggtt | gaaattacaaatgggcaaaaag | 58°C |
| 9 | CP11 | tgccgtatgagaaggaattaga | tctctcctccaaacattcattt | 58°C |
| 10 | CP12 | gggagggattgtagctcttt | ttggattttcccctacctaa | 57°C |
| 11 | CP13 | attgggaaccaaccattcg | tcaccaaccgcaggatataa | 59°C |
| 12 | CP14 | tcaatgttctcgtcgatagtc | tgggatagtgcaaattggt | 56°C |
| 13 | CP15 | atgcactcagcgaaaggat | tcctggtctgttcaaaagtct | 57°C |
| 14 | CP16 | tcaactatttcccccgcata | cacctccttgtccaaaggtt | 60°C |
| 15 | CP18 | ccgtcatgttttcgctttt | caattctcgttgattcttgg | 57°C |
| 16 | CP19 | taggggttgtgcgtccata | agcaggctaaaaactggtca | 58°C |
| 17 | CP20 | tgtgagattgtctgttggttg | gggctcgaaaatcaaaacat | 58°C |
| 18 | CP21 | atcgaccgaggaaggtacg | tcaaaaacccattgagtctgc | 60°C |
| 19 | CP22 | gttcgcgtgctctacgtgt | tgacacctgataaaggcaaga | 59°C |
| 20 | CP23 | aacaataggaagcaagctca | tccattccaacccacaaa | 57°C |

**Table 3: Similarity indices of 7 papaya varieties**

| | Arka Prabhat | Honey Dew | Red Lady | Solo | Arka Surya | Co 2 | Co 5 |
|---|---|---|---|---|---|---|---|
| Arka Prabhat | 1.00 | | | | | | |
| Honey Dew | 0.66 | 1.00 | | | | | |
| Red Lady | 0.76 | 0.90 | 1.00 | | | | |
| Solo | 0.47 | 0.61 | 0.61 | 1.00 | | | |
| Arka Surya | 0.61 | 0.66 | 0.66 | 0.66 | 1.00 | | |
| Co2 | 0.47 | 0.52 | 0.57 | 0.66 | 0.61 | 1.00 | |
| Co5 | 0.33 | 0.38 | 0.42 | 0.57 | 0.47 | 0.80 | 1.00 |

**Table 4: Description of the primers and SSR products of sevenpapaya genotypes**

| Sl. No | Marker Name | Allele Number | Gene diversity | PIC |
|---|---|---|---|---|
| 1. | CP02 | 3 | 0.4082 | 0.3182 |
| 2. | CP03 | 2 | 0.2449 | 0.2149 |
| 3. | CP04 | 2 | 0.4082 | 0.3249 |
| 4. | CP05 | 2 | 0.4898 | 0.3698 |
| 5. | CP06 | 3 | 0.3537 | 0.2882 |
| 6. | CP07 | 2 | 0.4082 | 0.3249 |
| 7. | CP09 | 2 | 0.4490 | 0.3474 |
| 8. | CP10 | 2 | 0.4082 | 0.3249 |
| 9. | CP11 | 2 | 0.2449 | 0.2149 |
| 10. | CP12 | 2 | 0.4490 | 0.3474 |
| 11. | CP13 | 4 | 0.3878 | 0.3086 |
| 12. | CP14 | 2 | 0.3673 | 0.2942 |
| 13. | CP15 | 3 | 0.4626 | 0.3549 |
| 14. | CP16 | 2 | 0.3265 | 0.2699 |
| 15. | CP18 | 1 | 0.0000 | 0.0000 |
| 16. | CP19 | 3 | 0.3537 | 0.2882 |
| 17. | CP20 | 1 | 0.0000 | 0.0000 |
| 18. | CP21 | 2 | 0.2449 | 0.2149 |
| 19. | CP22 | 1 | 0.0000 | 0.0000 |
| 20. | CP23 | 1 | 0.0000 | 0.0000 |
| Mean | | 2.1 | 0.3003 | 0.2403 |

CP 13 (Plate 4), which may be due to more number of repeat sites that have amplified and hence more polymorphic among the varieties. Thirty eight alleles were detected from 16 polymorphic SSR primers and 4 alleles from 4 monomorphic SSR primers. In all a total of 42 alleles were detected from the twenty primers used (Plate 1-4).The number of alleles per locus reported in our study is most likely a minimum value. The lower number of alleles can be due to the relatively few samples analyzed and to the different mating systems of these genotypes. The genotypes *viz.*, Arka Prabhat, Arka Surya, Red Lady, Solo are hermaphrodite; Co2 and Co5 are dioecious and Honey Dew variety is gynodioecious.

The similarity indices and consensus dendrogram were developed on the basis of scorable banding pattern of seven genotypes (0 for absence and 1 for presence). The similarity coefficient ranged between 0.33 to 1.00 (Table 3). The genotypes Arka Prabhat and Co-5 showed the lowest similarity index of 0.33.

The PIC values which denote allelic diversity and frequency among the genotypes had an average of 0.2403 /marker and ranged from 0.2149 to 0.3698 with the highest PIC obtained by CP 05 primer. The gene diversity was also calculated and ranged from 0.2449 to 0.4898 for the same primers and the highest gene diversity was with CP 05 with an average of 0.3003/ marker (Table 4).

Oliveira *et al.* (2010) genotyped 30 germplasm accessions and 18 landraces of papaya in Brazil. In total 59 primers amplified a polymorphic and easily scorable PCR product while 22 pairs amplified a monomorphic one. Considering

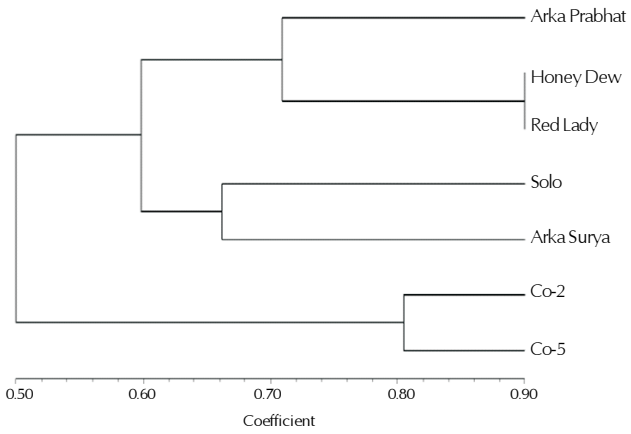



**Figure 1: UPGMA dendrogram representing relationship among seven papaya varieties**

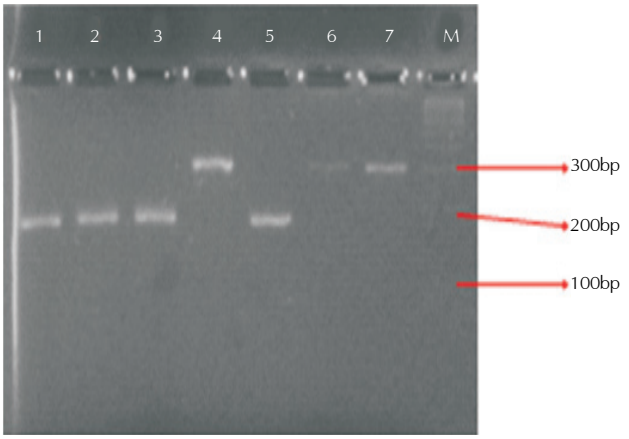



**Plate 1: Amplification of CP-05 SSR primer**

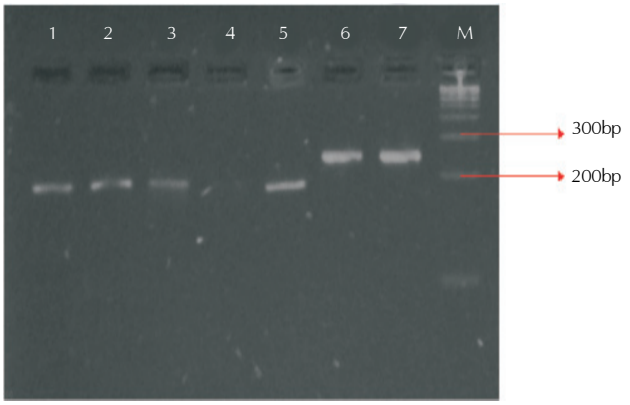



**Plate 2: Amplification of CP-12 SSR primer**

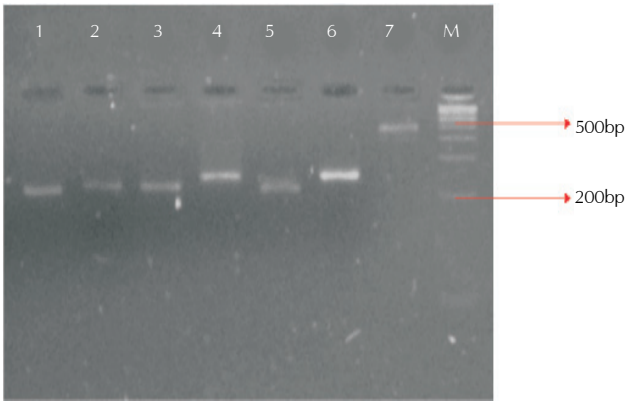



**Plate 3: Amplification of CP-15 SSR primer**

the 59 SSR loci analyzed in their study, a total of 48 genotyped individuals obtained from partial outcrossing and selfing germ plasm SSR markers detected a total of 237 alleles. The average number per locus was 4.02. The compound microsatellites seem to be more informative than dinucleotide and trinucleotide repeats in the present study.

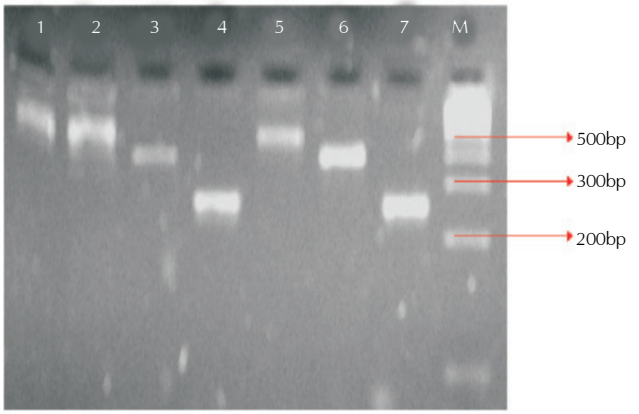
Genetic diversity analysis of forty two papaya accessions with seven SSR markers Kenya has given a PIC value ranging from 0.75-0.852 with average of 0.81, genetic similarity ranged from 0.764 to 0.932 with an average of 0.844 (Asudi *et al.,* 2013). Pairwise comparisions among these accessions exhibited greater than 0.802 genetic similarity in 96.9 per cent cases and less than 4 per cent showed genetic similarity lower than 0.802.

In a genetic diversity study with34 commercially popular papaya cultivars from India and abroad, 6 accessions of *Vasconcellea* species and 1 accession of *Jacaratiaspinosa*were carried out using SSR markers. The average allele number was 7 alleles/locus and the average PIC value was 0.735 per marker. Categorically the *Vasconcellea and Jacaratia* species had 54 alleles, the 7 non-Indian *Carica papaya* accessions had 70 and the 27 Indian accessions had 102 alleles. The average PIC value was 0.735 per marker. A total of 37 rare alleles were identified. *Jacaratia spinosa* had 17 rare alleles. Nineteen nullalleles were detected among the *Carica papaya* accessions. A *Carica papaya* accession from South Africa, Hortus Gold had 5 null alleles (Sengupta *et al*., 2013).

In an inter simple sequence repeat (ISSR) genetic fingerprinting of seventy two genotypes of *Carica papaya* from different parts of south and little Andaman islands, 24 primers produced 212 amplicons out of which 62 gave 29.2 % polymorphism (Sudha *et al.,* 2013).

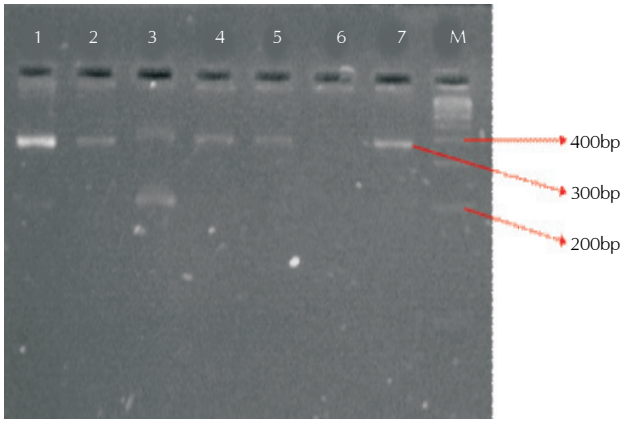
Madarbokus and Sanmukhiya (2012) in a RAPD diversity analysis of four papaya cultivars using sixty RAPD markers indicated minimum and maximum similarity values of 0.28 and 0.806.

The dendrogram constructed using SHAN clustering separated the seven genotypes of papaya into two major clusters, one with 5 genotypes and other with the two genotypes (Fig. 1). These two shared a common node at 50 % similarity level. The larger major cluster was further subdivided into two subclusters of which one subcluster contains 3 genotypes *viz.,* Arka Prabhat, Honey Dew and Red Lady and the other subcluster with two genotypes *viz.,* Solo and Arka Surya sharing a common node at 59 % similarity level. The remaining major cluster included Co-2 and Co-5 papaya cultivars sharing a common node at 80 % similarity level.The subcluster containing Arka Prabhat and other two cultivars Honey Dew and Red Lady share a common node at 70 % similarity level whereas, Solo and Arka Surya share a common node at




Lanes 1-8:1-Arka Prabhat, 2-Honey Dew, 3-Red Lady, 4- Solo, 5-Arka Surya, 6-Co-2, 7-Co-5 and 8- M- DNA ladder 100bp

**Plate 4: Amplification of CP-13 SSR primer**




Lanes 1-8: 1-Arka Prabhat, 2-Arka Surya, 3-Honey Dew, 4-Red Lady, 5-Solo, 6. Co-2 (Female), 7. Co-2(Male), 8. M-DNA ladder 100bp

**Plate 5: Amplification of RAPD-SCAR marker OP-Y7 for sex determination of papaya varieties**

similarity level of 66 %.

Two main clusters A and B with four and one cultivar respectively has been observed among 42 Kenyan papaya accessions using seven SSR markers (Asudi *et al.*, 2013). Cluster analysis of the genotypes of papaya in the Andaman islands using ISSR grouped them into three clusters with 37 % similarity and revealed that genotypes from various islands differ from each other of which those from Hut Bay and Neil island were distinct (Sudha *et al.*, 2013).

According to Sengupta *et al.* (2013) genetic similarity among the accessions of papaya ranged from 7% to 67% in a SSR marker analysis. In the dendrogram, the *Vasconcellea* and *Jacaratia spinosa* accessions separated as a distinct cluster from the rest of the *Carica papaya* accessions. The study indicated that the accessions of Indian *Carica papaya* cultivars included in their survey were genetically more diverse than the non-Indian *Carica papaya* cultivars.

Dendrogram of four Mauritian papaya cultivars using RAPD showed closer genetic relatedness between Ugandan female and Farc cultivars while Ramnan clustered together with Long Orange variety (Madarbokus and Sanmukhiya, 2012).

The data generated in the present study indicates that there is a great diversity of papaya cultivars. This needs to be exploited for developing cultivars for specific qualities like fruit shape, fruit size, increased carotenoids, resistance to disease like the papaya ring spot disease and other characteristics. Including more number of papaya varieties and using more number of SSR markers will help in better understanding of the genetic diversity in this species. The present study has revealed that the cultivars have clustered together based on the sexual nature of varieties and is interesting to study how this relates to sex determination in papaya.

**Sex determination of seven papaya cultivars**

The RAPD-SCAR marker developed by Chaves-Bedoya and Nunez (2007) was used in this study for papaya sex determination. The SCAR marker used for the validation of sex types in the collected papaya cultivars gave a band size of 369bp for male and hermaphrodite plants and no band was obtained for female plants. In Plate 5, the varieties Arka Prabhat, Arka Surya, Red Lady, Solo (Hermaphrodite) and Co2 (Male) showed a band size of 369bp; Honey Dew variety (Gynodioecious) showed two bands one of band size 369bp and other 200bp Co2 (female) showed no band. The results obtained were according to the sex types mentioned in Table 1.The 369 bp specific RAPD-SCAR marker developed by Chaves-Bedoya and Nunez (2007) has thus worked for sex determination of the Indian cultivars.Inclusion of more dioecious varieties and samples of all sex types in gynodioecious ones, will lead to a complete understanding of the sex types.

Several other RAPD-SCARS have been developed for sex determination in papaya like SCAR T12 and SCAR W11 in male and hermaphrodite plants which is tightly linked to sex 1gene that determines male and female plants in papaya (Deputy *et al.*, 2002). These markers have worked with an accuracy of 99.2 % in predicting hermaphrodite plants in a population of 182 $F_2$ plants from "Sun Up" and "Kapoho" cross.

Urasaki *et al.* (2002), have developed a 450 bp RAPD-SCAR marker named PSDM (Papaya Sex Determination Marker) found only in male and hermaphrodite plants in two important Hawaiian cultivars 'Sunrise Solo' and 'Waimanato Solo' as well as four Okinawa landraces IK-2, IK-7, IK-10 and TK-5 and a Mexican landrace Me-1 and four additional lines derived from crosses between the Hawaiian, Okinawa and Mexican cultivars (ON-7, ON-12, ON-34 and ON-36) in Okinawa, Japan and the authors opined that this marker is also linked to Sex-1 gene in papaya.

Parasnis *et al.* (1999) identified a minisatellite probe $(GATA)_4$ which is associated with sex determination of male plants in an experiment with the dioecious Co-1, Co-2, Co-4, Co-5, Co-6, MF-1, Pant-1, Washington, Pusa giant, Pusa dwarf, gynodioecious Disco and Sunrise cultivars and a wild species *Carica cauliflora* has provided molecular evidence for a putative Y-chromosome in papaya.

These reports suggest that RAPD-SCAR and microsatellite markers have been developed for sex determination in papaya over a wide range of cultivars across continents and is an

interesting finding as it may lead to the development of a single marker to differentiate male and female plants for all papaya cultivars in the future. This will help in papaya breeding, by aiding separation of male and female plants at early developmental stages and in papaya cultivation by saving time, space and labor cost otherwise required to grow plants of undesirable sexes. These markers may also help in an indepth analysis of the functional role and significance of genes like Sex-1 in papaya.

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