



Sex expression in papaya: morphological marker, molecular genetics and environments

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ABSTRACT

Papaya (*Carica papaya* L.) is a polygamous species and the plants are extremely diverse in their sexual systems. Three sex types are available in papaya viz., male, female and hermaphrodite and are controlled by a single gene with three alleles (m, M₁, M₂). The genotypes represent gynoeceous, androeceous and hermaphrodite individuals. Ninety percent of freshly dispersed pollen grains were viable in summer but viability dropped to about 45% in some lines and as low as 4.5% in others in winter. The extremes of humidity reduce the storage life of papaya pollen but under ideal (artificial) storage conditions it potentially remains viable for about 5-6 years. The stigma become receptive two days before anthesis and continued up to five days after anthesis but began to decline gradually upto five days after anthesis. Papaya plants produce fruit either through cross-pollination or self-pollination or parthenocarpy depending on their sex types. On an average 1,000 seeds are found in a single fruit, indicating that 1,000 viable pollen grains may fertilized receptive stigma. The sex types in papaya is found to be related with several morphological characters. Seed coat color, petiole thickness, stems color worked as a morphological marker for sex determination in papaya. The black and dark brown seed coat color exhibited higher frequency of the female and hermaphrodite plants. The range of 54–60° petiole orientation, 3.7–4.2 cm petiole thickness and 9.4–10.4 cm petiole length gave higher percentage of female and hermaphrodite plants. On the other hand, unique purple stem color was reported to express as hermaphrodite plants. In case of chemical identification of sex in papaya Almen reagent test, Ammonium molybdite test, Titanous chloride test gave 71%, 60% and 55% accuracy of femaleness respectively. Ethrel gave the most expected number (46.67%) but excessive Ethrel may also cause higher number of male. In case of Kinetin and IBB 100 ppm and 200 ppm gave higher percentage of female. Environment may also affect the sex expression of papaya. In along with these, several molecular markers may also be used to identify the sex type of papaya. Among them SSR and RAPD is mostly familiar and successful.

Keywords: *Carica papaya*, polygamous, gynodioecious, hermaphrodite, pistillode, sexual lability

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1 Introduction

Papaya (*Carica papaya* L.) belongs to the family Caricaceae and is a dicotyledonous, polygamous (having male, female or hermaphrodite flowers on the same plant), diploid species with a small genome of 372 Mbp/1C (Arumuganathan and Earle, 1991) and contains nine pairs of chromosomes (Bennett and Leitch, 2005). Papaya that is named as wonder fruit of the tropics has several medicinal and nutritional benefits. The genus name *Carica* is derived from the Latin name for a kind of fig which resemble as the leaves and fruits of *Carica papaya*; the specific epithet papaya probably comes from the common name for the fruit (Du and Telford, 1993). The papaya is a native of Central and South America. It is a member of the family Caricaceae. This family consists of 55 species (Dallwitz, 1980) and placed into four genera (Badillo, 1971), viz., *Carica*, *Cyclimorpha*, *Jacaratia* and *Jarilla*. However, a recent taxonomic revision proposed that some species formerly assigned to *Carica* were more appropriately classified in the genus *Vasconcellea* (Badillo, 2002). The highland papayas, *Vasconcellea*, are considered the nearest relatives to *Carica papaya* although the relationship is not close (Aradhya et al., 1999; Van Droogenbroeck et al., 2002). A more recent study (Van Droogenbroeck et al., 2004) actually suggested that there are two lineages within the Caricaceae family and that some members of *Vasconcellea* are more closely allied to *Carica papaya* than others.

The papaya plant is a semi-woody, latex-producing, usually single-stemmed, short-lived perennial herb. The relatively small genome of this species shows peculiarities in major gene groups involved in cell size and lignification, carbohydrate economy, photoperiodic responses, and secondary metabolites, which place the papaya in an intermediate position between herbs and trees (Ming et al., 2007). It exhibits palmately-lobed leaves and clustered at the top of plant (Morton and Dowling, 1987; OECD, 2005). Self-pollination in males, cross-pollination between males, and cross-pollination between males and hermaphrodites, can all be done using the sexually ambivalent males (SAMs) that produce perfect flowers during certain periods of the year. Male and hermaphrodite plants undergo various degrees of sex reversal, depending on seasonal changes and climate (Awada, 1958a). The female plant is the most stable form.

2 Floral Biology

2.1 Types of flowers

Papaya is polygamous species and possesses three sex forms viz., female, male and hermaphrodite (Fig. 1) (Yu et al., 2008). The flowers are grown on the inflorescence called Cyme (Storey, 1969), slightly fragrant,

fleshy and waxy, and yellow to cream in color. The inflorescence type varies according to the sex of the plants. Flowering occurs generally within 3-6 months after germination but it could be extended up to 9-12 months in some cases (Vashistha et al., 2016).

Hermaphrodite flower The perfect flower of papaya, also referred to as the elongate type, consists of five petals, five pairs of anthers, and an ovary. The petals are fused on the lower part of the flower (connate), to the point where the stamens are inserted, forming the corolla tube. The upper parts of the petals are free and slightly twisted. The ovary is superior, elongated, and composed of five carpels. Each pistil has five broad and flattened stigmata joined at their base, which may bend slightly backwards when the flowers open. There are five pairs of anthers, inserted into two whorls (diplostemonous androecium), but each member of a pair belongs to a different whorl. Stamens belonging to the antesealous have longer filaments than those in the antepetalous whorl (Decraene and Smets, 1999). Although the term hermaphrodite has been used to refer to papaya plants that bear perfect flowers, the correct term should be andromonoecy, which indicates the occurrence of staminate and hermaphroditic flowers on the same plant. The ratio of perfect to staminate flowers within an inflorescence may vary greatly due to the effects of genetic and environmental factors and may range from totally perfect to totally sterile. Female sterility in andromonoecious papayas is often expressed progressively, leading to reductions in ovary size, carpel number, and associated tissues and ultimately may lead to completely staminate flowers which contain only a pistillode (Nakasone and Lamoureux, 1982).

Female flower Female papaya flowers have five free petals and a rounded superior ovary (Decraene and Smets, 1999) that is five carpellate and hollow and exhibits parietal placentation (Fisher, 1980). In contrast to the hermaphroditic plants, females are completely stable and their flowers do not appear to undergo sex reversal due to environmental fluctuations.

Male flower Stamen arrangement in the male flowers is the same as in the hermaphrodite flower, surrounding a rudimentary pistil or pistillode. In some cases, due to genetic or environmental causes, some of the dominant flowers within the inflorescence may have fully developed pistils, resulting in a hermaphroditic flower and an overall male, fruit-bearing phenotype (Storey, 1953).

2.2 Anthesis of flowers

The flower opening starts from 6 am to 12 pm (Azad and Rabbani, 2004). The time of anthesis could be

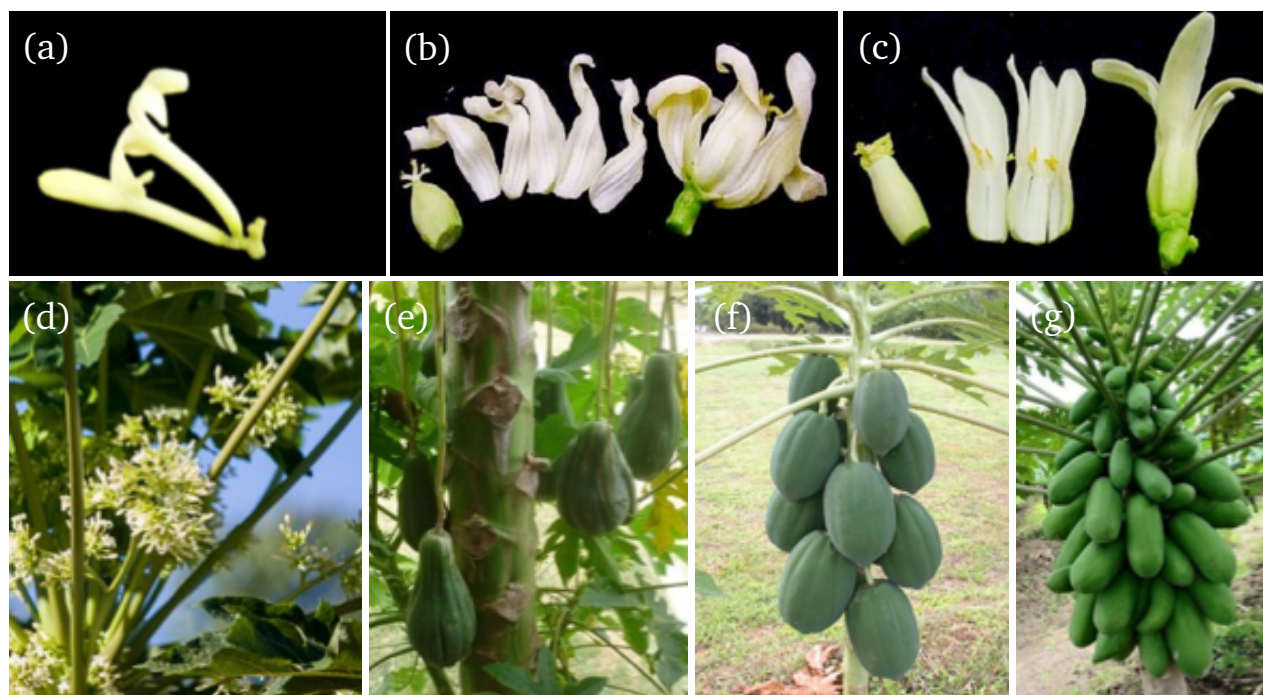


Figure 1. (a) Male flowers, (b) Female flowers, (c) Hermaphrodite flowers, (d) A male plant, (e) Male fruits, (f) Female fruits, and (g) hermaphrodite fruits of papaya

changed due to environmental difference as well as interaction of genotype.

2.3 Dehiscence of anthers

The anthers of *C. cauliflora* started dehiscence after 8 am. Azad and Rabbani (2004) reported the maximum anther dehiscence in *C. cauliflora* at 11-12 pm but minimum dehiscence was noticed in *C. papaya* cv. Shahi at the same time in Bangladesh. The anthers dehiscence of all species was increased with increasing the temperature up to 12 pm. These results revealed that high temperature speed up the dehiscence of anthers. Sharma and Bajpai (1969) reported that optimum anther dehiscence occurred at 12 to 1 pm for papaya. Khuspe and Ugale (1977) reported that maximum number of anthers dehiscid between 9 am to 12 pm in *C. papaya* cv. Washington.

2.4 Pollen viability

Pollen remains viable for 2 days before and after anthesis, with maximum viability on the day of anthesis. At room temperature, and 50% relative humidity, pollen remains viable for 48 hrs. Garrett (1995) reported that 90% of freshly dispersed pollen grains were viable in summer but that in winter, viability dropped to about 45% in some lines and as low as 4.5% in others. Allan (1963) found that extremes of humidity reduce the storage life of papaya pollen which, under ideal (artificial) storage conditions, potentially remains viable for about 5-6 years. Allan

(1963) also found that temperatures below 10°C significantly affect pollen viability, possibly as a consequence of degenerated pollen mother cells.

2.5 Stigma receptivity

Azad and Rabbani (2004) observed that the stigma become receptive two days before anthesis and continued up to five days after anthesis. Significant increase of number of fruit set was recorded on the day of anthesis. But it began to decline gradually upto five days after anthesis in all the species. No fruit set was found in all species at six days after anthesis and stigmas became dried.

2.6 Pollination

Fruit production in papaya plants may occur either through cross-pollination (out-crossing) or self-pollination or parthenocarpy depending on their sex types (Rodriguez et al., 1990; Nakasone and Paull, 1998; Louw, 2000). Environmental conditions, floral characteristics associated with the various flower types and flower receptivity may also affects cross-pollination (OECD, 2005). Pollen can be produced year round in papaya plant but pollen production by papaya plants varies depending on season and variety (Garrett, 1995). But general trend of quantities of pollen production decreased during winter or early spring (Magdalita et al., 1998). Wind pollination may also be important than insect pollination despite floral morphology in some countries (Nakasone and Paull,

1998; OECD, 2005). Very high papaya pollen counts (10-18% of total aeropollen) have been recorded in the outskirts of Calcutta (Chakraborty et al., 2005). Species in which both wind and insect pollination occur are described as having an amphiphilous pollination mechanism. Baker (1976) gave ample information to support the view that *Carica* is putatively moth pollinated. However, this view was recently put in doubt that several pollinators are involved, such as beetles, flies, and mosquitoes.

2.7 Fertilization and fruit setting

Abundance of pollen or pollinator efficiency may affect fertilization and fruit setting ultimately fruit production. On an average 1,000 seeds are found in a single fruit, indicating that 1,000 viable pollen grains may fertilized receptive stigma. The more seeds in a fruit, the larger the fruit grows (McGregor et al., 1976).

3 Genetics of sex expression

Papaya plants are remarkably diverse in their sexual systems (Barrett, 2002). Sex expression in papaya is controlled by a single gene, with three alleles which have a pleiotropic effect (Hofmeyer, 1938; Storey, 1953). In general, there are three sex types in papaya: male, female and hermaphrodite. Sex type in papaya is controlled by a single gene with three alleles i.e. m , M_1 , M_2 . The mm , M_1m and M_2m genotypes represent gynoeceous, androeceous and hermaphrodite individuals, respectively (Hofmeyer, 1938; Storey, 1938). The three sex types of papaya are inherited in unexpected ratios because male dominant alleles linked with a lethal factor (Table 1). These unexpected ratios have been becoming the topic of extensive studies. The progeny of self-fertilized hermaphrodite plants always segregate into 2:1 ratio of hermaphrodites and females. If female plants were fertilized by pollen from a male plant, then it segregate at the ratio of 1:1 male to female (Table 1). A similar ratio of 1:1 hermaphrodite to female obtained when female fertilized by pollen from a hermaphrodite plant. When male plants are self-fertilized occasionally (in optimal growing conditions some male flowers do not undergo their carpel abortion and form fruits), then male plants segregate at a ratio of 2 male: 1 female. When pollen of male plants fertilizes the female organ of hermaphrodite plants, then it segregate at a ratio of 1 male: 1 hermaphrodite: 1 female (Vashistha et al., 2016).

However, these sex types can't be determined before flowering stage which is a big hindrance for papaya cultivation. In an open-pollinated species such as papaya, the selection of the appropriate sex type of the progeny for commercial planting would be beneficial, since only the female and hermaphrodite plants

are grown for fruits (Magdalita and Mercado, 2003). Among these sex types, hermaphrodite plants are preferred for commercial cultivation in tropical regions due to their pyriform shaped fruits (Magdalita and Mercado, 2003). Male plants are not useful for economic purposes as they do not produced fruits and hence they should be removed from the field which increases production cost (Bedoya and Nuñez, 2007). So, to reduce the production cost sex determination prior to flowering stage is very helpful for papaya cultivation.

4 Sex modification in papaya

When hermaphrodite papaya plants are subjected to stresses such as high temperatures and water and nitrogen shortages, female sterility is exacerbated (Awada and Ikeda, 1957a; Arkle and Nakasone, 1984; Almeida et al., 2003). Hermaphrodite plants may be 'ambivalent', going through seasonal sex reversals (Storey, 1976). The proportion and type of flowers produced may vary even on the same plant (Villegas, 1997). Perfect papaya flowers may also undergo variable degrees of fusion between their stamens and the ovary (carpellody) (Decraene and Smets, 1999). In severe cases, the five antepetalous stamens are completely transformed into carpels, and the resulting flower resembles a female one, with a rounded ovary and free petals almost all along their length. This type of flower is also known as the "pentadria type". Intermediate carpellodic states are also common, in which only some of the stamens are completely or partially fused with the ovary, resulting in the development of misshapen fruits. Although the tendency to produce carpellodic flowers has a strong genetic component (Ramos et al., 2011; Storey, 1953), low temperatures, high soil moisture, and high nitrogen seem to favor this condition (Awada, 1953, 1958b; Awada and Ikeda, 1957b).

5 Sex determination in plants

There are no universal models supporting sex determination in plants. There are lots of hidden mechanisms for development of sex organ in the plant.

5.1 Development of reproductive organs

In the majority of the plants, male and female organ develop simultaneously and after a point growth of either of the organ may inhibited. These consequences are observed in *Melandrium album* (Grant et al., 1994), *Rumex acetosa* (Ainsworth et al., 1995) and *Pistacia vera* (Hormaza and Polito, 1996). In case of *Actinidia deliciosa* (Schmid, 1978) and *Asparagus officinalis* (Galli et al., 1993; Caporali et al., 1994) sexual differentiation

Table 1. Segregation ratio of crosses between different combinations of sex types [†]

Crosses				Offspring
Female (♀)		Male (♂)		
mm	×	M ₁ m	→	1 mm : 1 M ₁ m
mm	×	M ₂ m	→	1 mm : 1 M ₂ m
M ₂ m	×	M ₂ m	→	1 M ₂ M ₂ : 2 M ₂ m : 1 mm
M ₁ m	×	M ₂ m	→	1 M ₁ M ₂ : 1 M ₁ m : 1 M ₂ m : 1 mm
M ₁ m	×	M ₁ m	→	1 M ₁ M ₁ : 2 M ₁ m : 1 mm

[†] Mm = female; M₁m = hermaphrodite; M₂m = male; M₂M₂, M₁M₂, and M₁M₁ = lethal

takes place very late, and male and female flowers appear to be identical at first stage. In addition to timing, the inhibition of sexual development can also vary in character. In absence of cell division sexual development is inhibited i.e. *Rumex acetosa* (Ainsworth et al., 1995) and *Melandrium album* (Farbos et al., 1997), or necrosis of sexual organ cells, which is reported in *Asparagus officinalis* (Caporali et al., 1994) and *Actinidia deliciosa* (Harvey and Fraser, 1988).

5.2 Molecular basis of sex expression

A number of generalized hypotheses have been developed to explain this process. Frankel and Galun (1997) proposed the key gene theory to explain the sex expression mechanisms in plants. The theory proposed that the key gene activates a cascade of other genes with gene activation which leads to the development of the respective sex organs. A single-gene mechanism controls sex expression in plants such as *Asparagus officinalis* (Gao et al., 2007), *Ecballium elaterium* (Ainsworth, 2000), *Pistacia vera* (Hormaza et al., 1994) and *Carica papaya* (Storey, 1953).

5.3 Sex chromosomes

In plants, sex chromosomes or autosomes could be responsible for sex differentiation. But in animals, sex chromosomes are responsible for sex expression. Sex chromosomes have been identified in selected plant species, and their existence is merely suspected in other taxa. The identification of sex chromosomes in plants is problematic because most of them do not differ morphologically from autosomes or from one another (*Spinacia oleracea*, *Asparagus officinalis*) (Michalik, 2009). Sex chromosomes have been observed in a relatively small group of plants. In most cases, the presence of the Y chromosome enhances the maleness and suppresses the development of female organ as like that found in animals. The above mechanism is present in *Melandrium album*, *Asparagus officinalis* and *Spinacia oleracea* (Monika and Jakub, 2012). In some plant species, the ratio of the number of X chromosomes and autosomes is important for sex determination, for example, *Rumex acetosa* (Ainsworth, 2000),

Humulus lupulus (Shephard et al., 2004) and *Phoenix dactylifera* (Siljak-Yakovlev et al., 1996). Another two types of Y chromosomes have been found as XY1Y2 (male) and XX (female) (Dellaporta and Calderon-Urrea, 1993).

5.4 Labile sex

Sexual lability can be found in various plant taxa, but it's a rule for only ferns group (Korpelainen, 2007). In ferns, sex is determined by a gametophyte's age that is older gametophytes are hermaphroditic, while younger gametophytes are male and size that is taller gametophytes are capable of lifting up a zygote and a developing sporophyte (Korpelainen, 2007). Biotic factors, such as population density, also affect sex expression in the above species. Dense populations prefer for the development of male individuals whereas sparse populations produce hermaphrodites to boost fertilization (Tanurdzic and Banks, 2004). Environmental stresses such as drought, low temperature, less than optimal light, low nutrition, less than optimal pH and nitrogen-deficient soils favor maleness in spermatophytes (Korpelainen, 2007). In bryophytes, reverse phenomenon was observed where males seem to be more susceptible to environmental stressors (Longton, 1985, 1988; Cameron and Wyatt, 1990; Shaw et al., 1991; Bisang and Hedenäs, 2005). In addition to abiotic stresses, some physiological factors may also affect the sex expression such as auxins and gibberellins in *Bryum argenteum* favored maleness, whereas cytokines showed a clear preference for females (Korpelainen, 2007). *Ilex integra*, a dioecious tree that is native to East Asia, is an extraordinary plant in view of the above findings. Complete sex change, both from female to male and male to female, is observed in adult individuals of the above species but the reason remains unknown (Takagi and Togashi, 2012). Whatever the reason that causes sexual lability, it could be a manifestation of a plant's inability to preserve its genetically coded sex in a disturbed environment (Korpelainen, 2007) or it could be an adaptation mechanism that supports survival in a new habitat (Charnov and Bull, 1977).

5.5 Epigenetic inheritance of sex

Epigenetic inheritance that is based on inheritance unrelated to changes in the DNA sequence (Wierzbicki, 2004) is also responsible for sex expression and sex inheritance in plants. Epigenetic inheritance was reported in *Melandrium album*, a plant which changes its sex from male to andro-hermaphrodite when treated with the nucleoside analog of 5-azacytidine (5-azaC). This andro-hermaphroditic form can be re-appeared by the pollination of wild females with andro-hermaphrodite pollen and it's happened due to 5-azaC-induced hypo methylation of DNA (Monika and Jakub, 2012). Gene expression is inhibited by the hyper methylation of the promoter of the CmWIP1 gene responsible for pistil growth. The insertion of transposons is responsible for epigenetic change in the promoter region (Martin et al., 2009) whereas transposons are strongly methylated (Wierzbicki, 2004; Slotkin and Martienssen, 2007; Weil and Martienssen, 2008).

6 Sex identification in papaya

Prediction of papaya sex at seedling stage using morphological traits have been attempted by many researchers but success began to achieved with advancements in genomics, molecular tools and techniques. In this section, an attempt was taken to review the way of sex determination methods including morphological, cytological or isozyme and molecular markers based techniques in papaya.

6.1 Morphological marker

To raise an orchard identification of desirable plant population at early stage is very important and crucial fact for papaya. Several morphological characters such as seed coat colour, root morphology etc. found to be related with the sex types in papaya (Kumar, 1951). Soni et al. (2017) had done an exclusive experiment that was entitled with "Morphological Markers Related to Sex Expression in Papaya (*Carica papaya* L.)". In their experiment they used four different genotypes. They reported seed coat color, petiole thickness, stem color as a morphological marker. The black and dark brown color exhibited higher frequency of the female and hermaphrodite plants across the genotypes (Table 2). The reason behind the higher number of the black and brown seeds developed as productive plants might be the pollination of such flowers between female and hermaphrodite or selfing of the hermaphrodite (Hofmeyer, 1938; Storey, 1941). In case of petiole thickness Soni et al. (2017) reported that the lower the thickness the higher the probability of maleness (Table 2). Stem color can also be an indicator. At seedling stage light green

color stem may expressed as female plant at flowering stage. A unique purple stem color was also reported which expressed as hermaphrodite plants further. Among morphological traits, black and brown seed color was most reliable in predicting female and hermaphrodite plants (Table 2). The findings are in close conformity with report of (Kumar, 1951). However, results are in partial conformity with reports of Bojappa (1969) and Kumari (1989).

6.2 Molecular marker

Random amplified polymorphic DNA (RAPD) is most popular marker system for sex determination in papaya (Welsh and McClelland, 1990; Williams et al., 1990). PCR based DNA marker techniques such as AFLP (Vos et al., 1995), ISSR (Zietkiewicz et al., 1994), SSR (Akkaya et al., 1992) and SNP (Jordan and Humphries, 1994) have been used to develop gender or sex-linked markers in papaya (Table 3).

Random amplified polymorphic DNA (RAPD) Bedoya and Nuñez (2007) did an experiment on three Colombian papaya genotypes to identify their sex using a RAPD marker (OP-Y7900). Niroshini et al. (2008) identified OPC09 (1.7 kb) and OPE03 (0.4 kb) markers in male and hermaphrodite plants, respectively whereas OPE19 (2.18 kb) in female plants. Lemos et al. (2002) reported a RAPD marker BC210 (438bp) that can identify the hermaphrodite plants from a sample of hermaphrodites and female plants.

Sequence characterized amplified region (SCAR) SCAR markers are highly reproducible, sequence specific and simple to use which are developed by cloning the amplified bands of RAPD and then sequencing their ends. Deputy et al. (2002) used SCAR T12 and SCAR T1 as a positive control to predict correctly hermaphrodite papaya plants in a population of seedlings with an overall accuracy of 99.2%. Urasaki et al. (2002) identified a PSDM (Papaya Sex Determination Marker) i.e. 450 bp fragment in all male but not in the female plants. A SCAR that developed from this RAPD marker amplified fragments from the genomes of male and hermaphrodite plants, but not the female ones. Bedoya and Nuñez (2007) developed a SCAR marker from OP-Y7 (900 bp) RAPD marker. They reported from their experiment that the SCAR marker generated from the OP-Y7 (900 bp) can identify male and hermaphrodite plants from female plants. This result gave evidence that the SCAR marker is located in a region of the Y chromosome that is found only in male and hermaphrodite plants. Parasnis et al. (2000) and Urasaki et al. (2002) report SCAR markers that are specific for male and hermaphrodite plants

Inter Simple Sequence Repeat (ISSR) ISSR (Zietkiewicz et al., 1994) is a PCR based DNA fingerprinting technique that utilizes single primer con-

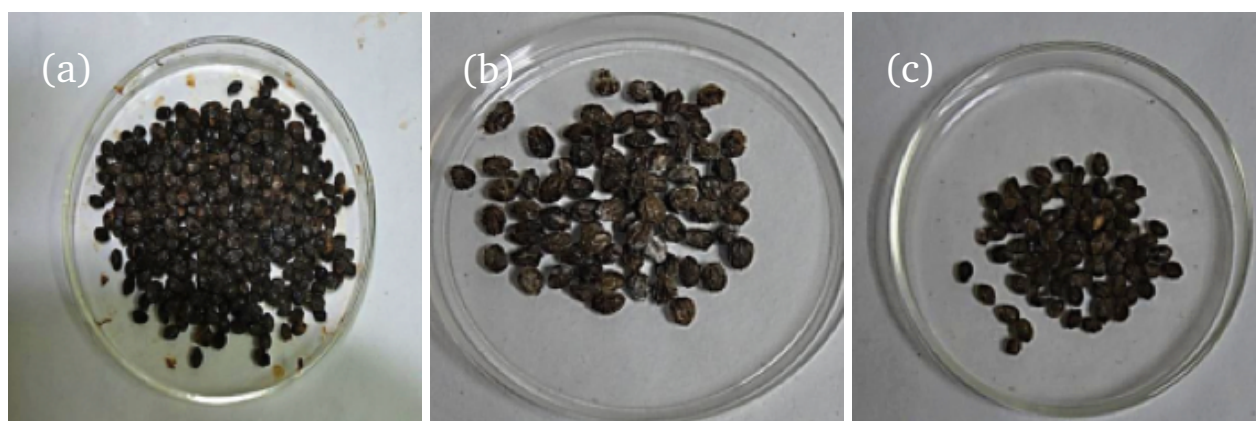


Figure 2. Different seed colors observed in papaya. (a) black, (b) dark brown, and (c) light brown seeds. Source: [Soni \(2015\)](#)

Table 2. Seed color, petiole thickness, petiole length, stem color, petiole orientation with stem and sex expression in papaya

Genotype	Seed color	Petiole thickness (mm)	Petiole length (cm)	Stem color	Petiole orientation (degree)
Pusa Nanha (F)	Black	3.7–4.2	9.5–10.4	–	54–60
Pusa Nanha (M)	Dark brown	3.1–3.6	9.5–10.4	–	50–54
P-7-2 × SAM (F)	Black	3.7–4.2	9.5–10.4	–	54–60
P-7-2 × SAM(M)	Dark brown	3.1–3.6	8.5–9.4	–	46–54
Red Lady (F)	Dark brown	3.1–3.6	–	–	50–54
Red Lady (H)	–	–	–	–	54–60
P-9-5 (F)	–	3.1–3.6	–	Light Purple	54–60
P-9-5 (H)	Dark brown	–	–	Green Purple	54–60

Modified from [Soni et al. \(2017\)](#)

Table 3. DNA molecular markers used for predicting the sex type in *C. papaya*

Marker type	Sex detection	Cultivar analyzed	Reference
SSR	H and M	Various	Parasnis et al. (1999)
RAPD	M	From India and USA	Parasnis et al. (2000)
SCAR	M	From India and USA	Parasnis et al. (2000)
SCAR	H and M	Sunrise, Kapoho, Rainbow, Sun UP	Deputy et al. (2002)
SCAR	H and M	Sunrise, Kapoho, Rainbow, Sun UP	Deputy et al. (2002)
RAPD	H and M	Various	Urasaki et al. (2002)
SCAR	H and M	Various	Urasaki et al. (2002)
SCAR	H and M	Sunrise	Urasaki et al. (2002)
Not described	H and M	Cariflora, Cavite, Sinta	Magdalita and Mercado (2003)
Not described	H	Cariflora, Cavite, Sinta	Magdalita and Mercado (2003)
RAPD	M	Catira, ILS 647, ILS 649	Bedoya and Nuñez (2007)
SCAR	H and M	Catira, ILS 647, ILS 649	Bedoya and Nuñez (2007)
RAPD	H	Sinrise, Calimosa, JTA, Tainung n [♀] 1	de Oliveira et al. (2007)
SSR	H	Tailandia, SS72/12, Tainung H	da Costa et al. (2011)
RAPD	M and F	Various	Reddy et al. (2012)

Modified from [Grewal and Goyat \(2015\)](#)

taining microsatellite sequences of 15–30 nucleotide (Gupta et al., 1996). Gangopadhyay et al. (2007) utilized three microsatellite probes (CAG)₅, (GACA)₄ and (CAA)₅ for sex-identification in papaya and only primer (GACA)₄ developed one female-specific band which was detected in all female and hermaphrodite plants. Parasnis et al. (1999) used a microsatellite probe (GATA)₄ that generated a 5 kb male-specific band. ISSR has some disadvantages such as low reproducibility and limited number of bands makes it less interesting for sex determination in papaya.

Amplified fragment length polymorphism (AFLP)

This technique is used for generating fingerprints of DNA of any origin or complexity. No AFLP marker is still available for sex determination in *C. papaya*, but it has been utilized in several other plant species. The AFLP method is rarely used for early sex diagnosis of seedlings among plants due to some drawbacks such as high cost, more time consuming and laborious analysis.

Simple Sequence Repeat (SSR) SSR are more precious molecular marker than other PCR-based markers like RAPD, ISSR and AFLP due to their sequence-specificity, multiallelic nature, co-dominant inheritance, abundance in the genome, high rate of transferability, high level of polymorphism and reproducibility (Powell et al., 1996; Zane et al., 2002; Thiel et al., 2003). In addition, it does not required high quality of DNA and performs well with low quantity of template DNA (10–100ng/reaction). Chiu et al. (2015) determined sex in all hermaphrodite cultivar (*Taichun Sunrise*; TS) and typical hermaphrodite cultivar (Taiwan Seed Station No.7; T7) of papaya and their F₁ progeny using SSR markers. Parasnis et al. (1999) reported a microsatellite sequence unique to males or hermaphrodites of several cultivars of papaya; however, they did not report detailed data on linkage.

Multiplex loop-mediated isothermal amplification (mLAMP)

Six male-hermaphrodite specific markers were developed for a rapid sex identification using multiplex loop-mediated isothermal amplification (mLAMP) to efficiently and precisely select hermaphroditic individuals in the seedling or early growth stage. The LM1-LAMP assay consisted of two sex-LAMP reactions for amplifying two male-specific markers (T12 and Cpsm90) in one reaction, and showed several advantages in terms of a rapid reaction time (<1h), isothermal conditions (less equipment required), a high efficiency (0.5 ng of DNA required in the reaction mixture), and an economical reaction system (5 µL in volume). The established method can be easily performed in the field by visual inspection and facilitates the selection of all hermaphroditic individuals in papaya production.

6.3 Biochemical marker

Choudhuri et al. (1957) differentiated the male and female plants of papaya based on leaf content of total carbohydrates, phosphorus, nitrogen, potash and chlorophyll a and b. Leaves of male plant were richer in CHO, phosphorus and Chlorophyll a and b whereas female plant contained higher nitrogen and potash (Choudhuri et al., 1957). Sing and Jindal (1972) observed lesser amount of free and bound phenolic in female plants than male one. They also reported that femaleness was enhanced with the application of TIBA. Todokovo (1930) observed that the pH range of male plants were 5.5–5.8 whereas it ranged from 4.0–5.4 in females. On the other hand Choudhuri et al. (1957) found higher pH in the leaves of female plants than males. It might be occurred due to different environmental conditions under which the plants were grown (Bojappa and Singh, 1974a). The amount of phenolic compounds was remarkably higher in male plants (Jindal and Singh, 1975). Sex forecasts would be possible through phenolic tests based on color reactions in vegetative (preflowering) seedlings of papaya at the nursery stage. The “Prussian blue” and “total phenolics” tests were found to be highly efficient in making sex forecasts of vegetative seedlings. The precision of sex forecasts in the “Prussian blue” test was 80% in female plants and 60% in male plants, while under the “total phenolics” test the precision was 86% and 77% in female and male plants respectively (Jindal and Singh, 1976). Female plants had higher levels of peroxidase activity than that in male (Nasrin et al., 2010).

6.4 Chemical identification of sex

For sex differentiation colorimetric tests were carried out by Singh et al. (1961). This tests could forecasted higher efficiency of femaleness due to the absolute sex stability of female plants as compared to male and hermaphrodite sex forms. The same test sometimes showed varied color reactions in the same sex form (Bojappa and Singh, 1974b). Maleness was identified by French blue, Cerulean blue or Chapri blue where as French blue, Hyacinth blue or Gentian blue were seen for femaleness and Enamel blue or Porcelain blue color for hermaphrodite in case of modified nitrous acid and mercuric nitrate tests. Female seedlings were identified more accurately (87%) than males (67%) by modified Almen reagent test (Singh et al., 1961). Bojappa and Singh (1974b) predicted femaleness and hermaphrodite at the level of 77% and 74% respectively in modified almen reagent test. Ferrous sulphate test gave 67% accuracy for femaleness where as 51% accuracy for femaleness was observed in case of Titanous chloride test (Singh et al., 1961). The percentage of sex predictions with Prussian blue test was 80% in female plants and 60% in male plants (Jindal and Singh, 1976). Rao et al. (1985) reported a

color change due to the application of modified semen agent to the leaves that correctly identified 92.5% of female plants, 72.5% of hermaphrodite and 70% of males. Mohan (2014) conducted an experiment that was entitled as “Validation of tests for sex determination in papaya (*Carica papaya*)”. She used four chemical test to identify the sex form of three different variety (Table 4). In case of Almen test overall the percentage of accuracy obtained for female sex form in three variety was 71% and for male it was 67% whereas 55% for male and 60% for female in Ammonium molybdate test. Ferric chloride test failed to distinguish male and female sex forms of papaya in her experiment. The accuracy level was 46% for male and 55% for female in case of Titanous chloride test.

6.5 Male-specific markers development

Identification of papaya sex types at juvenile phase can expedite breeding and production. However, there is no morphological difference among different sex types of papayas at the vegetative stage. Morphological identification of papaya sex types relies on flower morphology, but it only can be done after the plant flowers. Therefore, development of a simple, sensitive and accurate method for determination of papaya sex types is in high demand. The papaya genomic resources of HSY, MSY, and their X counterparts offered an unprecedented opportunity to develop male-specific markers precisely in the male-specific region. Two papaya markers, PMSM1 and PMSM2, were designed specifically to target the male-specific region (Liao et al., 2017). The PCR results indicate that both of them can be used to detect male papaya plants rapidly and accurately, demonstrating their potential for determining the sex type of papaya at the seedling stage and to study the gene regulatory network of sex determination.

7 PGR's effects on sex expression

In case of the sex expression of plants plant growth regulators have significant rules. Higida and Pascual (2015) observed the effect of different plant growth regulators on sex expression of papaya. Papaya plants applied with 100 ppm IBA at 0.25 mL per application resulted to more female flowers than those not applied with PGR but it produced lesser hermaphrodite (10%) and male (3.33%) flowers. IBA should be applied on the appearance of first to fourth true leaf. Factors that raise the auxin levels at the differentiating apex enhances femaleness and suppress maleness (Heslop-Harrison, 1957) as the application of IBA here. Naphthalene acetic acid (NAA) (100 ppm) also promoted female flower formation (Galun, 1959) in papaya. In case of hermaphrodite plants production, application of 200 ppm Ethrel gave the most expected number (46.67%) (Higida and Pascual,

2015, Table 5) but excessive Ethrel may also cause higher number of male (Ghani et al., 2013). Exogenous application of gibberellic acid on female and hermaphrodite flowers of papaya didn't yield any sex reversal phenotype but cause a significant increase in peduncle elongation and inflorescence branch number (Han et al., 2014). An increasing flower number per plant was also observed in female but not hermaphrodite or male.

8 Environmental effect

The basic sexes in papaya are genetically determined but certain male and hermaphrodite plants have been known to undergo sex reversal under the influence of environmental changes Storey (1958). Low temperature during the winter months in the subtropics promote femaleness in hermaphrodite plants in papaya (Table 6) The flowers revert from the Type 4 hermaphrodite that has 10 stamens to a Type 3 or carpelloid form with 6–9 stamens or the Type 2 (pentandria) having 5 stamens. This reduction in the number of stamens is brought about by the fusion of stamens to the ovary wall. At high elevations, Solo papaya has a greater number of Type 2 and Type 3 fruits that are not marketable (Awada, 1958a). At the other extreme, warm temperatures tend to promote the production of Type 4+ (barren) hermaphrodite flower resulting in sterility of the plants. Allan et al. (1987) reported that male trees also showed reversion to femaleness under cool temperatures. High soil moisture and nitrogen level promote vigorous plant growth and femaleness. Hermaphrodite trees stressed by drought produced more sterile Type 4+ flower, while consistently high moisture levels promote the production of hermaphrodite flower (Awada, 1961). Moisture and nitrogen level affects the vigour of plants bringing about sex reversal (Awada et al., 1979). Plant vigor in first year of growing season showed higher incidence of carpelloid than subsequent harvest (Chan, 1984).

Ramos et al. (2011) suggested that the selection of genotypes at winter and spring months from backcrossed generation will be resulted with a lower incidence of anomalies (malformations and sex reversal) and higher rates of normal flowers and marketable fruit. Awada and Ikeda (1957b) reported that plants that grown in the low irrigation plots produced solo type fruits in greater percentages than that in the high irrigation plots (Table 7). This indicated a relationship between the sex status and either of two factors that is the moisture level or growth of the papaya plant.

9 Important of sex expression

The study of sex type identification is valuable in papaya because sex of the papaya plant cannot be pre-

Table 4. Results of different chemical test on sex identification in three different papaya varieties

Chemical Test	Papaya variety	Sex form	Plants expected	No. of plants at flowering	Accuracy (%)	M and F (%)
Almen reagent test	Washington	M	9	6	66	M: 67 F: 71
		F	11	8	72	
	Pusa Dwarf	M	11	8	72	
		F	9	7	77	
	Pusa Nanha	M	8	5	62	
		F	12	8	66	
Modified Almen reagent test		–	–	–	–	F: 77–87 H: 74, M: 67
Ferrous sulphate test		–	–	–	–	F: 67
Ammonium molybdate test	Washington	M	12	6	50	M: 55 F: 60
		F	8	5	62	
	Pusa Dwarf	M	13	9	69	
		F	7	4	57	
	Pusa Nanha	M	15	7	46	
		F	5	3	60	
Titanous chloride test	Washington	M	7	3	42	M: 46 F: 55
		F	13	7	53	
	Pusa Dwarf	M	8	4	50	
		F	12	6	50	
	Pusa Nanha	M	11	5	45	
		F	9	5	55	

M = male, F =female, and H = hermaphrodite; Modified from [Mohan \(2014\)](#), [Bojappa and Singh \(1974b\)](#), and [Singh et al. \(1961\)](#)

Table 5. Effect of PGR on sex expression of papaya

PGR	Concentration	Female	Hermaphrodite
Ethrel	50	60	27
	100	60	32
	200	35	46
	500	–	60
Kinetin	50	50	35
	100	60	27
	200	60	38
IBB	50	62	30
	100	84	10
	200	65	35
NAA	100	–	62.5

Modified from [Higida and Pascual \(2015\)](#), [Mitra and Ghanta \(2000\)](#)

Table 6. Day-length and temperature effects on flower types for each locality [†]

Locality	Day length range (hr)	Mean min. temp. (Fr)	Mean max. temp. (Fr)	Flower types [‡] (%)			
				2	3	4	4+
Honolulu	10.88–13.45	69.7	80.8	4	8	70	18
Kainaliu	11.03–13.46	59.9	76.1	93	6	1	–
Makawao	10.98–13.55	58.3	72.0	53	42	5	–

[†] Source: Modified from [Awada \(1958a\)](#);

[‡] Type 4+: This type of flower has 10 functional stamens and it lacks functional pistil. It is the most staminate of the hermaphroditic types of flowers; Type 4: This type of flower possesses an elongate pistil and the resulting fruit is long-cylindrical. The petals are fused together for ¼ to ¾ of their length and form a fairly rigid corolla tube; Type 3: This type of flower is intermediate between types 2 and 4. It has six to nine functional stamens; Type 2: This type of flower has five functional stamens. This type of flower is least staminate and most pistillate of the hermaphroditic flowers.

Table 7. Effect of irrigation on percentage of fruit types per tree

Type of fruit	Percentage of fruit type/tree	
	Low irrigation	High irrigation
2 (Extremely misshapen)	9.71	15.64
3 (Less misshapen)	31.44	39.68
4 (Hermaphrodite)	58.85	44.68

Source: Modified from [Awada and Ikeda \(1957a\)](#)

dicted morphologically at early seedling stages. Papaya cultivars both commercial and backyard growers solely depend on seed as planting materials. Farmers usually need to plant three seeds per pit as they are not sure whether the plant will be male or female. Papaya seeds produce seedlings of unidentified sex, therefore farmers have to remove the male plants from the field and leave the female or hermaphrodite plants on the basis of floral morphology which can be performed only after three to four months from germination ([Ma et al., 2004](#)) as the papaya usually flowers 3-6 months after transplanting. After flowering it's found that all are male, it will be a loss for the farmers. Farmers need to be sure before flowering that a seedling is a female or a productive hermaphroditic plant, either of which will give a good harvest. If the prediction of sex of papaya could be done at early seedling stage, then an expected male and female plants ratios (5% males: 95% females) would be maintained by removing excess male plants. The determination of the sex type of papaya seedlings prior to the flowering stage would help to avoid the need for removing undesired sex types (e.g. males) from the field, thus saving labor, time and other resources. However, female plants require presence of small number (6-10%) of male plants in the field for fruit production ([Eustice et al., 2007](#)). Propagation of papaya by seed is still the most practical method of raising the crop, because it is efficient and economical. Knowledge of the sex type of papaya is important in selecting parents for use in hybridization work.

Crosses between females and hermaphrodites will give all fruit-bearing progenies. Among the three sex types hermaphrodite and female plants are grown for fruits that are preferred for consumption, while male plants are unwanted ([Urasaki et al., 2002](#)). In addition, the early detection or identification of the sex type of a particular papaya seedling would be advantageous for micropropagation, since the desired sex type can be selected. The prior detection of sex type will ensure that the resulting micropropagated plants are 100% either females or hermaphrodites ([Magdalita and Mercado, 2003](#)). Vegetative methods of propagation, such as the use of cuttings, grafting and tissue-cultured materials, are available but they are laborious and expensive.

10 Conclusions

Identification of desirable plant population at early stage is very important and crucial for papaya cultivation. Several morphological characters such as seed coat colour, root morphology etc. found to be related with the sex types in papaya. Black and dark brown seed coat color exhibited higher frequency of the female and hermaphrodite plants. Petiole thickness and stems color also worked as a morphological marker for sex determination in papaya. Almen reagent test, Ammonium molybdate test, Titanous chloride test also used to identify sex in papaya. Plant growth regulators such as Ethrel, Kinetin and IBB gave the

most expected number female flower in papaya but excessive use of Ethrel may cause higher number of male. Environment may also affect the sex expression of papaya. Recently molecular markers are also used to identify the sex type of papaya. The knowledge on sex expression and sex determination is important for papaya breeding as well as cultivation.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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