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Study of Flowering Behavior and Sex Determination in *Garcinia indica* (Thomas-Du Pettite) Choisy by Means of Molecular Markers

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Abstract: *Garcinia indica* (family-Clusiaceae) is highly endemic tree species from tropical rain forest of Western Ghats of India. It is cross-pollinated and polygamodioecious plant. Polygamodioecious is a condition in which trees have both bisexual and purely male flowers on certain branches of same tree. In *G. indica* there is no morphological or biochemical difference exists between male and female plants; other than flower characteristics. Distinction between male and female plants can not be done until it flowers i.e., about after 10-12 years. Determination of gender of a seedling at an early stage of development will enable the commercial cultivars to maintain the required ratio of male plants in the orchard for fruit production on large scale. Therefore, it is essential to differentiate the plants at seedling stage by molecular markers. Identified male and female plants collected from Dr. Balasaheb Sawant Agricultural University, Dapoli, Maharashtra. The genomic DNA was extracted from leaves and subjected to PCR amplification using RAPD and ISSR primers. Out of 92 RAPD and 28 ISSR primers screened only two RAPD (OPW-05, OPW-08) polymorphic bands in female plants of *G. indica*. There was only one ISSR primer (UBC-881) which produced polymorphic band only in male plants. Thus in conclusion, it can be stated that these three DNA markers are completely linked with specific sex in *G. indica* and can be used for screening of seedlings to determine their gender while plantation.

Key words: *Garcinia indica*, endemic plant, polygamodioecious, apomixis, sexual dimorphism, RAPD and ISSR markers

INTRODUCTION

The majority of flowering plants are hermaphrodite, developing perfect flowers that contain both pistils and stamens (Irish and Nelson, 1989). In dioecious species, individuals have either staminate or pistillate flowers and produce either male or female gametes, thus ensuring cross fertilization (Lazarte and Palser, 1979). In gynodioecious condition, some plants in same population are hermaphrodite and some plants are perfect female (Delannay, 1979). In androdioecious plants, male and hermaphrodite plants are present in same population.

In dioecious plants the sex identification, based on flower morphology is not possible until the season of maturity. Along with the flower morphology, certain other morphological characteristics like leaf morphology, seed coat colour, root morphology, also can be used as potential markers for gender differentiation (Magdalita and Mercado, 2003). However, these claims have not been proven scientifically.

There are reports in which biochemical assays can be used to determine the sex of the plant (Joshi *et al*., 2008). Such biochemical assay involves detection of certain enzymes like peroxidase in *Canabis sativa* L. (Truta *et al*., 2002), secondary metabolites like essential oils and phenolics in *Myristica fragrans* Houtt (Packiyasothy *et al*., 1991) etc. The difference in phenolics content of the male and female plant was also reported in *Pistacia vera* L. (Misirli and Ozekar, 1999). In recent years, molecular markers *viz*., RAPD, ISSR and AFLP have been used for sex determination in plants e.g. pistachio (Ehsanpour *et al*., 2008), *Trichosanthes* (Kumar *et al*., 2008), Guggul (Samantaray *et al*., 2010), Jojoba (Agrawal *et al*., 2007), etc.

Garcinia indica is polygamodioecious plant endemic to Western Ghats of Maharashtra, India (Rajasekharan and Ganeshan, 2002). Fruits of *G. indica* have bilious action. Hydroxycitric Acid (HCA) extracted from fruit rinds is used as an anti-obesity (Westerterp-Plantenga and Kovacs, 2002) and anti-cholesterol drug (Berkhout *et al*., 1990). Fat extracted from seeds is used in cosmetics as emollient (Patil, 2005).

In nature, seed raised progeny display male, female and bisexual plants in a ratio of 37, 55 and 8%,

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respectively. Male plants do not produce fruits whereas, the bisexual plants produce fruits very rarely which make them economically unproductive.

The plants are slow growing, perennials and the distinction between male, bisexual and female plants can be done only after flowering (about 10-12 years). Also there are no morphological or biochemical traits that will differentiate the plants at an early juvenile stage (Karnik, 1978).

Hence, systematic study of flowering behavior in *G. indica* was undertaken. The study also includes the use of molecular markers viz., RAPD and ISSR for sex determination in *G. indica*.

MATERIALS AND METHODS

Study of flower morphology: Various floral types were collected from Dr. Balasaheb Sawant Agricultural University, Dapoli, Ratnagiri district, Otavane, Talkat and Shiroda in Sawantwadi, Sindhudurg district and from Bapat's garden and Joshi's garden at Dive-agar in Raigad district in January-February and April-May (2009-10). Morphological characteristics of male, female and bisexual flowers collected from various populations viz., colour of calyx and corolla, stigmatic surface, number of stamens, receptacle length were studied.

Collection of plant material for DNA extraction: Identified male and female plants were selected for sex determination from Dr. Balasaheb Sawant Agricultural University Dapoli, Ratnagiri district. Four apical leaves were collected in May and June (2009-10) from each plant and cryopreserved using liquid nitrogen until DNA extraction.

Development of molecular markers: DNA was extracted by modified CTAB method (Thatte *et al*., 2012). In all, 92 RAPD primers (Kit A, B, C, D, W; Operon Technologies, Alameda, CA, USA) and 28 ISSR primers (UBC-kit 09; 17 bp long) obtained from Bangalore Genei Pvt. Ltd, India were screened to determine the sex specific banding pattern. PCR amplification reaction was carried out in 25 μL volume in a thermal cycler (Applied Biosystems, USA) using standardized protocol (Thatte, 2012).

ISSR analysis: First denaturation took place at 94°C for 5 min. Forty five cycles were repeated with initial denaturation at 94°C for 30 sec. Primer annealing temperature varied from 50 to 56°C for 45 sec and extension at 72°C for 2 min. The final extension step was carried out at 72°C for 7 min. The amplification products were electrophoresed on 2% agarose gel in 1X TAE buffer. The size of the amplicons was determined using standards (100 bp DNA ladder: Bangalore Genei Pvt. Ltd., Bangalore, India). DNA fragments were visualized under UV light after staining with Ethidium bromide (0.1 μg mL^{-1} of gel solution) and documented in Gel-Doc (Alpha-innotech, CA, USA).

RESULTS

Floral morphology: The male flowers collected in orchard were long pedicellate; occur in cluster, about 1 cm in length, with green sepals. Petals were 4 to 5 yellow-red in colour. Numerous fertile stamens are present on central hemisphere receptacle. The carpel was absent. Another type of male flower showed numerous fertile stamens, non functional carpel or as rudimentary carpel at the center of receptacle (Fig. 1a, b).

Typical female flower observed at Dapoli had comparatively short pedicel. Petals were 4 yellowish in colour. The flowers bear functional pistil 1.5-2.5 mm in diameter was surrounded by staminodes arranged in 4 tufts. The other type of female flower collected at Dapoli was having pistil 3.5 mm diameter which was surrounded by only 2 tufts of staminodes (Fig. 2a, b). In bisexual

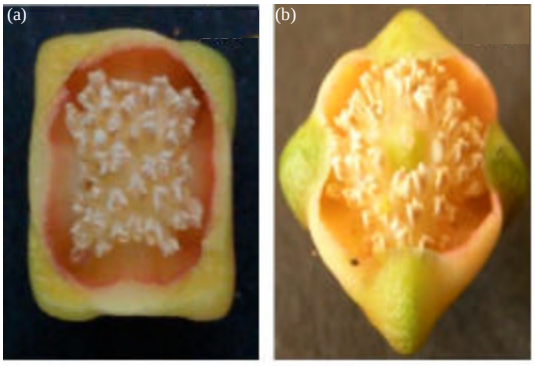

Fig. 1(a-b): Male flower (a) Typical and (b) With rudimentary pistil

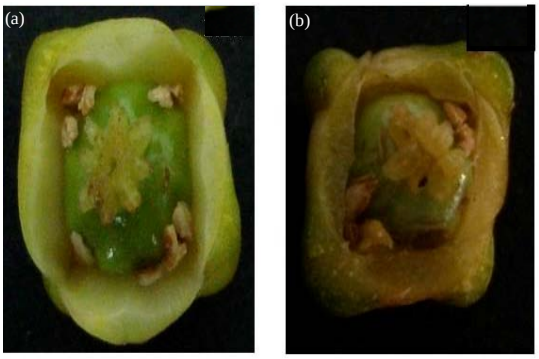

Fig. 2(a-b): Female flower (a) Typical and (b) With staminodes in 2 tufts

flowers, stamens are arranged in 8 tufts. One of the bisexual flowers found at Otavane; a region of Sawantwadi had stamens arranged in ring around the pistil. These flowers had red calyx and red corolla (Fig. 3a, b).

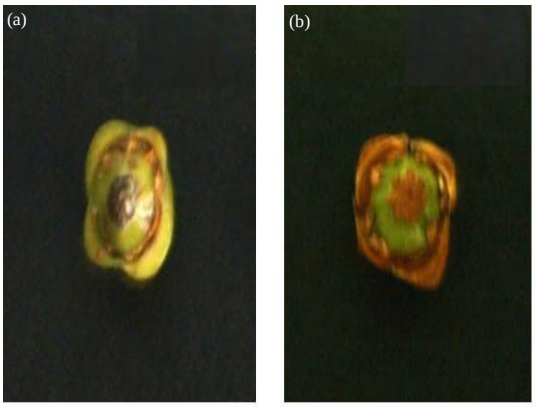

Fig. 3(a-b): Bisexual flowers with stamens arranged in (a) 8 tufts and (b) In ring

Molecular marker analysis for sex determination: The identified 7 male and 8 female plants of *G. indica* were collected from Dr. Balasaheb Agricultural University, Dapoli and were screened for the identification of sex linked molecular marker using 92 RAPD and 28 ISSR primers.

RAPD analysis: Out of 92 RAPD primers screened 78 primers gave a reproducible RAPD pattern. The number of amplification products varied from 2 to 13 bands per primer and size ranging between 300 to 1800 bp. Among 92 primers screened, only 2 primers (OPW-05 and OPW-08) showed polymorphic bands, which could differentiate male and female plants of *G. indica*.

Figure 4a represents the amplification pattern produced by the primer OPW-05 (5'-GGCGGATAAG-3'). Lanes 1 to 3 and 5 to 7 represent the male plants, lane 4 represent bisexual plant and lanes 8 to 24 represent the female plants. This primer has generated an 1100 and 1200 bp band in most of the tested female individuals and in bisexual plant. This band was completely absent in all male individuals and few female plants.

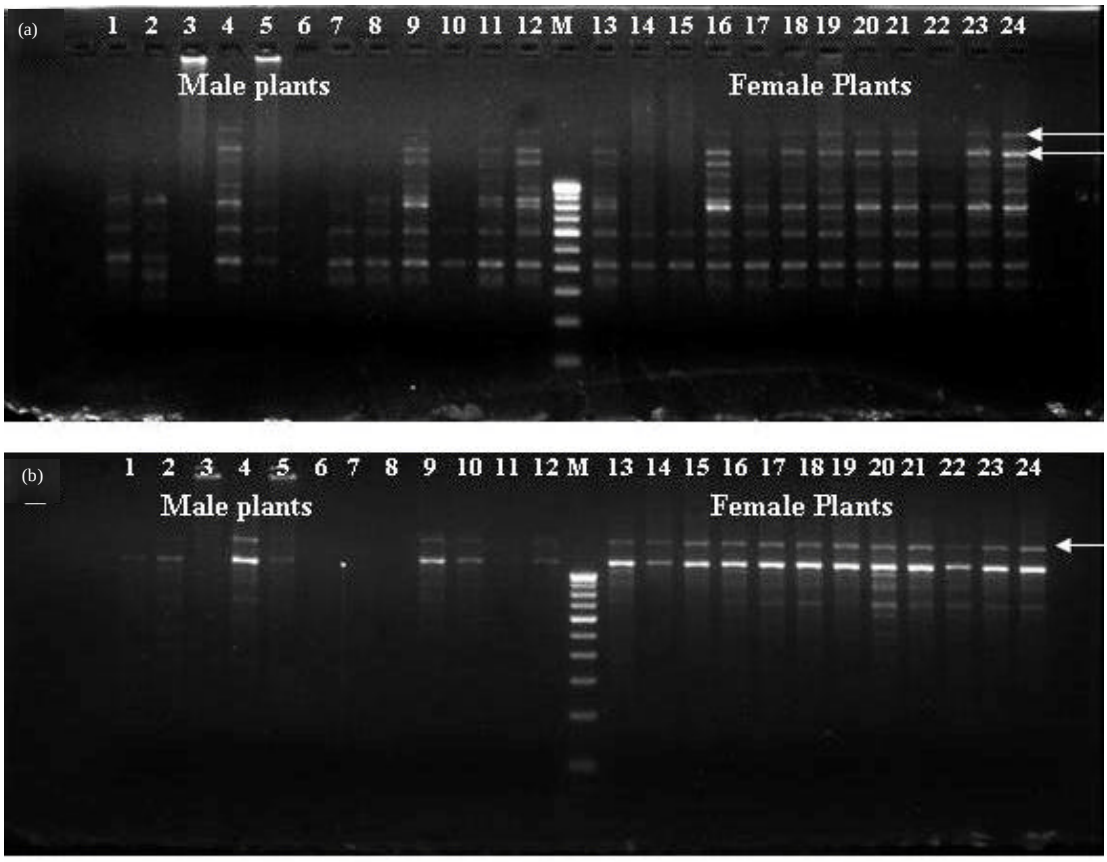

Fig. 4(a-c): Continued

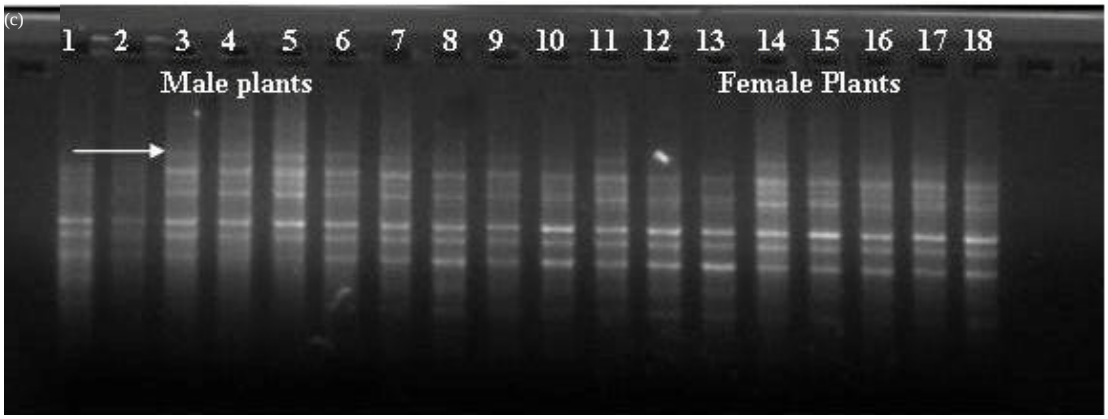

Fig. 4(a-c): Sex specific molecular markers differentiating male and female plants of *G. indica* (a) Amplific pattern produced by OPW-05 primer, (b) amplification pattern produced by OPW-05 primer and (c) amplification pattern produced by UPC-881 primer, Lanes 1-3 and 5-7: Male plants, Lane 4: Bisexual plant and lanes 8-24: Female

Figure 4b represents the amplification pattern generated by the primer OPW-08 (5'-GACTGCCTCT-3'). Lanes 1 to 3 and 5 to 7 represent the male plants, lane 4 represent bisexual plant and lanes 8 to 24 represent the female plants. A 1500 bp band was observed in all female and bisexual plants, which is absent in all male plants. The female plant (lane 8 and 11) did not show amplification with the primer.

ISSR analysis: Out of 28 ISSR primers used herein for gender differentiation, only 2 primers were found to be sex specific. Fig. 4c represents the amplification pattern generated by the primer UBC-881 (5'- GGGTGG GGTGGGGTG-3'). A 1200 bp band was absent in most of the female plants (Lanes 8 to 18) but observed in all male plants (Lanes 3 to 7) except first two males.

DISCUSSION

Extensive studies have been done on flowering, mode of pollination and fruiting etc., in several species of *Garcinia*. In *Garcinia mangostana*, flowers arise from the tip of young shoots mostly single to three. The flower is 4-6 cm. in diameter; it produces 14-18 stamens 5-6 mm in length; however do not bear fertile pollen grains. The fruit set always occurs by obligate apomixis (Sobir and Poerwanto, 2007).

Detailed study of reproduction on *Garcinia atroviridis* is done by Pangsuban *et al.* (2007). It is a plant endemic to Malaysia. The plants are dioecious, staminate flowers are produced in peduncles of short raceme but pistillate flowers are solitary. They classified 2 gender classes of *G. atroviridis* based on their floral feature and fruit production. The trees with functional anthers, which produce few fruits or no fruits, were distinguished as Low Fruiting (LF). Trees with rudimentary anthers producing high number of fruits were distinguished as High Fruiting (HF).

G. indica can also be categorized into 3 classes viz., no fruit yielding (functional males), low fruit yielding (Bisexual) LF and high fruit yielding (functional females or HF). There are very few reports regarding the flower morphology and pollen behavior in *G. indica*. All types of plants bear staminate flowers in the month of December to February. The male flowers are elongated with long receptacle, 4 sepals and 4 petals with reddish yellow colour. Stamens are numerous, fertile; cohere at the base forming anthophore. Carpels are absent or small rudimentary pistil is observed. Somewhat later (February to April), the female or high fruiting plants produce functionally female flowers. These flowers are rarely produced in male (LF) plants. The flowers are comparatively broader, with short pedicel, sepals were 4 green in colour, corolla was generally pale yellowish. They bear comparatively less number of stamens or staminodes, which are arranged in to 2, 4 or 8 tufts surrounding the pistil. Ovary is comparatively larger with 4-8 functional ovules in axile placentation. Large fruiting or bisexual plants bear flowers with a ring of stamens/ staminodes around the carpel. The size of the carpel is comparatively small. Petals are reddish in colour (Fig. 3b). These plants produce spindle shaped fruits in very low yield.

Much earlier Karnik (1978) made detailed study of flowering and fruiting in Kokum with the help of beautiful diagrammatic sketches. They described 11 floral types in *G. indica* which can be arranged in increasing functionality of pistils and decreasing functionality of

stamens. For the first time they reported presence of bisexual plant and they stated occurrence of 37% males and 55% female plants in natural population of *G. indica*. The bisexual plants were only 8%.

It necessitates sex determination of *G. indica* at an early stage of development so that commercial cultivars can maintain desired 1:10 ratio of male plants in the orchard. There are no vegetative or morphological traits like leaf morphology that could differentiate male and female plants of *G. indica*. Similarly there are also no biochemical parameters like phenolics content that helps in gender differentiation (Thatte, 2012). The sexual dimorphism was studied in *Telfairia occidentalis* Hook by Ndukwu *et al.* (2005) using morphological markers. Vegetative morphological traits like leaf length, breadth, petiole length and flower morphology has been used to differentiate between male and female plants. It was found that the leaves of the females appeared to be longer and wider than those of the males, but there was no significant difference exists in such vegetative traits (Ndukwu *et al*., 2005).

Therefore, it is an immense important to identify the gender of a species at juvenile stage so that the cultivation of large number of plants of productive gender in an orchard is possible. Till date, no other method of sex determination other than the flower morphology is existed. In recent years, attempts have been made to understand the genetic basis of the sex determination in plants, which can make the use of molecular markers viz., RAPD, ISSR, AFLP to identify the sex-linked DNA marker in plants. Gangopadhyay *et al.* (2007) have found OPB-01 male specific and OPB-05 was female specific marker in *Carica papaya*. They have also used 3 ISSR primers out of which $(GACA)_4$ primer generated a band in all females and hermaphrodite plants of *C. papaya*. This band was absent in all male plants (Gangopadhyay *et al*., 2007).

The present study revealed that the female specific DNA fragment of size 1100 bp produced by the primer OPW-05 and 1500 bp produced by OPW-08 is tightly linked with the female sex locus and is useful for sex determination in *G. indica*. Among ISSR primers, UBC-881 has produced the male specific amplicon of 1200 bp size. The two male plants (lane 1 and 2) did now show this band and few females (lanes 14 to 17) showed presence of feeble band of amplification. The presence of 1200 bp band in few female plants indicated that these plants must be in transitory stage of female from male. These plants show presence of staminodes with fertile pistil. Some times the stamens are arranged in 2 or 4 tufts surrounding the fertile ovary (Karnik, 1978).

RAPD and ISSR primers are known for lesser reproducibility and less stringent. Therefore, putative sex linked RAPD markers is converted to SCAR marker and used further to determine the gender of a plant (Stehlik and Blattner, 2004).

Vinod *et al.* (2007) have studied the sex determining loci in *Pandanus fascicularis* using DNA based molecular markers. RAPD and ISSR primers were used initially to find out the banding profile polymorphic between the sexes. Such sex specific fragment was then gel eluted, cloned sequenced and specific primers were designed. This Sequence Characterized Amplified Region (SCAR) were continued to amplify the male specific 976 bp band which is absent in all female genotypes.

Prasanthi *et al.* (2010) studied the Polygamodioecious plant *Simarouba glauca* belonging to family Simaraubaeae to determine the sex specific molecular marker using RAPD and SCAR markers for improved production. This plant is economically important as it contains 55-60% of edible oil. They analysed DNA samples of 32 male and 27 female plants with 250 RAPD primers. Only one primer among 250 was found to be associated with maleness in *S. glauca*. The amplified band was then transformed into SCAR marker and retested with male and female plants. The same 950 bp band was generated in only male and andromonoecious plants.

Therefore in present study, it can be concluded that the large number of RAPD and ISSR primers needs to be screen with more number of identified male and female plants. The putative sex linked marker can then be converted to SCAR marker to get more reproducibility of results. Attempts are made in V. G. Vaze College, to determine the karyomorphology in *G. indica*. So that sex specific SCAR markers can be derived from sex specific chromosomes.

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