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Sex Detection in Micropropagated Plants and Natural Seedlings of *Garcinia indica* using RAPD and ISSR Markers

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Abstract

Garcinia indica (Kokum) is an economically important endemic tree species of the Western Ghats region of India. It has three sex types viz. male, female and bisexual that could be identified only at the emergence of flowers after seven to eight years of vegetative growth. RAPD and ISSR markers were used for the detection of sex (male or female) in micropropagated plants and natural seedling populations of *G. indica.* Amplification of genomic DNA using primers RAPD OPT 01 (5'-GGGCCACTCA-3') and ISSR UBC 881 (5'-GGGTGGGGTGGGGTG-3') have produced a specific band in standard male plants and that particular band was absent in female plants. Hence, these two primers were used for screening large number of tissue cultured plants and natural seedling populations. The results indicated the presence of specific band in some of the tissue cultured plants and as well as in the natural seedlings indicating them as male plants, while this specific band was absent in remaining plants screened indicating them as female. Determination of sex in seedlings stage of *G. indica* is essential to establish desired ratio of male and female plants in field for large scale cultivation.
Key Words: *Garcinia indica;* RAPD; ISSR; Sex determination.

Introduction

Garcinia (Clusiaceae) is a large pantropical genus comprising of dioecious trees and shrubs. It exhibits a wide spectrum of floral form (Sweeney, 2008; Sharma *et al*., 2013). *Garcinia indica* (Thouars) Choisy is an underexploited, economically important, evergreen, endemic tree species of the Western Ghats region of India (Anerao *et al*., 2013; Braganza *et al*., 2012). It is endangered in Southern India due to deforestation, urbanization and habitat destruction (Chauhan *et al*., 2012). The fruits are used in various traditional preparations and have high value appreciation when processed (Daniel and Dudhade, 2006; Braganza *et al*., 2012). They are anti-bilious, possess anti-helmintic properties contain important biochemical constituents like garcinol and hydroxycitric acid that have antioxidative, antiglycation, free radical scavenging activity,

anti-cancer action (Kaur *et al*., 2012; Braganza *et al*., 2012), act as anti-obesity agent and an anti-cholesterol drug (Anerao *et al*., 2013; Thatte *et al*., 2012). The anthocyanin pigment extracted from the fruit rinds is a natural source for the red colour.

Besides being stated by Karnik (1978) that *G. indica* has eleven floral types. Thatte and Deodhar (2012) and Devi *et al*. (2013 characterized three main floral types i.e. male (staminate), female (pistillate) and bisexual (hermaphrodite). The flowers are insect pollinated (Thatte *et al*., 2012; Anerao *et al*., 2013) or may be wind pollinated (Haldankar *et al*., 2012; Patil *et al*., 2012). The flowering period begins from November to February and fruiting occurs during April to first week of June (Braganza *et al*., 2012; Devi *et al*., 2013).

Molecular markers were employed in the detection of sex in several dioecious plants such as *Pistacia sp.*(Kafkas *et al*., 2001), *Carica papaya* (Lemos *et al*., 2002; Urasaki *et al*., 2002; Reddy *et al*., 2012), *Ginkgo biloba* (Ling *et al*., 2003), *Borassus flabellifer* (George *et al*., 2007), *Pandanus tectorius* (Panda *et al*., 2010), *Piper betle* (Samantaray *et al.,* 2011), *Trichosanthes dioica* (Kumar *et al.,* 2012), *Momordica dioica* (Patil *et al*., 2012; Baratakke *et al*., 2013), etc. Detection of sex in *Garcinia indica* was studied by Sawardekar *et al*. (2011) wherein the male, female and hermaphrodite plants were screened with 20 RAPD primers. Primer OPA-05 revealed specific band in male plants and not in female plants. Similarly, Thatte and Deodhar (2012) used RAPD primers and shown the production of polymorphic bands in female plants and ISSR primer produced polymorphic bands in male plants of *G. indica*.

The determination of sex in *G. indica* is necessary for the farmers to establish seedling orchards, as the male plants are of not much use unlike the fruit yielding female trees. It is not possible to distinguish sex types in seedling stage until the onset of flowering which normally takes about 7-8 years. This study aimed at determining the sex (male or female plants) in tissue cultured plants (*in vitro* mass propagated plants) and natural seedling populations of *G. indica* using RAPD and ISSR markers.

Materials and methods

Plant materials: Ripened fruits were collected from the natural source of the Western Ghats regions. Seeds were separated from the pulp, sun dried, sown in pots, grown under polyhouse condition for raising seedlings. Baskaran and Krishnan (2011) carried out *in-vitro* regeneration and mass multiplication of *G. indica* for the production of elite clones. Leaf samples from these *in vitro* propagated plants were collected and used for isolation of DNA (Plate 1G). Similarly, leaf samples from natural seedling populations were collected and used for DNA isolation (Plate 1F). At the same time, Leaf samples from

male and female plants of *G. indica* variety "Konkan Amruta" were collected and used for DNA isolation and these samples kept as standards. All the leaf samples were cleaned and stored in -80^{0}C deep freezer until DNA isolation.

Table 1. Details of RAPD primers used.

Primer Name	Sequence (5'-3')	Tm	Previous reports
OPA 05	AGGGGTCTTG	32.0^{0}C	Sex detection in *G. indica* (Sawardekar *et al*., 2011)
OPAS 12	GGGTGTGTAG	32.0^{0}C	Sex determination in *Carica papaya* (Deputy *et al*., 2002)
OPD 11	AGCGCCATTG	32.0^{0}C	Genetic diversity studies in *G. indica* (Sahasrabudhe *et al*., 2010)
OPD 13	GGGGTGACGA	34.0^{0}C	Genetic diversity studies in *G. indica* (Sahasrabudhe *et al*., 2010)
OPT 01	GGGCCACTCA	34.0^{0}C	Sex determination in *Carica papaya* (Deputy *et al*., 2002)
OPW 08	GACTGCCTCT	32.0^{0}C	Sex determination in *G. indica* (Thatte and Deodhar, 2012)
OPW 11	CTGATGCGTG	32.0^{0}C	Sex determination in *Carica papaya* (Deputy *et al*., 2002)
STD	ATTTGATCGC	21.0^{0}C	Random primer

Table 2. Details of ISSR primers used.

Primer Name	Sequence (5'-3')	Tm	Previous reports
UBC 807	AGAGAGAGAGAGAGAGT	50.0^{0}C	Selection of male and female plants in *Simmondsia chinensis* (Sharma *et al*., 2008)
UBC 881	GGGTGGGGTGGGGTG	57.0^{0}C	Sex determination in *G. indica* (Thatte and Deodhar, 2012)

Isolation and purification of genomic DNA: The genomic DNA from the respective plant leaf samples were extracted using CTAB method according to Sahasrabudhe *et al*. (2010) and Baskaran (2012) with some modifications. The quality of the plant DNA samples were further checked by running on 0.8 % (w/v) agarose gel with 1X TBE buffer and stained with ethidium bromide. The gel images were visualized using a gel documentation system (Bio-Rad EZ

System, USA). Similarly the purity of the isolated DNA samples were checked using UV spectrophotometer by taking absorbance at 260 and 280 nm. Pure DNA samples were screening using various primers.

PCR amplification: PCR amplification was carried out with primers obtained from Pharmaids & Equipments, Bicholim, Goa. List of RAPD and ISSR primers used during this study is provided in Table 1 & 2. Amplification was performed in a 25 µl reaction volume that contained 13 µl of MiliQ water, 2.5 µl *Taq* buffer, 0.5 µl *Taq* DNA polymerase, 2 µl of 10 mM dNTPs mixture, 2 µl 25 mM $MgCl_2$, 3 µl of the respective primer and 2 µl of the respective DNA plant sample. The amplification was carried out in a PCR thermal cycler (Eppendorf, Hamburg, Germany). For RAPD analysis the first cycle consisted of initial denaturation of template DNA at 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, primer annealing step and extension of newly synthesized DNA strand at 72°C for 2 minutes. This was followed by a final extension step of 10 minutes at 72°C (Baskaran, 2012). For ISSR analysis the first cycle consisted of initial denaturation of template DNA at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, primer annealing step and extension of newly synthesized DNA strand at 72°C for 2 minutes and followed by a final extension step of 10 minutes at 72°C (Baskaran, 2012). The amplified products were run in electrophoresis unit by using 1.8% (w/v) agarose gel containing ethidium bromide in 1X TBE buffer for about 2.5 h. The size of the amplified products was determined using 100 bp DNA ladder. The DNA fragments were visualized under UV light using gel documentation system (Bio-Rad EZ System, USA).

Results and Discussion

G. indica is perennial woody tree that grows up to the height of 20-30 m, conical or pyramidal in shape with spreading or drooping branches (Plate 1A). Male flowers are borne in clusters that may be terminal or axillary. The flowers are generally consists of a long pedicel, with numerous fertile stamens; tetrasporangiate with four anther locules; petals are yellow to red in colour, anthers are borne in a circular fashion around the main axis of the flower (Plate 1B, E). Numerous pollen grains are present in each of the anther locules. The male flower also contains many laticifers. Female flowers may be sessile or borne on short pedicels. They are generally bundled in two or three, of which only one remains; hence appear solitary. They consist of staminodes in phalanges that are present on four sides around the pistil, sepals 4, smaller than inner petals, green; petals 4, yellow to pinkish, ovary superior, 4-8 locular, axile placentation; About eight ovules are seen in each flower. The ovules are of orthotropous type, micropyle, chalaza and funiculus found as straight line. Laticifers are seen surrounding the ovules. Fruits a berry, smooth and many

seeded (5-6) (Plate 1C, D). Thatte and Deodhar (2012) and Devi *et al*. (2013) reported three flower types viz. staminate (do not yield fruits), pistillate (high fruit yielding) and bisexual plants produce fruits very rarely and are poor in yield (low fruit yielding). Bisexual flowers showed a decrease in masculine characters but development of more feminine characters (Haldankar *et al*., 2012). Hence, only female trees are more beneficial in terms of fruit yield.

Plate 1. A. *Garcinia indica* female plant; B. Longitudinal section (L.S) of male flowers; C. Opened female flower showing staminodes; D. L.S. of female flower; E. Male flowers (lower row) and female flowers (upper row); F. Seedlings from natural population; G. In-vitro mass propagated plants of *G. indica* grown in polyhouse used for isolation of genomic DNA.

Eight RAPD and two ISSR primers were used for analysis of distinguishing male and female plants of *G. indica*. The banding patterns in the gel were analyzed for presence or absence of specific bands in male and female plants. Among the eight RAPD primers screened, OPA 05 primer, OPAS 12, OPW 11, and OPT 01 primer have produced clear visible banding pattern and two other

primers viz. STD and OPW 08 produced faint band. The primers OPD 11 and OPD 13 did not show any amplified bands. Therefore, four primers OPA 05, OPAS 12, OPW 11 and OPT 01 were used for the analysis of DNA isolated from standard male and female plants, tissue cultured (*in vitro* derived plants) and natural seedling populations of *G. indica.*

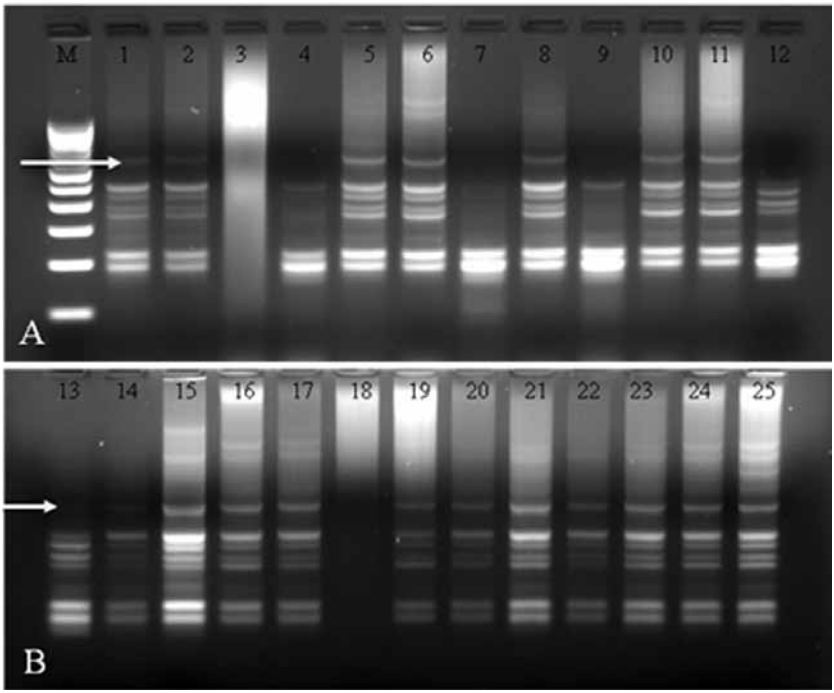

Plate 2. A & B. RAPD primer (primer OPT 01) amplified banding patterns showing the presence of specific band in male and absence of specific band in female plants of *Garcinia indica*. Lane M is 100 bp ladder; Lane 1, 2 male plants, Lane 3, 4 female plants; Lane 5-19 tissue cultured samples; Lane 20-25 seedling from natural populations.

Sawardekar *et al*. (2011) used RAPD primer OPA 05 (5'-AGGGGTCTTG-3'; Tm: 32°C) to distinguish male specific bands in *G. indica* and this particular band is absent in female plants. However, during this study no distinct band for male and absence of band in female plants were not observed. A total of 21 plant samples were screened out of which four were standard male plants, three standard female plants, eight tissue cultured plants and six natural seedlings. The primer yielded a total of 141 bands.

Total of 25 samples were screened with RAPD primer OPAS 12 (5'-GGGTGTGTAG-3'; Tm: 32°C), out of which two were standard male

plants, two standard female plants, 16 tissue cultured plants and five natural seedlings. The amplification yielded a total of 222 bands. However, no sex determining bands could be identified with this primer. RAPD primer OPW 11 (5'- CTGATGCGTG-3'; Tm: 32°C) was used for the evaluation of 25 plant samples, two standard male plants, two standard female plants, 15 tissue cultured plants and six seedlings. A total of 114 amplified bands were obtained that included both faint and prominent bands. This primer also could not detect any specific bands among the screened plants.

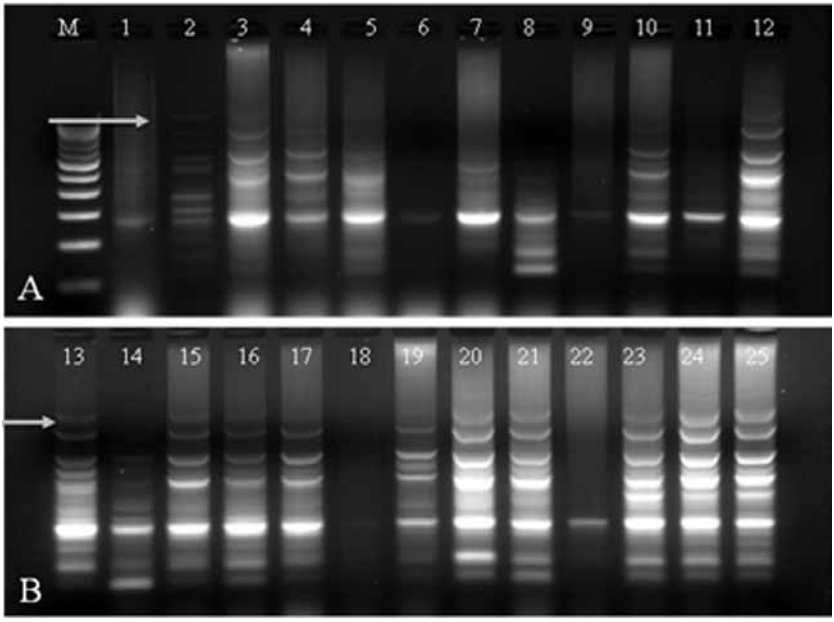

Plate 3. A & B. ISSR primer (primer UBC 881) amplified banding patterns showing the presence of specific band in male and absence of specific band in female plants of Garcinia indica. Lane M is 100 bp ladder; Lane 1-4 male plants, Lane 5-8 female plants; Lane 9-18 tissue cultured samples; Lane 19-25 seedling from natural populations.

Detection of sex in male and female plants of *G. indica* was achieved by using the primer OPT 01 (5'- GGGCCACTCA-3'; Tm: 34°C). During this screening, two standard male plants, two standard female plants, 15 tissue cultured sample plants and six seedling samples were examined. A band of approximate size of 790 bp was detected in standard male plants and the presence of same band was observed in some of the tissue cultured plants and seedling populations indicating that those plants are male plants (Plate 2). This same band was absent in the standard female plants and some of the other pants

screened indicating them as female plants. Hence, this particular RAPD primer can detect sex in *G. indica* and it could be used for large scale screening to distinguish male, female plants at seedling stage. This primer generated a total of about 222 amplified bands (Plate 2).

Detection of sex in male and female plants of *G. indica* was achieved by using the primer OPT 01 (5'- GGGCCACTCA-3'; Tm: 34^{o}C). During this screening, two standard male plants, two standard female plants, 15 tissue cultured sample plants and six seedling samples were examined. A band of approximate size of 790 bp was detected in standard male plants and the presence of same band was observed in some of the tissue cultured plants and seedling populations indicating that those plants are male plants (Plate 2). This same band was absent in the standard female plants and some of the other pants screened indicating them as female plants. Hence, this particular RAPD primer can detect sex in *G. indica* and it could be used for large scale screening to distinguish male, female plants at seedling stage. This primer generated a total of about 222 amplified bands (Plate 2).

ISSR primers UBC 807 and UBC 881 were used for screening the DNA samples of four standard male, four standard female, ten tissue cultured plants and seven natural seedling populations of G. indica. ISSR primer UBC 807 yielded about 217 bands, however no distinction of male and female bands could be observed. However, ISSR primer UBC 881 distinguished male and female plant by producing a specific band in male plants and that particular band was absent in female plants (Plate 3). A total of 193 bands were obtained. This primer was also used earlier for determination of sex in G. indica (Thatte and Deodhar, 2012).

Among eight RAPD primers used during this investigation, only OPT 01 (5'-GGGCCACTCA-3) produced a distinct band of ~ 790 bp in the standard male plants, some tissue cultured plants and some seedling samples indicating them as males. This same band was absent in standard female plants and remaining plants examined indicating them as female. Among the two ISSR primers, UBC 881 primer produced specific band in standard male and the same band was absent in female plants of *G. indica*. It is concluded that the RAPD primer OPT 01 and ISSR primer UBC 881 can amplify and produce specific band in male plants and the same band was found to be absent in female plants. Hence, these two primers are effective in distinguishing male and female plants of *G. indica*. As Kokum (*G. indica*) is predominantly cultivation in Konkan regions which occupies an area of about 1000 hectares and present annual production of fruits of about 4500 metric tonnes (Haldankar *et al*., 2012; Patil *et al*., 2012). There is a continuous increase in demand as it is evident by the market trends and export scenario. Also, it is reported that 50% chance of occurrence of males in sexually propagated plants (Haldankar *et*

al., 2012). Hence, this present study of determination of sex in early seedlings stage is very important in order to establish desired ratio of male and female plants in field for large scale cultivation of Kokum.

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References

1. **Anerao J, Desai N, Patel B and Deodhar M.** 2013. Karyomorphology of *Garcinia indica* (Thomas-Dupetite) Choisy. *J Trop Agri* 51 (1-2): 140-143.
2. **Baskaran M.** 2012. *Micro-propagation, biochemical and histochemical studies of Garcinia indica Choisy (Kokum)*. Ph.D., thesis submitted to Goa University, Goa, India.
3. **Baskaran M and Krishnan S. 2011. High frequency plant regeneration from the mature seeds of *Garcinia indica***. *Biol Plantarum* 55 (3): 554-558.
4. **Baratakke RC, Patil CG, Poornima B and Sankannavar SH.** 2013. Molecular tool for sex identification (female) in *Momordica dioica* Roxb. with reference to medicinal values. *Int J Res Ayurveda Pharm* 4 (4): 487-490.
5. **Braganza M, Shirodkar A, Bhat DJ and Krishnan S.** 2012. *Resource Book on Kokum*, Western Ghats Kokum Foundation, Panaji, Goa. India. 133 pp.
6. **Daniel JN and Dudhade PA.** 2006.Analysis of economic characteristics of value chains of three underutilized fruits of India. Research Report No. 3. International Centre for Underutilized Crops, Colombo, Sri Lanka. 22 pp.
7. **Deputy JC, Ming R, Ma H, Liu Z, Fitch MMM, Wang M, Manshardt R and Stiles JI.** 2002. Molecular markers for sex determination in papaya (*Carica papaya* L.). *Theor Appl Genet* 106: 107-111.
8. **George J, Karun A, Manimekalai R, Rajesh MK and Remya P.** 2007. Identification of RAPD markers linked to sex determination in Palmyrah (*Borassus flabellifer* L.). *Curr Sci* 93(8): 1075-1077.
9. **Haldankar PM, Haldavnekar PC and Kulkarni MM.** 2011. Present scenario of Kokum cultivation in Maharashtra. 3rd National Seminar on Kokum, "Post-harvest Technologies and Value-added Products Marketing". 6-7 May 2011. Goa University, Goa, pp. 8-11 (Abs).
10. **Haldankar PM, Pawar CD, Kshirsagar PJ and Kulkarni MM.** 2012. Present status and future thrust areas in production technology of kokum. In: *Resource Book on Kokum*. Braganza M, Shirodkar A, Bhat DJ and Krishnan S (Eds.), 43-53. Western Ghats Kokum Foundation, Panaji, Goa, India.
11. **Kafkas S, Cetiner MS and Perl-Treves R.** 2001. RAPD markers linked to sex in the genus *Pistacia*. In: *XI GREMPA Seminar on Pistachios and*

Almonds. Ak BE (Ed.). Zaragoza: CIHEAM, pp 251 -255 (Cahiers Options Méditerranéennes; no. 56).

12. **Kaur R, Chattopadhyay SK, Tandon S, Sharma S.** 2012. Large scale extraction of the fruits of *Garcinia indica* for the isolation of new and known polyisoprenylated benzophenone derivatives. *Industrial Crops and Products* 37: 420-426.
13. **Kumar S, Singh BD and Sinha DP.** 2012. RAPD markers for identification of sex in pointed gourd (*Trichosanthes dioica* Roxb.). *Ind J Biotech* 11: 251-256.
14. **Lemos EGM, Silva CLSP and Actis H.** 2002. Identification of sex in *Carica papaya* L. using RAPD markers. *Euphytica* 127: 179-184.
15. **Ling J, Rui-Lin Y, Mao-Xue L and Chen S.** 2003. Identification of a sex-associated RAPD marker in *Ginkgo biloba*. *Acta Botanica Sinica* 45 (6): 742-747.
16. **Panda KK, Sahoo B, Das AB and Panda BB.** 2010. Use of RAPD markers to detect sex differences in *Pandanus tectorius* Parkinson, an important bioresource plant in Orissa, India. *Intl J Biodiver Sci Ecosys Services & Management* 6(1-2): 28-34.
17. **Patil CG, Baratakke RC and Sandigwad AM.** 2012. Development of RAPD based SCAR marker for sex identification in *Momordica dioica* Roxb. *Israel J Plant Sci* 60: 457-465.
18. **Patil BP, Gawankar MS, Sawant DS, Pujari KH and Jambhale ND**. 2012. Cultivation of "Konkan Amruta" and Search for Elite Varieties. In: *Resource Book on Kokum.* Braganza M, Shirodkar A, Bhat DJ and Krishnan S. (Eds.), Western Ghats Kokum Foundation, Panaji, Goa, India.
19. **Reddy SR, Krishna RB and Reddy KJ.** 2012. Sex determination of Papaya (*Carica papaya*) at seedling stage through RAPD markers. *Research in Biotech* 3(1): 21-28.
20. **Priya Devi S, Thangam M, Ramchandrudu K, Ashok Kumar J and Singh NP.** 2013. Genetic diversity of Kokum (*Garcinia indica*) in Goa-Tree and fruit characters. Technical Bulletin No. 33, ICAR, Goa. 40 pp.
21. **Sahasrabudhe A and Deodhar M.** 2010. Standardization of DNA extraction and optimization of RAPD-PCR conditions in *Garcinia indica*. *Int J Bot* 6 (3): 293-298.
22. **Samantaray S, Phurailatpam A, Bishoyi AK, Geetha KA and Maiti S.** 2011. Identification of sex-specific DNA markers in betel vine (*Piper betle* L.). *Genet Resour Crop Evol* DOI.10.1007/s10722-011-9707-4.
23. **Sawardekar SV, Gokhale NB, Mote MS, Joshi SN and Sawant SS.** 2011. Sex detection of kokum (*Garcinia indica* Choisy) by RAPD markers. *Indian J Genet*, 71(1): 82-83.
24. **Sharma K, Agarwal V, Gupta S, Kumar R and Prasad M**. 2008. ISSR marker assisted selection of male and female plants in a promising dioecious crop: Jojoba (*Simmondsia chinensis*). *Plant Biotech Rep* 2: 239-243.

25. **Sharma PHB, Handique PJ and Devi HS.** 2013. A historical and taxonomic overview of *Garcinia* L. and its reproductive ecology. *Folia Malaysiana* 14 (1): 63-76.
26. **Sweeney PW.** 2008. Phylogeny and floral diversity in the genus *Garcinia* (Clusiaceae) and relatives. *Int J Plant Sci* 169(9): 1288-1303.
27. **Thatte KS and Deodhar MA.** 2012. Study of flowering behaviour and sex determination in *Garcinia indica* (Thomas- Du Pettite) Choisy by means of molecular markers. *Biotechnology,* 1-6. DOI: 10.3923/biotech.
28. **Thatte KS, Khandekar RG and Deodhar MA.** 2012. Assessment of diversity in *Garcinia indica* (Dupetit-Thouars.) Choisy using morphological and molecular markers. *J Trop Agri* 50 (1-2): 30-36.
29. **Urasaki N, Tokumoto M, Tarora K, Kayano Ban T, Tanaka H, Oku H and Terauchi ICR.** 2002. A male and hermaphrodite specific RAPD marker for papaya (*Carica papaya* L.). *Theor Appl Genet* 104: 281-285.