RESEARCH NOTE

Development of male-specific SCAR marker in *Garcinia morella* (Gaertn.) Desr.

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Introduction

Garcinia morella (Gaertn.) Desr. (family Clusiaceae) is popularly known as 'Indian gamboge' is a fruit yielding tree of tropical rain forests of Western Ghats of India (Anonymous 1956). It also occurs in Sri Lanka and Indo-China Himalayan regions. It is a multipurpose tree grown as a plantation crop along with *Garcinia indica* (Kokum) and *Garcinia cambogia* (Malabar tamarind). The fruit rinds are used as a condiment and for garnish. Bioactive compounds such as moreollin (Subba Rao *et al.* 1978), gambogic acid (Tang *et al.* 2011) have been isolated from the fruits and the bark, respectively, and evaluated for their antibiotic and anticancer properties.

The trees are dioecious and the distinction between male and female trees can be made only at the flowering stage, after 10–12 years. It is cultivated as a plantation crop and the sex determination in this plant at an early juvenile stage will be useful for planning the male and female tree ratio in the orchards and it also enables the tree improvement programme. Recently, molecular tools were employed in dioecious taxa for early identification of sex and understanding the developmental and the evolutionary pathways of sexual dimorphism. Specific molecular markers can be deduced from unique, single-copy segments of the genome and can be considered codominant and can be used in sex determination. Sequence characterized markers (SCAR) which are based on randomly amplified polymorphic DNA (RAPD) analysis are locus-specific, more reliable and more reproducible for molecular identification (Paran and Michelmore 1993). SCAR marker linked to sex-specific genes have beensuccessfully used in sex identification of many dioecious plants including *Carica papaya* (Bedoya and Nunez 2007), *Phoenix dactylifera* (Dhawan *et al.* 2013). In this study, we have developed male specific SCAR markers from fragments amplified by RAPD primers for sex identification in *Garcinia morella*.

Materials and methods

Garcinia morella (Gaertn.) Desr. collections were made from its natural distribution range in the Western Ghats region of India (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). The individual trees were identified as males or females during the flowering season and young leaves were collected from 20 individual trees, consisting of 10 males and 10 females. Genomic DNA was extracted using cetyltrimethyl ammonium bromide (CTAB) method. One hundred and fifty decamer primers (series A, B, C, D, E, H, J, K, L; Bioserve Biotechnologies, Hyderabad, India) were screened for the sex-specific fragments on the two DNA bulks of males and females made by pooling equal volumes of DNA from each individual. The RAPD-PCR analysis was performed in a 25 μL reaction mixture containing 50 ng genomic DNA, 10 mM Tris HCl, 25 mM $MgCl_2$, 1 mM primer, 1 mM dNTP's and 0.3 U DNA *Taq* polymerase. The amplification conditions were as follows: initial denaturation at 94°C for 5 min, subsequent 40 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 2 min and a final extension at 72°C for 5 min. The fragments were resolved using 1.5% agarose gel and visualized using gel documentation system. One polymorphic male sex-specific band amplified by RAPD primer OPN-15 was excised and purified with Axy Prep DNA gel extraction spin columns (Axygen, California, USA) according to manufacturer's instructions. The isolated DNA fragment was cloned into the pTZ57R/T vector using InsTA clone PCR cloning kit (Thermo Fisher,

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Waltham, USA) according to manufacturer's instructions and transformed into ultra-competent *Escherichia coli* strain DH5α prepared by Inoue method. The plasmid was isolated from the transformed cells using plasmid isolation kit (Bioserve Biotechnologies, India) using spin column method according to the manufacturer's instructions and was sequenced by M13 universal primers in both forward and reverse directions using ABI3700 DNA analyzer (Applied Biosystems, Foster City, USA). Based on the sequence of the unique male-specific RAPD fragment, a pair of SCAR oligonucleotide primers, which could amplify 634 bp of genomic DNA of *G. morella* was designed by visual inspection and was commercially synthesized (Bioserve Biotechnologies, India). The SCAR primer pair was used for PCR amplification of all 20 collected male and female samples of *G. morella*. The 25 μL reaction mixture contained 50 ng of genomic DNA, 5 pmol of each primer, 25 mM $MgCl_2$, 15 mM Tris-HCl buffer with $MgCl_2$, 5 mM dNTP's (Fermentas, Waltham, USA) and 0.3 U *Taq* polymerase (Bangalore Genei, Bangalore, India). The thermo cycler conditions were 94°C for 4 min, followed by 40 cycles at 94°C for 30 s, 66°C for 30 s, 72°C for 30 s and final extension of 72°C for 5 min. PCR amplifications were repeated at least twice. Further, we verified reproducibility and reliability of SCAR primers on additional 24 known male and female plants.

Results and discussion

Of the 150 decamer primers screened, only one primer OPN-15 (5′-CAGCGACTGT-3′) differentiated the male and female samples of *G. morella*. Validation of this marker was done with individual male and female samples, and it was present exclusively in male samples but not in female samples (figure 1). This male specific fragment amplified by primer OPN-15 was excised, purified, cloned and sequenced. Homology searches using BLAST algorithm of the 634 bp sequence showed no similarity with any of the known sequences of NCBI database. Based on the sequence of the unique male specific RAPD fragment, a pair of SCAR oligonucleotide primers specifically amplifying the 634 bp fragment was designed (table 2 in electronic supplementary material, figure 2). Using this primer pair, PCR was performed with individual male and female samples. A single, distinct and brightly resolved band of 634 bp was obtained in all male samples but was absent in all female samples (figure 3). Hence, the RAPD primer $OPN15_{634}$ was successfully converted into SCAR primer MOR-634. The sequence of the male specific fragment $OPN15_{634}$ (GenBank accession no. KJ809108) revealed a 634 bp fragment (figure 2) with 46.8% G + C and 53.2% A + T content. The reproducibility and reliability of the designed SCAR primers was verified by analysing 24 samples with known sexes (figure 4). The designed SCAR primers could identify the male and female samples correctly.

The application of molecular markers such as RAPD, AFLP and ISSR (Danilova and Karlov 2006; Mwase *et al.* 2007; Esfandiyari *et al.* 2011) for sex determination in dioecious species which have a long juvenile and vegetative stage have been greatly used in recent years. Even though the techniques like RAPD, AFLP and ISSR are rapid, inexpensive tools showing a high amount of polymorphism, they are not preferred by researchers as their reproducibility, sensitivity to reaction conditions and their reliability are the causes of their concern. In contrast, a SCAR marker is much more advantageous as it is insensitive to the reaction conditions; it can identify a single-specific locus making it more reproducible (Jiang and Sink 1997) and also has the capability of becoming a codominant marker (Paran and Michelmore 1993). This primer is 19–25 bp long with a high annealing temperature of 60–67°C making it more specific and stable. In this study, a new SCAR marker was developed from RAPD marker

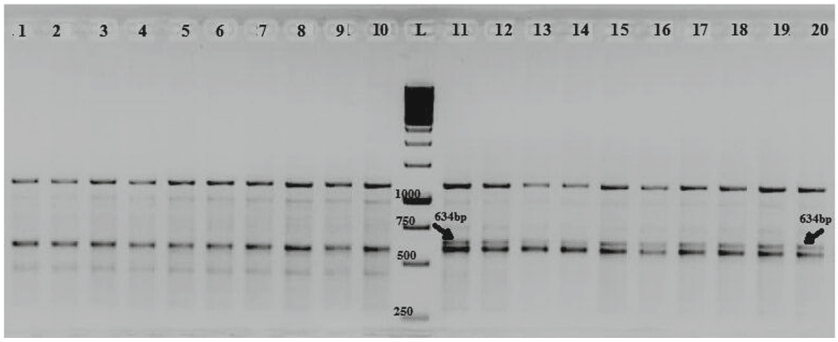

Figure 1. Amplification profiles of RAPD primer, OPN-15 in 10 male and 10 female individuals of *G. morella*. Lane marked with 'L' contains 1 kb ladder. From left to right: 1, GMF1; 2, GMF2; 3, GMF3; 4, GMF4; 5, GMF5; 6, GMF6; 7, GMF7; 8, GMF8; 9, GMF9; 10, GMF10; 11, GMM1; 12, GMM2; 13, GMM3; 14, GMM4; 15, GMM5; 16, GMM6; 17, GMM7; 18, GMM8; 19, GMM9; 20, GMM10. Arrowheads represent male specific bands.

Figure 2. Nucleotide sequence (634 bp) of the cloned male-specific RAPD fragment. Square boxed ends show the sequence of OPN-15 decamer primer used for initial amplification of the fragment. Arrowhead with nucleotide sequence defines the forward and reverse SCAR primers.

$OPN15_{634}$ and was used for sex differentiation in *G. morella*, which has a very long vegetative phase of 10–12 years. In this study, a single male-specific fragment was obtained, suggesting that the DNA segment involved in sex determination is not large but involves a single gene or a few tightly-linked genes (Jiang and Sink 1997). If heteromorphic sex chromosomes would be determining the sex of this species, a large number of sex-specific fragments would have appeared and prevented the detection of a single male or female marker.

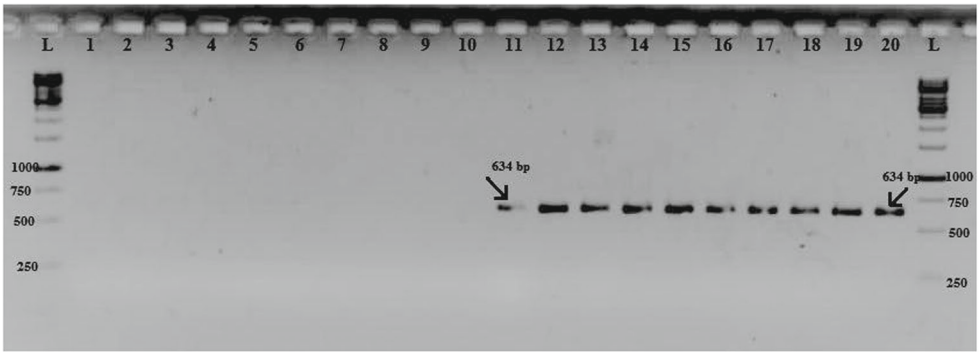

Figure 3. SCAR analysis carried out with MOR-634 SCAR primers showed amplification at 634 bp in male samples but not in female samples. Lane marked with 'L' contains 1 kb ladder. From left to right: 1, GMF1; 2, GMF2; 3, GMF3; 4, GMF4; 5, GMF5; 6, GMF6; 7, GMF7; 8, GMF8; 9, GMF9; 10, GMF10; 11, GMM1; 12, GMM2; 13, GMM3; 14, GMM4; 15, GMM5; 16, GMM6; 17, GMM7; 18, GMM8; 19, GMM9; 20, GMM10. Arrowheads represent male specific bands.

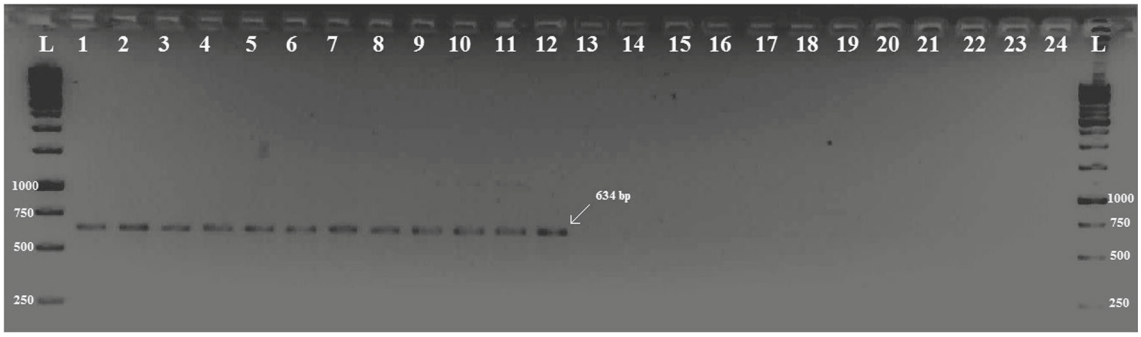

Figure 4. SCAR analysis carried out to check the reproducibility and reliability of the developed SCAR primers on sex known samples of *G. morella*. Lane marked with 'L' contains 1 kb ladder. From left to right: 1, GMM11; 2, GMM12; 3, GMM13; 4, GMM14; 5, GMM15; 6, GMM16; 7, GMM17; 8, GMM18; 9, GMM19; 10, GMM20; 11, GMM21; 12, GMM22; 13, GMF11; 14, GMF12; 15, GMF13; 16, GMF14; 17, GMF15; 18, GMF16; 19, GMF17; 20, GMF18; 21, GMF19; 22, GMF20; 23, GMF21; 24, GMF22. Arrowheads represent male-specific bands.

In conclusion, a new SCAR marker MOR-634 has been developed for the RAPD fragment $OPN15_{634}$ for identification of sex in *G. morella* at the juvenile stage. The primer pairs designed from the sequence of male specific fragment amplified only in male samples and thus can be used as an efficient, highly reliable and reproducible molecular marker for sex determination at the early juvenile stage. This can be adopted by plant breeders in various breeding programmes as a potential tool for sex determination in *G. morella* before planting in the fields.

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