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Short communication

# An *APETALA3 MADS*-box linked SCAR marker associated with male specific sex expression in *Coccinia grandis* (L). Voigt

Biplab Kumar Bhowmick[a], Satyabrata Nanda[b], Sanghamitra Nayak[b], Sumita Jha[a], Raj Kumar Joshi[b,*]

[a] *Centre of Advanced Study, Department of Botany, University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, India*
[b] *Centre of Biotechnology, Siksha O Anusandhan University, Kalinga Nagar, Ghatikia, Bhubaneswar 751003, India*

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ABSTRACT

*Coccinia grandis* L. (Family – Cucurbitaceae), a perennial dioecious herb with heteromorphic sex chromosomes has the quality of a model plant for analysis of sexual evolution in angiosperms. Yet, little is known about the physical side, genetic orientation and substitution behavior of key gender-defining factors in this important plant species. Screening of genomic DNA with RAPD primers was used for sex diagnosis and gender specificity of *C. grandis* in this study. Bulk analyses of pooled DNA from male and female genotypes resulted in the isolation of a putative male specific sex marker *CgMSM*. A sequence characterized amplified region (SCAR) marker *CgY1* designed from *CgMSM* amplified an 829 bp fragment in the male *C. grandis* but not in the female plants. Southern blotting confirmed it as a single copy locus in the male genome. Further, the *CgY1* marker accurately identified 11 male and 6 female genotypes from 17 individuals of *C. grandis* with unknown sex. Sequence homology revealed high similarity between *CgMSM* (*CgY1*) and the putative MADS-box domain of the *APETALA3* (*AP3*) *MADS-box* gene which is responsible for development of petals and stamens in flowering plants. Thus, the SCAR marker *CgY1* could be used as an efficient tool for early sex identification in *C. grandis* and forms the basis for characterization of genes associated with evolutionary pathways in sexual dimorphism.



## 1. Introduction

Since sex is the queen of problems in evolutionary biology, understanding the factors behind sex expression has immense importance both in basic and applied research. Plant kingdom majorly follows hermaphroditism where male and female sex organs coexist in the same blossom. The sex determination that is most common in animals (heteromorphic XY or WZ sex chromosomes), evolved rarely in plants and is known in only 5–6 genera of angiosperms (Ming et al., 2007). Further, separate sexes (dioecy) evolved in around six percent of the 240,000 flowering plant species (Renner and Ricklefs, 1995), usually from perfect-flowered or monoecious ancestors (Vyskot and Hobza, 2004). Different sexual behavior in plants ascertains outcrosses to promote genetic variation and better adaptability of the species. Although it is not clear that all organisms with separate sexes have a distinctive sexual morphology, theoretical evidences suggest selective aggregation of sex-linked genes under suppressed recombination on one of the gonosomes. This resulted in physical distinction of the male and female sex chromosomes (Charlesworth, 2002). In other words, sexual evolution started with the growth of a chromosome with sex-determining genes within a recombination suppressed region.

The family Cucurbitaceae is an ideal case where different sexual patterns occur depending on the organization of gamete producing structures. Among several cucurbits, the genera *Bryonia* and *Ecballium* display male heterogamety and have homomorphic sex chromosomes (Ming et al., 2011; Volz and Renner, 2008) while *Coccinia* and *Trichosanthes* possess heteromorphic sex chromosomes (Ming et al., 2011). *Coccinia grandis* (Ivy gourd) is the sole species of the genus *Coccinia* with invasive occurrence across sub-Saharan Africa, tropical Asia, Neotropics and India (Shaina and Beevy, 2011). It has wonderful medicinal properties apart from its dietary uses (Perry, 1980). A fairly small genome size, an active Y sex determination system and presence of physically distinct and large Y chromosome makes it a model plant for evolutionary analysis of key gender-determining genes (Bhowmick et al., 2012; Sousa et al., 2013). To gather insight into the possible bearing of such genes, it will be necessary to sequence a long sex-linked region to

* Corresponding author. Tel.: +91 9437684176.
*E-mail addresses:* rajkumar.joshi@yahoo.co.in, rajjoshi@soauniversity.ac.in (R.K. Joshi).

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study its substitution behavior and visualize the physical position. The foremost measure is to develop markers that could observe the demeanor of such factors in the sex chromosomes. However, no effort has been made for generating a molecular marker in this evolutionarily significant cucurbit in spite of having enigmatic cytogenetic features. Sex-linked marker in *C. grandis* would immediately open avenues for unveiling the mechanism of sexual behavior, physiology and sexual dimorphism in Cucurbitaceae.

Several molecular genetics tools such as RAPD, ISSR, SSR and AFLP have been applied to define the hereditary base of gender expression in different flora species (Sarmah and Sarma, 2011; Aleksandrov et al., 2011; Costa et al., 2011; Agrawal et al., 2011; Milewicz and Sawicki, 2013). Within Cucurbitaceae, markers linked to sex loci has been identified in dioecious species of *Bryonia* (Oyama et al., 2009), *Trichoxanthes* (Kumar et al., 2012; Nanda et al., 2013) and *Momordica* (Patil et al., 2012; Panigrahi and Mishra, 2012). The use of these molecular markers largely depends on their reproducibility, simplicity, specificity or randomness. Although developed in the early 1990s, RAPD markers still enjoys the highest popularity for analysis of sex determination as they are simple, cheaper and less time consuming. Screening with RAPD markers for sex identification has been carried out in many economically and evolutionarily important dioecious plants such as *Seline dioica* (Di Stilio et al., 1998), *Asparagus officinalis* (Gao et al., 2007), *Simmondsia chinensis* (Agrawal et al., 2007), *Phoenix dactylifera* (Younis et al., 2008), *Trichosanthes dioica* (Kumar et al., 2008), *Commiphora wightii* (Samantaray et al., 2010) etc. The amplified sex-linked RAPDs often results in sequenced characterized amplified regions (SCARs) to produce an exact and reproducible amplification of sex specific loci (Esfandiyari et al., 2011; Khadke et al., 2012). RAPD-SCAR markers have been successfully applied in sexual dimorphism studies to identify gender associated traits in many plants such as *Carica papaya* (Parasnis et al., 2000), *Salix viminalis* (Gunter et al., 2003), *Eucommia ulmoides* (Xu et al., 2004), *Piper longum* (Manoj et al., 2005), *Gingko biloba* (Liao et al., 2009), *Acuba japonica* (Maki, 2009), *Pistachia atlantica* (Esfandiyari et al., 2011) etc. In the present work, we describe the development of a reliable sex-linked SCAR marker associated with male specific expression in *C. grandis* and its subsequent employment in the distinction of male and female genotypes.

## 2. Materials and methods

### 2.1. Plant material and DNA isolation

30 male and 16 female plants of *C. grandis*, with well defined sex, were collected from isolated areas of Kolkata, North 24 Praganas, and Siliguri districts of West Bengal, India. Individual plants were raised and sustained in the experimental garden of Dept. of Botany, Calcutta University for sex marker study. Seventeen accessions of *C. grandis* with unknown sex type were also picked up randomly at a later date from the same collection site as well as from the neighboring state of Orissa for validation of sex specific marker (see supplemental material). Young tender leaves were used for DNA isolation using the standard CTAB procedure of Doyle and Doyle (1990). Quantification and purity of the DNA was determined with a UV–vis spectrophotometer (Thermo Scientific) and 0.8% agarose gel electrophoresis. DNA was diluted to a final concentration of 50 ng/μL for use in polymerase chain reaction (PCR).

### 2.2. Male-linked RAPD marker screening

Prior to RAPD amplification, the isolated DNA was subjected to bulk segregant analysis (Michelmore et al., 1991). Two separate DNA bulk samples were prepared one for each sex by pooling equal quantity of genomic DNA from male and female genotypes respectively. A selected set of 19 randomly amplified polymorphic DNA (RAPD) markers was used for PCR amplification of DNA samples to ascertain sex specific polymorphism. RAPD amplification was carried out in a final volume of 25 μL PCR master mix containing 50 ng of genomic DNA, 200 μM dNTPs, 1 μM of each primer, 1 unit of *Taq* DNA polymerase (Fermentas Inc, MD, USA) and 10× *Taq* Polymerase buffer A containing 16 mM $(NH)_2$ $SO_4$, 67 mM Tris–HCl and 25 mM $MgCl_2$. PCR programming included an initial denaturation of 1 min at 94 °C followed by 45 cycles of 94 °C for 1 min, annealing at 37 °C for 1 min and denaturation at 72 °C for 2 min. A final extension was performed at 72 °C for 7 min. Amplified products were separated on a 1.5% agarose gel, stained with 0.5 μg/mL ethidium bromide and visualized under a UV light source in a Gel Doc EZ Imager (BioRad, USA). Amplified bands specific to male individuals and their bulk were identified as putative male linked markers.

### 2.3. RAPD fragment isolation and screening

The identified male linked RAPD marker was isolated and sequenced. Four parallel amplification reactions were pooled and concentrated before separation in a 1% agarose mini gel (Bagalore Genei, India). The target fragment was excised and dissolved in 50 μL of 10 mM Tris solution at 70 °C for 30 min. A repeat PCR reaction was done utilizing the same grounds with the collected supernantant as the template DNA. The amplified product was separated on a 2.5% agarose gel. The desired male specific target fragment was eluted and purified using the Wizard SV gel and PCR cleanup system (Promega, USA). The cleaned PCR product was ligated into a pGEM-T easy vector using the pGEM-T easy vector system (Promega, USA) according to the manufacturer's instructions. Competent *Escherichia coli* JM109 strain cells transformed with recombinant DNA were selected using a Luria Broth (LB) agar blue/white selective medium supplemented with 80 μg/mL X-Gal, 0.5 mM IPTG and 100 μg/mL ampicillin. Colony PCR with universal M13F and M13R primers resulted in the selection of ten positive white colonies. Plasmid isolation was done using the Wizardplus miniprep DNA purification system (Promega, USA). Plasmid products were sequenced using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer) on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequence editing was performed using the GeneDoc software, to remove the primer and vector sequences. The National Centre for Biotechnology Information Center's BlastN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for GenBank database search. Sequence alignment, comparison and homology study was carried out using Clustal-Omega software (Sievers et al., 2011).

### 2.4. SCAR marker development and sex type testing

The sequences of RAPD male linked markers with appropriate length were aligned and a set of sequenced characterized amplified region (SCAR) primers were designed using the Primer Premier Software Ver 5.0 (Premier Biosoft, USA). Sex specificity of the SCAR primers was tested with genomic DNA of five male and five female plants *C. grandis* and further applied to identify the sex type of seventeen unknown accessions. A touchdown PCR amplification was performed in a total volume of 25 μL containing 2 μL of 1× PCR Buffer, 1.5 μL of 15 mM $MgCl_2$, 2 μL of dNTPs (200uM), 1 μL each of the forward and reverse SCAR primers (10 μmol/L), 1 μL of *Taq* DNA Polymerase (1 U/μL) and 25 ng of template DNA. The touchdown amplification program was optimized as follows: 94 °C for 5 min, 20 cycles of denaturation at 94 °C for 30 s, annealing range from 65 to 55 °C (with 0.5 °C reductions per cycle) for 45 s, polymerization at 72 °C for 2 min followed by next 15 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, polymerization at 72 °C