Sex detection of *Carica papaya* and *Cycas circinalis* in pre-flowering stage by ISSR and RAPD

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The sex of *Carica papaya*, an angiosperm and *Cycas circinalis*, a gymnosperm was studied using ISSR and RAPD techniques in pre-flowering stage. One female-specific band generated from ISSR profile using primer $(GACA)_4$ was detected in papaya, which seems to have importance from agricultural point of view. Sequencing of a male-specific RAPD band (PCR with primer OPB 01) in *C. circinalis* revealed homology with putative retro elements of diverse plants, probably indicating its use in the detection of male *C. circinalis* in future.

Keywords: *Carica papaya*, *Cycas circinalis*, pre-flowering stage, sex detection.

SINCE sex is the queen of problems in evolutionary biology[1], understanding the molecular factor(s) behind sex expression has immense importance both in basic and applied research. The evolution of sex in plants, in particular, has been hypothesized variously as the plants display a great variety of sexual phenotypes[2]. In general, there are three major sex strategies in angiosperms, viz. hermaphrodite, monoecious and dioecious. The evolution of dioecy directly from a hermaphrodite species is considered unlikely since the occurrence and establishment of two independent mutations, one for male and the other for female sterility, must occur simultaneously and the mutant genes (or multiple loci) must be tightly linked so that the generation of hermaphrodites does not occur by recombination[3]. The sexuality of gymnosperms is different from that of angiosperms, as the salient feature that distinguishes angiosperms from gymnosperms is the presence of carpels completely enclosing the ovules. Another typical feature that distinguishes flowers from the reproductive cones of gymnosperms, however, is the fact that male (stamens) and female (carpels) reproductive organs are usually united in flowers (or secondarily separated, as in the unisexual flowers of monoecious and dioecious angiosperms), while they are primarily separated in different structures in gymnosperms.

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For detecting the sex at pre-flowering stage, two plants of commercial importance, viz. *Carica papaya* and *Cycas circinalis* were selected. *C. papaya*, a polygamous member of family Caricaceae, shows a unique assemblage of all three possible sex forms, viz. hermaphrodite, male and female. Determination of sex in papaya is of utmost importance from the commercial agricultural point of view, since the sexuality cannot be distinguished prior to floral initiation. In an out-breeding system like papaya, 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 fruit[4]. In the case of gymnosperms, the study material was *C. circinalis*, family Cycadaceae, which is dioecious. Sexuality in cycads is apparently controlled by sex chromosomes, unlike most angiosperms (http://www.plantoftheweek.org/week199.shtml), but occasional sex reversal either due to hormonal influence and/or physical/mechanical damage/wounding has also been documented (http://www.plantapalm.com/vce/biology/sexchange.htm). Specific molecular information about sex detection in *Cycas*, however, is not extensively available in the literature though few molecular markers for sex determination in papaya are available[5].

Two hundred saplings of a local variety of *C. papaya* L. of unidentified sexuality were planted in the Experimental Farm of Bose Institute, Kolkata to raise a population. The plants were studied in detail during their transition from the vegetative to the reproductive stage, and the flowering time as well as ratio of male, female and hermaphrodite plants were recorded. Male and female cone-bearing plants (three each) of *C. circinalis* L. were procured from local nursery and used as study material.

For RAPD and ISSR analysis, samples from newly-emerging leaves were used. DNA was extracted using the CTAB method[6]. DNA concentration in the samples was adjusted to 25 mg dm^{-3} for PCR reaction in each sample. RAPD analysis was performed according to the method of Williams *et al.*[7] using ten oligonucleotide (decamer) primers, OPA 01–OPA 05 and OPB 01–OPB 05 (Operon Tech., Alameda, USA). Amplifications were carried out in a thermal cycler (Perkin Elmer System – 2400, Norwalk, USA) with an initial denaturation of 120 s at 94°C and the temperature profile of each cycle was as follows: 60 s denaturation at 94°C, 60 s annealing at 35°C and 120 s for extension at 72°C. The reaction continued for 45 cycles followed by 300 s hold at 72°C to ensure that the primer extension was completed. The PCR reaction mixture of 0.025 cm^3 consisted of 1X buffer; 0.2 mM dATP, dCTP, dGTP, dTTP; 2 mM $MgCl_2$; 0.2 μM of primer; 100 ng of template DNA and 1 unit of Taq DNA polymerase (Roche).

Three non-anchored oligonucleotide ISSR primers: $(CAG)_5$, $(CAA)_5$ and $(GACA)_4$ (synthesized by Isogen), were used for amplification[8]. To optimize the reaction conditions, several PCR parameters were tested, including DNA concentration (0.5–150 ng/reaction, 14 values), primer

concentration (10–500 pmol/reaction, 9 values), $MgCl_2$ concentration (0–10 mM, 11 values), dNTP concentration (20–700 mM each, 8 values), Taq DNA polymerase (0.5–2 units/reaction, 5 values) and number of cycles (15–40, 11 values). Reactions without DNA were used as negative controls. The optimum annealing temperature was determined for each ISSR primer from a minimum of five temperatures. Amplifications were carried out in two thermal cyclers (MJ Research and Perkin Elmer) with an initial denaturation of 90 s at 94°C and the temperature profile of each cycle was as follows: 60 s denaturation at 94°C, 60 s annealing at specific temperature (as standardized for each primer) and 240 s for extension at 72°C. The reaction continued for 27 cycles followed by 420 s hold at 72°C to ensure that the primer extension reaction was completed. The best patterns were amplified in the presence of 12 ng genomic DNA, 100 pmol of primer, 2.5 mM of $MgCl_2$, 200 µM of each of the four dNTPs, 1.25 units of Taq polymerase, 1X enzyme buffer and 27 PCR cycles. Among the PCR parameters, annealing temperature was found to be the most crucial and stringent: 60°C for $(CAG)_5$, and 50°C for both $(CAA)_5$ and $(GACA)_4$. Amplified products were electrophoresed in 1.8% agarose gel with DNA molecular weight marker X (0.07–12.2 kbp, Roche) as size markers for RAPD profiling. ISSR profiling was resolved in both 1.8% agarose gel and 6.0% polyacrylamide gel.

RAPD-derived unique male and female-specific bands of *C. circinalis* were eluted and cloned by conventional TA cloning method in pGEM-T Easy vector (Promega). Sequencing of the cloned DNA fragments was done by Sanger's dideoxy method using both forward and reverse M13Φ primers (Amersham kit). Sequence homology was studied by BLAST searching[9].

Among the two hundred papaya saplings in the experimental plot, one hundred and fifty-one were reared till maturity and the relative flowering time of the three forms was noted. The hermaphrodite plants flowered earliest (mean flowering days 59) followed by male (mean flowering days 71.5) and female (mean flowering days 80). Though distinct phenotypically with three sex morphs, no polymorphism was detected between profiles of the randomly selected male, female and hermaphrodite plants of papaya, generated from RAPD analysis with ten random primers (result not shown). RAPD, though initially widely adopted for efficient fingerprinting technique, gradually paved the way for more advanced molecular techniques[10]. However, development of sex-associated SCAR marker originating from RAPD is also available[5,11].

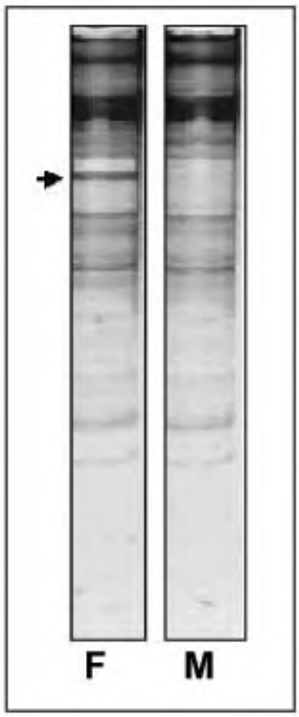

Figure 1. ISSR profiles [primer $(GACA)_4$] of male (M) and female (F) plants of *Carica papaya* in polyacrylamide gel.

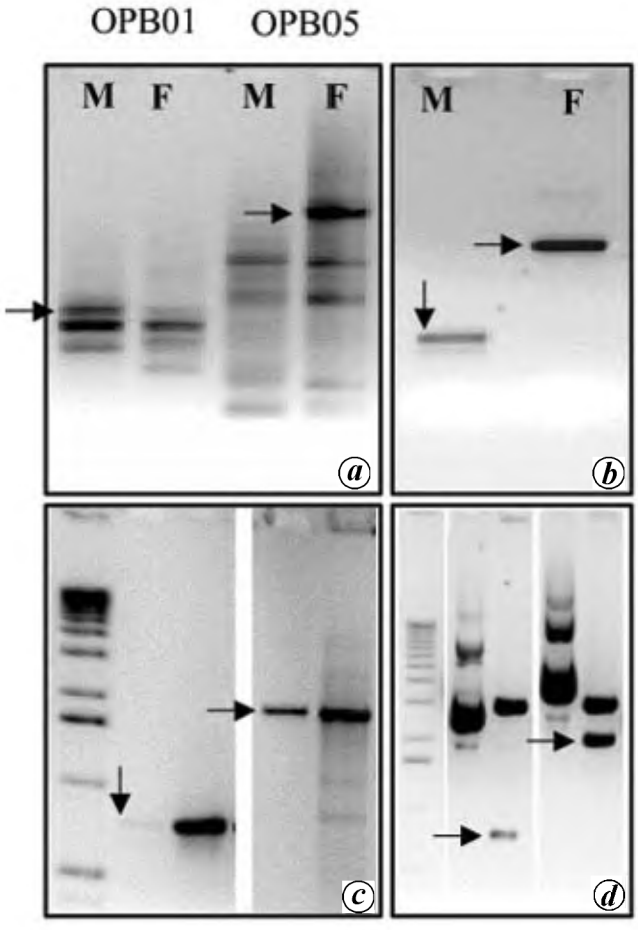

Figure 2. Cloning of RAPD derived male (M)- and female (F)-specific bands of *Cycas circinalis*. ***a***, RAPD profiles. ***b***, Purified eluted bands. ***c***, Purified bands with representative colony PCR products. ***d***, Purified plasmids from clones and their respective restriction enzyme (*Eco*R1) digested fragments showing inserts (all bands of interest are marked with arrows).

Adoption of ISSR fingerprinting for determination of sex using three primers, however, resulted in one female-specific band in the case of primer $(GACA)_4$, which was present in all the female and hermaphrodite plants but was absent in male plants of papaya (Figure 1). The two other ISSR primers used for the present study ($(CAG)_5$, $(CAA)_5$), however, did not result in any alteration of banding profile in male, female or hermaphrodite plants. Male-specific ISSR marker, however, has been reported[12] in papaya using primer $(GATA)_n$. Recent studies indicate that sex in papaya is governed by a single gene with three alleles[13]. A high-density linkage map of papaya revealed severe suppression of recombination around the sex-determination locus with a total of 225 markers co-segregating with sex types[14]. In terms of agriculture, the discovery of markers linked to sex chromosomes will help farmers to selectively grow hermaphrodite papayas.

Sexual dimorphism of *C. circinalis* was readily distinguishable in RAPD profiles generated from a number of random primers under study, among which the profiles of primers OPB 01 and OPB 05 were noteworthy, since they represent one male-specific (686 bp) and another female-specific (2048 bp) band respectively (Figure 2). Sequencing of these two cloned DNA fragments, followed by BLASTX searching, revealed maximum homology with reverse transcriptase of *Ginkgo biloba* (score 69.3 bits; NCBI accession no. AAY87195) followed by putative retroelement pol poly protein of *Arabidopsis thaliana* (score 59.3 bits; NCBI accession no. AAC61290) and putative poly protein of *Oryza sativa* (japonica cultivar-group; score 58.5 bits; NCBI accession no. AAU90089) in case of male-specific DNA fragment (NCBI accession DQ386640, dated 22.02.2006), while the female-specific DNA fragment did not result in any significant match. Homology of male-specific sequence with retroelement calls for further investigation towards isolation of full-length sequence of this DNA fragment to be used as a marker for male *C. circinalis* in future. Diversity, evolution and genome organization of retroelements have been studied in a wide range of gymnosperms, especially the conifers[15,16], but assigning of direct relationship to sex is yet to be established.

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Free-radical scavenging activities of Himalayan rhododendrons

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Reactive oxygen species can damage cellular biomolecules leading to degenerative diseases. Phenols, a major group of phytochemicals with antioxidant properties, can help inactivate them. To find the antioxidant potential of the genus *Rhododendron*, its 21 species were studied for their total phenolic content (TPC), flavonoids and antioxidant activity (AOA). TPC varied from 37.3 to 208.9 mg/g, flavonoids from 11.5 to 137.1 mg/g and AOA from 30.4 to 97.4%. *R. baileyii*, *R. camellie-*

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