# Identification of RAPD markers linked to sex determination in palmyrah (*Borassus flabellifer* L.)

Palmyrah (*Borassus flabellifer* L.), a tropical palm, adorns the dry landscape of the semi arid regions of Tamil Nadu (TN), Andhra Pradesh, Orissa, West Bengal, Bihar, Karnataka and Maharashtra. It is a multipurpose tree of great utility and occurs extensively in TN. It is exploited for food from the fruit and tuberous seedlings; beverage and sugar from the sap; fibre from the fruits and leaves for brushes, cordage weaving and plaiting; and trunk wood for construction and fuel. Due to its multifarious uses, the Government of TN has declared it as a 'state tree'[1].

Palmyrah is a dioecious palm with the great majority of its economic products such as immature endosperm, mesocarp pulp, tuberous seedlings obtained only from female palms. But sweet sap from the inflorescence, toddy, palm sugar, brush fibre and wood are obtained irrespective of whether the palms are male or female. However, differences in their yield or quality have been reported. Thus female palms are supposed to yield more toddy on tapping from the inflorescence[2] and the female tree gives better and hard timber than the male tree, and is also more expensive[3].

The palms are slow-growing perennials and have no distinguishing features to identify the sex until flowering. The palm commences flowering only after 12 to 15 years of maturity. On account of the dioecious nature and long juvenile period, farmers have hesitated in planting this multipurpose tree. Breeding and crop improvement would be highly facilitated if gender could be determined at the seedling stage itself. This would help farmers while selecting the seedlings and maintain an optimum sex ratio at plantation.

Currently there is no method to distinguish between male and female plants prior to flowering in palmyrah. Molecular markers can be utilized to diagnose and select a genotype based on linked DNA markers, long before the phenotype is apparent. This is particularly important in palmyrah palm, which has a long juvenile period. Random amplified polymorphic DNA (RAPD) markers have been used for determining sex by bulk segregant analysis in *Pistacia vera*[4,5], *Atriplex garrettii*[6], *Trichosanthes dioica*[7] and *Salix viminalis*[8,9]. Using this method, Mulcahy *et al.*[10] identified markers specific to the Y-chromosome in *Silene latifolia*. The present investigation was carried out to differentiate between pistillate and staminate palmyrah genotypes based on RAPD markers.

The material for this study comprised of 30 male and 30 female leaf samples collected from Kerala (Kasaragod), Karnataka (Mangalore) and TN (Killikulam), 10 males and 10 females from each state. Immature leaflets from adult pistillate and staminate trees of palmyrah were collected and placed in polybags and transported to the laboratory in an icebox where they were kept at 4°C prior to DNA extraction.

Total genomic DNA was extracted using the SDS method, whereby 2 g of immature leaf tissue was ground in liquid nitrogen and transferred to extraction buffer containing 1% sodium dodecyl sulphate (SDS). The contents were incubated at 60°C for 1 h, and cooled and extracted with an equal volume of 24 : 1 chloroform : isoamyl alcohol mixture. The supernatant was transferred to a new tube and DNA was precipitated with equal volume of ice-cold isopropanol. The DNA-containing tube was centrifuged at 3000 rpm for 1 min, the isopropanol was removed and the DNA pellet was rinsed with 70% alcohol thrice. The pellet was dried in laminar airflow for 3 h and then suspended in 500 µl TE buffer (pH 8.0) and stored at 4°C for three days to dissolve it completely. RNAse (0.05 µg/ml) was added to 500 µl of crude DNA and incubated at 37°C for 1 h. Equal volume of chloroform : isoamyl alcohol (24 : 1) was added to the solution and mixed thoroughly without vortexing and centrifuged at 14,000 rpm for 15 min at 4°C. The upper aqueous phase was transferred to a fresh tube to which 700 µl of ice-cold ethanol and 20 ml of 3 M sodium acetate (pH 5.2) were added. The contents were mixed by inverting the tube and then incubated at –20°C for 1 h. DNA was precipitated by centrifuging at 14,000 rpm for 2 min at 4°C and washed with chilled 70% ethanol twice. The pellet was air-dried and dissolved in 200 µl of TE buffer (pH 8.0) and stored at –20°C. DNA concentration was determined for each sample spectrometrically at 260 nm.

Sex-pooled DNA samples were prepared for bulk segregant analysis by mixing equal quantities of the DNA of 30 male individuals (male bulk) and 30 female individuals (female bulk) separately. RAPD reactions were performed with 180 random decamer oligonucleotide primers of the series OPBA, OPBE, OPM, OPAB, OPAH, OPA, OPC, OPE and OPAF (Operon, USA). The PCR reaction mixture consisted of 20 ng genomic DNA, 200 µM of dNTPs, 10 pmol/µl of each primer, 1× *Taq* DNA buffer and 0.5 units of *Taq* DNA polymerase, in a final volume of 15 µl in sterile ultra-pure water and overlaid with one drop of mineral oil. PCR was performed in Eppendorf Master Gradient thermal cycler with initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min and extension at 72°C for 1 min, finally ending with one cycle of 72°C for 7 min. After amplification, the PCR product was stored at –20°C till electrophoresis. Amplified products were mixed with 3 µl of 6X gel-loading dye (0.25% bromophenol blue and 40% (w/v) sucrose in $H_2O$) before loading. The PCR products were separated by agarose gel (1.5%) electrophoresis in 1× TBE buffer. Ethidium bromide-stained gels were documented using Syngene UK Gel Documentation and Analysis System. RAPD primers were first screened in pooled DNA samples. Primers that detected polymorphism between the two sex pools were tested in the 60 individual plants.

Out of 180 RAPD primers screened, only three (OPBE-12, OPBA-13 and OPA-06) showed polymorphism between male and female sex pools of palmyrah. The male bulk was prepared by mixing ten male samples each from Kerala, TN and Karnataka; likewise the female bulk was also prepared. OPBE-12 produced a 1100 bp band only in male bulk, OPBA-13 produced 500 bp band only in female bulk, and OPA-06 produced 600 bp band only in male bulk.

The experiment was repeated thrice with individual male and female DNA samples and it was observed that the

band of size 1100 bp produced by the primer OPBE-12 was found to be male-associated and it was completely absent in all the female entries; the band was not present in all the male palms tested.

OPBA-13 produced a female-associated band of 500 bp; however, it was also present in one of the male amplicons when tested individually. OPA-06 with the sequence 5′GGTCCCTGAC3′, displayed a band at 600 bp that expressed polymorphism between male and female bulk. When tested with DNA from 30 individual male plants and 30 individual female plants used to make the bulk, this fragment was present in all male plants and absent in all female plants (Figure 1 *a*–*c*). The result was confirmed by repeating the experiment thrice. This DNA marker is completely linked to male sex in palmyrah and can be used for screening seedlings with the objective of sex determination during planting.

The problem with markers produced by OPBE-12 and OPBA-13 is that they are not completely linked to the sex-determining locus. Kafkas *et al.*[5] reported the possibility of some markers which are not tightly linked to a sex-determining locus. Some individuals may possess marker-phenotype of the opposite sex.

Obtaining a marker linked to a gene or genomic region through RAPD analysis depends to a large extent on chance, because random sequences are used as PCR primers. Hormaza *et al.*[4] screened 1000 primers in *Pistacia vera* for sex determination and found only one female-associated marker, which was absent in males. They suggested that the low frequency of sex-linked bands may indicate that the DNA segment(s) involved in sex determination is small and probably involves a single gene, or few genes.

Claudate *et al.*[6] identified a 2075-bp male-specific band after screening 158 RAPD primers in dioecious *Atriplex garrettii*. They tested DNA from ten male, ten female and hermaphrodite plants individually and reported that the male-specific fragment was present in hermaphrodites, all but one of the male plants and was absent in all female plants. A female-specific DNA fragment of size 416 bp was identified in dioecious nutmeg by Ganeshaiah *et al.*[11], after screening 60 RAPD primers.

Singh *et al.*[7] could identify a female sex-associated 567 bp RAPD marker in pointed gourd (*Trichosanthes dioica* Roxb.), which is completely absent in males, after screening 100 decamer primers. In pointed gourd, the male and female plants strictly maintain their respective sexual phenotypes, enabling the identification of completely sex-associated markers.

The present study revealed that the male-specific DNA fragment of size 600 bp produced by the primer OPA-06, is tightly linked to the male-sex locus and is useful for sex determination in palmyrah.

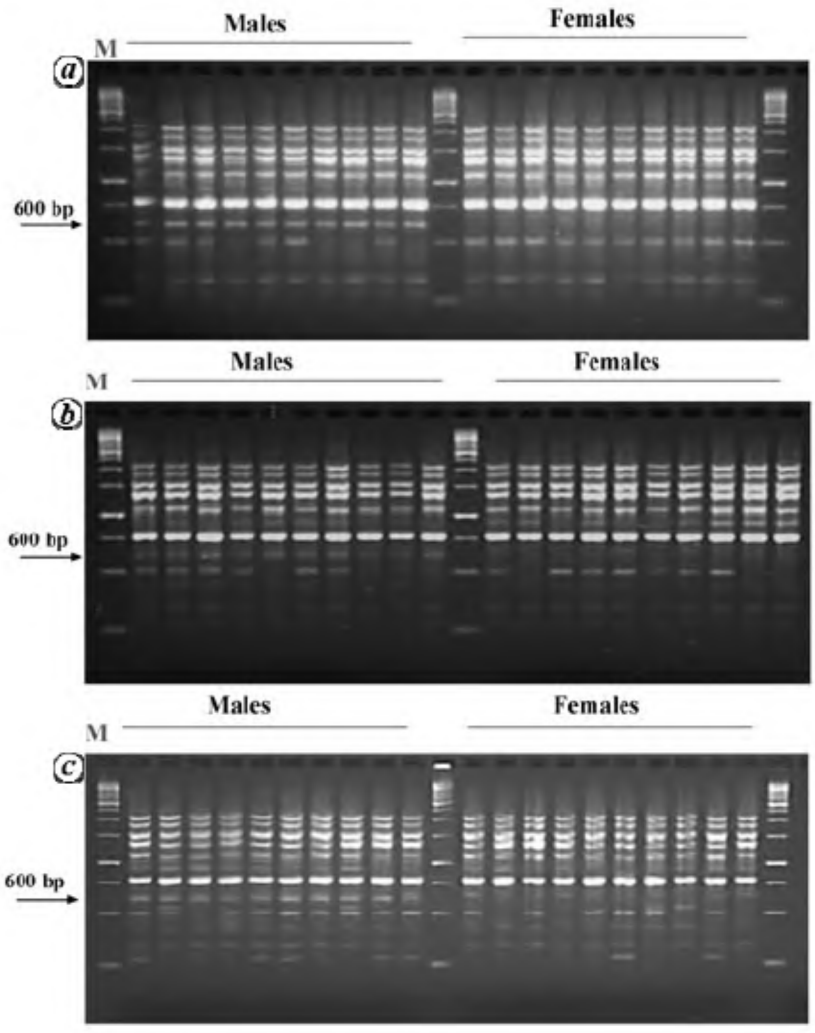


**Figure 1.** RAPD banding profile of 10 males and 10 female palmyrah individuals collected from (***a***), Kerala (Kasaragod), (***b***) Karnataka (Mangalore) and (***c***) Tamil Nadu (Killikulam) using primer OPA-06. The lanes marked 'Males' and 'Females' represent DNA amplified from individual male and female plants. The 600 bp male-specific band is indicated with an arrow. M, 1 kb ladder.


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# Molecular cloning of rubber elongation factor protein cDNA from *Hevea brasiliensis* Muell. Arg. and its heterologous expression in *Escherichia coli* and *Nicotiana tabacum**

*Hevea brasiliensis*, commonly known as rubber tree, is presently the world's sole commercial source of natural rubber (*cis*-1,4-polyisoprene). Natural rubber, an important biomacromolecule for many industrial purposes in the world, is produced by over 2000 plant species distributed among four of the six superorders of Dicotyledonae[1]. Despite the availability of petroleum-based synthetics, natural rubber is highly valued because no synthetic substitute has comparable elasticity, resilience and resistance to high temperature[2]. Rubber represents 30–50% by weight of the latex exuded by mature *Hevea* trees in regular tappings and constitutes more than 90% of the total latex solid[3].

Natural rubber from *H. brasiliensis* consists of long chains of *cis*-polyisoprene which are synthesized through the mevalonate pathway from acetyl CoA derived from glycolysis[3]. Polymerization of isopentenyl pyrophosphate by prenyltransferase assisted by the Rubber Elongation Factor (REF) gives rise to long chains of *cis*-polyisoprene which form aggregates called rubber particles surrounded by a lipoproteic membrane. Rubber biosynthesis takes place on the surface of rubber particles suspended in the latex produced in special latex vessels called laticifers[4]. Kush *et al.*[5] have shown the differential expression of several rubber biosynthesis-related genes in the latex of *Hevea*. The REF, an enzyme involved in rubber biosynthesis, is highly expressed in laticifers[6]. The primary protein structure of REF has been elucidated[7]. The amount of REF in the whole latex is proportional to the rubber content. As such, if the amount of REF protein is correlated with rubber yield, there is a possibility that this relationship could be used to select clones that are able to sustain high rubber biosynthetic efficiency. Therefore, overexpression of *REF* gene may enhance latex yield in transgenic plants by genetic engineering. To achieve this goal, it is essential to clone and characterize REF cDNA from high latex-yielding Indian *Hevea* clone. More recently, cloning and characterization of a genomic DNA coding for *REF* gene and its promoter sequence has been reported[8].

Here we report cloning and characterization of *REF* gene from latex of Indian *Hevea* clone RRII105; the cDNA was inserted into an expression vector for heterologous expression in *Escherichia coli*. In addition, this gene was also overexpressed in tobacco plants via *Agrobacterium*-mediated genetic transformation.

Total RNA was isolated from mature rubber trees of RRII 105 clone following the method of Venkatachalam *et al.*[9]. REF cDNA was synthesized by RT–PCR according to the manufacturer's instructions (Promega). PCR was carried out with *REF* gene-specific primers (5′-CGA TTA TGG CTG AAG ACG AAG ACA ACC-3′ and 5′-GGC CAA TAA TTC AAT TGG CCC TTT ATT C-3′) using the following parameters: 94°C for 4 min followed by 30 cycles at 94°C for 1 min, 55°C for 1.30 min, 72°C for 2 min and a final extension at 72°C for 10 min. The amplified cDNA was cloned into pUC19 vector and sequenced.

For heterologous protein expression, the REF cDNA was cloned between *Bam*HI and *Eco*RI sites of pGEX-2T vector to generate a recombinant plasmid with *REF* gene designated as pGEX-REF, according to vendor's instructions (Amersham, UK). The recombinant plasmid was transformed into *E. coli* competent cells (DH5$\alpha$) according to standard protocols[10] and induced with 0.1 mM IPTG (isopropyl-$\beta$-D-thiogalacto-pyranoside) to express the foreign protein. The bacterial proteins were analysed on 12% SDS–PAGE[11]. The fusion protein was purified, transferred to polyvinylidene fluoride (PVDF) membrane and detected by an enhanced chemilumeniscence system (ECL, Amersham, UK). The membrane was also incubated in buffer (TTBS) containing polyclonal antibodies raised against native REF protein followed by incubation with goat anti-rabbit IgG–ALP conjugate and visualized using BCIP/NBT.

*Hb*REF cDNA was cloned into the binary vector pBIB and the recombinant plasmid was then mobilized into *Agrobacterium tumefaciens* strain, LBA 4404

*The sequence described in this work has been deposited in the NCBI GenBank database under accession number AY120685. The first two authors contributed equally to the work.