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Molecular identification of sex in *Simarouba glauca* by RAPD markers for crop improvement strategies

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ABSTRACT

Due to lack of morphological methods to identify sex at early stage in the plants with long juvenile period the application of molecular markers is expected to facilitate breeding program. The objective of this study is to identify molecular markers linked to sex determination of the plant *Simarouba glauca* which assists in crop improvement program. Random amplified polymorphic DNA primers were tested on dioeceious and hermaphrodite plant *Simarouba glauca*. A set of eighty five RAPD primers were screened out of which only five primers were found to be associated with sex. The primer OPU-10 is male specific and OPD-19 primer is female specific. Another primer OPU-19 produced a unique amplification in only hermaphrodite individuals. Female and hermaphrodite specific primer OPS-05 amplified an amplicon in female and hermaphrodite and was absent in male plant. Primer OPW-03 produced amplicon specific to male and hermaphrodite plants and was absent in female plants.

1. Introduction

Due to gradual depletion of world's petroleum reserves and impact of environmental pollution of increasing exhaust emission, there is an urgent need to develop alternative energy resources such as biodiesel fuel. Vegetable oil is a promising alternative because it has several advantages, viz it is renewable, environment friendly and produced easily in rural area, where there is acute need for modern forms of energy. Therefore in recent years several researches have been studied to use vegetable oil as fuel in engine as biodiesel [27,28]. Various vegetable oils, palm oil, soybean oil, sunflower oil, rapeseed oil, canola oil, jatropa oil, pongamia oil have been used to produce biodiesel fuel and lubricants [29,30]. Biodiesel is usually produced by transesterification of vegetable oils or animal fats with methanol or ethanol [31].

Biodiesel producers are looking for alternative feed stock which are non-agricultural, high oil content seed and non-food crops. *Simarouba* species has the ability to substitute the requirement of low cost feed stock with the potential for high oil seed production and added benefit of an ability to grow on marginal land. This property supports the suitability of *Simarouba* species for the sustainable biodiesel industry [12]. Simarouba yields +3 t biofuel/ha/year at 5 m × 5 m plant spacing with proper nutrition and irrigation. Simarouba belongs to Simaroubaceae family, is indigenous to the Amazon rainforest and other tropical areas in Mexico, Cuba, Haiti, Jamaica, and Central America. It was brought to India from Latin America in 1960s. It can be grown anywhere from the sea coast to evaluations of 1000 m in tropical climatic condition. Simarouba is commonly known as paradise tree, dysentery bark. The leaves and bark have amoebicide, antidiarrheal, analgesic, antibacterial, antileukemic, antimalarial properties [2]. Wood is used to make furniture [9]. *Simarouba glauca* is a tree born oilseed crop. The seeds of Simarouba are economically very important since they contain 65–75% of oil. Simarouba is polygamodioecious with three types of plants pistillate (female flowers), staminate (male flowers) and andromonoecious (male dominated bisexual flowers) [12]. The waiting time from sowing to flowering is long. Usually it flowers after 5–7 years of planting hence growers need to ensure the seedling's sex for good harvest. The determination of the sex of Simarouba seedling prior to the flowering stage would avoid the need for removing undesired sex (male) plants from the field. Only 5% male or andromonoecious plants in a field are sufficient for efficient pollination. Identification of sex types prior to propagation, especially in polygamodioecious plant species with a long juvenile cycle such as Simarouba, would result in higher fruit production and increased profitability. There is no method available to distinguish male, female, and hermaphrodite plants in pre-flowering stage in Simarouba. Molecular markers could be utilized to diagnose sex-linked DNA markers. RAPD markers have shown their reliability for determining sex in *Pistacia vera*[11],*Atriplex garrettii* [5],

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Trichosanthes diocia [22], *Salix viminalis* [3], *Piper longum* [16], *Borassus flabellifer* [8], *Simmondsia chinensis* [1], *Carica papaya*, and *Cycas circinalis* [7], *Commiphora wightii* [21]. The aim of present study is to indentify RAPD markers associated with sex determination in Simarouba.

2. Materials and methods

2.1. Plant material

Fresh leaf sample each of two accessions of both female and hermaphrodite were collected from University of Agricultural Sciences, Bangalore (UASB) and a male from University of Agricultural Sciences, Dharwad (UASD), India. The samples were stored at −80 °C until use. Leaf samples were collected from male, female and hermaphrodite plants after complete observation of flower types and these were used for DNA extraction.

2.2. Genomic DNA isolation

Total genomic DNA was isolated from leaf tissues from five accessions (one male, two female and two hermaphrodites) with the minor modifications in CTAB method [20]. About 0.3 g of leaf tissue was ground to a fine powder in liquid nitrogen and mixed with 700 μl of CTAB (cetyltrimethylammonium bromide) extraction buffer (100 mM Tris–HCl pH 8, 1.4 M NaCl, 20 mM EDTA (pH 8), 2% CTAB, 1% β-mercaptoethanol, 1% PVP). The mixture was first incubated at 65 °C for 30 minutes, and then an equal volume of a phenol:chloroform:isoamylalcohol (25:24:1) mixture was added, followed by centrifugation at 4000 rpm for 30 minutes at 4 °C. The aqueous phase was decanted and transferred to a new micro tube to reduce impurity between the two phases. The extraction steps were repeated using the same phenol:chloroform:isoamylalcohol (25:24:1) mixture. The last aqueous phase was mixed with two-thirds volume of isopropanol and stored at −20 °C for at least 2 h to precipitate the DNA, then centrifuged at 4000 rpm for 15 minutes. The nucleic acid precipitate was washed with 70% ethanol, air dried, and suspended in 50 μl of TE buffer. DNA was treated with RNaseA (Quiagen, USA) for eradication of RNA followed by two washings with chloroform:iso-amyl-alcohol (24:1; v/v) before actual use. Subsequently, quality and quantity were checked by running the dissolved DNA in 0.8% agarose gel and uncut λ DNA (Bangalore Genei, Bangalore, India) of known concentration. The extracted DNA was diluted in ddH_2O to 50 ng/μl and subjected to RAPD-PCR analysis.

2.3. RAPD marker analysis

Eighty five 10-base primers (Operon Technologies, Alameda, USA) were used for polymerase chain reaction (PCR) for screening of known sex to ascertain their potential of clear amplification in polymorphism and also the reproducibility. The RAPD-PCR reactions were performed in 25 μl volumes in 100 μl PCR tubes (Tarson Pvt., Ltd., India). The reaction mixture contained 30 ng of template DNA, 1× amplification buffer (10 mM of Tris–HCl – pH 8, 50 mM of KCl, 1.8 mM of $MgCl_2$ and 0.01 mg/ml gelatine), 2.5 mM each of dCTP, dGTP, dATP, and dTTP, 5 pM primers and 1 U *Taq* DNA polymerase (Bangalore Genei, Pvt., Ltd., India). The reactions were performed in a Master Cycler Gradient 5331 (Eppendorf version 2.30. 31-09, Germany) with an initial denaturation step at 94 °C for 4 minutes, followed by 35 cycles of 94 °C for 1 minute, 37 °C for 1 minute, 72 °C for 2 minutes. The final extension step was at 72 °C for 10 minutes. The reactions were then cooled and held at 4 °C. The RAPD-PCR products were separated on 1.5% (w/v) agarose (Sigma–Aldrich, USA) gel at 5 V/cm in 1 × TBE (89 mM Tris–HCl, 89 mM boric acid and 2 mM EDTA, pH 8.0) buffer. The agarose gels were stained with 0.5 μg ml^{-1} ethidium bromide visualized under UV light and photographed on a digital gel-documentation system (SYNGENE). The molecular weights of the RAPD amplicons were estimated with a 100 bp DNA ladder (New England).

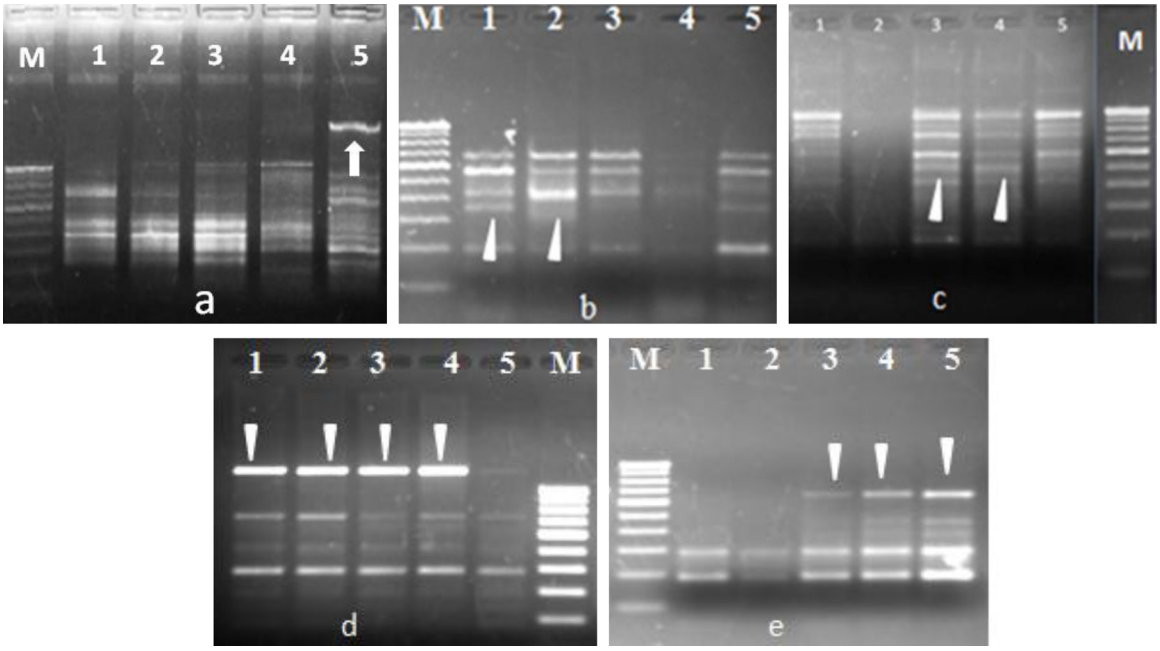

Fig 1. RAPD banding profile of male, female and hermaphrodite *Simarouba glauca* plants obtained by the primers OPU-10, OPD-19, OPU-19, OPS-05 and OPW-3. (a) OPU-10; arrow indicates the unique band present in male. (b) OPD-19; arrow indicates the unique band of 350 bp present in female. (c) OPU-19; arrow indicates the unique band of 500 bp present in hermaphrodite. (d) OPS-05; arrow indicates the unique bands present in female and hermaphrodite. (e) OPW-03; arrow indicates the unique band of 600 bp present in male and hermaphrodite. M; 100 bp ladder; lanes 1 and 2 female individuals; lane 3 and 4 hermaphrodite individuals; lane 5 male individual.

3. Results and Discussion

A set of 85 decamer primers were used to amplify the genomic DNA of male, female, and hermaphrodite individuals of which 16 primers showed reproducible results. Five primers OPU-10 (5ACCTCGGCAC3), OPD-19(5CTGGGGACTT3), OPU-19 (5GTCAGTGCGG3), OPS-05(5TTTGGGGCCT3) and OPW-03 (5GTCCGGAGTG3) produced unique amplicon for sex differentiation. Among these five decamer primers three primers, OPU-10, OPD-19, and OPU-19 showed sex specificity of male, female and hermaphrodite respectively. The primer OPU-10 produced a unique band in male individual DNA which was absent in female and hermaphrodite in the region above 1 kb DNA marker banding pattern (Fig. 1a). OPD-19 primer produced 350 bp unique amplicon in female individual's DNA that was completely absent in male and hermaphrodite (Fig 1b). Similarly, another unique band of 500 bp specific to hermaphrodite amplified by OPU-19 primers was observed which was not present in male and female DNA markers (Fig. 1c). OPS-05 primer was specific to both female and hermaphrodite and was absent in male plant in the region above bands produced by 1 kb DNA ladder (Fig. 1d). On the other hand 800 bp amplicon produced by OPW-03 is specific to both male and hermaphrodite and was absent in female plants (Fig. 1e). Previous study revealed some female specific sex-linked markers in *Pistachio vera* (OPA-08_{945}), *Salix viminalis* (UBC-345_{560}), and *Trichosanthes diocia* (OPC-07_{567}), *Commiphora wightii* (OPN-06_{1280}), *Pistacia* (BC1200), *Garcinia indica* (OPW-05_{1100} and OPW-08_{1200}) by [3,6,11,21–23] respectively. Male and harmaphrodite specific primers OPB-01 (Carica papaya); OPN-16 (Commiphora wightii); OPA-08 (*Simarouba glauca*); OPG 05 (*Simmondsia chinensis*) reported by [1,7,18,21] respectively. The OPS-05 primer could also be used to discriminate male from female and hermaphrodite plants. Similarly, female plants could also be differentiated from male and hermaphrodite plants by the primer, OPW-03. Several constraints have been faced with Simarouba cultivation by its growers. The very long waiting time from planting to harvesting, and it flowers after 5–7 years of plantation. Apart from this, there is no available method for characterization of male and female plants. Realizing these inherent problems, it is essential to identify the sex of this plant at the seedling stage prior to its plantation to the field, so that desired ratio of male and female plants can be achieved, and resources like planting space, fertilizers, water and the labor costs can be devoted to the cultivation of the desired sex (female plants and male plants) [1]. Thus, an increase in the number of fruit-bearing plants per hectare of land would directly increase the total yield in the field making its cultivation more profitable. The development of molecular strategies for early sex detection of dioecious plants has been a priority in breeding programs for their greater economic potentials. The use of molecular markers to distinguish the sexes has been employed since the genetic mechanism of sex determination is not available [4,26]. Molecular marker based technology has been proved a reliable strategy for detection of sex-associated markers in dioecious and bisexual plants. The RAPD marker technique is the cheapest, user friendly and reliable tool [25] used for efficient fingerprinting of many plants. In addition, SCAR markers originating from RAPD markers were also developed for distinguishing the sex specificity in many plant species [4,10,15,16,19,24].

4. Conclusion

RAPD markers in Simarouba could help farmers to select the best seedlings and maintain an optimum sex ratio in plantations as well as save time and costs in Simarouba breeding programs. Further, the male specific OPU-10, female specific OPD-19_{350} and hermaphrodite specific OPU-19_{500} could be used to convert more reliable SCAR markers [17] for better reproducibility. Such reliable SCAR marker has been achieved in *Mercurialis annua, Carica papaya,* and *Cannabis sativa* [14,15,24]. The availability of markers linked to sex-associated genes would allow cloning the gene/s involved in this process and this information will help in the development of gene specific markers. It is possible to differentiate male, female, and hermaphrodite plants of Simarouba precisely and rapidly using the RAPD markers.

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