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# RAPD Analysis for Detection of Genetic Variability and Sex in *Givotia rottleriformis* Griff.

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## ABSTRACT

*Givotia rottleriformis* Griff. is an economically important dioecious tree species known for the softwood used in making toys. Knowledge of genetic variation in a dioecious tree species is important for devising strategies for its successful management and conservation. Studies were conducted to examine the genetic variation in *Givotia* plants using RAPD (random amplified polymorphic DNA) analysis and to identify molecular marker(s) linked to sex, if any. RAPD analysis was initially performed using 32 random decamer primers in DNA bulks of 5 male and 5 female plants. Out of 32 random primers tested, 24 resulted in DNA amplification of male and female plants whereas no amplification was observed with the remaining 8 primers used. Analysis of individual male and female plants with 24 random primers revealed a total of 142 amplified bands of which 86 were polymorphic accounting for an average polymorphism of 52.9%. The highest number of amplified bands (11) was generated from primer OPAL-08, 8 of which were polymorphic; the highest number of polymorphic bands (10) was generated from primer OPG-16. Cluster analysis constructed from pooled RAPD data using Jaccard's similarity coefficient showed grouping of males and female plants into three clusters at a 70% similarity level. Twelve random primers which produced sex-specific bands in DNA bulks of males and females when tested in individual male and female plants exhibited a variable banding pattern except for primer OPT-17, which amplified a 1000-bp band in all 5 females and also in 1 male thus exhibiting partial association with sex.

**Keywords:** Euphorbiaceae, dioecious species, RAPD markers, genetic variation, sex identification
**Abbreviations:** **AFLP**, amplified fragment length polymorphism; **BSA**, bulk segregant analysis; **CTAB**, cetyltrimethylammonium bromide; **DNA**, deoxyribonucleic acid; **dNTP**, deoxyribonucleotide triphosphate; **EDTA**, ethylenediaminetetraacetic acid; **ISSR**, inter simple sequence repeats; **NaCl**, sodium chloride; **PCR**, polymerase chain reaction; **PVP**, polyvinylpyrrolidone; **RAPD**, random amplified polymorphic DNA; **RFLP**, restriction fragment length polymorphism; **SCAR**, sequence-characterized amplified region; **Tris-HCl**, tris(hydroxymethyl)aminomethane hydrochloride

## INTRODUCTION

*Givotia rottleriformis* Griff. is an economically important tree species belonging to the Euphorbiaceae family and is known for its light softwood. This tree is found in dry deciduous forests of India. The wood of this tree is useful for making toys, boxes, catamarans, fancy articles and for purposes where lightness is an advantage. In India, many of the small-scale industries depend on this wood (Reddy *et al*. 2001). The tree is also known for its medicinal properties and the bark powder and seeds are used for treating rheumatism, dandruff and psoriasis (Thammanna and Narayana Rao 1990). The oil extracted from the seeds is used in lubricating fine machinery. The exploitive collection of this tree species, particularly for its softwood, coupled with problems of propagation through seeds and vegetative cuttings has resulted in a considerable decrease in natural populations. Thus, there is an immediate need for effective collection, growing plantations and conservation strategies to be adapted in this species.

Successful management and conservation of forest tree species depend on accurate assessment of genetic diversity among individuals in a population (Renau-Morata *et al*. 2005). In studies on genetic variation, the most efficient and most accurate are DNA markers as they are not affected by the environment and the stage of plant development. A number of polymerase chain reaction (PCR)-based DNA markers, including random amplified polymorphic DNA (RAPD) (Williams *et al*. 1990), amplified fragment length polymorphism (AFLP) (Zabeau and Vos 1993), restriction fragment length polymorphism (RFLP) (Botstein *et al*. 1980), simple sequence polymorphic DNA (Tautz 1989), and inter simple sequence repeat (ISSR) (Zietkiewicz *et al*. 1994) have been used to characterize genetic diversity and identify sex in different plant species (reviewed in Teixeira da Silva *et al*. 2005). Among these, RAPD is technically simple, inexpensive, and requires no previous sequence information on the target genome (Williams *et al*. 1990). The reproducibility problem faced with RAPDs can be overcome if factors such as DNA quantity and experimental conditions are strictly maintained across different sets of reactions (Ulloa *et al*. 2003). RAPDs have been successfully used in analysis of genetic variation, genetic mapping, early determination of sex, molecular phylogenetics, genetic fidelity and marker assisted selection in several plant species (Bardakci 2001; Salem *et al*. 2007; Kumar *et al*. 2009). To the best of our knowledge, there has been no attempt made to characterize the genetic variability of *G. rottleriformis*, an important softwood tree species.

*G. rottleriformis* is a dioecious tree species and highly heterogeneous due to open pollination. The female plants of *Givotia* have greater economic value than males as the seeds have oil and medicinal properties. There are no morphological characters that can be used to distinguish sex prior to flowering in this species. The inability to determine sex at early stages of vegetative growth is a disadvantage, especially in raising plantations with a desired sex ratio. In addition to agronomic benefits, addressing the issue of sex determination is of importance in *Givotia*, as the mechanism of sex determination is not known. In dioecious plant

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**Table 1** RAPD markers identified for sex differentiation in dioecious tree species.

| Plant species | No. of primers tried | No. of primers that produced sex specific bands | Total no. of plants analyzed | | | Primer name(s) |
|---|---|---|---|---|---|---|
| | | | Males | Females | Hermaphrodites | |
| *Borassus flabellifer* L. | 180 | 1 | 30 | 30 | -- | OPA-06 |
| *Calamus simplicifolius* C.F. Wei | 1040 | 1 | 20 | 20 | -- | S1443 |
| *Carica papaya* L. | 80 | 1 | 7 | 7 | -- | OPF-02 |
| *Carica papaya* L. | 152 | 1 | -- | 10 | 10 | BC-210 |
| *Carica papaya* L. | 25 | 1 | 6 | 7 | 4 | IBRC-RP07 |
| *Carica papaya* L. | 32 | 1 | 4 | 3 | 2 | OPY-07 |
| *Carica papaya* L. | 100 | 2 | 10 | 10 | 10 | OPC-09 |
| | | | | | | OPE-03 |
| *Commiphora wightii* (Arnott.) Bhandari | 60 | 3 | 5 | 5 | 3 | OPN-06 |
| | | | | | | OPN-16 |
| | | | | | | OPA-20 |
| *Cycas circinalis* | 10 | 2 | 3 | 3 | -- | OPB-01 |
| | | | | | | OPB-05 |
| *Encephalartos natalensis* (Dyer and Verdoorn) | 140 | 1 | 31 | 38 | -- | OPD-20 |
| *Eucommia ulmoides* Oliv. | 560 | 1 | 5 | 5 | -- | OPF-08 |
| *Ginkgo biloba* | 1200 | 1 | 30 | 30 | -- | S1478 |
| *Myristica fragrans* Hout. | 60 | 1 | 10 | 10 | -- | OPE-11 |
| *Phoenix dactylifera* L. | 30 | 3 | 3 | 4 | -- | A10 |
| | | | | | | A12 |
| | | | | | | D10 |
| *Pistacia vera* L. | 700 | 1 | 30 | 29 | -- | OPO-08 |
| *Simarouba glauca* DC. | 70 | 1 | * | * | -- | OPD-20 |

| Plant species | Sex-associated band(s) detected | | | Reference |
|---|---|---|---|---|
| | Males | Females | Hermaphrodites | |
| *Borassus flabellifer* L. | Absent | 600 bp present | -- | George *et al*. 2007 |
| *Calamus simplicifolius* C.F. Wei | 500 bp present | Absent | -- | Yang *et al*. 2005 |
| *Carica papaya* L. | 800 bp present | Absent | -- | Parasnis *et al*. 2000 |
| *Carica papaya* L. | -- | Absent | 438 bp present | Lemos *et al*. 2002 |
| *Carica papaya* L. | 450 bp present | Absent | 450 bp present | Urasaki *et al*. 2002 |
| *Carica papaya* L. | 900 bp present | Absent | Absent | Chaves-Bedoya and Nuñez 2007 |
| *Carica papaya* L. | 1700 bp present | Absent | 1700 bp present | Niroshini *et al*. 2008 |
| | 400 bp present | Absent | 400 bp present | |
| *Commiphora wightii* (Arnott.) Bhandari | Absent | 1280 bp present | Absent | Samantaray *et al*. 2010 |
| | Absent | Absent | 400 bp present | |
| | Absent | 1140 bp present | 1140 bp present | |
| *Cycas circinalis* | 686 bp present | Absent | -- | Gangopadhyay *et al*. 2007 |
| | Absent | 2048 bp present | -- | |
| *Encephalartos natalensis* (Dyer and Verdoorn) | Absent | 850 bp present | -- | Prakash and Van Staden 2006 |
| *Eucommia ulmoides* Oliv. | Absent | 569 bp present | -- | Xu *et al*. 2004 |
| *Ginkgo biloba* | 682 bp present | Absent | -- | Ling *et al*. 2003 |
| *Myristica fragrans* Hout. | Absent | 416 bp present in 9 females | -- | Shibu *et al*. 2000 |
| *Phoenix dactylifera* L. | Absent | 490 bp present | -- | Younis *et al*. 2008 |
| | 370 bp present | 750 bp present | -- | |
| | 675 bp present | 800 bp present | -- | |
| *Pistacia vera* L. | Absent | 945 bp present | -- | Hormaza *et al*. 1994 |
| *Simarouba glauca* DC. | Absent | 900 bp present | -- | Simon *et al*. 2009 |

* The complete paper was not available and hence the information regarding the total number of male and female plants analyzed could not be furnished

species, the development of molecular strategies for the early identification of sex has been a priority in breeding programs in order to increase their economic potential and better understand the developmental as well as evolutionary pathways of dimorphism (Shibu *et al*. 2000; Agrawal *et al*. 2007; Sharma *et al*. 2008). Sex determination in dioecious plants may often be genetic or environmental and only a small proportion of them have evolved sex chromosomes (Kumar *et al*. 2008). Genetic sex determination may be due to a single locus, multiple tightly linked loci on autosomes, multiple unlinked loci on autosomes, or several genes located on heteromorphic chromosomes (Parrish *et al*. 2004). To our knowledge, there is no information on sex chromosomes in male and female plants of this species.

RAPD markers have been used to determine sex in various dioecious tree species as indicated in **Table 1**. To date, there are no published reports on the development of molecular markers for the identification of sex of *Givotia* plants. Therefore, it is worthwhile to develop a molecular marker for early detection of sex of this species. The present paper reports for the first time the genetic variability in *Givotia rottleriformis* Griff. plants and the potential of RAPD markers for the identification of sex of this species.

## MATERIALS AND METHODS

### Plant material

Young leaves were collected from 10-12 year-old trees of *G. rottleriformis* at Nallamala forest, Mahaboobnagar District, Andhra Pradesh during June 2010. The sex of the plants was identified based on the observations of male and female flowers. The leaves collected separately from 5 male and 5 female trees were immediately placed in polythene covers in an ice box until they were brought to the laboratory and then subsequently placed in a -80°C freezer prior to DNA extraction. The leaf samples collected from male plants were labeled as 1M, 2M, 3M, 4M and 5M and female plants as 1F, 2F, 3F, 4F and 5F.

## Genomic DNA isolation

DNA was extracted from young leaves obtained from 5 male and 5 female trees of *Givotia* by the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1990) with a few modifications. Young leaves (1 g) were ground to a powder in liquid nitrogen using a mortar and pestle. The fine powder was resuspended in 2 ml preheated DNA extraction buffer [2% CTAB (Sigma, St. Louis, USA), 2% PVP (polyvinylpyrrolidone, MW 40,000, Himedia, Mumbai, India), 1.4 M NaCl (sodium chloride, Himedia), 10 mM EDTA (ethylenediaminetetraacetic acid, SRL, Mumbai, India), 100 mM Tris-HCl, pH 8.0 and 0.2% (v/v) β-mercaptoethanol, Himedia]. The samples were incubated at 65°C for 30 min followed by the addition of an equal volume of chloroform and isoamyl alcohol (24: 1) and then centrifuged at 12,000 rpm for 10 min. After centrifugation, the supernatant was taken and the DNA was precipitated by the addition of an equal volume of chilled isopropanol and then placed at -20°C for 30 min. The DNA was collected by centrifugation at 10,000 rpm for 10 min, washed with 70% ethanol, air dried at room temperature and resuspended in 500 µl TE buffer (10 mM Tris, 1 mM EDTA buffer, pH 8.0). Subsequently, it was treated with 0.5 µl of 10 mg/ml RNaseA (Sigma) and incubated for 1 h at 37°C followed by the addition of chloroform: isoamylalcohol (24: 1, v/v). After centrifugation, the supernatant was taken and 1/10 volume of 3M sodium acetate (pH 4.8) was added and then the DNA was precipitated after adding 500 µl pre-chilled isopropanol and placed at -20°C for 15 min and centrifuged at 12,000 rpm for 15 min. The DNA pellet was washed in 70% ethanol, air dried at room temperature and resuspended in 200 µl TE buffer (10 mM Tris, 1 mM EDTA buffer, pH 8.0). Genomic DNA was quantified using a Nanodrop® ND-1000 spectrophotometer (Wilmington, Delaware, USA) at 260 nm and the quality was estimated by the $A_{260}/A_{280}$ ratio. The integrity and concentration was further checked by running the dissolved DNA on a 1.0% agarose (Sigma) electrophoresis gel. The resuspended DNA was then diluted in TE buffer to a concentration of 10 ng/µl and used for PCR amplification.

## RAPD analysis

A total of 32 random primers were initially used to screen the two bulked DNA samples prepared from males and females. Subsequently, 24 random primers which generated amplified bands with the bulked DNA samples of males and females were used to screen the individual DNA samples used to create both bulks for analyzing the genetic variation and identifying the molecular markers linked to sex. The samples of DNA bulks of males and females were prepared by combining equal amounts of genomic DNA from each of 5 males and 5 females, respectively.

RAPD reactions (Williams *et al*. 1990) were performed in a Master Cycler PCR thermocycler (Eppendorf, Hamburg, Germany). The reaction mixture of 20 µl contained 2 µl 10X PCR Buffer (MBI Fermentas, Vilnius, Lithuania), 0.5 µl of 10 mM dNTP (deoxyribonucleotide triphosphate, MBI Fermentas), 2 µl of 10 ρmol/µl primer (Bioserve, Hyderabad, India), 2.5 µl of template DNA (25 ng), 0.2 µl of *Taq* polymerase (5 U/µl, MBI Fermentas), and sterile milli-Q water. PCR amplification was carried out with pre-denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec, primer annealing at 37°C for 1 min and primer extension at 72°C for 2 min followed by 42 cycles of amplification and final extension at 72°C for 5 min; finally amplified products were maintained at 4°C. After completion of PCR, 4.0 µl of 6X loading dye (MBI Fermentas) was added to the 20 µl of amplified products, which were separated on a 1.4% agarose gel in 1X Tris-acetate buffer at 100 V for 2 h. DNA was stained with ethidium bromide (0.5 mg/l, Sigma) and visualized in a UV transilluminator (Uvitec, Cambridge, UK). Gel photographs were scanned through a Gel Documentation System (Syngene, Frederick, USA). The PCR experiments were carried out in triplicate at different times and only the repetitive PCR products were scored. The size of amplified bands was estimated using a 100-3000 or 100-10,000 bp DNA ladder (MBI Fermentas).

RAPD markers were scored for the presence (1) or absence (0) of bands for each primer by examining gel images. Bands with the same mobility were considered as identical fragments and received equal values regardless of their staining intensity. All bands (mono- and polymorphic) were taken into account to calculate similarity so as to avoid over- or underestimation of the distance (Gherardi *et al*. 1998). Pair-wise similarity matrices were generated by Jaccard's coefficient of similarity (Jaccard 1908) by using the SIMQUAL format of NTSYS-pc (Rohlf 1997). A dendrogram based on similarity coefficients was constructed by using the unweighted pair group method using arithmetic averages (UPGMA) and a sequential agglomerative hierarical nested clustering (SAHN) was obtained (Sneath and Sokal 1973).

# RESULTS AND DISCUSSION

## Genetic variability in *Givotia* plants detected by RAPD analysis

The variability available in a population needs to be detected and documented as it is a prerequisite for a selection programme and for devising conservation strategies. Molecular markers such as RAPDs, RFLPs, SSRs and ISSRs have been used for characterizing the genetic diversity in plants belonging to the Euphorbiaceae *viz*., *Hevea brasiliensis* (Besse *et al*. 1994; Varghese *et al*. 1997; Lakawipat *et al*. 2003; Gouvea *et al*. 2010), *Manihot esculenta* (Asante and Offei 2003; Fregene *et al*. 2003), *Jatropha curcas* (Singh *et al*. 2010) and *Ricinus communis* (Bajay *et al*. 2009; Gajera *et al*. 2010). This is the first report to utilize RAPD markers for determining the genetic variability in *Givotia* plants growing at one location. Of the 32 primers screened in the bulk DNA samples of males and females, only 24 produced distinct bands whereas 8 primers (OPA-17, OPAW-07, OPD-14, OPG-08, OPK-09, OPK-10, OPK-16 and OPT-18) did not result in any amplification. The 24 random primers that resulted in amplified bands were used for characterizing the genetic variability of *Givotia* plants at the molecular level. Among the 24 random primers used, 22 resulted in polymorphic banding pattern whereas two *viz*., OPG-06 and OPH-03, produced 4 and 3 bands, respectively, which were monomorphic (**Table 2**). A total of 142 amplified bands were produced by 24 primers of which 86 amplified bands were polymorphic accounting for an average polymorphism of 52.9%. The number of amplified bands ranged from 2 to 11 with a molecular size ranging from 150-2200 bp for different primers tested. The Operon primer, OPAL-08 yielded a maximum number of 11 bands (**Fig. 1**) whereas a minimum number of amplified bands (2) was generated from primer OPC-06. The highest level of polymorphism (100%) was produced by primers OPG-16 and OPS-05, which produced 10 and 5 polymorphic bands, respectively. Primer OPC-07 produced 6 amplified bands, of which only one was polymorphic resulting in a low level of polymorphism (16.7%). The average number of amplified bands/primer was 5.9 whereas the average number of polymorphic bands/primer was 3.58. The intensity of banding varied among *Givotia* plants (**Figs. 1, 2**). In addition, unique amplified bands were generated from 5 primers in male plants (1M, 2M, 3M and 4M) but were absent in other plants. For example, primer OPAL-08 generated a unique band of 900 bp in one male plant (**Table 2; Fig. 1**). Another primer, OPA-18, produced two unique bands of sizes 500 and 750 bp in two male plants that were absent in other plants (**Table 2; Fig. 2**). The present results suggest the occurrence of genetic changes in the genome of *Givotia* plants analyzed which is expected due to its out-breeding nature. Gajera *et al*. (2010) observed a high level of polymorphism (80.2%) using 30 random primers in castor (*Ricinus communis* L.) genotypes. In contrast, a low level of polymorphism (42.0%) was detected with 400 RAPD primers in Indian accessions of *Jatropha curcus* which has been interpreted due to the few introductions that have spread across the country, primarily through vegetative propagation (Basha and Sujatha 2007). Ganesh Ram *et al*. (2008) detected a high level of polymorphism (80.2%) across 8 species of *Jatropha* using 26 RAPD primers thus making it inevitable to exploit wild relatives to broaden the

**Table 2** RAPD data obtained with 24 random primers from genomic DNA of *Givotia* plants.

| Primer | Sequence of the primer (5′-3′) | Size range (bp) | Total number of amplified bands | Total number of polymorphic bands | % Polymorphism | Unique bands |
|---|---|---|---|---|---|---|
| OPA-04 | AAT CGG GCT G | 250-1300 | 5 | 1 | 20.0 | -- |
| OPA-18 | AGG TGA CCG T | 200-1500 | 9 | 7 | 77.8 | 750 bp band in 1M; 500 bp band in 4M |
| OPAB-06 | GTG GCT TGG A | 400-1050 | 6 | 3 | 50.0 | -- |
| OPAK-14 | CTG TCA TGC C | 500-1100 | 3 | 2 | 66.7 | -- |
| OPAL-08 | GTC GCC CTC A | 250-2200 | 11 | 8 | 72.7 | 900 bp band in 1M |
| OPB-12 | CCT TGA CGC A | 150-1400 | 9 | 8 | 88.9 | -- |
| OPC-06 | GAA CGG ACT C | 475-1000 | 2 | 1 | 50.0 | -- |
| OPC-07 | GTC CCG ACG A | 325-900 | 6 | 1 | 16.7 | -- |
| OPC-08 | TGG ACC GGT G | 480-1900 | 8 | 5 | 62.5 | 1300 bp band in 3M |
| OPC-09 | CTC ACC GTC C | 500-1700 | 3 | 1 | 33.3 | -- |
| OPC-10 | TGT CTG GGT G | 450-1200 | 3 | 1 | 33.3 | 1200 bp band in 4M |
| OPF-03 | CCT GAT CAC C | 600-1500 | 4 | 1 | 25.0 | -- |
| OPF-11 | TTG GTA CCC C | 230-1600 | 8 | 3 | 37.5 | -- |
| OPG-06 | GTG CCT AAC C | 400-1400 | 4 | 0 | 0.0 | -- |
| OPG-16 | AGC GTC CTC C | 350-1800 | 10 | 10 | 100.0 | 1000 bp in 2M |
| OPG-17 | ACG ACC GAC A | 350-1100 | 6 | 5 | 83.3 | -- |
| OPH-03 | AGA CGT CCA C | 600-1400 | 3 | 0 | 0.0 | -- |
| OPK-01 | CAT TCG AGC C | 390-2100 | 5 | 2 | 40.0 | -- |
| OPK-07 | AGC GAG CAA G | 350-1800 | 7 | 6 | 85.7 | -- |
| OPS-05 | TTT GGG GCC T | 300-1000 | 5 | 5 | 100.0 | -- |
| OPT-17 | CCA ACG TCG T | 300-2000 | 9 | 7 | 77.8 | -- |
| OPZ-01 | TCT GTG CCA C | 300-900 | 4 | 1 | 25.0 | -- |
| OPZ-06 | GTG CCG TTC A | 300-1200 | 7 | 6 | 85.7 | -- |
| OPZ-10 | CCG ACA AAC C | 350-2000 | 5 | 2 | 40.0 | -- |
| | | Total | 142 | 86 | Average 52.9 | |

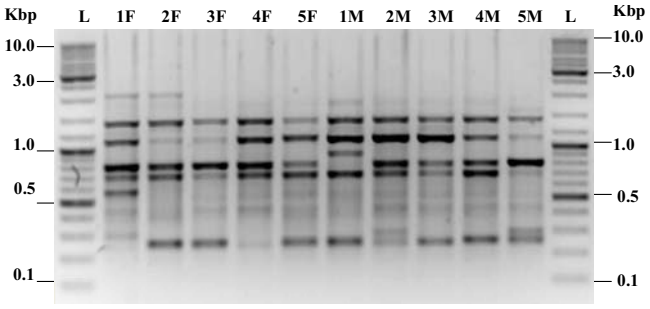


**Fig. 1 PCR amplification profiles of DNA samples of female and male *Givotia* plants produced with RAPD primer OPAL-08.** Lanes designated as L represent DNA ladder (100 – 10,000 bp), lanes 1F, 2F, 3F, 4F and 5F represent individual females, lanes 1M, 2M, 3M, 4M and 5M represent individual males.

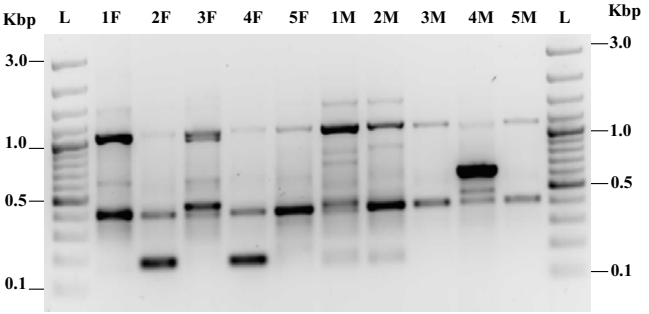


**Fig. 2 PCR amplification profiles of DNA samples of female and male *Givotia* plants produced with primer OPA-18.** Lanes designated as L represent DNA ladder (100 – 3000 bp), lanes 1F, 2F, 3F, 4F & 5F represent individual females, lanes 1M, 2M, 3M, 4M & 5M represent individual males.

genetic base of *J. curcus*. The polymorphism in amplified bands might have resulted from changes in either the sequence of the primer binding site (e.g. point mutations) or changes which altered the size or prevented successful amplification of target DNA (e.g. insertions, deletions, inversions) (Williams *et al*. 1993). Chromosomal crossing-over during meiosis may result in a loss of primer attachment pair sites in the offspring leading to novel RAPD patterns in the offspring (Smith *et al*. 1996). The results of our study corroborate the fact that dioecious tree species, being long-lived, predominantly out-crossed, and often wind-pollinated and dispersed have usually a large proportion of their total neutral genetic variation within populations (Hiltfiker *et al*. 2004). The genetic variation detected by RAPDs in *Givotia* plants may be the combined result of its mating system, wind pollination, seed dispersal, and survival and germination rate of seedlings which in turn are affected by the environment and the intra- and inter-specific competition prevailing among them, as reported in *Hagenia abyssinica* (Asmare 2005).

Cluster analysis was done on the basis of Jaccard's similarity co-efficient generated from pooled RAPD data of 142 amplified bands using 24 primers. Similarity coefficients ranged from 0.642 to 0.829, with the highest similarity of 0.829 observed between male plants 3M and 4M (**Table 3**). Mean RAPD locus similarities between male and female plants was found to be 0.704, with an average of 0.714 for all pair-wise comparisons. Cluster analysis revealed the grouping of *Givotia* plants into 3 clusters at a 70% similarity level (**Fig. 3**). The first cluster comprised of 4 female plants (1F, 2F, 5F and 4F) and 4 male plants (3M, 4M, 5M and 2M) whereas the second and third cluster comprised of 1 male plant (1M) and 1 female plant (3F), respectively. The first cluster was sub-divided into two sub-clusters. The first sub-cluster consisted of 3 female plants (1F, 2F and 5F) with 2 female plants (2F and 5F) forming another sub-cluster within it. The second sub-cluster comprised 4 male plants (3M, 4M, 5M and 2M) and 1 female plant (4F) and had three sub-clusters within it. Thus, cluster analysis revealed a lack of grouping of *Givotia* plants based on sex with the RAPD primers employed. Sharma *et al*. (2009) reported that most of the genotypes of dioecious jajoba (*Simmondsia chinensis* L. Schneider) formed small clusters based on their sex when analyzed by RAPD data, but a dendrogram based on ISSR data showed a more complicated genetic variation pattern with interspersed male and female genotypes.

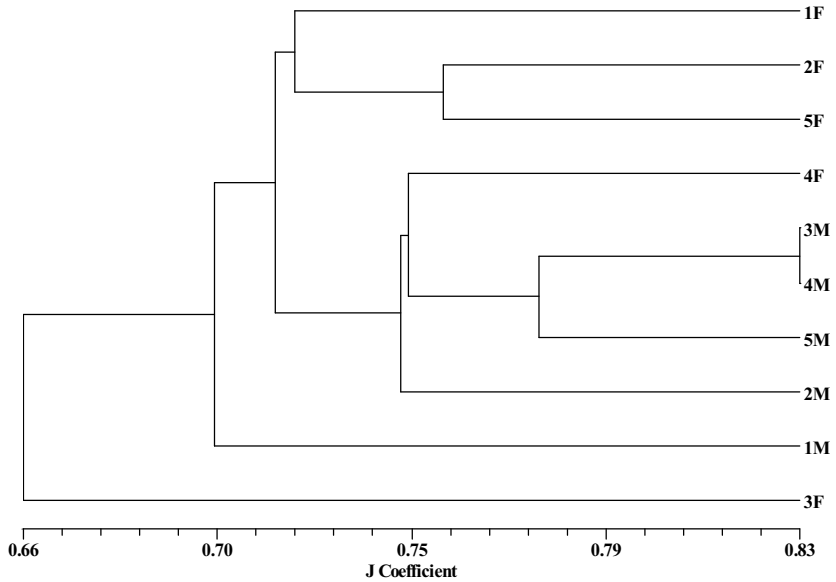


**Fig. 3 Dendrogram of male and female plants of *Givotia* based on Jaccard's similarity coefficient** (1M, 2M, 3M, 4M and 5M represent individual male plants and 1F, 2F, 3F, 4F and 5F represent individual female plants).

**Table 3** Jaccard's similarity matrix of male and female *Givotia* plants revealed by RAPD analysis with 24 random primers.

| | 1Female | 2Female | 3Female | 4Female | 5Female | 1Male | 2Male | 3Male | 4Male | 5Male |
|---|---|---|---|---|---|---|---|---|---|---|
| 1Female | 1.000 | | | | | | | | | |
| 2Female | 0.732 | 1.000 | | | | | | | | |
| 3Female | 0.649 | 0.643 | 1.000 | | | | | | | |
| 4Female | 0.712 | 0.737 | 0.714 | 1.000 | | | | | | |
| 5Female | 0.709 | 0.752 | 0.695 | 0.714 | 1.000 | | | | | |
| 1Male | 0.675 | 0.683 | 0.661 | 0.708 | 0.705 | 1.000 | | | | |
| 2Male | 0.697 | 0.692 | 0.642 | 0.744 | 0.684 | 0.736 | 1.000 | | | |
| 3Male | 0.757 | 0.721 | 0.652 | 0.761 | 0.713 | 0.707 | 0.729 | 1.000 | | |
| 4Male | 0.717 | 0.712 | 0.661 | 0.765 | 0.719 | 0.713 | 0.807 | 0.829 | 1.000 | |
| 5Male | 0.690 | 0.714 | 0.646 | 0.709 | 0.771 | 0.701 | 0.694 | 0.787 | 0.759 | 1.000 |

## RAPD analysis of *Givotia* plants for identification of molecular markers linked to sex

Dioecious plants represent only a small proportion of all plant species and they serve as very good models in the study of sex determination and evolution. In the majority of dioecious species it is difficult to ascertain the sex of seedlings using morphological or chemical methods. Identification of sex at the seedling stage will enormously facilitate the cultivation and breeding by saving time, space and labor cost, otherwise it would be required to grow plants of undesirable sexes (Urasaki *et al*. 2002). Since the sex of *Givotia* cannot be determined from the examination of morphological characteristics until flowering, it would be particularly useful to rapidly determine sex at early stages using DNA markers. Much effort has been made by several research groups to identify sex-linked molecular markers in various dioecious tree species using RAPDs (**Table 1** and the references therein), ISSRs (Gangopadhyay *et al*. 2007; Ehsanpour *et al*. 2008; Sharma *et al*. 2008; Younis *et al*. 2008), AFLPs (Parrish *et al*. 2004), SSRs (Parasnis *et al*. 1999) and SCARs (Deputy *et al*. 2002; Urasaki *et al*. 2002; Xu *et al*. 2004; Yakubov *et al*. 2005; Chaves-Bedoya and Nuñez 2007; Liao *et al*. 2009). In the present study, bulk segregant analysis (BSA) in combination with RAPD has been used for identifying sex-linked markers in *Givotia.* The basic principle of BSA is that the individuals that share a common trait are grouped together so that genomic regions associated with that trait can be studied against a randomized background of unlinked loci (Michelmore *et al*. 1991; Parrish *et al*. 2004). The bulked DNA samples of males and females were screened with 24 random primers in order to identify the amplified bands specific to males and females. The number of amplified bands ranged from 1 to 8 with different primers from the bulk DNAs of male and female plants of *Givotia* (**Table 4**). The total number of amplified bands in the bulk DNA samples was 100 which were less than the total number of amplified bands in individual plants (142); this could be due to less template DNA of individual plants available for amplification in bulk DNA. Sweeney and Dannenberger (1994) reported that bulked DNA samples do not reflect all of the diversity existing within or between cultivars in perennial ryegrass. Thus the amplification products from bulked samples are not simply the sum of amplification products from individual plants.

Of the 24 random primers used in the present study, 12 produced polymorphic bands in bulked DNA samples of males and females whereas monomorphic bands were produced from 12 primers (**Table 4**). Twelve primers generated 26 polymorphic bands in bulk DNA samples of which 17 were specifically present in females and 9 were specifically present in males. Primer OPAL-08 produced of the most amplified bands (8) in bulked DNA samples, 7 of which were monomorphic and one which was specifically present in females (**Fig. 4**). Maximum number of amplified bands (4) specific to females was produced by primer OPT-17 in bulked DNA samples. Primer OPZ-10 generated 3 amplified bands specific to females and one amplified band specific to males in bulked DNA samples (**Fig. 4**). Of the different primers tested, a higher number of bands (2) specific to males was generated by primers OPG-16 and OPK-01. Similarly, Zhao *et al*. (1999) identified few genetic markers for sex identification of *Acer negundo* using bulked DNA samples of 5 males and 5 female plants. Yang *et al*. (2005) tested a total of 1040 RAPD 10-mer primers in *Calamus simplicifolius* and observed that the majority of reactions resulted in monomorphic banding patterns, demonstrating high genomic similarity between males and females of this

**Table 4** Amplification product profile generated from 24 random primers with bulk DNA samples of male and female plants of *Givotia*.

| Primer | Sequence of the primer (5'-3') | No. of amplified bands | | | Total No. of amplified bands | Total No. of polymorphic bands |
|---|---|---|---|---|---|---|
| | | specific to females (bp) | specific to males (bp) | present in males and females | | |
| OPA-04 | AAT CGG GCT G | 0 | 0 | 4 | 4 | 0 |
| OPA-18 | AGG TGA CCG T | 1 | 1 | 2 | 4 | 2 |
| OPAB-06 | GTG GCT TGG A | 0 | 0 | 5 | 5 | 0 |
| OPAK-14 | CTG TCA TGC C | 0 | 0 | 3 | 3 | 0 |
| OPAL-08 | GTC GCC CTC A | 1 | 0 | 7 | 8 | 1 |
| OPB-12 | CCT TGA CGC A | 1 | 0 | 6 | 7 | 1 |
| OPC-06 | GAA CGG ACT C | 0 | 0 | 2 | 2 | 0 |
| OPC-07 | GTC CCG ACG A | 0 | 0 | 1 | 1 | 0 |
| OPC-08 | TGG ACC GGT G | 2 | 1 | 2 | 5 | 3 |
| OPC-09 | CTC ACC GTC C | 0 | 0 | 3 | 3 | 0 |
| OPC-10 | TGT CTG GGT G | 1 | 0 | 2 | 3 | 1 |
| OPF-03 | CCT GAT CAC C | 0 | 0 | 4 | 4 | 0 |
| OPF-11 | TTG GTA CCC C | 2 | 0 | 3 | 5 | 2 |
| OPG-06 | GTG CCT AAC C | 0 | 0 | 3 | 3 | 0 |
| OPG-16 | AGC GTC CTC C | 1 | 2 | 3 | 6 | 3 |
| OPG-17 | ACG ACC GAC A | 0 | 0 | 5 | 5 | 0 |
| OPH-03 | AGA CGT CCA C | 0 | 0 | 3 | 3 | 0 |
| OPK-01 | CAT TCG AGC C | 0 | 2 | 3 | 5 | 2 |
| OPK-07 | AGC GAG CAA G | 0 | 1 | 2 | 3 | 1 |
| OPS-05 | TTT GGG GCC T | 0 | 0 | 4 | 4 | 0 |
| OPT-17 | CCA ACG TCG T | 4 | 0 | 1 | 5 | 4 |
| OPZ-01 | TCT GTG CCA C | 0 | 0 | 3 | 3 | 0 |
| OPZ-06 | GTG CCG TTC A | 1 | 1 | 2 | 4 | 2 |
| OPZ-10 | CCG ACA AAC C | 3 | 1 | 1 | 5 | 4 |
| | Total | 17 | 9 | 74 | 100 | 26 |

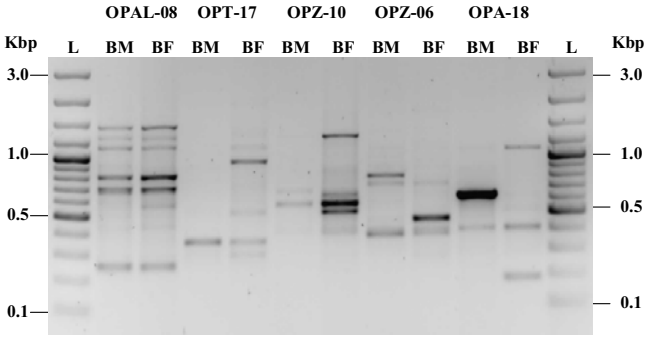


**Fig. 4 PCR-based DNA amplification profiles generated from different RAPD primers in bulk DNA of male and female *Givotia* plants.** Lanes designated as L represent DNA ladder (100 – 3000 bp), BM represent bulk DNA samples of males, BF represent bulk DNA samples of females. The amplified product profiles were generated from the primers, OPAL-08, OPT-17, OPZ-10, OPZ-06 and OPA-18, respectively in bulk males (BM) and bulk females (BF).

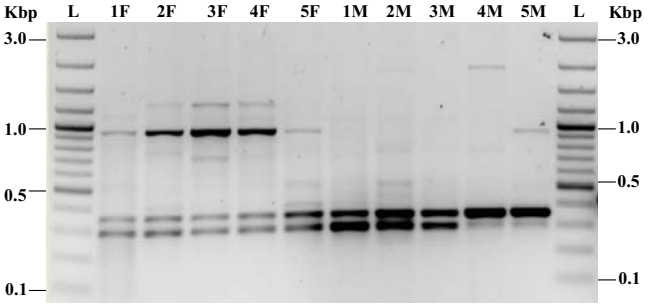


**Fig. 5 RAPD band patterns of five individual female and male *Givotia* plants generated from the primer OPT-17.** Lanes designated as L represent DNA ladder (100 – 3000 bp), lanes 1F, 2F, 3F, 4F and 5F represent the individual females and lanes 1M, 2M, 3M, 4M and 5M represent individual males.

species.

The chances of any RAPD marker being linked to a gene or a genomic region of interest is mainly dependent on genomic size, type of gene or genomic region and on the type of population used for marker analysis (Kumar *et al*. 2008). In the present study, 12 random primers that produced polymorphic bands in DNA bulks of males and females, when tested in individual male and female plants, showed a variable banding pattern in the presence of 11 primers with the amplified bands being observed in both males and females. Therefore the polymorphic bands produced by these primers in the bulked DNA samples are not reliable markers for sex determination as they fail to produce the bands specifically in all males or females when tested in individual plants. Only one primer, OPT-17 showed a partial association with sex producing a 1000-bp amplified band present in all 5 females and one male and absent in 4 other males tested (**Fig. 5**). This result suggests the possibility that the marker is not tightly linked to a sex-determining locus and as a result of recombination the males might have the alleles of the opposite sex. The existence of a high amount of variability in natural populations may also be one of the reasons for the failure to identify markers tightly linked to loci. Shibu *et al*. (2000) reported that primer OPE-11 produced a sex-specific fragment of 416 bp in the dioecious tree, nutmeg (*Myristica fragrans* Houtt.) which was present in 9 out of 10 females and was absent in all male plants collected from two different plantations. Gebler *et al*. (2007) reported that primer OPB-20 produced a band of about 700 bp which was present in all female plants, and in 4 phenotypically male plants of asparagus (*Asparagus officinalis* L.). In the male plants, the band of 700 bp amplified by primer OPB-20 showed a much lower intensity in comparison with female phenotypes possibly because it is linked to the X chromosome. Thus, in female probes with an XX karyotype the template DNA is duplicated and hence the intensity of the band is double in relation to an XY karyotype. Sex determining mechanisms in dioecious plants may be extremely diverse involving heteromorphic sex chromosomes or the allelic constitution at a single locus or several loci possibly interacting in an epistatic manner (Dellaporta and Calderon-Urrea 1993; Lebel-Hardenack and Grant 1997). Sex chromosome based sex determination in dioecious plants might involve the active Y-system or the

X-to-autosome ratio system (Lebel-Hardenack and Grant 1997). The sex-specific markers might represent either the amplified DNA from a sex chromosome or DNA polymorphisms (e.g. point mutations) that are linked to individual sex genes (Welsh and McClelland 1990; Williams *et al*. 1990; Ainsworth 2000). Ling (2003) found one male-associated band after screening 8372 RAPD bands of 1200 primers in *Ginkgo biloba*. The low frequency of sex-linked bands indicated that the DNA segments involved in sex determination are very small and probably represents a single gene or very few genes (Hormaza *et al*. 1994). They concluded that except for the heteromorphic chromosome, the larger the genome was, the more random primers could be needed to find a sex-specific RAPD marker. Further screening of a large number of males and females with more primers might help in identifying molecular marker(s) tightly linked to sex in *Givotia*. Once a tightly linked marker is identified, it could be converted to a SCAR (sequence-characterized amplified region) marker to increase the accuracy and reliability of sex identification. It would also facilitate in identification and isolation of the gene(s) involved in the process of sex determination.

In conclusion, the present study revealed the usefulness of RAPD in detecting genetic variation and also led to the identification of molecular markers exhibiting partial association with sex in *Givotia* plants. The results obtained in the study could further pave the way for characterizing the genetic diversity among and within populations of *G. rottleriformis* growing at different locations and the development of markers for early identification of its sex as well as gaining insight into the mechanism of sex determination. Such studies have important implications in devising appropriate strategies for its breeding, management and conservation.

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