MOLECULAR CHARACTERIZATION OF GENETIC VARIABILITY AND *IN VITRO* PROPAGATION OF *GIVOTIA ROTTLERIFORMIS* GRIFF.

A thesis submitted to the University of Hyderabad for the award of Ph.D. degree in Plant Sciences

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DECLARATION

I, **Mrs. T. Latha** hereby declare that this thesis entitled **"MOLECULAR CHARACTERIZATION OF GENETIC VARIABILITY AND *IN VITRO* PROPAGATION OF *GIVOTIA ROTTLERIFORMIS* GRIFF."** submitted by me under the guidance and supervision of **Prof. G. Padmaja** is a bonafide research work which is also free from plagiarism. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma. I hereby agree that my thesis can be deposited in Shodganga/INFLIBNET.

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CERTIFICATE

This is to certify that this thesis entitled **"MOLECULAR CHARACTERIZATION OF GENETIC VARIABILITY AND *IN VITRO* PROPAGATION OF *GIVOTIA ROTTLERIFORMIS* GRIFF."** is a record of bonafide work done by **Mrs. T. Latha**, a research scholar for Ph.D. programme in Plant Sciences, Department of Plant Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision. The thesis submitted is original and plagiarism free. The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

(Prof. G. Padmaja)
(Supervisor)

(Head of the Department) **(Dean of the School)**

Dedicated to my parents-in-law & husband

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T. Latha

LIST OF ABBREVIATIONS

ABI	Applied Biosystems
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
BAP	6-benzylaminopurine
BLAST	Basic Local Alignment Search Tool
bp	base pair
cm	centimetre
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
dNTP	deoxy Nucleotide Tri Phosphate
EDTA	Ethylenediamine Tetra Acetic Acid
Fig.	Figure(s)
gm	Gram
GA_3	Gibberellic Acid
$HgCl_2$	Mercuric Chloride
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
ISSR	Inter Simple Sequence Repeat
Kg	Kilogram (s)
Km	Kilometer
KN	Kinetin
l	Litre
LB	Luria-Bertani
M	Molar
mg	Milligram
min	Minutes
ml	Millilitre
mm	Millimetre
MS	Murashige and Skoog's
MW	Molecular Weight
µl	Microlitre
mM	Millimolar
nm	Nanometre
$CaCl_2$	Calcium chloride
$MnCl_2$	Manganous chloride
NAA	α-Naphthalene Acetic Acid
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ng	Nanogram
OD	Optical Density
ºC	Degree Celsius
%	Percent
PCA	Principal Coordinates Analysis

PVP	Polyvinylpyrrolidone
PCR	Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
SE	Standard error
Sec	Second
Taq	*Thermus aquaticus*
TE	Tris- EDTA
UPGMA	Unweighted Pair Group Method Based on Arithmetic Average
UV	Ultraviolet
V	Volts
v/v	Volume by Volume
w/v	Weight by Volume
w/w	Weight by Weight
WPM	Woody Plant Medium
IPTG	Isopropyl-beta-D-thiogalactopyranoside
NAA	α-napthaleneacetic acid
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
Xgal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
µM	Micromolar
h	hour
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
µg	Microgram

CONTENTS

1. INTRODUCTION

1.1. Economic importance of *Givotia*:

Givotia rottleriformis Griff. is an economically important tree species belonging to Euphorbiaceae family known for the light and softwood. It is a medium sized tree distributed in the tropics and subtropics of Asia and Srilanka. It is found in forest regions of West Bengal, Tamil Nadu, Andhra Pradesh, Karnataka and coastal Sri Lanka (Madhava Chetty *et al.,* 2008). In India, many of the small scale industries depend on this wood (Reddy *et al.,* 2001). The wood is fairly durable for making toys, trays, carved figures and other fancy articles which are lacquered and painted. The famous Kondapalli, Nirmal and Bengal toys are made from the softwood of *Givotia* (Siva Rama Krishna and Sujatha, 2012). It is also used for making boxes, panels for palkhis, catamarans and other purposes where lightness is an advantage. The seeds of *Givotia* yield oil, which is useful for lubricating machinery. The seeds and bark powder are reported to have medicinal properties and used for treatment of diseases (rheumatism, dandruff and psoriasis) (Thammanna and Narayana Rao, 1990). In Ayurveda, *Givotia* bark is used for setting of bone fractures (Madhira Geetha and Vijayalakshmi, 2013). The endosperm of *Givotia* is mixed in milk and given to children for three days to improve digestion by Pa'l'liyar tribes of southern Tamil Nadu.

1.2.1. Need for genetic diversity studies in *Givotia*:

The natural plant population of *Givotia rottleriformis* is gradually diminishing because of large-scale and destructive exploitation of these plants by toy making artisans coupled with long seed dormancy and poor natural regeneration (Rao *et al*., 1999). The problem is further compounded by wild life damage and difficulty in propagating the species through vegetative methods. This has led to concerns for the long term sustainability of harvesting and the conservation of this valuable toy making tree species. There have been efforts made by the forest department as well as local professionals to raise the plantations through seeds and stem cuttings but have not been successful. Thus multiplication and conservation of this species is the need of the hour.

Milligan *et al.* (1994) reported that, in any programme aimed at conservation of the species, it is important to understand the genetic variation present in the population, structure

of populations and also the major factors that influences the distribution pattern of such variations. Such studies will facilitate in devising strategies to protect the genetic diversity and conservation of this species. During the process of identifying the source material for any plantation, it is important to select the highly diverse populations that are suitable for gene conservation. In studies on genetic variation, the most efficient and most accurate are the DNA markers. These markers are not biased by the environmental factors, the phase of ontogenic development of analyzed individuals, the kind of tissue used for the analysis, and the regulation of gene activity as well as modifications taking place after transcription and translation.

The genetic structure of any population is determined by movement of pollen and seeds (Dow and Ashley, 1996). *Givotia* being a dioecious tree species harbours high level of heterozygosity due to cross pollination. The flowers of maternal parent receive pollen grains from different sources thereby generating half-sib seedling progeny of a particular genotype thus constituting natural recombinants. Identification and selection of plus trees with desirable traits are an integral part of any tree improvement programme for producing good quality progenies. The key to success in such programmes is the knowledge related to diversity existing within plus trees as well as its progenies. Recently, a few studies have been conducted with half-sib progenies to investigate the issue of relatedness in open-pollinated families with the help of DNA markers in different plant species (Dow and Ashley, 1996; Asolkar *et al*., 2011; De Greef *et al*., 1998). Molecular characterization of half-sib progenies along with its maternal plants would be useful in assessing the genetic similarities and variation and would unravel the pollination patterns in any population. Such information would be valuable for the development of highly productive plantations from seed progenies of plus trees or future breeding programmes or conservation activities.

1.2.2. Dioecy and sexual dimorphism in plants:

Majority of flowering plants in nature are hermaphroditic that exhibit a variety of reproductive systems with perfect flowers (Barrett, 2002), but approximately 7.6% of genera and 6% of 240,000 species of angiosperm present are dioecious in nature (Renner and Ricklefs, 1995). These plants have male and female flowers on completely different plants. The dioecious nature exhibited by plants provides an excellent opportunity for the examination of

trade-offs in resource allocation that is related to plant reproduction (Cepeda-Cornejo and Dirzo, 2010). Also, higher costs of reproduction paid by female plants compared to male plants (Delph, 1999; Obeso, 2002) can lead to sexual dimorphism in attributes related to resource acquisition. In general, the evolution of dioecious nature include the avoidance of inbreeding depression (Lloyd, 1975) and changes that occur within the resource allocation that permit female plants to produce twice as many seeds than hermaphrodites (Charlesworth, 1999).

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. Molecular markers have been used to determine sex in various dioecious tree species. There has been no attempt made towards 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.

1.2.3. Molecular markers for genetic diversity studies and rapid identification of sex:

A number of polymerase chain reaction (PCR)-based DNA markers, including restriction fragment length polymorphism (RFLP) (Botstein *et al.*, 1980), simple sequence polymorphic DNA (Tautz, 1989), amplified fragment length polymorphism (AFLP) (Zabeau and Vos, 1993), random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) and inter simple sequence repeat (ISSR) (Zietkiewicz *et al.*, 1994) have been used to identify varieties or germplasm, assess genetic diversity or relationships, tag or map or pyramid desirable genes, early selection of superior genotypes and to protect released varieties (Joshi *et al.,* 1999; Thottappilly *et al.,* 2000).

RAPD technique is technically simple, inexpensive, and requires no previous sequence information on the target genome (Williams *et al.,* 1990). It has been widely used for different applications owing to their ability to detect DNA variation at many loci using small amounts of tissue and relatively quick to perform. Inter Simple Sequence Repeats (ISSRs) are microsatellite-derived useful in genetic fingerprinting experiments and similar to RAPDs since

both require no prior knowledge of the genome, cloning or specific primer design, yet has higher reproducibility than RAPDs because of high annealing temperature, and the cost of the analysis is lower than the cost of RFLP and AFLPs (Zietkiewcicz *et al.*, 1994). Both RAPDs and ISSRs markers 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.

The sequence-characterized amplified region (SCAR) markers, generally developed from ISSR and RAPD fragments, are locus-specific and more reliable and more reproducible for molecular identification (Paran and Michelmore, 1993). RAPD derived SCAR markers have been successfully used for plant identification at inter-and intra-species levels (Roh *et al.*, 2006; Choi *et al.*, 2008). SCAR markers have been developed for identification of different varieties, cultivars and different populations of *Jatropha curcas* (Mastan *et al.*, 2012). SCAR markers linked to sex-specific genes have been developed in many dioecious plants like *Carica papaya* (Chaves-Bedoya and Nunez, 2006), *Cannabis sativa* (Mandolino *et al.*, 1999), *Pistacia vera* (Yakubov *et al.*, 2005), *Asparagus officinalis* (Gao *et al.*, 2007), *Cycas circinalis* (Gangopadhyay *et al.*, 2007), *Coccinia grandis* (Bhowmick *et al.*, 2014), *Pistacia atlantica* and *Pistacia khinjuk* (Esfandiyari *et al.*, 2011), *Garcinia gummi-gutta* (Joseph *et al.*, 2014), and *Ginkgo biloba* (Liao *et al.*, 2009).

1.3. Propagation of *Givotia:*

Propagation of *Givotia rottleriformis* through conventional methods remains problematic through seeds as well as stem cuttings. The seeds of *Givotia* exhibit prolonged seed dormancy of 1-1½ years and propagation through seeds is hampered by a low germination rate. The hard seed coats along with high phenolics present in the seeds are mainly responsible for long seed dormancy (Reddy *et al.*, 2001) in natural conditions although the exact factors involved in seed dormancy have not been investigated so far. Previous attempts to break seed dormancy and to initiate seed germination by giving different treatments have not been very successful. Naidu *et al.* (2001) used scarification methods such as treatment with hot water and feeding to goats, acid treatment which ended without success. Treatment of the seeds with GA_3 was not effective in breaking the seed dormancy as the seedlings failed to germinate (Kiran *et al.,* 2001). The effectiveness of GA_3 in promoting *in vivo* seed germination has been reported by Rambabu *et al.* (2006), but these methods could not be adapted for raising the plantations on large scale.

Vegetative propagation through softwood stem cuttings of *Givotia* has been achieved but the success rates were found to be highly dependent on the age of the stem cuttings, season specificity and treatment with IBA (Reddy *et al*., 2001; Babitha, 2002). Additionally, obtaining sufficient number of softwood cuttings from the trees in the forests is difficult and labour intensive thus restricting their multiplication at commercial level. Thus there is a need to adopt efficient *in vitro* propagation approaches for mass cultivation of this species.

In vitro techniques hold great importance in overcoming the difficulties associated with the propagation of the species using conventional methods. The success of using *in vitro* methods for rapid multiplication or genetic transformation relies on the availability of efficient regeneration systems employing organogenesis or somatic embryogenesis. *In vitro* tree propagation methods are relatively difficult with explants obtained from mature trees as compared to seedling derived explants. The major difficulties faced in successful establishment and proliferation of cultures are leaf drop, browning of culture media, slow multiplication percentage, internodal elongation, rooting of the shoots and infection by endophytic bacteria or fungi (Guitierrez *et al*., 2011). A greater attention is therefore required to overcome these problems as they also would affect the survival of plants during acclimatization.

In vitro regeneration has been successfully achieved using different explants in a few genera of Euphorbiaceae such as *Emblica*, *Givotia*, *Hevea*, *Jatropha*, *Manihot* and *Ricinus* (Rajesh *et al*., 2009). It is well known from the literature that the process leading to plant regeneration in any plant species are influenced by various factors such as genotype, explant type, developmental stage of the explant, culture media, growth regulators and culture conditions. Until now, there are three published papers on *in vitro* culture of *Givotia rottleriformis*. Rambabu *et al.* (2006) reported 100% germination frequency from zygotic embryos of *Givotia* on MS (Murashige and Skoog's) basal media with 3% sucrose and addition of tyrosine resulted in promoting seedling growth in the basal medium. *In vitro* germination and micropropagation of *Givotia* using shoot tip explants of *in vitro* germinated seedlings has been achieved, and *in vitro* generated seedlings as well as tissue culture raised plantlets have been established in the soil (Samuel *et al,*. 2009). Samuel *et al.* (2012) examined the effect of temperature pre-treatment on *Givotia* seeds to overcome the zygotic embryo dormancy that resulted in rapid *in vitro* development of emblings. To the best of our knowledge, there are no reports on micropropagation using explants collected from mature trees of *Givotia*. The *in vitro*

regeneration potential of epicotyl and hypocotyl explants derived from seedlings of *Givotia* has not been investigated.

The most crucial concern during *in vitro* propagation is the genetic integrity of the clonally propagated plants with respect to the mother plants. The concept of producing genetically stable plants has been questioned due to detection of somaclonal variants in micropropagated plants (Rani *et al.,* 2000; Devarumath *et al.,* 2002). The explant type, the donor genotype, conditions of physical culture, the composition of the culture medium and the duration between successive subcultures affect the frequency of variants (Vasil and Vasil, 1981; Ahuja, 1987). It is important to establish the genetic uniformity of micropropagated plants before they are used for commercial purposes. The clonal fidelity of the micropropagated plants can be examined by phenotypic or allozyme or molecular analysis. Phenotypic identification based on description of the morphological and physiological characteristics of the desired traits requires an in-depth observation of the plants until maturity. This is not practical in tree species due to long generation cycles. Moreover, the absence of visible variations does not preclude the absence of all variation among micropropagated plants. Cryptic somaclonal variation can be identified using allozyme markers but these markers are limited by both number and amount of polymorphism and their developmentally regulated expression. These days, molecular markers have overcome the problems associated with phenotype-based and allozyme analysis. DNA markers, especially RAPDs have proved useful for this purpose owing to their ability to analyze DNA variation at many loci using small amounts of tissue (William *et al*., 1990). RAPDs have been applied for characterization of micropropagated plants of different forest trees (Rahman and Rajora, 2001; Nas *et al*., 2004). Therefore, in the present study, the RAPD method was employed to ascertain the genetic stability of the micropropagated plants of *Givotia*.

1.4. Importance of oils and fatty acids:

Fats and oils are one of the most important components of present human food since many years ago. The available forms of oils in the diet are as fatty acids. Fatty acids represent 30-35% of total energy intake in many industrial countries and the most important dietary sources of fatty acids are vegetable oils, dairy products, meat products, grain and fatty fish or fish oils (Rustan and Drevon, 2005). The third most important agricultural products after meat

and cereal are oil seeds that are important products for global trading. Vegetable oils are important not only for nutritional purposes but also as raw materials for a wide range of industrial products, which include fuels, skin care products, lubricants, water-paint binder and soap making. Human consumption accounts for 80% of oil consumption in the world (Luhs and Friedt, 1994). About 6% is used as animal feed, and the remaining is used in industrial applications. As per the data available from the Solvent Extractors Association of India (SEA), India's consumption of edible oil has been increased to around 17.5 million metric ton (mmt) in 2012 to 2013 from 11.6 mmt in 2003 to 2004 with a compounded annual growth rate (CAGR) of 4.6% during the period under consideration. It is estimated that demand is likely to reach 23 mmt by 2019 to 2020.

As the world population is expected to double by the turn of the present century, it is anticipated that a serious global crisis will arise for the need of oil for edible and non-edible purposes. Consequently the programme of screening uncultivated seed oils has been initiated in many advanced countries with a view to discover new seed oils and fatty acids which may lead them to practical utilization. The utilization of oil as a source of energy depends on its well digestible property and also its absorption into the body (Tannenbaum, 1979). The quality of the oil, flavour and odours depend on the fatty acid composition (Leon *et al.,* 2004). A number of reports have shown that the oil content and concentrations of fatty acids in the seed oil are strongly affected by the climate, crop season, environmental conditions, production process and degree of maturation.

Fatty acids and/or their derived metabolites are also recognized as signalling molecules central to various biological processes (Savchenko *et al*., 2010). They are known to affect cellular activities through changes in membrane lipid composition and the generation of a diversity of bioactive derivatives. Structural properties of fatty acids such as their chain length and their degree of desaturation, largely determine the nature of these processes. Generally, fatty acids (FAs) are classified as saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. Oils that contain higher monounsaturated fatty acids (MUFAs) and lower saturated fatty acids (SFAs) are preferred because of the beneficial effect of MUFAs on serum cholesterol levels (Abouzar Hashempour *et al.,* 2010). MUFA are fatty acids with a single double bond in the molecule. Oleic acid (C18:1n-9) is the most abundant MUFA in the diet (Alonso *et al.,* 2005). Stability of oils is mainly influenced by the fatty acid

composition and PUFAs have been found to play a major role in the rancidification of most of the oils (Leon *et al.,* 2004). Several studies suggest that it is important that the proper amount of antioxidants is included in the diet with the PUFA to decrease the risk of lipid peroxidation. Literature showed that seeds of *Givotia* contain oil which is used in industry for lubricating fine machinery. However, there is no published information related to the oil content and fatty acid composition in seeds of *Givotia*. Thus there is a need to explore the potential of seeds of *Givotia* with respect to oil content and fatty acid composition.

The present study aimed at characterizing the genetic variability in different accessions of *Givotia* collected from forest regions using RAPD and ISSR markers. In addition, the molecular diversity in male and female plants was assessed using RAPD and ISSR markers in order to explore the possibility for the identification of sex specific marker. *In vitro* regeneration potential of nodal and shoot tip explants from mature trees and epicotyl and hypocotyl explants of *in vitro* germinated emblings was examined and the micropropagated plants derived from nodal explants were assessed by RAPD method. The study also focused on characterizing the variability for fruit and seed traits, seed oil content of different accessions and fatty acid composition of *Givotia* seed oil.

The specific objectives of the work are as follows:

- Assessment of genetic variability in *Givotia rottleriformis* trees growing at different forest regions using RAPD and ISSR markers.
- Molecular characterization of genetic variation in male and female trees and identification of molecular marker(s) linked to sex, if any.
- Development of methods for rapid *in vitro* propagation using different explants and genetic fidelity analysis of *in vitro* raised plantlets using RAPD markers.
- Analysis of fruit and seed traits, seed oil content of different accessions and fatty acid profile in seed oil of *Givotia*.

2. REVIEW OF LITERATURE

2.1.1. Softwood plants:

Forests are considered as an integral part of the world's ecosystems consisting approximately 80,000 to 100,000 different tree species. It is estimated that 31% of land area globally is occupied by different tree species (Food and Agricultural Organization (FAO), United Nations). Despite the high number of known plant species present in the nature, approximately 450 different tree species present in the forests are actively taken part in domestication purposes through various tree improvement programmes (FAO: http://www/fao.org/docrep/meeting/028/mg468e.pdf). In the present developing world, people are excessively depending on forest tree products such as wood-based fuels or non-timber based products for health and nutrition for their sustainable livelihoods.

The family of Euphorbiaceae includes nearly 322 genera and 8910 species (Bingtao Li *et al*., 2008) and several of these have their own economic importance and therefore contribute to the floristic wealth of tropical and subtropic countries of the planet (Rajesh *et al*., 2009). The genus *Givotia* belonging to Euphorbiaceae, consists of four closely related species with geographically separated distributions in India, Srilanka and Africa. *Givotia gosai* is found in Ethiopia, Somalia and Kenya; *Givotia stipularis* and *Givotia madagascariensis* are found in Madagascar; *Givotia moluccana* or *Givotia rottleriformis* Griff. are confined to India and Srilanka (http://www.kew.org).

There are only a few softwood producing plants in the world. A wide range of tree species such as *Gardenia latifolia*, *Gyrocarpus americanus*, *Wrightia tinctoria*, *Givotia rottleriformis, Santalum album*, *Dalbergia sissoo* and *Cedrus deodara* are known for timber of commercial value used traditionally in the toymaking sector (Aggarwal *et al*., 2013). The wood of these species are used for making toys throughout India and especially in the north, northeast, central and south, depending on the availability of raw materials. The major species known for the softwood and used for toy making in Southern India are *Gardenia latifolia*, *Gyrocarpus americanus*, *Wrightia tinctoria* and *Givotia rottleriformis* (Rao *et al*., 1999). Among these four species, *Givotia rottleriformis* Griff. has been considered to be the best as it produces high quality softwood with excellent properties suitable for toy making. The toys made from these species depict countries rich history, mythologies, legends, folklore and plant and animal life (Fig. 1a-f). In addition, the

wood of this species is used for making panels, boxes and catamarans where lightness is required. The softwood is used for making beautiful toys particularly by artisans by Kondapalli village in Krishna district and Nirmal town in Adilabad district of Andhra Pradesh. The toys made from this species have very good demand in domestic and international markets and are exported to countries like the United States, Japan, West Germany and Singapore. In natural habitat, the population of *Givotia* is gradually declining due to large scale destructive harvesting of trees for softwood either for toy making or for other commercial uses. The problem is further compounded by poor natural regeneration of the seed, wild life damage and difficulty in propagating through vegetative methods. This has led to concerns for the long term sustainability of harvesting and the conservation of this species.

2.1.2. Distribution and description of *Givotia*:

Givotia rottleriformis Griff. is a medium sized tree (Fig. 2a) mainly found in dry deciduous forests of India. It is available in limited pockets in forests of Tamil Nadu, Andhra Pradesh, West Bengal, Karnataka and coastal Sri Lanka (Madhava Chetty *et al.,* 2008). It is commonly known as Tella Poliki, Konda Ponaku or Yella Dabba in Telugu.

The leaves of *Givotia* are alternate, rounded and cordiform, toothed, base 5-7 nerved, with many veins. Leaf fall is commonly seen in March-April. Petiole is stout, 4-6 inches in length with typically a couple of glands on it. *Givotia* bark is brown, smooth shedding in circular thick bosses. Bark yields red blood latex after injury (Fig. 2b). Flowers are dioecious, axillary and sub-terminal raceme cymes with 4-8 inches long (Fig. 2c). Male flower is 1/6 inch in diameter. Female flower is 1/4 inch in diameter (Fig. 2d). It flowers within the hot season *i.e.* during April to May and fruiting is observed from June. Fruit is single seeded subglobose drupe like ¾ -1 inch in diameter. The mature fruits have three layers of pericarp consisting of epicarp, mesocarp and stony hard endocarp (Fig. 2e). The shoot base of the seedlings is tuberous having well developed roots (Fig. 2f).

2.1.3. Need of research in *Givotia*:

The species is gradually dwindling due to intensive logging for its high quality wood which has multiple uses. The long seed dormancy, poor natural regeneration and lack of efficient methods for its propagation either by seeds or vegetative cuttings have compounded the problem. The knowledge of molecular genetic diversity is also important for facilitating

breeding in *Givotia* towards development of high yielding trees as well as conservation of valuable genotypes. There is no published report on chromosome number of this species. The pollination mechanisms in *Givotia* are still unknown. The sex identification procedures remain to be investigated. Development of efficient tissue culture methods is important for propagation of plus trees of *Givotia*. There is also a need to analyze the variability that is present in different seed traits, percentage of oil and fatty acid composition of seed oil for tapping it as a source of edible or industrial oil.

2.2.1. Molecular markers:

Molecular markers are naturally occurring polymorphism which includes proteins and nucleic acids that are detectably different (Thottappilly *et al.,* 2000). Phenotypic traits that are used for estimating genetic diversity are generally affected by environmental conditions. Fast advances in genome research and molecular biology led to the use of DNA markers which have complemented the classical methods. The advantage of using DNA markers is due to their capacity to detect genetic diversity at a better level of resolution than other methods (Sergio and Gianni, 2005).

2.2.2. Random amplified polymorphic DNA (RAPD):

RAPD marker detects the polymorphic sequences in deoxyribonucleic acid by employing a single random primer (oligonucleotide primer, principally 10 bases long) (William *et al.,* 1990). These oligonucleotides function as forward and reverse primers and amplify fragments from 1-10 genome sites at the same time. These polymorphisms are thought of primarily due to the variation in primer annealing sites. Kumar *et al.* (2009) reported that every amplicon is derived from a genomic region that contains 2 short segments in inverted orientation, on opposite strands that are complementary to the primer. 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). Due to its technical simplicity and speed, 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 using molecular markers.

2.2.3. Inter simple sequence repeats (ISSR):

ISSRs are DNA fragments of approximately 100-3000 bp set between adjacent, oppositely adjusted microsatellite regions. Zietkiewicz *et al.* (1994) primarily reported ISSRs by utilizing primers that supported microsatellites. ISSRs are amplified by PCR utilizing microsatellite core sequences as primers with some selective nucleotides that anchor into the non-repeat adjacent regions (16-18 bp). ISSR markers do not need any previous data of the SSR targets sequences, are reproducible as a result of their primer length, extremely tight as a result of its annealing temperature and were found to produce polymorphic fingerprints (Kumar *et al.,* 2009). As a result of the multilocus procedure profiles obtained, ISSR analysis was applied in studies involving genetic identity, parentage, taxonomic categorization studies of closely connected species, clone and strain identification and helpful in genetic mapping studies (Verma *et al.,* 2007; Kallamadi *et al.*, 2015).

2.2.4. Molecular markers in genetic diversity:

The major key component in the long term survival of the species is genetic diversity that is maintained by the nature in all the species. It forms the basis and provides the raw material for survival and evolution of species and individuals, especially during environmental changes and disease occurrences (Erikson *et al.*, 1993). 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).

There has been greater attention paid to study the extent of genetic variation in *Jatropha* and castor belonging to Euphorbiaceae. Basha and Sujatha (2007) identified population-specific bands for the accession from Kerala (2 RAPD markers), Neemuch-1 from Rajasthan (1 each of RAPD and ISSR markers) and the non-toxic genotype from Mexico (17 RAPD and 4 ISSR markers), for use as diagnostic markers in genotyping. Ganesh Ram *et al.* (2008) reported that 18 random primers gave reproducible amplification banding patterns of 112 polymorphic bands out of 134 bands scored accounting for 80.2% polymorphism among 8 species of *Jatropha*. Basha *et al.* (2009) reported narrow genetic variation among accessions from different regions of the world and rich diversity among Mexican genotypes in terms of phorbol ester

content and distinct molecular profiles. Molecular characterization of 72 *J. curcus* accessions representing 13 countries has been carried out by Basha *et al.* (2009) which revealed polymorphism of 61.8% and 35.5% with RAPD and ISSR primers, respectively.

Cai *et al.* (2010) investigated the genetic diversity of Chinese *Jatropha* germplasm by using 15 ISSR primers which revealed 75.2% polymorphism. Rafii *et al.* (2012) used eight primers for analyzing the genetic diversity in 48 accessions of *J. curcas* and found that they grouped together according to their geographical origin. Gautam Murty *et al.* (2013) compared RAPD, ISSR and DAMD (directly amplified minisatellite DNA) markers for genetic diversity assessment between accession of *Jatropha curcas* and its related species. They reported that all the markers are equally efficient for diversity studies in *Jatropha*.

The extent of genetic diversity in 31 accessions of castor representing seven geographic areas in the world was assessed using RAPD, ISSR and SCoT (start codon targeted polymorphism) primers (Kallamadi *et al.*, 2015). The study indicated modest level of genetic variability in the accessions analyzed and also identification of accessions with several unique bands. Vivodik *et al.* (2014) assessed the genetic variability among the set of 40 castor genotypes using 8 RAPD markers. The dendrogram based on hierarchical cluster analysis grouped the genotypes into two main clusters and only two genotypes could not be distinguished.

2.2.5. Molecular markers in sex identification:

In dioecious plant 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; 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). RAPD markers have been used to determine sex in various dioecious tree species as indicated in Table (A). To our knowledge, there is no information on sex chromosomes in male and female plants of this species.

2.2.6. SCAR markers for identification of species:

The reproducibility of RAPD is affected by many factors; the quality of template DNA, the concentration of primer and template, the different sources of DNA polymerase etc. The sequence characterized amplified region (SCAR), commonly converted from RAPD, and is a locus-specific or population specific method that amplify bands at higher annealing temperature with more reliability and more reproducibility for molecular identification (Paran and Michelmore, 1993). Apart from RAPD markers, SCAR markers have been developed from other more reproducible markers like AFLP (Vos *et al.,* 1995), SSR (Weber and May, 1989) and ISSR (Zeikiewicz *et al.,* 1994). When compared with SCAR markers developed with RAPD, the development of SCAR markers from other markers is costly, difficult for handling and time consuming. SCAR primers target the unknown sequence of the genomic DNA. Even though SCARs are not highly polymorphic they help in rapid marker development. SCAR markers are not affected by the presence of introns that could eliminate the primer binding sites. Compared to random primers, SCAR primers exhibited several advantages in map-based cloning, mapping studies, homology studies among related species, detection of sub-species, marker assisted selection and sex identification. The specificity, annealing temperatures and annealing time are partially dependent on SCAR primer length and its GC (guanine-cytosine) content (Vanichanon *et al.,* 2000).

SCAR markers have been successfully developed for identification of species or varieties or as a diagnostic marker in different species of Euphorbiaceae. Theerakulpisut *et al.* (2008) developed species-specific SCAR markers for identifying and differentiating morphologically similar *Phyllanthus* species. SCAR primers efficiently amplified 408, 501 and 319 bp bands unique to *P. amarus*, *P. debilis* and *P. urinaria*, respectively. Basha and Sujatha (2007) identified polymorphic ISSR markers that could differentiate the Indian accessions from the Mexican genotype and two of them were converted to SCAR markers. The SCAR primer pair ISPJ1 amplified a 543 bp fragment in all the Indian populations, while ISPJ2 with a specific amplicon of 1096 bp was specific to the Mexican genotype. Subsequently, three SCAR markers (RSPJ-1, RSPJ-2 and ISPJ-3) were developed to differentiate non-toxic Mexican genotypes from the toxic genotypes collected from the rest of the world (Basha *et al.*, 2009). Mastan *et al.* (2012) selected four markers specific for toxic and non-toxic varieties, purified, cloned, sequenced, and designed primers out of which one set of primers could able to

discriminate the non-toxic with toxic *Jatropha* by giving expected 430 bp size amplification in non-toxic variety.

2.2.7. SCAR markers for sex identification:

SCAR markers are produced for identification of the sex of several plants at juvenile stage before flowering. Codominant SCAR markers are produced by digesting the monomorphic amplified fragments with different restriction enzymes (Paran and Michelmore, 1993). In contrast, Jiang and Sink (1997) reported that by changing the annealing temperature, two different results were obtained with SCAR markers in male and female plants. While 60°C annealing temperature resulted in amplification of locus M in both the parents and 67°C annealing temperature resulted in amplification of only one band in males and none in females. Mandolino *et al.* (1999) developed a SCAR marker from the 400 bp amplified fragment generated from OPA-08 primer for a precise, early and rapid identification of male plants during breeding programs of dioecious and monoecious hemp (*Cannabis sativa* L.). Parasnis *et al.* (2000) developed a male-specific SCAR marker in papaya by cloning a male-specific RAPD (831 bp) fragment and designing longer primers. Further a simplified and highly accurate sex diagnostic assay was developed using SCAR markers for mass screening of papaya seedlings.

Gao *et al.* (2007) presented the development of SCAR which generated a 928 bp marker for correctly screening homozygous mm female plants of *Asparagus officinalis* plants. However, the results of Southern blot analysis indicated that S368-928 fragment was present in both male and female genomes. Chaves-Bedoya and Nunez (2006) found a RAPD marker of 900 bp in male plants, but not in females or hermaphrodites which was used to develop SCAR that amplified fragments from the genomes of male and hermaphrodite plants but not the female plants of *Carica papaya*.

Liao *et al.* (2009) conducted studies for sex identification in *Ginkgo biloba* and showed that SCAR primer GBA amplified a single 571 bp band in male samples but not in female samples, whereas the primer GBB generated a 688 bp band only in the female individuals. Esfandiyari *et al.* (2011) used SCAR-PCR in sex determination of two wild *Pistacia* seedlings. According to the results, a 300 bp fragment was amplified in all female trees which was absent in male trees. Dhawan *et al.* (2013) cloned and sequenced a fragment of -1.0 kb amplified by OPA-02 and SCAR primer was designed that amplified a fragment of 354 bp in only male

genotypes of date palm (*Phoenix dactylifera*). Joseph *et al.* (2014) screened 150 randomly amplified polymorphic DNA primers in *Garcinia gummi-gutta* and only one of them (OPBD20) showed different amplification band pattern associated with sex type. The sex-linked DNA fragment was converted to SCAR which amplified a 556 bp band in male samples but not in female ones. It was noted that the fragment from both sexes were amplified at relatively low annealing temperature.

2.3.1. Need for *in vitro* propagation of *Givotia*:

Givotia rottleriformis can be propagated by seeds and stem cuttings. The propagation of *Givotia* through seeds is very difficult due to long seed dormancy associated with poor natural regeneration. Various researchers have attempted to overcome the problems of propagation of *Givotia* by using conventional methods. Several treatments such as sulphuric acid, hot water and feeding to goats were used to break seed dormancy in *Givotia* which were unsuccessful (Naidu *et al.*, 2001). Kiran *et al.* (2001) conducted experiments to break the seed dormancy of this tree species by pre-treatment of the seeds with GA_3 for different durations. They observed that high concentration of gibberellic acid (2000 ppm) induced germination at a frequency of 40%, when the seeds were treated for 72 hrs. Germination commenced and continued up to 40 days, and subsequently no seedlings emerged. Murthy (1992) conducted experiments to vegetatively propagate this tree species through stem cuttings, which did not yield positive results. Reddy *et al.* (2001) evaluated the effects of three auxins, IAA, IBA and NAA and seasons on rooting of stem cuttings of *Givotia* and reported a higher percentage of rooting in the presence of NAA whereas controls did not show any rooting response in both the seasons. Babitha *et al.* (2002) observed rooting in the stem cuttings of *Givotia* treated with IBA but could not be exploited on large scale due to scarcity of stem cuttings. Moreover, this method is not practicable as the efficiency of root induction is majorly influenced by environmental conditions during rooting (Nautiyal *et al.*, 1992).

Tree improvement by conventional breeding is a slow process because of the long juvenile period and high heterozygosity (Singh *et al.*, 2002). Propagation of woody trees through tissue culture confers advantages in terms of fast multiplication, production of disease-free plants, season-independent production of plants, quick release of improved cultivars and germplasm conservation. *In vitro* approaches have been successfully used in the large scale

propagation and genetic improvement of the tree species (Pena and Sequin, 2001; Peacock, 1995).

Most investigators working with trees have found that seeds and juvenile tissues are more amenable for *in vitro* manipulation than explants taken from trees in the adult growth phase. In order to achieve cloning, it is desirable to utilize explants from selected plus trees as they result in production of genetically similar plants. In some cases, axillary bud or shoot tip based micropropagation has also resulted in somaclonal variation (Rani and Raina, 2000; Rahman and Rajora, 2001). Micropropagation using explants from trees grown in natural habitats has certain limitations. The availability of suitable explants, collection, establishment of aseptic cultures due to high level of contamination, phenolic exudation and recalcitrance of these explants for regeneration and low rates of multiplication restrict the use of these explants for mass plant production. Alternatively, the explants obtained from *in vitro* germinated seedlings or emblings can be used as source of explants for micropropagation. The use of seedlings for micropropagation would lead to generation of genetically variable progeny in dioecious species owing to cross pollination and existence of high level of heterozygosity. The seedlings can be preferred when it is advantageous to have genetic diversity in the population. Previous studies have demonstrated that adventitious shoot formation directly from the excised organs without a callus phase, although less reliable than axillary bud proliferation, is definitely a better approach than callus methods for propagation of plants (Borcheria *et al*., 2009).

A number of factors restrict tissue culture response of tree explants, including explanting season, age of the donor tree, and type of primary explant. Excessive phenolic exudation, basal callusing of shoot explants, vitrification, and shoot tip necrosis also reduce the efficiency of tree micropropagation (Timofeeva *et al*., 2010). Nevertheless, *in vitro* propagation has been achieved in several tree species using nodal explants or shoot tip explants. Quraishi *et al*. (1996) determined the suitable medium and season for *in vitro* plantlet production from juvenile nodal explants of *Cleistanthus collinus*. They observed that April to June was the best time for initiating shoot cultures with MS medium containing 1.1 μM BAP being the best medium for shoot multiplication. Phulwaria *et al*. (2011) had overcome the problem of phenolic exudation from nodal explants of mature trees of *Terminalia bellirica* by treating them with antioxidant solution of ascorbic acid and citric acid. Mohan Reddy and Saritha (2013) achieved clonal propagation of *Gardenia latifolia*, a toy-making tree species using mature nodal

explants on MS medium fortified with cytokinins (BAP/KN/2-ip) (1.0-5.0 mg/l) in combination with IAA (0.5 mg/l). Shoot tips were found to be superior to nodal segments in the micropropagation of *Acacia catechu* (Kaur and Kant, 2004) and *Sorbus domestica* (Arrillaga *et al*., 1991).

There have been numerous reports on *in vitro* regeneration from different explants in *Jatropha* and *Ricinus* which are economically important oil producing tree species of Euphorbiceae family. Datta *et al*. (2007) achieved higher rates of multiplication from nodal explants of *Jatropha* using MS medium supplemented with 22.22 µM BAP and 55.6 µM adenine sulphate followed by subculture on MS medium with 2.3 µM KN, 0.5 µM IBA and 27.8 µM adenine sulphate. Misra *et al*. (2010) used different antioxidants for solving the problem of tissue browning and necrosis in *Jatropha curcus*. They reported that medium comprising 25 mg/l reduced glutathione and 10 mg/l ascorbic acid was beneficial for the growth of shoots. Sharma *et al*. (2011) found that the shoot buds induced from hypocotyl explants of different genotypes of *Jatropha* on the medium containing 0.5 mg/l TDZ elongated upon transfer to medium containing 2 mg/l KN and 1.0 mg/l BAP. Mve *et al*. (2013) successfully regenerated plants from node explants of *J. curcus* following a procedure of bud aggregate induction on MS medium supplemented with 25 mg/l citric acid, 12.2 mg/l adenine sulfate, 15 mg/l L-arginine, 2.46 µM IBA and enriched with different balances of BAP and L-glutamine. Sujatha and Reddy (1998) achieved high rate of plant regeneration using embryonic tips (an average of 40 shoots per explant). *In vitro* regeneration was achieved from hypocotyls and cotyledonary explants with formation of adventitious shoots from cortex tissues 22-24 per explants (Ahn and Chen, 2008). Regeneration has been reported in castor (Athma and Reddy, 1983; Sujatha and Reddy, 1998; Kansara *et al*., 2010). Successful *in vitro* plant regeneration has also been described for fusarium wilt resistant castor parental line SKP-84 through apical meristem excised from 5-7-days old *in vitro* grown seedling and the plantlets were found to be genetically stable as revealed by RAPD (Kansara *et al*., 2010). The low regeneration, low multiplication rates, non-reproducibility and involvement of pre-existing meristems impede their application for transformation or large scale multiplication in *Jatropha* (Mve *et al*., 2013).

2.3.2. Field performance of micropropagated plants:

Most of the studies pertaining to micropropagation of plants have focused on development of methods for rapid multiplication. Relatively there have been few reports on the performance of micropropagated plants in the field. Jamieson and Nicerson (2003) reported that enhanced rhizome production in the micropropagated and seedlings plants of lowbush blueberry was not always associated to a greater fruit production. Rahman *et al.* (2003) observed differences in the root system architecture of loblolly pine seedlings and micropropagated plantlets. They reported that mean relative growth rates for height were greater for plantlets during the first growing season, but comparable thereafter. Kumar *et al.* (2004) reported that tissue culture raised plants of *Tagetes erecta* exhibited superior field performance in terms of plant height, number of secondary branches and number of leaves and plant spread, while the leaf chlorophyll contents were equal to that of seedling plants. Litwinczuk *et al.* (2005) evaluated the performance of highbush blueberries (*Vaccinium* X *corymbosum*) cv. 'Herbert' propagated by cuttings and obtained by micropropagation of axillary and adventitious shoots of 1-year-old *in vitro* cultures or 11-year-old cultures. The study revealed that culture age had more significant influence than shoot source for the variation observed among micropropagation systems. Osorio *et al.* (2007) observed enhanced growth in micropropagated plantlets of Carob tree than seedlings, with the greatest number of branches and a larger length increase in the main stem. Based on the results it was suggested that the pre-acclimation, in pots, to external environmental conditions might have improved field survival, plant vigour and enhanced the endurance to adverse conditions of micropropagated carob trees. The results of Dibi *et al.* (2010) showed a gain in volume of the trunk from 9.93% to 16.83% and rubber production per tree of 3.5% to 32.35% due to the use of *in vitro* plantlets of *Hevea brasiliensis*.

2.3.3. Genetic fidelity studies of micropropagated plants:

The plants regenerated from tissue culture are generally expected to be genetically similar to the parent plant. However, phenotypic variations have frequently been observed amongst regenerated plants which have been referred as somaclonal variants by Larkin and Scowcroft (1981). Since then somaclonal variation has been widely documented in tissue culture-raised plants at morphological, chromosomal, biochemical and molecular levels in many

plant species and has been extensively reviewed (Karp, 1991). Changes to the growth regulators habituations, explant type and repeated subcultures are primarily known to be associated with genetic instability in plants. Such modifications included gene methylation changes, DNA rearrangements and alterations in copy number. It has been emphasized that the failure to observe gross changes or abnormalities in morphology of plants does not rule out the possibility of genetic variations and thus reliable methods are required for identification of genetic variability. Among the various methods available for analyzing the genetic variability, molecular markers have been preferred. RAPDs have been successfully used to assess the genetic variation induced by tissue culture in a number of tree species including those belonging to Euphorbiaceae.

Rady *et al.* (2006) used 9 primers which generated 142 bands of which 7.74% showed polymorphism among *in vitro* propagated plants from shoot tip explants of *Gypsophila paniculata* The analysis of bands showed 92.3% similarity. Lakshmanan *et al.* (2007) found that the morphological changes detected in the regenerated plants of *Musa acuminata* were not associated with genetic modifications. It was suggested that the lack of polymorphism did not rule out the genetic changes as undetected changes might have occurred as a consequence of point mutations occurring outside the priming site. Cuesta *et al.* (2010) suggested the use of RAPD marker for somaclonal variation assessment in *Pinus pinea* as it was the only one able to detect variation at interclonal level while ISSR, AFLP and SAMPL (Selective amplified microsatellite polymorphism length) showed monomorphic amplification profiles.

Sharma *et al.* (2011) reported that in micropropagated plantlets of *J. curcas* generated by axillary bud proliferation using nodal segments at the subculture, 4 out of a total of 177 bands were polymorphic, but in the 8^{th} and 16^{th} subcultures, no polymorphisms were detected. Leela *et al.* (2011) optimized an accession-independent micropropagation protocol of *Jatropha* using axillary nodal explants on MS medium supplemented with a combination of growth regulators, glutamine and citric acid and using RAPD analysis confirmed the similarity of the clones with their respective mother plants. Field evaluation studies by scoring 13 quantitative phenotypic characters and evaluation of genetic fidelity among the regenerants by RAPD using 10 selected random primers revealed morphological and genetic uniformity of plants originated from a single mother tree of *Morinda citrifolia* (Sreeranjini and Siril, 2014). Thus there have been contrasting reports in different species with respect to the genetic integrity of cloned plants

even when employing axillary based or direct regeneration methods. These contrasting observations necessitate the characterization of micropropagated plants for their genetic fidelity before they are used for large-scale multiplication and mass plantations.

2.4.1. Need for investigating fruit and seed traits, oil content and fatty acid composition of seed oil of *Givotia*:

In the recent years, major attention has been paid to characterize the phytochemicals in stem, leaves and bark extracts of *Givotia*. Hari babu *et al.* (2011) carried out phytochemical and anti-microbial tests with dried and powdered leaves of *Givotia rottleriformis*. They reported that steroidal and flavonoid type compounds were present in extracts of ethyl acetate and methanol and microbial tests were positive for selected organisms. The study of Baskar Ananda Raj *et al.* (2011) revealed the presence of amino acids, flavonoids, alkaloids, proteins, carbohydrates, tannins and phenolic compounds in stem extracts of *Givotia moluccana*. Madhira Geetha and Vijayalakshmi (2013a) studied the pharmacognostic and phytochemical characteristics of *Givotia* bark and the phytochemical analysis had revealed the presence of flavonoids, alkaloids, saponins, carbohydrates and proteins. Madhira Geetha and Vijayalakshmi (2013b) demonstrated that ethanol extract of *Givotia* was found to be very safe up to 2000 mg/kg of body weight by acute toxicity model study in Swiss albino mice. Janardhan *et al.* (2013) reported that methanolic extract of *Givotia rottleriformis* had very high antioxidant property which was five to six times more than the ascorbic acid (standard). It has been reported that the crude extract can be used for the jaundice therapy. Despite the presence of oil in seeds of *Givotia* which is known to have industrial applications, there have been no reports on seed oil content and the composition of fatty acids in seed oil.

2.4.2. Importance of fats and oils:

Vegetable oils obtained from plants are essential in meeting global nutritional demands and are utilized for many food and other industrial purposes (Idouraine *et al.*, 1996). Vegetable oils are preferred over the solid animal fats because of health benefits. Oils contain higher proportion of unsaturated fatty acids, while solid fats contain more saturated fatty acids, which increased the low density lipoprotein level of the blood which is considered harmful for human health (Lucas, 2000). Of the various sources of oils and fats, edible vegetable oils constitute half of the world production of oils and fats. Idouraine *et al.* (1996) stated that the

conventional sources of vegetable oil no longer meet the ever increasing demands of domestic and industrial sectors. Of about 2, 50,000 species of plants, seed oils of about 13,000 plants have been screened for fatty acid analysis. India has huge unexploited plant genetic resources that can be screened for seed oils which might lead to the development of better substitute for conventional oils. There is a need to identify new sources of edible oils to meet the increasing demands for vegetable oils. Research work on oils in the past has been limited due to difficulty in separation of oils into simpler components. The development of chromatographic techniques, especially gas-liquid chromatography together with advances in spectroscopy, has led to revolutionary changes in the oil seed research.

Seed oils are not only known to have nutritional value but also have cosmetic and medicinal properties. In many instances, the seed oils containing essential fatty acids used for cooking and eating have also been used for maintaining healthy skin and hair. These oils especially consisted of lipids that had the property to condition, nourish, soothe, moisturize and return elasticity to the hair and skin. Recent research has shown that essential fatty acids have anti-inflammatory and anti-irritancy activities.

In majority of the oil yielding plant species, the most prevalent saturated fatty acids are palmitic acid with 16 carbon atoms (C16:0); stearic acid (C18:0) with 18 carbon atoms (C18), and the unsaturated fatty acids with 18 carbon atoms (C18) that include oleic acid (C18:0) having one double bond, linoleic acid (C18:2) having two double bonds, and α-linolenic acid (C18:3 α) having three double bonds. Plants containing a relatively large amount of these fatty acids, such as soybean, groundnut, oil palm, sunflower, rapeseed, and coconut palm are cultivated as fat or oil source plants (also referred as oil plants).

Palmitic acid is a major component of the palm oil from palm trees but can also be found in meats, cheeses, butter, and dairy products. Stearic acid or octadecanoic acid occurs in many animal and vegetable fats and oils, but it is more abundant and present up to 30% in animal fat than vegetable fat typically <5%. The important exceptions are cocoa butter and shea butter, where the stearic acid content is 28-45% (Rogers *et al.,* 200l). Stearic acid is used to produce dietary supplements, in fireworks, stearic acid is often used to coat metal powders such as aluminum and iron for preventing oxidation allowing compositions to be stored for a longer period of time. Stearic acid is mainly used in the production of detergents, soaps, and

cosmetics such as shampoos and shaving cream products. Oleic acid is a mono-unsaturated fatty acid naturally found in many plants and in some animal products. Oils rich in monounsaturated fatty acids (e.g. oleic acid) are generally more stable to oxidative rancidity and stable as deep frying oils (Mohammed *et al.*, 2003). It has many applications as plant-based lubricants. It has been shown to have many health benefits by reducing the risk of coronary artery and heart disease (Bockisch, 1998). Linoleic acid is one of the essential fatty acid but human body cannot synthesize it using other food components (Burr *et al.,* 1930). It is abundant in many oil seeds such as safflower, sunflower, corn and soybean oils. Linoleic acid has anti-inflammatory, acne reductive, and moisture retentive properties when applied topically on the skin (Darmstadt *et al.,* 2002).

The biological significance of conjugated fatty acids has been demonstrated over the years. The most intensively studied in recent times have been conjugated linoleic acid and conjugated linolenic acid. α-eleostearic acid is a conjugated fatty acid and one of the isomers of octadecatrienoic acid, often simply called eleostearic acid although there is also a β-eleostearic acid. These two are present naturally in milk and dairy products resulting of the ruminal microbial metabolism on fatty acid, present in foods, but they are also found in some plant seed oils (Van Nieuwenhove *et al.*, 2012). The tung seed oil has 82% of α-eleostearic acid where as bitter gourd seed oil has 60% α-eleostearic acid. The high degree of unsaturation and drying property of tung seed oil is due to the presence of α-eleostearic acid (Burr *et al.,* 1932). It has been widely used as a drying ingradient in paints, varnishes, coatings and finishes (Pyrde, 1979).

2.4.3. Genetic variation in seed oil content and fatty acids in tree species:

The oils stored in seeds serve as a source of energy for the embryo during the heterotrophic stage. There has been great progress made in molecular understanding of the origin of the fatty acids in seeds and their diversification mechanisms (Baker *et al.*, 2007). Considerable variation in oil content of *J. curcus* was generated by genetic and environmental factors, including rainfall and soil fertility (Mishra, 2008). Wang *et al.* (2008) compared the oil content and fatty acid composition in samples of *J. curcas* collected from three regions of China and India, found 12 fatty acids and reported differences between accessions. The work of Ovando-Medina *et al.* (2011) showed that the variation in the oil content and eleven fatty acids

was very high in populations of Mexican *J. curcas* demonstrating that they are useful markers in estimating the genetic diversity. Ghosh and Singh (2011) found significant variation among zones and among different provenances within zones, for all traits of seed and seedlings of *J. curcas*. The study of Atangana *et al.* (2011) revealed that highly significant between- and within-tree variation characterized fruits and seed characters in *Allanblackia floribunds* whereas between-site phenotypic variation was not significant. The presence of stearic acid increased its value in cosmetics. Khan *et al.* (2012) observed differences in the fatty acid composition of seed oils of *Acacia* within the same species collected from different geographical areas. Significant variations in fatty acid composition were noted within the same species and between species of *Acacia* (Khan *et al.*, 2012). Luh Arpiwi *et al.* (2012) reported that seed oil of *Millettia pinnata* contained nine fatty acids and by weight more than half was oleic acid. Similar observations have been reported in *Millettia pinnata* with respect to seed traits, oil content and fatty acid composition which varied across trees (Arpiwi *et al.*, 2012).

Hitherto there are no reports on seed oil content and fatty acid composition of seed oil of *Givotia*. Thus studies were carried out to estimate and compare the oil content in seeds of different accessions and fatty acid composition in seed oil of *Givotia*.

3. MATERIALS AND METHODS

3.1. Plant material:

Mature dry fruits and young leaves of *Givotia rottleriformis* were collected from plants growing at different forest regions of Telangana and Andhra Pradesh *viz*., Nallamalla forest, Pacharla, Kurnool District; Nallamalla forest, Balapally, Chitoor District; Kondapalli forest, Kondapalli, Vijayawada, Krishna District, one region of Telangana *viz*., Nallamala forest, Achampet, Mahaboobnagar District and one region of Karnataka *viz*., Devarayanadurga, Tumkur District and used for genetic diversity and germination studies. The leaf samples collected from male and female trees growing at Nallamalla forest, Achampet, Mahaboobnagar District were used initially for analyzing the genetic variation and detection of molecular markers linked to sex, if any. Leaf samples were also collected from twenty each of male (1M-20M) and female trees (1F-20F) growing at Mulugu Forest Research Centre, Medak District, Telangana and used for studies related to sex identification. The sex of the plants was identified based on the observations of male and female flowers. The leaf samples collected from male plants were labelled as 1M, 2M, 3M, 4M, 5M and female plants as 1F, 2F, 3F, 4F, 5F. The samples collected from different regions *viz*., Achampet, Pacharla, Balapally, Kondapalli and Devarayanadurga were labelled as GA, GP, GB, GK and GD, respectively. The leaf samples collected from candidate plus tree and its half-sib progenies of Balapally region were labelled as M (mother) and HS1-HS10 (half-sibs), respectively.

The stem cuttings and shoot tips collected from mature trees of *Givotia* growing at Mulugu Forest Research Centre, Medak District, Telangana were used for *in vitro* propagation. The mature dry fruits were collected and stored at room temperature until further use. Geographical location, distribution of *Givotia* trees and the sampling sites are shown in Fig. 3a & b. The distances between *Givotia* populations used for sample collection in the study are listed in Table 1. The forest regions at which the accessions were collected were separated by a minimum distance of 330 km (Achampet and Pacharla; Devarayanadurga and Balapally) and maximum distance of 740 km (Devarayanadurga and Kondapalli).

Young leaves were collected from nine micropropagated plants derived from nodal explants along with the donor plant of *Givotia*. The leaf samples collected from the trees were

immediately placed in polythene covers in an ice box until they were carried to the laboratory and then subsequently placed in -80°C deep-freezer before DNA extraction.

3.1.1. RAPD and ISSR analysis of genetic variability in different accessions of *Givotia:*

Genetic variation was analyzed in 50 accessions growing at five different forest regions with 10 accessions from each region using molecular methods (RAPD and ISSR). Thirty two random primers and 15 ISSR primers were initially screened and 22 random primers and 14 ISSR primers that resulted in amplification were used to assess the DNA polymorphism in different accessions.

3.1.2. RAPD and ISSR analysis of half-sib progeny of plus tree:

The genetic variability in the candidate plus tree growing at Balapally region and its half-sib progenies was assessed using 12 RAPD and 11 ISSR primers. The half-sib progenies were raised *in vitro* by culturing the zygotic embryo axes of seeds of the plus tree and the leaf samples were obtained from plantlets after six months of establishment in the glasshouse.

3.2.1. RAPD and ISSR analysis of genetic variation studies in male and female plants:

The seed orchard of *Givotia* established by Forest Department at Mulugu Forest Research Centre was used for recording the observations on different morphological characteristics in male and female trees. As per the information provided by the Forest Department, this is the only seed orchard that was successfully established out of many trails conducted. The seeds germinated after 1 ½ years of sowing in the soil although efforts made to raise seed orchards subsequently was not successful.

The observations on different morphological characteristics such as average number of leaves/branch, average number of nodes/branch, average length of internodes in cm, leaf length and width in cm, petiole length in cm, trunk girth diameter at breast height in cm were taken on eight-year-old male and female *Givotia* trees growing at Mulugu Forest Research Centre.

The genetic variability in 5 each of male and female trees of *Givotia* growing at Nallamalla forest, Achampet, Mahaboobnagar District was assessed using RAPD and ISSR methods. Thirty two random primers were initially used to screen the two bulk DNA samples

prepared from 5 each of males and females for RAPD analysis. Subsequently, 24 random primers which generated amplified bands with the bulk 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. The RAPD data obtained with the primer OPT-17 was further validated by screening 20 plants each of male and female plants growing at Mulugu Forest Research Centre.

3.2.2. DNA extraction and quantification:

DNA extraction was carried out from young leaves of different samples of *Givotia* by the cetyltrimethylammonium bromide (CTAB) method as described by Doyel and Doyel (1990) with a few modifications. Young leaves (1 gm) were taken and grounded into powder in liquid nitrogen using mortar and pestle. The fine powder was resuspended in 2 ml pre-heated 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 using 500 µl pre-chilled isopropanol and placed at -20°C for 15 min and centrifuged at 12,000 rpm for 15 min. Ethanol (70%) wash of the DNA pellet was carried out followed by air drying at room temperature. The DNA pellet was then resuspended in 200 µl TE buffer (10 mM Tris, 1 mM EDTA buffer, pH 8.0). Quantification of genomic DNA was done 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 concentrated DNA was again diluted in Tris-EDTA buffer to 10 ng per µl and used for PCR amplification.

3.2.3. PCR conditions for RAPD and ISSRs:

RAPD reactions (Williams *et al.,* 1990) were performed in a Master Cycler PCR machine (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, Vilnius, Lithuania), 2 µl of 10 picomoles/µl primer (Bioserve, Hyderabad, India), 2.5 µl of template DNA (25 ng), 0.2 µl of Taq polymerase (5 Units/µl, MBI Fermentas, Vilnius, Lithuania), and sterile milli-Q water. The first step in PCR was carried out with pre-denaturation at 94°C for 3 min, denaturation step was set at 94°C for 30 sec, primer annealing temperature was set at 37°C for 1 min and primer extension was set at 72°C for 2 min followed by 42 cycles of amplification and final extension step was carried out at 72°C for 5 min, finally amplified products were hold at 4°C. After completion of the PCR, 4.0 µl of 6X loading dye (MBI Fermentas, Vilnius, Lithuania) was added to the 20 µl amplified products and the amplified products were separated on 1.4 % agarose in 1X Tris-acetate buffer at 100 V for 2 h. Staining of DNA was done with ethidium bromide (0.5 mg/l, Sigma, St. Louis, USA) and visualized in UV transilluminator (Uvitec, Cambridge, UK). Gel images were scanned through Gel Documentation System (Syngene, Frederick, USA). The PCR experiments were carried out thrice at different times and only the repetitive PCR products were scored. The size of amplified bands was determined using the DNA ladder, 100-3000 bp (MBI Fermentas, Vilnius, Lithuania) or 100-10,000 bp (MBI Fermentas, Vilnius, Lithuania).

For ISSR analysis, the reaction mixture of 20 µl contained 2 µl 10X PCR buffer (Fermentas International), 0.5 µl of 10 mM dNTP, 2 µl of 10 picomoles/µl primer, 2.0 µl of template DNA, 0.2 µl of Taq polymerase (5 Units/µl) and sterile water. PCR amplification was carried out with pre-denaturation at 94°C for 4 min, denaturation at 92°C for 30 sec, primer annealing at 53°C for 1 min and primer extension at 72°C for 1 min followed by 40 cycles of amplification and final extension at 72°C for 10 min. Finally

amplified products were hold at 4°C. After completion of the PCR, 4.0 µl of 6X loading dye (Fermentas) was added to the 20 µl amplified products and the amplified products were separated on 1.4 % agarose (Sigma) gel in Tris-acetate buffer. DNA was stained with ethidium bromide (0.5 mg/l) and visualized in UV light with the use of the Gel Documentation System. The PCR experiments were carried out twice and only the repetitive PCR products were scored. The DNA ladder (100-3000 bp) was used as a molecular weight marker.

RAPD and ISSR markers were scored for the presence considered as '1' or absence as '0' for each primer by examining the 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). Dendrograms based on un-weighted pair group method using arithmetic averages (UPGMA) were constructed for the two marker systems and a sequential agglomerative hierarchical nested clustering (SAHN) was obtained (Sneath and Sokal, 1973). The significance of genetic similarity matrix data generated with RAPD and ISSR markers was determined using Mantel test (Mantel, 1967). Principal coordinate analysis was carried using the NTSYS-pc software and the ordination displayed in both two and three dimensions.

3.2.4. Development of SCAR markers from selected RAPD fragment:

To convert the selected region-specific or sex-specific RAPD fragment to a SCAR marker, the amplified fragment was excised from the gel, cloned in a vector followed by sequencing.

3.2.4.1. Gel elution and competent cell preparation:

The selected bands were excised from agarose gel and the DNA was eluted using Qiaquick Gel Extraction Kit (Qiagen, Germany) as per manufacturer's instructions. The eluted DNA was PCR amplified using the specific primers and checked for the corresponding product on agarose gel electrophoresis before cloning.

The single colony of DH5α from freshly streaked plate was taken and inoculated on LB (Luria broth) medium (pH 7.4). From the overnight culture, 2 ml was added to 200 ml of LB broth and was kept in shaker at 37ºC until an OD (Optical Density) of 0.4-0.6 was reached. The culture was incubated in ice for 30 min and then centrifuged at 4000 rpm for 15 min at 4ºC. The pellet was dissolved in 200 ml of filter sterilized buffer A (100 mM $CaCl_2$, 70 mM $MnCl_2$ and 70 mM sodium acetate, pH 5.5) and was centrifuged at 3700 rpm for 30 min. The resulting pellet was dissolved in buffer B (buffer A with 20% glycerol) in appropriate quantity (3-5 ml) and 100 µl was aliquoted in each eppendorf tube, frozen in liquid nitrogen and stored at -80ºC until further use.

3.2.4.2. Ligation:

The eluted DNA was cloned into cloning site of the pTZ57R/T cloning vector (2868 bp) as described in InsT/A clone TM PCR product cloning kit (K1214, MB1, Fermentas, U.S.A.). Restriction digestion of the vector and ligation of the DNA insert was performed according to manufacturer's protocol. Ligation mixture was incubated at 4°C for 6 h.

3.2.4.3. Transformation and screening of recombinant or positive clones:

The whole ligated mixture (15 µl) was added to 100 µl of DH5α competent cell suspension. The mixture was incubated on ice for 30 min. It was subjected to heat shock at 42°C for 90 sec and immediately kept on ice for 30 min. Transformed bacterial cell suspension was grown in 1 ml of LB broth for 1 h at 37°C in a rotary incubator shaker at 200 rpm. After incubation, the culture was centrifuged at 10,000 rpm for 1 min and the pellet was resuspended in 100 µl of LB broth. The resuspended culture was spread using sterile spreader on LB agar plate containing ampicillin 100 µg/ml, 50 µl of Xgal (20 mg/ml dissolved in difloromethylornithine) and 40 µl of isopropyl-beta-D-thiogalactopyranoside (IPTG; 24 mg/ml dissolved in milliQ). The plate was incubated at 37°C for 12-16 h. The plate was transferred to 4°C for 2-3 h for blue colour development. The positive colonies were screened through blue white selection (α-complementation) and the white colonies were selected for colony PCR.

3.2.4.4. Colony PCR and master plating:

Colony PCR was performed by streaking white colonies on LB agar plate (master plate) containing ampicillin 100 µg/ml, 50 µl of Xgal (20 mg/ml dissolved in difloromethylornithine) and 40 µl of isopropyl-beta-D-thiogalactopyranoside (IPTG; 24 mg/ml dissolved in milliQ) and other colonies were used for PCR. Each 20 µl reaction mixture consisted of 2.0 µl of 10X PCR buffer, 0.5 µl of 10 mM dNTP mix, 0.2 µl of *Taq* polymerase (5 U/µl), 2 µl of 10 picomoles/µl primer and sterile milli-Q water and mixed well. The master mix aliquot PCR tubes were placed on ice and individual white colony was resuspended. The PCR conditions used were initial denaturation at 94°C, 3 min; denaturation at 94°C, 30 sec; primer annealing 37°C, 1 min; extension 72°C, 2 min; 42 cycles followed by final extension at 72°C, 5 min. The positives colonies identified from PCR were selected for plasmid isolation.

3.2.4.5. Plasmid isolation and purification:

The plasmid DNA was purified from 5 independent transformed clones using Quicklyse Mini prep kit (Qiagen, Maryland, USA).

3.2.4.6. Restriction digestion:

The restriction digestion of plasmids and PCR products were performed according to standard procedures (Sambrook *et al*., 1989) using *Eco*RI and *Hind*III in appropriate buffer. The digested product was analysed in the 1.5% agarose gel.

3.2.4.7. Sequencing of plasmids and analysis of sequencing result:

The confirmed positive plasmids through restriction digestion were purified through column and sent for sequencing to the Ocimum Biosolutions Pvt. Ltd.

The FASTA format nucleotide sequence obtained from Ocimum Biosolutions Pvt. Ltd. was analyzed for homology in the GenBank database using the BLASTN analysis. FASTA of nucleotide sequence was pasted in the dialog box indicating "enter accession number (s), or FASTA sequences" and non-redundant data base selected for the analysis. Results were predicted based on the following parameter; maximum score, total score, query coverage, E value and indent. The unique amplified fragment was also submitted to NCBI

database, U.S.A. Further, the sequence of the amplified fragment was used to design specific primer pairs using Primer3 too.

3.2.4.8. Designing primers for SCAR markers and validation:

The SCAR marker sequences were designed by identifying the original 10 bp sequence of the RAPD primer and adding the next approximately 10 bp in the DNA sequence. These SCAR primers were synthesized by Bioserve Biotechnologies (Hyderabad, India). PCR amplification was achieved in 20 µl reaction as described above for RAPDs, except that the RAPD primer was replaced by the specific primer pair of SCAR primers. PCR products were analysed by agarose gel (1.5%) electrophoresis. The efficiency and sensitivity in detection of SCAR markers were analyzed by screening the different accessions collected from Achampet and Devarayandurga regions for determining the possibility of identifying region-specific marker. The SCAR markers designed based on the RAPD amplicon of male and female plants were further validated by screening 10 plants each of male and females of Mulugu Forest Research Centre.

3.3.1 *In vitro* propagation from nodal explants:

The stem cuttings from young or medium thick green or old brown colour shoots were collected from mature trees growing at Mulugu Forest Research Centre during rainy season (July-September) and placed in polythene covers. The nodal explants of 3-4 cm with axillary buds were excised from the stem cuttings either immediately or after 2 days of placing at room temperature and then disinfected. The nodal explants were taken in a sterile conical flask and washed with tap water for 15 min (2-3 washes). The explants were subsequently treated with 2% bavistin for 20 min. They were treated with antioxidants (100 mg/l each of ascorbic acid and citric acid) for 5 min. They were rinsed with sterile double distilled water and surface disinfected with 70% ethanol for 5 min followed by treatment with 0.1% $HgCl_2$ (w/v) containing 1 or 2 drops of Tween-20 for 8 min and washed 4-5 times with sterile distilled water. The nodal explants with axillary buds were cultured on MS (Murashige and Skoog's, 1962) or Woody Plant Medium (Lloyd and McCown, 1980) with or without BAP (0.5 and 1.0 mg/l) either singly or in combination with 0.1 mg/l NAA for evaluating the response for axillary bud sprouting. The frequency and the duration for axillary bud sprouting were determined after 30 days of

culture. The explants were cut at the base and the nodal regions with the sprouted axillary bud were then subcultured on the same medium. The average number of the shoots and length of shoots (cm) was recorded after 30 days of subculture. The shoot tips (0.5-1.0 cm) excised from axillary shoots were cultured on MS medium with different concentrations of BAP (0.25, 0.5, 1.0 and 2.0 mg/l) for evaluating the response of shoot multiplication. The average number of shoots induced per explant and average length of the shoots (cm) was recorded after 30 days of culture.

3.3.2. Shoot regeneration from epicotyl and hypocotyl explants:

The mature dried fruits from the trees growing at the Nallamalla forest, Pacharla, Kurnool District of Andhra Pradesh were collected during March 2009 and stored at room temperature until further use. The dried fruits were treated at 55°C for 24 h and the epicarp and mesocarp were removed. The seeds with stony endocarp were soaked in double distilled water for 24 h and surface sterilized with 70% (v/v) ethanol for 3 min followed by treatment with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 15 min and rinsed 3-4 times with sterile double distilled water. The zygotic embryo axes were excised from the temperature-treated seeds and cultured for germination on MS medium with half-strength major salts containing 0.25 mg/l KN, 0.1 mg/l IBA, 3% (w/v) sucrose and 0.8% agar (w/v) (Qualigens, India). The cultures of zygotic embryo axes were initially placed in dark for a week and then transferred to light for germination.

The regeneration potential of epicotyl and hypocotyl explants obtained from emblings of different ages (10, 20 and 30-day-old) was tested by culturing on MS medium with 1.0 mg/l BAP. The epicotyl and hypocotyl explants were excised from 20-day-old emblings of 5-6 cm and placed on MS full-strength basal medium containing 3% sucrose and 0.8% (w/v) agar or MS full-strength medium supplemented with different concentrations of BAP (0.25, 0.5, 1.0 and 2.0 mg/l) or KN (0.25, 0.5, 1.0 and 2.0 mg/l) for studying their effects on shoot regeneration. The epicotyl and hypocotyl explants obtained from 20-day-old seedlings were cut into two halves (one half closer to cotyledonary node, and the other half closer to shoot apex for epicotyl explants and root node for hypocotyl explants) and cultured on MS medium with 1.0 mg/l BAP for determining their regenerative capacities. The effect of orientation of explants was studied by placing them vertically or horizontally on the medium containing 1.0

mg/l BAP. In another experiment, the response of shoot regeneration from epicotyl and hypocotyl explants was tested after culturing on MS medium with 1.0 mg/l BAP and 0.5 mg/l NAA. Four explants were placed in each culture bottle having dimensions of 60 X 110 mm and containing 40 ml of medium. The observations on percentage of shoot regeneration, average number of shoots/explant and average length of shoots in cm was recorded after four weeks of culture.

3.3.3. *In vitro* multiplication of shoots:

The shoots regenerated from epicotyl and hypocotyl explants on initiation medium were transferred as a clump and repeatedly cultured on medium with 0.5 mg/l or 1.0 mg/l BAP for inducing shoot proliferation. Thereafter, either the shoot clumps or single shoots are placed on medium with 0.5 mg/l BAP which favoured shoot multiplication and elongation. Shoots of 2-3 cm were separated from the clumps and placed on medium with 0.25 mg/l BAP for 3 weeks before root induction. Shoot multiplication was carried out by placing one shoot clump per culture bottle containing 40 ml of medium. The data on the average number of shoots per explant and average length of shoots (cm) was recorded after 30 days of culture on medium with 0.5 mg/l BAP and 1.0 mg/l BAP.

3.3.4. Root induction from *in vitro* regenerated shoots:

The regenerated shoots of 3-4 cm originating from epicotyl and hypocotyl explants were given a pulse treatment by dipping the base of shoots for 10 sec with 10 mg/ml IBA or 10 mg/ml IAA and then placed in culture bottles containing MS basal medium or MS medium containing 0.1 mg/l IBA or 0.1 mg/l IAA, 3% (w/v) sucrose and 0.8% agar (w/v) for root induction. The average root induction percentage, the average number of roots/shoot and average length of roots (cm) were recorded after 5 weeks.

The axillary shoots of 3-4 cm originating from nodal explants were given a pulse treatment by dipping the base of shoots for 10 sec with 10 mg/ml IAA or 10 mg/ml IBA and then placed in culture bottles containing MS basal medium alone or with 0.1 mg/l IBA, respectively for root induction.

The cultures for all experiments were maintained at 26 ± 2°C followed by 16 h photoperiod. The photosynthetic photon flux density (PPFD) inside the culture room was of 83.6 $\mu Em^{-2}s^{-1}$ provided by white fluorescent tubes.

3.3.5. Acclimatization of *in vitro* regenerated plants:

The emblings produced from cultured zygotic embryo axes or micropropagated plants originating from nodal explants of mature trees that were separated from the medium were washed with tap water until the agar sticking to the roots was removed. They were placed in plastic pots containing autoclaved potting mixture. The potting mixture was used in the ratio of 3:1:1:1 (soil: sand: peat moss: farm yard manure) and acclimatized in culture room for 30 days by covering with polythene covers and then transferred to glasshouse. The temperature in the glassware ranged from 26°C to 31°C with a light intensity of 500–1,700 $\mu mol\ m^{-2}\ s^{-1}$ and 12 h photoperiod. The polythene covers were removed after 30 days of acclimatization of the plantlets in glasshouse. Later on the plantlets were transplanted into earthen pots containing soil and farmyard manure in the ratio of 3:1. The plantlets were irrigated with tap water every 2 days. The percentage of survival of the plants was recorded after 2 months of transfer to the greenhouse. The data on the percentage of survival of plants, height of the plants (cm), number of leaves, leaf length (cm) and leaf width (cm) was recorded after six months of transfer to soil. The measurements of leaf length and leaf width were taken from the third fully expanded leaves of the plantlets. The emblings and micropropagated plants that were established in the glasshouse were subsequently transplanted to soil and farmyard manure (3:1 v/v) in open field during rainy season (July-October) and the survival frequency was determined.

3.3.6. Statistical analysis:

In all the experiments, the minimum number of explants used per treatment was 10 and the experiments were repeated thrice at different time points. The data obtained in the study was analysed using one-way analysis of variance and pair-wise means were compared using Newman-Keul's multiple comparisons test ($p \leq 0.05$) using SigmaStat 5.1 software.

3.3.7. Genetic fidelity studies in micropropagated plants using RAPDs:

Ten RAPD primers that detected polymorphism in different accessions were used for assessing the genetic stability of micropropagated plants in comparison to donor plant. The bands were scored as described previously in Section 3.2.1.

3.4.1. Variation in fruit and seed traits, seed oil content and fatty acid composition of seed oil of *Givotia*:

Mature fruit samples were collected from different trees of *Givotia* growing in five forest regions of Andhra Pradesh, Telangana and Karnataka. A total of 25 individual accessions representing 5 forest regions with 5 accessions in each forest region were used for analyzing seed oil content and fatty acid analysis in seed oil. The dried fruits were collected separately from each tree, placed in plastic cover and the identity was maintained. The fruits were air dried until constant weight at room temperature under similar conditions. The seeds were extracted mechanically from mature dried fruits by removal of pericarp. A total of 100 mature fruits and seeds for each accession were used and the weight of ten mature fruits and seeds for each accession was determined. The diameter of randomly selected mature dry fruits and seeds of each accession was measured with ten seeds for each accession. The average values and standard errors for different fruit and seed traits were based on five accessions for each forest region.

The seeds isolated from mature dried fruits of five forest regions were used for *in vitro* germination studies. Temperature treatment was given by placing the seeds at 55ºC for 24 h and the seeds that were kept at room temperature were used as controls. The zygotic embryo axes were excised from temperature-treated or control mature seeds and cultured on MS medium (half-strength major salts) with or without 0.25 mg/l KN and 0.1 mg/l IBA for inducing germination. The germination percentage was scored after 30 days after placing on the medium. A minimum of ten seeds of each accession was used per treatment and averaged across five trees of each forest region and standard error was calculated.

3.4.2. Extraction of seed oil:

The seeds were extracted mechanically from mature dried fruits by removal of pericarp and used for estimation of oil content in each accession with five accessions in each

forest region. The procedure used for extraction of seed oil is presented through a flow chart in Fig. 49. In brief, the seeds were dried at 55°C for 24 h in hot air oven. The seeds of each accession were weighed to 5 gm and ground separately in mortar and pestle. The powdered mill was collected in cellulose extraction thimbles (internal diameter x internal length was 25 mm x 80 mm; Whatman Schleicher and Schuell (Cat No. 2800258) and was inserted in the centre of the extractor. The oil was extracted from the ground kernels either with hexane or petroleum ether using the Soxhlet extraction method. Finally the oil was obtained after removing the solvent from the extract with the help of rotary vacuum evaporator. The result obtained was expressed as the percentage of oil in the dry matter of seed powders. The extracted oil was then stored in -20°C for further experiments. The fatty acids present in the seed oil were compared with the other edible and non-edible oils.

3.4.3. Fatty acid analysis in seeds of *Givotia* by GC and GC-MS analysis*:*

Fatty acid (FA) composition of the oil extracted from one-year-old seeds of *Givotia* collected from Achampet region was determined as their corresponding methyl esters. Preparation of fatty acid methyl esters (FAMEs) was carried out according to the modified ISO method (BS EN 5508, 1995) with minor modifications. About 5 ml of hexane was added to 2-3 gm of seed and heated gently. Subsequently, 0.5 ml of 12% (w/v) methanolic potassium hydroxide (KOH) was added and heated for 10 min (trans-esterification reaction). The resulting mixture was centrifuged at 5000 rpm for 5 min as a result of which two layers are formed. The pellet was discarded and the supernatant was collected and analyzed in gas chromatography (GC).

The fatty acid composition of the seed oil was analysed by using Gas Chromatography system (6890N series of Agilent Technologies, Palo Alto, CA, USA) fitted with a column (DB-225, 30 m length, 0.25 mm inner diameter and 0.25 μm thickness, J & W Scientific, USA) packed with 50% cyanopropylphenyl-dimethyl-polysiloxane and also connected to an Agilent 5973, Mass Spectrometer operating system. The fatty acid composition of seed oil was determined by retention times by means of comparing them with authentic standards analyzed under the same conditions using GC.

Structural assignments were based on mass spectrometric fragmentation. The results of the fragmentation pattern were interpreted, confirmed by comparison of retention times

with the fragmentation pattern of authentic compounds and the spectral data obtained from the Wiley and NIST libraries. Fatty acid composition was reported as proportion of each fatty acid to the total fatty acid in each sample. The experiments were repeated 3 times at different points of time.

4. RESULTS

The major goal of the present study has been to characterize the genetic variation in 50 accessions of *Givotia* collected from 5 different forest regions (10 accessions from each region) using RAPD and ISSR methods. The genetic relatedness and differences of half-sib progenies raised from candidate plus tree were assessed using RAPD and ISSR primers. Further, the molecular genetic variation in male and female plants was analyzed using RAPD and ISSR primers in order to determine the possibility of identifying molecular marker linked to sex. The study also focused on optimizing media and growth regulators for plant regeneration from explants obtained from mature trees as well as *in vitro* raised emblings for achieving rapid propagation. The genetic uniformity of the micropropagated plants originating from nodal explants was assessed using RAPD primers and their performance was evaluated in comparison to emblings. In addition, the variability in fruit and seed traits, seed germination and seed oil content of different accessions was determined and fatty acids present in the *Givotia* seed oil were analyzed. The results obtained in this regard are presented below:

4.1.1. Genetic diversity detected by RAPD analysis in *Givotia* accessions growing at five different regions:

In *Givotia rottleriformis* Griff., there has been no study conducted so far to examine the extent of molecular genetic variation in plants growing in their natural habitat, despite high economic importance of this species. This is the first study carried out to estimate the genetic variation in 50 accessions of *Givotia* collected from 5 different forest regions (Fig. 4a-e) using RAPD and ISSR methods. Out of 32 RAPD primers initially screened, 22 primers produced bands whereas no amplification was observed with 10 primers. The polymorphism generated by different random primers ranged from 54.5-100%. Out of 22 random primers tested, fifteen primers *viz*., OPG-16, OPG-06, OPG-17, OPK-07, OPC-10, OPC-08, OPC-07, OPF-03, OPA-04, OPAB-06, OPAL-08, OPT-17, OPZ-01, OPZ-06 and OPA-18 generated 100% polymorphism in different accessions analyzed (Table 2). The lowest polymorphism of 54.5% was generated by the primer OPC-06. On an average, 12.5 loci were generated per primer with 12.1 loci being polymorphic. The size of the amplicons ranged from 150-2100 bp

for different primers. The primer, OPAL-08 produced the highest number of loci (23) as well as highest number of amplified fragments (283) in the accessions used. The lowest number of loci (5) was generated by the primer OPG-06 whereas as the lowest number of amplified bands (110) was produced by the primer OPC-06 of which 60 were polymorphic. A total of 4497 bands were generated of which 4147 bands were polymorphic. Higher percentage of polymorphic loci were observed in accessions of Balapally (85.9%) followed by Pacharla (68.5%), Achampet (65.0%) whereas it was 60.4% and 59.6% in Kondapalli and Devarayanadurga accessions, respectively. RAPD profiles obtained with the primers OPAK-14 and OPZ-01 in *Givotia* accessions of different regions are depicted in Fig. 5a-e and Fig. 6a-e, respectively.

Out of 274 loci generated by 22 random primers in 50 accessions of *Givotia*, only 7 loci produced by primers *viz*., OPC-09 (1700 bp), OPC-06 (490 bp), OPC-03 (1450 bp), OPA-04 (900 bp), OPAK-14 (500 bp), OPZ-10 (575 bp) and OPB-12 (490 bp) were found to be monomorphic irrespective of the forest region from which they were collected. The primer OPK-07 amplified a band of 400 bp present in all accessions of Balapally but absent in accessions of other forest regions. The random primers, OPT-17 and OPC-08 produced bands of 350 bp and 490 bp, respectively in all accessions of Pacharla, Balapally, Achampet, Kondapalli but absent in accessions of Devarayanadurga region.

A dendrogram constructed using the UPGMA method grouped the 22 accessions into two major clusters at Jaccard's similarity coefficient value of 0.26. Overall, the accessions belonging to each forest region grouped into one cluster. The first major cluster was sub-divided into further sub-clusters with increase in Jaccard similarity coefficient values whereas the second main cluster comprised the accessions of Balapally region. The accessions of Achampet and Pacharla grouped into one sub-cluster whereas Kondapalli and Devarayanadurga formed separate sub-clusters (Fig. 7a).

4.1.2. Genetic diversity detected by ISSR analysis in *Givotia* accessions:

In this study, out of 15 ISSR primers screened, 14 ISSR primers resulted in DNA amplification which was used for characterizing the genetic variability in *Givotia* accessions growing at different forest regions. The number of loci generated varied from 6-18 with the size of the amplified bands varying from 200-1900 bp (Table 3). On an average, 10.5 loci were

generated per primer of which 9 loci were polymorphic resulting in 85.7% polymorphic loci. The percentage of polymorphism varied from 41.2-100% with the primers tested. Four ISSR primers, UBC-824, UBC-825, UBC-826 and UBC-834 revealed 100% polymorphism whereas lowest polymorphism of 41.2% was observed with UBC-810 primer. The highest number of loci (18) was generated by the primer UBC-825 which was all polymorphic. The lowest number of loci (6) was generated by the primers UBC-815 and UBC-820 of which 4 and 5 were polymorphic, respectively. The total number of amplified bands using 14 ISSR primers in 50 accessions was 3864 of which 2864 were polymorphic resulting in 74.1% polymorphism. Analysis of polymorphic loci in accessions of different regions revealed relatively higher percentage (65.5%) of polymorphic loci in accessions of Balapally followed by accessions of Devarayanadurga (50.4%). The accessions of Pacharla and Kondapalli displayed 45.3% and 43.8% of polymorphic loci, respectively whereas Achampet accessions exhibited lowest percentage (35.1%) polymorphic loci. It was observed that 8 ISSR primers generated 18 loci *i.e.* 4 from UBC-810 (400, 300, 250, 200 bp); 4 from UBC-812 (490, 410, 320, 200 bp); 2 from UBC-808 (510, 310 bp); 2 from UBC-815 (400, 300 bp); 1 from UBC-820 (300 bp); 2 from UBC-835 (700 bp, 300 bp); 1 from UBC-846 (390 bp) and 2 from UBC-899 (550, 300 bp) which were found in all 50 accessions analyzed. The banding pattern produced by the ISSR primers, UBC-808 and UBC-835 are shown in Fig. 8a-e and Fig. 9a-e, respectively.

The cluster analysis was performed by UPGMA method which showed grouping of *Givotia* accessions into 2 major clusters having a similarity coefficient of 0.53 (Fig. 10). The first main cluster was sub-divided into sub-clusters, Ia and Ib representing Achampet and Pacharla accessions. The second main cluster grouped the accessions into 3 sub-clusters with sub-cluster IIa comprising Balapally accessions, sub-clusters IIb-1 and IIb-2 comprising Kondapalli and Devarayanadurga accessions. A clear grouping was observed with plants of each region with higher diversity observed among accessions of Balapally region as compared to accessions of other forest regions.

4.1.3. Genetic diversity detected by combined RAPD and ISSR markers in *Givotia* accessions:

The RAPD and ISSR data were combined for UPGMA dendrogram analysis. The 50 accessions formed 2 main clusters at 0.38% similarity level (Fig. 11). The similarity coefficients

of the 50 accessions of *Givotia* based on 22 RAPD markers and 14 ISSR markers ranged from 0.26 to 0.83. Cluster 1 formed sub-cluster with increase in similarity coefficient values. The sub cluster I was further sub-divided into two sub-clusters Ia-1 and Ia-2 comprising accessions of Achampet and Pacharla regions and the sub-clusters Ib and Ic consisting of accessions of Kondapalli and Devarayanadurga, respectively. The accessions of Balapally were included in the main cluster II and exhibited greater genetic diversity as compared to the accessions of other regions. Dendrogram constructed based on the combined data of RAPD and ISSR was more similar to that obtained with RAPD data. Mantel test values showed a positive correlation between the two marker types. The correlation coefficient (r) was 0.523 between RAPD and ISSR, which showed goodness of fitness between the marker systems.

The PCA (Principal coordinate analysis) based on combined RAPD and ISSR polymorphisms grouped the *Givotia* accessions into four groups in both two-dimensional and three-dimensional graphs (Fig. 12 & 13). The cluster I included the accessions of GA and GP while GK, GD and GB formed separate single clusters, II, III and IV, respectively. Eigen values obtained reveal the percentage of variation of PC1, PC2, PC3 and PC4 as 15.1, 12.73, 10.3 and 9.34.

4.1.4. SCAR markers generated from cloned RAPD fragments for validation:

During the analysis of molecular genetic diversity with different random primers, two RAPD primers *viz*., OPC-08 and OPT-17 produced 490 bp and 350 bp bands present in all accessions Achampet, Pacharla, Balapally and Kondapalli but absent in Devarayanadurga of Karnataka (Fig. 14a & b). The amplified fragment was eluted from the gel followed by the purification and then cloned into pTZ57R/T (Insta) T/A cloning vector. After ligation, the transformation of plasmids into competent cells was performed. The transformed cells were selected based on blue-white screening of the colonies. The plasmid DNA was isolated from white cells and the presence of DNA insert in the cloning vector was confirmed by double digestion with *Eco*RI and *Hind*III restriction enzymes. Out of five colonies tested, the plasmid isolated from one white colony yielded the expected fragment of 490 bp confirming the presence of insert in the vector (Fig. 14c). The cloned DNA fragment was sequenced (Fig. 14d) and deposited in NCBI GenBank (Accession No.: KF772203.1). BLAST search of 490 bp sequence did not show any sequence similarity with those available in the data base. The

nucleotide sequence of selected amplicon obtained from OPC-08 primer is, therefore, considered to be unique.

Similarly, the primer OPT-17 primer generated a band of 350 bp present in all accessions of Achampet, Pacharla, Balapally and Kondapalli but absent in Devarayanadurga of Karnataka (Fig. 15a & b). The band was eluted and amplified by PCR before cloning into vector (Fig. 15c). After cloning, the plasmid was isolated and confirmed by double digestion with restriction enzymes which yielded 350 bp fragment (Fig. 15d). The cloned DNA fragment was sequenced and deposited in NCBI Genbank (JX026068.1) (Fig. 15e). The sequence of the 350 amplicon generated with OPT-17 primer exhibited highest similarity of 91% with *Jatropha curcus* uncharacterized LOC105640258, m-RNA. It also matched (86% similarity) with the clone-3 hypothetical protein gene, partial coding sequence (pds) of *Ricinus* during BLAST analysis.

SCAR primers designed based the nucleotide sequence of 490 bp amplified fragment were screened on 10 accessions each of Pacharla and Devarayanadurga for validation (Table 4). However, amplification was observed in all the accessions analyzed indicating that it is not region-specific. Similarly, the SCAR primers developed based on the nucleotide sequence of 350 bp amplified fragment (Table 4) when screened on 10 accessions each of Pacharla and Devarayanadurga resulted in amplification in all the accessions analyzed indicating its inability to distinguish the accessions of Devaranadurga from other accessions (Fig. 16a & b).

4.1.5. Genetic variability detected in half-sib progeny of candidate plus tree of *Givotia* by RAPD primers:

A candidate plus tree was identified in the present study at Balapally region during the process of sample collection. The candidate plus tree had increased trunk girth of 55 cm at breast height as compared to other trees observed in that region. The half-sib progenies of the candidate plus tree were raised *in vitro* by culturing the zygotic embryo axes and established in the glasshouse. This is the first study to examine the variation in half-sib progenies and its maternal tree using RAPD and ISSR methods. The twelve RAPD primers generated 106 loci in 11 samples analyzed of which 76 loci were found to be polymorphic (Table 5). The percentage of polymorphism ranged from 1.5-100% with the highest polymorphism produced by the primer OPE-20 and the lowest (1.5%) by OPB-12. The number of loci ranged from 5

(OPAL-20 and OPG-14) to 16 (OPAI-12) with the average number of loci/primer being 8.8. The amplicon sizes obtained from different primers ranged from 150-2025 bp. The RAPD banding pattern obtained with random primers OPB-07, OPAI-12, OPB-12 and OPT-17 in half-sib progeny analysis are depicted in Fig. 17a & b and 18a & 18b, respectively.

Cluster analysis separated the 10 half-sibs and 1 maternal tree into one main cluster and outlier respectively at 0.44 Jaccard's similarity coefficient (Fig. 19). The major cluster was sub-divided into 2 sub-clusters at 0.75 similarity coefficients with the first sub-cluster comprising 6 half-sibs and the remaining grouped into second sub-cluster. The half-sib progenies exhibited greater similarities among themselves as compared to the maternal plant as evident from the grouping.. The average similarity coefficients among the half-sibs was 0.771 with the maximum similarity of 0.896 observed between HS3 and HS5 and the least similarity of 0.663 between HS1 and HS2 (Table 6). The similarity coefficients of half-sibs in comparison to the maternal tree ranged from 0.385 to 0.500 with an average value of 0.444. The half-sib HS5 exhibited lowest similarity of 0.385 whereas the half sib HS7 exhibited highest similarity of 0.50 to the maternal tree (Table 6).

Examination of the banding pattern generated by RAPD primers revealed that four RAPD primers *viz*., OPG-16, OPT-17, OPV-08 and OPB-07 generated bands only in maternal tree and absent in all 10 half-sib progeny analyzed (Table 7). On the other hand, two RAPD primers, OPV-08 and OPAI-12 generated unique bands of 550 bp and 625 bp in HS9 and HS10, respectively. Of the 12 primers employed, 11 primers generated 30 monomorphic bands present in maternal tree and all half-siblings.

4.1.6. Genetic variability detected in half-sib progeny of candidate plus tree of *Givotia* by ISSR primers:

The genetic variability among ten half-sibs and its maternal tree was characterized using 10 ISSR primers. The number of loci generated ranged from 4-12 whereas the polymorphic loci varied from 1-8 (Fig. 20a & b: 21a & b). Of the 76 loci detected across all 11 samples analyzed, 51 loci were polymorphic (Table 8). All the ISSR primers used resulted in polymorphic bands with the highest percentage of polymorphism (81.7%) generated by the primer UBC-825 and the least (18.5%) by UBC-810. Out of 566 bands amplified, 291 bands were polymorphic accounting to 51.4% polymorphism.

Cluster analysis based on the pooled data from 10 ISSR primers grouped the 10 half-siblings into one main cluster while the maternal tree was found as an outlier at Jaccard's similarity coefficient of 0.56 (Fig. 22). The main-cluster was sub-divided into two sub-clusters I and sub-cluster II with maximum number being grouped in sub-cluster I. There were greater similarities observed among half-sib progenies as compared to the maternal tree (Table 9). The similarity coefficients ranged from 0.623 to 0.850 in half-sibs with an average similarity coefficient of 0.735 (Table 9). On an average, the half-sibs shared about 0.563 similarities with the maternal parent.

The ISSR primers employed in the study generated few bands present in all half-siblings but absent in maternal tree (Table 10). Two ISSR primers UBC-815 and UBC-810 produced 3 bands and 1 band, respectively present in all half-siblings but absent in maternal tree. All the ISSR primers generated monomorphic bands which ranged from 1 to 4 in the samples analyzed.

4.1.7. Genetic variability detected in half-sib progeny of candidate plus tree using combined RAPD and ISSR data:

The dendrogram constructed based on combined data from 12 RAPD and 10 ISSR primers showed one main cluster consisting of 10 half-sib progenies and an outlier consisting of maternal parent at 0.49 similarity coefficient (Fig. 23). Thus in both individual and combined analysis, the half-sib seedlings grouped into one cluster although the similarity coefficient values differed between each other and also with maternal tree. The half-sib HS5 displayed least genetic similarity (0.385) to the maternal tree in RAPD analysis whereas in ISSR analysis it exhibited highest similarity of 0.645 to the maternal tree. The half-sibs showed an average similarity coefficient of 0.563 with the maternal parent in combined RAPD and ISSR analysis (Table 11). The clustering pattern of half-sibs obtained with combined RAPD and ISSR analysis was more similar to that obtained with RAPD data. It was noticed that the banding patterns of few of the half-siblings were similar with some RAPD and ISSR primers but exhibited polymorphic bands with other primers.

4.2.1. Genetic variability in male and female plants of *Givotia* detected by RAPD analysis:

The morphological characteristics of male and female eight-year old trees growing at Mulugu Forest Research Centre were examined. The trunk girth (cm) at breast height in male trees was 32.9 cm as compared to 34.9 in female trees. The average number of nodes per branch in male and female trees was found to be 14.6 and 15.2, respectively. The average length of internodes, number of leaves per branch, leaf length and width, and petiole length in male trees did not show significant differences from those of female trees. Thus, there were no prominent differences noticed among male and female trees with respect to the characteristics evaluated (Table 12). Since the sex of *Givotia* cannot be determined from the examination of morphological characteristics until flowering, it would be particularly useful to rapidly determine the sex at the early stages using DNA markers. In the present study, bulk segregant analysis in combination with the randomly amplified polymorphic DNA has been used for identifying sex-linked markers in *Givotia*. The bulk DNA samples of males and females were screened with 24 random primers in order to identify the bands specific to males and females. The number of bands ranged from 1 to 8 with different primers from the bulk DNAs of male and female plants of *Givotia* (Table 13).

Of the 32 primers screened in the bulk DNA samples of males and females, only 24 primers 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. Of the 24 random primers used in the present study, 12 primers produced polymorphic bands in bulk DNA samples of males and females whereas monomorphic bands were produced from 12 primers (Table 13). Twelve primers generated 26 polymorphic bands in bulk DNA samples of which 17 were found to be specifically present in females and 9 were found to be specifically present in males. The primer OPAL-08 produced highest number of bands (8) in bulk DNA samples of which 7 were found to be monomorphic and one was found to be specifically present in females (Fig. 24a). The primer OPZ-10 generated 3 bands specific to females and one band specific to males in bulk DNA samples (Fig. 24a). Maximum number of bands (4) specific to females was produced by the primer OPT-17 in bulk DNA samples (Fig. 24b). The primer OPG-17 produced 5 bands present in both males and females (Fig. 24c).

In the present investigation, the pattern of genetic variation in male and female trees growing at Achampet region of Telanagana was examined using RAPD and ISSR analysis. The 24 random primers that resulted in bands were used for characterizing the genetic variability of *Givotia* accessions at the molecular level (Fig. 25b; Fig. 26a & b; Fig. 27 a & b). Among the 24 random primers used, 22 primers resulted in polymorphic banding pattern whereas two primers *viz*., OPG-06 and OPH-03 produced 4 and 3 loci respectively, which were found to be monomorphic (Table 14). A total of 142 loci were produced by 24 primers of which 86 loci were found to be polymorphic with 60.6% loci being polymorphic. The number of loci ranged from 2 to 11 with a molecular size ranging from 150-2200 bp for different primers tested. Of 974 bands amplified by RAPD primers, 404 bands were polymorphic giving 41.5% polymorphism. The Operon primer, OPAL-08 yielded maximum number of 11 loci whereas minimum number of loci (2) was generated from the primer OPC-06. The percentage of polymorphism varied from 4.8% to 100% for the different primers. Highest level of polymorphism (100%) was produced by the primers OPG-16 and OPS-05 which produced 39 and 22 polymorphic bands, respectively. The primer OPAB-06 produced 6 loci of which 3 were found to be polymorphic resulting in 50% polymorphic loci (Fig. 26b). The average number of loci/primer was found to be 5.9 whereas the average number of polymorphic loci per primer was found to be 3.6. It was observed that few bands amplified by some primers showed considerable differences in the intensities in *Givotia* plants. In addition, unique bands were generated from 5 primers in male plants (1M, 2M, 3M and 4M) and absent in other plants. For example, the primer OPAL-08 generated a unique band of 900 bp in one male plant (Table 13; Fig. 26a). Another primer OPA-18 produced two unique bands of sizes 750 bp and 500 bp in two male plants and absent in other plants (Table 14; Fig. 27a).

Cluster analysis was done on the basis of Jaccard's similarity co-efficient based on the pooled RAPD data of 974 bands using 24 primers. Similarity coefficients ranged from 0.642 to 0.829, with the highest similarity of 0.829 observed between male accessions 3M and 4M (Table 15). Mean RAPD locus similarities between male and female accessions was found to be 0.704, with an average of 0.714 for all pair-wise comparisons. Cluster analysis revealed grouping of *Givotia* plants into 3 clusters at 70% similarity level (Fig. 28). The first cluster comprised of four female accessions (1F, 2F, 5F and 4F) and four male accessions (3M, 4M, 5M and 2M) whereas the second and third cluster comprised of one male accession (1M) and

one female accession (3F), respectively. The first cluster of the major cluster was sub-divided into 2 sub-clusters. The first sub-cluster consisted of three female accessions (1F, 2F and 5F) with two female accessions (2F and 5F) forming another sub-cluster within it. The second sub-cluster comprised four male accessions (3M, 4M, 5M and 2M) and one female accession (4F) and having sub-clusters within it. Thus cluster analysis revealed lack of grouping of *Givotia* accessions based on the sex with the random primers employed.

4.2.2. Genetic variability in male and female *Givotia* plants detected by ISSR analysis:

Screening of the 12 ISSR primers on the bulk DNA samples did not reveal any differences between male and female trees (Fig. 29a). These primers were further tested on 5 individual male and female plants to determine their usefulness in detecting genetic variability. Among the 12 ISSR primers used, polymorphic banding pattern was detected in 11 primers whereas one primer UBC-810 produced 4 bands, which were found to be monomorphic. The percentage of polymorphism varied from 11.7% to 75.6% with different primers with the highest produced by the primer UBC-880 (Table 16). The number of loci ranged from 4 to 11 and the molecular weight of the amplified bands ranging from 200-2000 bp. The average number of loci/primer was found to be 7.4 whereas the average number of polymorphic loci/primer was found to be 3.1. The primer UBC-835 yielded maximum number of 11 loci whereas minimum number of loci (4) was generated from the primer UBC-810. Out of 730 bands amplified by 12 ISSR in all plants, 210 bands were polymorphic resulting in 28.8% polymorphism. The ISSR profiles obtained with the primers UBC-808, UBC-846 and UBC-824 are shown in the Fig. 29b, Fig. 30a and Fig. 30b, respectively.

Cluster analysis was performed using Jaccard's similarity co-efficient values from pooled ISSR data of 730 bands using 12 primers. Similarity coefficients matrices, calculated from ISSR markers to generate a dendrogram ranged from 0.655 to 0.923 between male and female plants (Table 17). Mean ISSR locus similarities between male and female plants was found to be 0.808 which was higher than that of RAPDs. Cluster analysis revealed grouping of *Givotia* plants into 3 clusters at 83.5% similarity level (Fig. 31). The first cluster comprised of five female plants (1F, 2F, 3F, 4F and 5F) and three male plants (1M, 2M, and 3M) whereas the second and third clusters comprised of two male plants (4M and 5M), respectively. The first cluster was sub-divided into two sub-clusters with increase in similarity coefficient values.

Thus cluster analysis revealed lack of grouping of *Givotia* plants based on the sex with the ISSR primers employed.

4.2.3. Genetic variability in male and female plants of *Givotia* detected by combined RAPD and ISSR analysis:

Cluster analysis based on the combining data of RAPD and ISSR markers generated a dendrogram that failed to separate the male and female into two distinct clusters. Similarity coefficients ranged from 0.672 to 0.836, with the highest similarity of 0.836 observed between male plants 4M and 2M (Table 18). Mean locus similarities between male and female plants were found to be 0.760 for all pair-wise comparisons. Cluster analysis based on combined data revealed grouping of *Givotia* plants into 3 clusters at 74.5% similarity level (Fig. 32). The first cluster comprised of four female plants (1F, 2F, 4F and 5F) and four male plants (2M, 3M, 4M and 5M) whereas the second and third cluster comprised of one male plant and one female plant (1M and 3F), respectively. The first cluster was sub-divided into two sub-clusters. Differences were noticed with respect to the position of male and female plants in the dendrograms obtained from individual RAPD, ISSR and combined analysis. Thus cluster analysis did not group the plants according to sex of the plants.

The Mantel test statistic (Z) and the product moment correlation (r) were calculated to estimate the degree of relationship between the similarity matrices obtained with combined RAPD and ISSR data and the correlation was not significant (-0.078).

4.2.4. Screening of male and female plants for identification of molecular marker linked to sex:

In the present study, twelve 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 bands being observed in both males and females. Therefore, the polymorphic bands produced by these primers in the bulk 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. The total number of bands in the bulk DNA samples was found to be 100 which were lesser than the total number

of bands in individual plants (142) which could be due to lesser amount of template DNA of individual plants available for amplification in bulk DNA. During screening of 5 male and 5 female individual plants with OPT-17, there was partial association of 1000 bp band with sex which was present in all 5 females and one male and absent in other 4 males tested (Fig. 25b).

Subsequently, the OPT-17 primer was tested in 20 each of male and female plants growing at Mulugu Forest Research Centre to study the association of 1000 bp band with sex (Fig. 33). However, the 1000 bp band was faintly present in 1 female and absent in rest of the plants. But the OPT-17 primer resulted in an amplified product of 550 bp in 13 females out of 20 females and faintly present in 6 males out of 20 males analyzed.

4.2.5. Screening of SCAR primers in male and female plants for validation:

The 550 bp band produced by OPT-17 primer was eluted, amplified by PCR, cloned in the vector and confirmed by double digestion with restriction enzymes which yielded a 550 bp product (Fig. 34a & b). The cloned DNA fragment was sequenced and BLASTN analysis of the nucleotide sequence of the 550 bp amplified fragment did not show any matches with known sequence in the NCBI database (Fig. 34c & d). SCAR primers were designed based on the nucleotide sequence of the 550 bp amplicon and tested on 20 each of male and female plants (Table 19). However, the SCAR primers generated the 550 bp band in all male and female trees and did not show any association with sex.

4.3.1. *In vitro* propagation from nodal explants of *Givotia*:

The nodal explants obtained from green shoots of medium thickness exhibited axillary bud sprouting whereas those obtained from tender green shoots or mature brown shoots failed to respond after culturing on MS basal medium. The rates of contamination were very high and varied from 60-80% irrespective of the maturity of the nodal cuttings. The browning of explants due to phenolic exudation was the major problem affecting the success of axillary bud sprouting from the nodal explants. The browning problem was reduced by placing the nodal cuttings at room temperature for 2 days and incubating the explants in antioxidant solution (100 mg/l each of ascorbic acid and citric acid) for 5 min before sterilization of the explants. The axillary bud sprouting from the nodal explants varied from 21.8-39.6% on the

media tested (Fig. 35a & b). The nodal explants cultured on MS or WPM basal medium exhibited sprouting at a frequency of 24.8% and 23.3%., respectively after 30 days of culture (Fig. 36a & b). Axillary bud sprouting was achieved at a higher frequency of 39.6% on WPM medium fortified with 1.0 mg/l BAP as compared to other media tested (Fig. 36c & d). The frequency of axillary bud sprouting decreased (22.7-23.5%) on MS or WPM supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA. Multiple shoot induction was observed from the axillary bud region of the nodal explants after subculture on the same medium (Fig. 36e & f). The average number of shoots induced per explant remained higher (3.8) on MS medium with 1.0 mg/l BAP as compared to WPM containing 1.0 mg/l. BAP (Fig. 35b). The average length of the shoots varied from 0.5-1.8 cm on the media tested (Fig. 35b). There was abundant callusing observed from the base of the axillary shoots after subculture on WPM with 1.0 mg/l BAP as compared to MS medium with 1.0 mg/l BAP. Hence, MS medium was further used with BAP for shoot multiplication

The shoot tips excised from the axillary shoots were cultured on MS medium with BAP for evaluating the response of shoot proliferation. Of the different concentrations (0.25-2.0 mg/l) of BAP used in MS medium, 1.0 mg/l BAP induced higher frequency (59.8%) of multiple shoot induction (Fig. 37a) with induction of 3.4 shoots per explant with an average shoot of 1.2 cm (Fig. 37b & c). As repeated subcultures in the presence of 1.0 mg/l BAP increased the callus formation from the base of the cultures, MS medium with 0.5 mg/l BAP was considered to be suitable for shoot multiplication as well as shoot elongation (Fig. 37d & e).

Although the contamination rates were lower from shoot tips excised from mature trees as compared to nodal explants, they failed to respond and turned brown after 30 days of culture.

4.3.2. Effect of age of emblings on *in vitro* shoot regeneration from epicotyl and hypocotyl explants:

In the present study, the epicotyl and hypocotyl explants obtained from 20-day-old emblings exhibited maximum shoot regeneration in terms of frequency (82.6-87.6%) as well as number of shoots/explant (5.2-7.3) as compared to 10-day-old or 30-day-old emblings on MS medium with 1.0 mg/l BAP (Fig. 38a & b). The epicotyl and hypocotyl explants cultured on

growth regulator free medium failed to respond for regeneration irrespective of the age of the emblings.

4.3.3. Influence of explant position and orientation on shoot regeneration:

The morphogenic potential of the proximal or distal regions of epicotyl and hypocotyl explants varied. Maximum percentage of shoots was induced from the epicotyl and hypocotyl explants from the proximal region close to the cotyledonary nodes when placed vertically on the medium with 1.0 mg/l BAP. The hypocotyl explants close to root node exhibited callus formation with induction of 2-3 shoots whereas the proximal region close to cotyledonary node responded efficiently with induction of 5-6 shoots per explant. Only 2-3 shoots were induced from the epicotyl explants close to shoot tip in comparison to 7-8 shoots from the region close to the cotyledonary node. Occasionally, 10-12 shoots were also induced from a single epicotyl explant. Very few shoots (2-3) were induced from the epicotyl and hypocotyl explants (either the region close to cotyledonary node or distal region) when placed horizontally on medium containing 1.0 mg/l BAP. Hence, for the subsequent experiments on the effects of different growth regulators on shoot regeneration, the proximal region of the epicotyls and hypocotyls close to cotyledonary node were used.

4.3.4. Effect of plant growth hormones on shoot regeneration from epicotyl and hypocotyl explants:

The shoot regeneration response from epicotyl and hypocotyl explants was tested after culture on medium supplemented individually with BAP or KN. High frequency shoot regeneration was observed from epicotyl and hypocotyl explants on medium supplemented with BAP compared to KN (Fig. 39a & b). Among the different concentrations of BAP tested, the best response in terms of frequency of shoot regeneration (82.6-87.6%) and average shoot number per explant (5.2-7.3) was observed on medium constituting 1.0 mg/l BAP compared to other combinations tested. Shoot buds were induced directly from the explants after 7-8 days of culture. Epicotyl explants displayed higher capacity for shoot regeneration with induction of 7.3 shoots per explant as compared to 5.2 shoots per explants in hypocotyls on initiation medium containing 1.0 mg/l BAP (Fig. 39a & b; Fig. 40 a

& b). Higher concentrations of BAP (2.0 mg/l) in the medium decreased the regeneration frequency and enhanced the callus formation from the base of epicotyl and hypocotyl explants. The shoot regeneration frequency from epicotyl and hypocotyl explants varied from 51.4-73.8% on medium supplemented with different concentrations of KN (0.25-1.0 mg/l) with induction of 2.0-3.8 shoots per explant after 30 days of culture. The average length of the shoots induced on medium containing BAP and KN varied from 0.2-1.8 cm after 30 days of culture (Fig. 39b). Incorporation of 0.1 mg/l NAA on medium containing 1.0 mg/l BAP increased the callus formation from the base of the explants and decreased the shoot growth.

Shoot multiplication was achieved at a higher rate after subculture on medium with 1.0 mg/l as compared to 0.5 mg/l BAP. The average number of shoots induced per explant from hypocotyls was lower than epicotyls in the initiation medium but no differences were noticed in terms of shoot multiplication rate after they were subcultured on medium with 1.0 mg/l BAP which stimulated induction of 6-7 new shoots per explant (Fig. 40c & d). Only 1-2 shoots from the shoot clump increased to about 2 cm whereas the remaining exhibited slow growth on medium containing 1.0 mg/l BAP. On the other hand, subculture on medium with 0.5 mg/l BAP favoured shoot elongation and induction of 4-5 new shoots. Two subcultures (at 30-day intervals) on medium containing 0.5 mg/l BAP resulted in recovery of 4-5 shoots of 2-3 cm per explant and well expanded leaves (Fig. 40e & f). In order to achieve higher rates of shoot multiplication, the explants were initially subcultured on medium with 1.0 mg/l BAP followed by 2 subcultures on medium with 0.5 mg/l BAP which resulted in induction of 10-12 shoots of 2-3 cm per explant. The shoots of 2-3 cm were separated from the shoot clumps and placed on medium with 0.25 mg/l BAP for 3 weeks in order to prevent or minimize callus formation from the base of shoots during root induction. After separating the elongated shoots, the remaining shoot clumps were subcultured on MS medium with 0.5 mg/l BAP for promoting shoot multiplication as well as shoot elongation. This process of repeated subcultures on medium with 0.5 mg/l BAP was carried out for 6 cycles for recurrent production of shoots without any loss in multiplication capacity.

4.3.5. Root induction from *in vitro* regenerated shoots:

Roots were induced from the regenerated shoots on medium containing 0.1 mg/l IAA or IBA whereas those cultured on medium without plant growth regulators failed to form

roots. Significant differences in root induction frequency (37.8-38.2%) were not observed in the presence of IBA and IAA but the average number of roots (2.8) and the average length of roots (5.5 cm) were higher on medium containing IBA as compared to IAA (Fig. 41a & b). Among the different treatments examined, pulse treatment with IBA for 10 sec improved the rooting response from the shoots when cultured on MS medium with 0.1 mg/l IBA. The shoots that were pulse treated with IBA formed roots at 44.3% frequency with induction of 4.9 roots/shoot with an average root length of 5.9 cm after 5 weeks of culture on medium with 0.1 mg/l IBA (Fig. 41a & Fig. 42a). The response of rooting from the shoots that were pulse treated with IAA was found to be better (39.6%) on basal medium with induction of 3.5 roots/shoot with an average root length of 5.8 cm after 5 weeks of culture (Fig. 42b). Pulse treatment of the shoots with IAA decreased the root induction frequency and root growth, and enhanced the callus formation from the base of the shoots when cultured on MS medium with 0.1 mg/l IAA. Roots started to appear after 10-12 days of culture for the different treatments used. The shoot growth in cultures that exhibited rooting was rapid with well developed shoots of 5-6 cm and leaves whereas the shoots that remained un-rooted exhibited slow growth. Occasionally, the un-rooted or rooted shoots exhibited callusing at the base of the shoots irrespective of the treatment used. The growth of the roots was affected whenever callusing was observed at the juncture of shoots and roots and these plants fail to establish in the soil.

The frequency of rooting from the shoots originating from the nodal explants collected from mature tree was also evaluated after pulse treatment with IBA or IAA followed by culture on MS with IBA and MS basal medium, respectively. The shoots pulse treated with IBA rooted with a frequency of 41.3% while those that were pulse treated with IAA rooted with a frequency of 40.7% on MS basal medium (Fig. 42c & d). The rooting response from the pulse treated shoots in liquid media containing 0.1 mg/l IBA was evaluated. However, the base of the shoots exhibited bulging without any root induction (Fig. 42e).

4.3.6. Establishment of acclimatized plants in soil:

The micropropagated plants or emblings with three to four completely expanded leaves and well-developed roots were transferred to pots containing potting mixture and acclimatized initially in culture room for 4 weeks and then moved to glasshouse. The plants

resumed growth with emergence of new leaves after 15-20 days of transfer to pots. After acclimatization for 4 weeks in glasshouse (Fig. 43a), plants were transferred to larger pots for further growth (Fig. 43b). The emblings and micropropagated plants survived at frequency of 80% and 70%, respectively in the glasshouse and were transplanted with 90-95% success rate in field during rainy season (Fig. 43c & d).

The performance of micropropagated plants and emblings with respect to different traits was studied after 12 months of transfer to soil in the greenhouse. There were no significant differences noticed with respect to average height of the plants, average internodal distance and average number of leaves in micropropagated plants in comparison to the emblings (Fig. 43e). However, the micropropagated plants had larger length (15.7 cm) and width (19.5 cm) as compared to the leaf length (11.1 cm) and width (19.5 cm) of the emblings.

The scheme of micropropagation used in the study resulted in recovery of 6-7 plants per single nodal explant derived from mature tree in 6 months duration (Fig. 44). When epicotyls and hypocotyl explants of *in vitro* germinated emblings were used as explants, about 12-16 plants per embling were obtained in 6 months duration (Fig. 45).

4.3.7. RAPD analysis of micropropagated plants:

DNA markers such as RAPD have been widely used to detect somaclonal variations in tissue culture raised plants in several plant species. A total of 10 RAPD primers that gave clear and reproducible bands were used to evaluate the genetic stability of the micropropagated plants after 12 passages in culture and after three months of transfer to soil. The number of loci varied from 4 to 11 with the amplified fragment sizes ranging from 190-3000 bp for the primers tested. Ten RAPD primers produced 67 loci, with an average of 6.7 loci per primer (Table 20). The primer OPV-17 generated highest number of loci (11) as well as the highest number of bands (110) whereas the lowest number of loci (4) and bands (40) was produced by the primers OPP-03 which were found to be monomorphic among the regenerated plants. The primers OPF-3 produced 6 lcoi, of which 5 were monomorphic and one band corresponding to 800 bp was polymorphic present in 3 micropropagated plants and absent in 6 micropropagated plants (Fig. 46a). Similarly, the primer, OPZ-06 produced 7 monomorphic and one polymorphic band (800 bp) present in 3 micropropagated plants and absent in 6 micropropagated plants. The OPC-05 primer produced 6 monomorphic bands

present in all micropropagated plants (Fig. 46b). Out of 658 amplified fragments, 650 were monomorphic in tissue culture raised plants and comparable to donor plant whereas 8 were polymorphic resulting in a low polymorphism of 1.2%. Thus RAPD analysis revealed a high percentage (98.8%) of genetic similarity in micropropagated plants.

4.4.1. Variation in fruit and seed traits and seed germination in *Givotia* accessions:

Variation was observed in mature dry fruit and seed traits and seed germination among accessions of *Givotia* collected from five forest regions which were found to be significant (Fig. 47a & b; Fig. 48). Considerable variability was observed with respect to the average weight of 10 mature dry fruits with the highest (56.0 gm) in Achampet accessions and the lowest (29.4 gm) in Balapally accessions. The average diameter of fruits varied from 18.7 to 23.0 mm with the highest fruit diameter recorded in Achampet accessions as compared to other accessions. The average weight of 10 seeds ranged from 7.3 to 9.9 gm with highest observed in Achampet accessions whereas lowest values were observed for Balapally and Devarayanaduga accessions which were not significantly different. The average weight of 10 seeds did not differ significantly among Kondapalli and Pacharla accessions. The average seed diameter varied from 12.2 to 11.1 mm with relatively large seed diameter being observed for Achampet and Devarayanadurga accessions. The total percentage of fruit weight to seed weight varied from 17.5-24.8% whereas fruit diameter to seed diameter varied from 52.2-59.7% for the different accessions analyzed. Overall, the accessions of Achampet exhibited larger fruit weight as well as seed weight in comparison to other seed sources.

4.4.2. Seed oil content of *Givotia* accessions:

The total oil content present in the seeds was estimated using solvent extraction method (Fig. 49). The crude oil was yellow in colour and was liquid at room temperature (26±5°C)) and solidified when kept at refrigerator (4°C). Hexane extraction yielded marginally higher oil yield (22%) from the bulk seeds compared with petroleum ether (20%) and thus hexane was used in the subsequent experiments for estimation of oil content in different accessions. The oil percentage in seeds of different accessions ranged from 18.2% to 22% with the highest oil percentage observed in Achampet accessions followed by

Devarayanadurga accessions whereas significant differences in oil percentage was not observed among accessions of Balapally, Kondapalli and Pacharla accessions (Fig. 48).

4.4.3. *In vitro* germination from zygotic embryo axes of *Givotia* accessions:

The seeds of all the accessions were found to be dormant as they failed to germinate either *in vitro* or in soil even after 3 months. The germination percentage of zygotic embryo axes was calculated after culture on MS medium (half-strength major salts) medium. A low germination response (5.2-13.2%) was observed from the zygotic embryo axes of all the accessions with the lowest value (5.2%) observed from accessions of Kondapalli accessions (Fig. 50). In all the accessions analyzed, the temperature treatment (55ºC) of the seeds for 24 h enhanced the germination response (16.3-25.6%) from cultured zygotic embryo axes. Incorporation of 0.25 mg/l KN and 0.1 mg/l IBA in MS medium had a beneficial effect, with the germination percentage from zygotic embryo axes ranging from 25.3% to 35.5% for different *Givotia* accessions analyzed (Fig. 50 & 51). In the present study, the maximum germination percentage (40.3-70.3%) was achieved from the temperature treated seeds upon culture on MS medium with 0.25 mg/l IBA and 0.1 mg/l KN. Among all the accessions analyzed, the higher germination response was recorded in Kondapalli (70.3%) followed by Pacharla (53.1%), Achampet (50.2%), Balapally (47.1%) and Devarayanadurga (40.3%) accessions. Thus significant differences in germination percentage was observed from different accessions although in all cases temperature treatment of seeds followed by culture of zygotic embryo axes on medium with KN and IBA proved to be superior for obtaining high germination percentage. Germination occurred asynchronously and the germination response with respect to root and shoot emergence was not uniform from the seed lots of the same accession as well as different accessions for the treatments used. The germination was initiated after 7-12 days from zygotic embryo axes of untreated seeds when cultured on MS medium containing IBA and KN whereas in temperature treated seeds initiation was observed within 4-8 days of culture.

4.4.4. Fatty acid composition of seed oil of *Givotia* accessions:

The prevalent fatty acids in methyl ester extract of seed oil of *Givotia* as detected by GC-MS analysis were oleic acid (18:1), linoleic acid (18:2), stearic acid (18:0), palmitic acid (16:0) and α-eleostearic acid (Fig. 52). A group of five conjugated linoleic acids were observed in GC with the retention values ranging from 16.044 to 16.708 among which α-eleostearic acid is the only fatty acid that could be identified and characterized. The seed oil of *Givotia* was composed of approximately 8.21% palmitic acid, 9.8% stearic acid, 10.43% oleic acid, 25.14% linoleic acid, 7.55% eleostearic acid and 38.92% of other conjugated fatty acids. Thus the seed oil was composed of 18.0% saturated fatty acids, 10.43% monounsaturated fatty acids and 71.6% polyunsaturated fatty acids. Comparison of the component fatty acids with that of other edible and non-edible seed oils (as reported in literature) showed that it has high percentage of conjugated fatty acids with eleostearic acid comprising 7.54% of conjugated fatty acids whereas tung (*Vernicia fordii*) seed oil was characterized by the presence of high percentage of α-eleostearic acid (Table 21). The oleic acid and linoleic acid was relatively lower as compared to sunflower seed oil The composition of fatty acids in seed oil of *Givotia* also differed from seed oils of *Jatropha* and *Hevea brasiliensis* belonging to Euphorbiaceae.

Table A: 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)	Sex-associated band(s) detected			Reference
			Males	Females	Hermaphrodites		Males	Females	Hermaphrodites	
Borassus flabellifer L.	180	1	30	30	--	OPA-06	Absent	600 bp present	--	George *et al.* 2007
Calamus simplicifolius C.F. Wei	1040	1	20	20	--	S1443	500 bp present	Absent	--	Yang *et al.* 2005
Carica papaya L.	80	1	7	7	--	OPF-02	800 bp present	Absent	--	Parasnis *et al.* 2000
Carica papaya L.	152	1	--	10	10	BC-210	--	Absent	438 bp present	Lemos *et al.* 2002
Carica papaya L.	25	1	6	7	4	IBRC-RP07	450 bp present	Absent	450 bp present	Urasaki *et al.* 2002
Carica papaya L.	32	1	4	3	2	OPY-07	900 bp present	Absent	Absent	Chaves-Bedoya and Nuñez 2007
Carica papaya L.	100	2	10	10	10	OPC-09 OPE-03	1700 bp present 400 bp present	Absent Absent	1700 bp present 400 bp present	Niroshini *et al.* 2008
Commiphora wightii (Arnott.) Bhandari	60	3	5	5	3	OPN-06 OPN-16 OPA-20	Absent Absent Absent	1280 bp present Absent 1140 bp present	Absent 400 bp present 1140 bp present	Samantaray *et al.* 2010
Cycas circinalis	10	2	3	3	--	OPB-01 OPB-05	686 bp present Absent	Absent 2048 bp present	-- --	Gangopadhyay *et al.* 2007
Encephalartos natalensis (Dyer and Verdoorn)	140	1	31	38	--	OPD-20	Absent	850 bp present	--	Prakash and Van Staden 2006
Eucommia ulmoides Oliv.	560	1	5	5	--	OPF-08	Absent	569 bp present	--	Xu *et al.* 2004
Ginkgo biloba	1200	1	30	30	--	S1478	682 bp present	Absent	--	Ling *et al.* 2003
Myristica fragrans Hout.	60	1	10	10	--	OPE-11	Absent	416 bp present in 9 females	--	Shibu *et al.* 2000
Phoenix dactylifera L.	30	3	3	4	--	A10 A12 D10	Absent 370 bp present 675 bp present	490 bp present 750 bp present 800 bp present	-- -- --	Younis *et al.* 2008
Pistacia vera L.	700	1	30	29	--	OPO-08	Absent	945 bp present	--	Hormaza *et al.* 1994
Simarouba glauca DC.	70	1	*	*	--	OPD-20	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

Table 1: Geographical location and distance between populations of *Givotia rottleriformis* Griff.

Location	Region	Latitude ^{o}N	Longitude ^{o}E	Distance between populations (Km)			
Andhra Pradesh	Pacharla (GP)	15	78	GP	GB	GA	GK
	Balapally (GB)	17	80	360			
Telangana	Achampet (GA)	16	78	330	446		
Andhra Pradesh	Kondapalli (GK)	17	82	470	493	354	
Karnataka	Devarayanadurga (GD)	13	77	452	331	488	740

Table 2: Amplification product profiles obtained with RAPD primers by analyzing 50 accessions of *Givotia* growing at different forest regions

S. No.	Primer	Loci generated/Polymorphic loci					TL	TPL	Total AB	Total PB	% polymorphism
		GA	GP	GB	GK	GD					
1.	OPG-16	11/11	4/2	13/12	3/0	11/8	15	15	209	209	100
2.	OPG-06	4/0	4/1	3/0	4/2	3/3	5	5	171	171	100
3.	OPG-17	6/4	5/4	8/7	2/0	9/4	11	11	186	186	100
4.	OPK-01	5/2	5/4	8/6	2/0	7/3	9	8	190	140	73.7
5.	OPK-07	7/4	6/5	11/11	7/6	11/3	15	15	218	218	100
6.	OPC-09	3/2	3/1	5/2	2/1	10/6	10	9	151	101	66.9
7.	OPC-06	2/1	5/4	7/6	2/1	7/6	10	9	110	60	54.5
8.	OPC-10	5/4	4/3	5/5	2/0	14/12	15	15	145	145	100
9.	OPC-08	9/6	7/2	14/12	9/7	9/9	17	17	243	243	100
10.	OPC-07	6/2	6/1	4/3	1/0	7/4	10	10	188	188	100
11.	OPH-03	3/0	5/1	5/3	5/3	5/4	6	5	174	124	71.3
12.	OPF-03	5/5	8/7	8/8	3/2	11/5	12	12	191	191	100
13.	OPA-04	5/1	6/5	10/10	7/5	9/6	12	12	201	201	100
14.	OPAB-06	6/3	3/0	5/5	6/4	9/4	10	10	196	196	100
15.	OPAL-08	9/6	12/12	22/21	11/9	15/11	23	23	283	283	100
16.	OPAK-14	4/2	4/3	5/4	4/2	7/2	8	7	181	131	72.4
17.	OPT-17	8/7	6/6	14/14	4/3	8/4	17	17	185	185	100
18.	OPZ-01	5/2	10/9	4/4	7/7	11/4	13	13	248	248	100
19.	OPZ-10	7/4	10/6	8/5	7/4	8/4	14	13	253	203	80.2
20.	OPZ-06	12/12	12/8	8/8	8/6	12/5	15	15	266	266	100
21.	OPB-12	9/5	8/3	8/6	7/1	9/5	11	10	281	231	82.2
22.	OPA-18	12/10	13/13	9/6	3/1	11/9	16	16	227	227	100
Total % PB		143/93 65.0%	146/100 68.5%	184/158 85.9%	106/64 60.4%	203/121 59.6%	274	267 97.4%	4497	4147	92.2

GA - *Givotia* Achampet; GP - *Givotia* Pacharla; GB - *Givotia* Balapally; GK - *Givotia* Kondapalli; GD - *Givotia* Devarayanadurga; TL - Total loci; TPL - Total polymorphic loci; AB - Amplified bands; PB - Polymorphic bands

Table 3: Amplification product profiles obtained with ISSR primers by analyzing 50 accessions of *Givotia* growing at different forest regions

S. No.	Primer	Loci generated/Polymorphic loci					TL	TPL	Total AB	Total PB	% polymorphism
		GA	GP	GB	GK	GD					
1.	UBC-810	5/1	6/2	6/2	5/1	6/1	7	3	255	105	41.2
2.	UBC-812	8/2	8/4	8/2	8/1	9/1	9	5	382	182	47.6
3.	UBC-808	7/1	9/6	7/0	7/0	7/5	9	7	322	222	68.9
4.	UBC-815	3/0	4/1	5/3	4/2	5/3	6	4	180	80	44.4
5.	UBC-824	9/5	4/3	7/6	5/3	4/3	10	10	181	181	100
6.	UBC-825	15/14	7/7	17/13	16/15	17/13	18	18	396	396	100
7.	UBC-826	6/3	3/0	11/9	7/5	8/4	12	12	246	246	100
8.	UBC-820	2/0	3/1	5/4	4/1	4/3	6	5	149	99	66.4
9.	UBC-823	6/2	6/2	6/4	5/3	5/4	7	6	224	174	77.7
10.	UBC-834	7/1	4/2	7/5	4/1	12/3	13	13	295	295	100
11.	UBC-835	10/0	8/2	9/5	5/1	10/4	12	9	377	227	60.2
12.	UBC-846	9/3	11/8	10/8	8/4	13/10	15	14	317	267	84.2
13.	UBC-880	5/0	5/0	10/8	7/0	11/4	12	11	304	254	83.5
14.	UBC-899	5/2	8/1	11/9	4/2	4/0	11	9	236	136	57.6
Total % polymorphism		97/34 35.1%	86/39 45.3%	119/78 65.5%	89/39 43.8%	115/58 50.4%	147	126	3864	2864	74.1

GA - *Givotia* Achampet; GP - *Givotia* Pacharla; GB - *Givotia* Balapally; GK - *Givotia* Kondapalli; GD - *Givotia* Devarayanadurga; TL - Total loci; TPL - Total polymorphic loci; AB - Amplified bands; PB - Polymorphic bands

Table 4: Sequences of SCAR primers designed from cloned RAPD amplicons generated with OPC-08 and OPT-17 primers in specific accessions of *Givotia*

S. No.	Primer	Primer sequence 5' to 3'
1.	490 FP	CTT TTC CCT TTC CCT CCA TC
2.	490 RP	AGA CCG TGT TTG GAT TCT GG
3.	490 FP	AAC CCT CAA CAG CGG CCG C
4.	350 FP	CCG ATT CCA ACG TCG TCC
5.	350 RP	CCA ACG TCG TGG CAT GGG

Table 5: Amplification product profiles obtained with RAPD primers in half-sib progeny of candidate plus tree of *Givotia*

S. No.	Primer	Size range (bp)	Total loci	Total polymorphic loci	Total AB	Total PB	% polymorphism
1.	OPG-16	320-2000	10	9	57	46	80.7
2.	OPC-08	275-1900	10	7	76	43	56.6
3.	OPT-17	300-1000	6	4	34	12	35.3
4.	OPB-12	150-1350	7	1	67	1	1.5
5.	OPV-08	150-2025	13	12	86	75	87.2
6.	OPW-17	250-1500	10	8	70	48	68.6
7.	OPAI-12	200-1600	16	14	100	78	78.0
8.	OPAL-20	150-1100	5	2	52	19	36.5
9.	OPE-20	1300-150	9	9	66	66	100
10.	OPB-07	300-1550	12	8	98	54	55.1
11.	OPG-14	600-2000	5	1	49	5	10.2
12.	OPK-03	500-1550	3	1	27	5	18.5
Total number of bands		150-2025	106	76	782	452	57.8

AB - Amplified bands; PB - Polymorphic bands

Table 6: Jaccard's similarity matrix of candidate plus tree (M) of Balapally and its 10 half-sib progeny revealed by RAPD analysis with 12 random primers

	M	HS1	HS2	HS3	HS4	HS5	HS6	HS7	HS8	HS9	HS10
M	1.000										
HS1	0.479	1.000									
HS2	0.464	0.663	1.000								
HS3	0.412	0.788	0.744	1.000							
HS4	0.433	0.812	0.747	0.854	1.000						
HS5	0.385	0.762	0.760	0.896	0.827	1.000					
HS6	0.427	0.831	0.722	0.852	0.831	0.872	1.000				
HS7	0.500	0.786	0.787	0.805	0.786	0.778	0.805	1.000			
HS8	0.447	0.756	0.709	0.774	0.798	0.768	0.795	0.709	1.000		
HS9	0.444	0.706	0.767	0.744	0.726	0.759	0.765	0.763	0.711	1.000	
HS10	0.440	0.738	0.757	0.823	0.759	0.818	0.823	0.753	0.702	0.779	1.000

Table 7: Banding pattern in half-siblings and maternal tree of *Givotia* obtained with RAPD primers

S. No.	Primer	Number of bands (Molecular weight)		
		Present in all	Present in maternal tree only	Unique to half-sibling
1.	OPG-16	1 (320 bp)	1 (1450 bp)	--
2.	OPC-08	3 (800 bp, 650 bp, 450 bp)	--	--
3.	OPT-17	2 (350 bp, 300 bp)	1 (1000 bp)	--
4.	OPB-12	6 (1350bp, 1000 bp, 700 bp, 600 bp, 500 bp, 150 bp)	--	--
5.	OPV-08	1 (475 bp)	1 (850bp)	1 in HS9 (550 bp)
6.	OPW-17	2 (1500 bp, 250 bp)	--	--
7.	OPAI-12	2 (1000 bp, 650 bp)	--	1 in HS10 (625 bp)
8.	OPAL-20	3 (1100 bp, 400 bp, 150 bp)	--	--
9.	OPE-20	--	--	--
10.	OPB-07	4 (1000 bp, 650 bp, 500 bp, 400 bp)	3 (1100 bp, 750 bp, 700 bp)	--
11.	OPG-14	4 (2000 bp, 1500 bp, 1200 bp, 600 bp)	--	--
12.	OPK-03	2 (1550 bp, 1000 bp)	--	--

Table 8: ISSR profiles of candidate plus tree (M) and its 10 half-sib progeny of *Givotia*

S. No.	Primer	Size range (bp)	Total loci	Total polymorphic loci	Total amplified bands	Total polymorphic bands	% polymorphism
1.	UBC-823	400-1800	8	4	55	11	20.0
2.	UBC-808	300-1800	12	8	93	49	52.7
3.	UBC-834	250-1850	8	6	59	37	62.7
4.	UBC-812	100-1050	9	8	47	36	76.6
5.	UBC-815	220-1220	11	8	93	60	64.5
6.	UBC-899	300-1580	7	5	40	18	45.0
7.	UBC-810	200-500	5	1	54	10	18.5
8.	UBC-880	350-800	4	1	43	10	23.3
9.	UBC-825	250-1600	9	8	60	49	81.7
10.	UBC-826	350-450	3	2	22	11	50.0
Total number of bands		100-1850	76	51	566	291	51.4

Table 9: Jaccard's similarity matrix of candidate plus tree (M) and 10 half-sib progeny of *Givotia* revealed by ISSR markers

	M	HS1	HS2	HS3	HS4	HS5	HS6	HS7	HS8	HS9	HS10
M	1.000										
HS1	0.567	1.000									
HS2	0.484	0.677	1.000								
HS3	0.545	0.850	0.741	1.000							
HS4	0.492	0.710	0.778	0.807	1.000						
HS5	0.645	0.820	0.712	0.770	0.717	1.000					
HS6	0.538	0.758	0.737	0.797	0.772	0.738	1.000				
HS7	0.629	0.803	0.695	0.845	0.729	0.814	0.780	1.000			
HS8	0.563	0.787	0.623	0.738	0.683	0.767	0.677	0.810	1.000		
HS9	0.581	0.783	0.672	0.763	0.707	0.793	0.729	0.746	0.759	1.000	
HS10	0.581	0.646	0.672	0.705	0.707	0.651	0.729	0.689	0.645	0.724	1.000

Table 10: Banding pattern obtained with ISSR primers in half-siblings and maternal tree of *Givotia*

S. No.	Primer	Number of bands (Molecular weight)	
		Present in all	Present in all half-siblings and absent in maternal tree
1.	UBC-823	4 (1100 bp, 1000 bp, 800 bp, 500 bp)	--
2.	UBC-808	4 (1500 bp, 700 bp, 400 bp, 300 bp)	--
3.	UBC-834	2 (390 bp, 250 bp)	--
4.	UBC-812	1 (400 bp)	--
5.	UBC-815	3 (1200 bp, 400 bp, 300 bp)	3 (600 bp, 500 bp, 450 bp)
6.	UBC-899	2 (500 bp, 300 bp)	--
7.	UBC-810	4 ((420 bp, 300 bp, 250 bp, 200 bp)	1 (500 bp)
8.	UBC-880	3 (700 bp, 550 bp, 350 bp)	--
9.	UBC-825	1 ((500 bp)	--
10.	UBC-826	1 (400 bp)	--

Table 11: Jaccard's similarity matrix of candidate plus tree (M) of *Givotia* and its 10 half-sib progeny revealed by RAPD and ISSR markers

	M	HS1	HS2	HS3	HS4	HS5	HS6	HS7	HS8	HS9	HS10
M	1.000										
HS1	0.516	1.000									
HS2	0.473	0.669	1.000								
HS3	0.466	0.814	0.743	1.000							
HS4	0.457	0.769	0.759	0.835	1.000						
HS5	0.487	0.786	0.739	0.841	0.780	1.000					
HS6	0.472	0.800	0.728	0.829	0.807	0.813	1.000				
HS7	0.553	0.793	0.746	0.821	0.762	0.793	0.794	1.000			
HS8	0.494	0.769	0.671	0.759	0.750	0.768	0.745	0.750	1.000		
HS9	0.500	0.738	0.725	0.752	0.718	0.774	0.750	0.755	0.730	1.000	
HS10	0.497	0.698	0.720	0.771	0.738	0.743	0.783	0.725	0.678	0.756	1.000

Table 12: Morphological characteristics of eight-year-old male and female trees of *Givotia*

Character	Male trees	Female trees
Average no. of nodes/branch	14.6 $\pm$ 0.9	15.2 $\pm$ 0.4
Average no. of leaves/branch	15.6 $\pm$ 0.7	15.4 $\pm$ 0.4
Average length of internodes (cm)	2.9 $\pm$ 0.3	2.4 $\pm$ 0.3
Leaf width (cm)	17.6 $\pm$ 0.4	16.9 $\pm$ 0.2
Leaf length (cm)	16.2 $\pm$ 0.5	16.5 $\pm$ 0.2
Petiole length (cm)	19.6 $\pm$ 1.4	20.3 $\pm$ 1.5
Trunk girth (cm)	32.9 $\pm$ 1.1	34.9 $\pm$ 1.9

Table 13: Amplification product profile generated from 24 random primers with bulk DNA samples of male and female plants of *Givotia*

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

Table 14: RAPD data obtained with 24 random primers from genomic DNA of male and female *Givotia* plants

S. No.	Primer	Size range (bp)	Total loci	Total polymorphic loci	Total amplified bands	Total polymorphic bands	% polymorphism	Unique bands
1.	OPA-04	250-1300	5	1	49	9	18.4	--
2.	OPA-18	200-1500	9	7	39	19	48.7	750 bp band in 1M ; 500 bp band in 4M
3.	OPAB-06	400-1050	6	3	45	15	33.3	--
4.	OPAK-14	500-1100	3	2	22	12	54.5	--
5.	OPAL-08	250-2200	11	8	82	52	63.4	900 bp band in 1M
6.	OPB-12	150-1400	9	8	51	41	80.4	--
7.	OPC-06	475-1000	2	1	14	4	28.6	--
8.	OPC-07	325-900	6	1	58	8	13.8	--
9.	OPC-08	480-1900	8	5	55	25	45.5	1300 bp band in 3M
10.	OPC-09	500-1700	3	1	24	4	16.7	--
11.	OPC-10	450-1200	3	1	21	1	4.8	1200 bp band in 4M
12.	OPF-03	600-1500	4	1	37	7	18.9	--
13.	OPF-11	230-1600	8	3	66	16	24.2	--
14.	OPG-06	400-1400	4	0	40	0	0	--
15.	OPG-16	350-1800	10	10	39	39	100	1000 bp in 2M
16.	OPG-17	350-1100	6	5	37	27	73.0	--
17.	OPH-03	600-1400	3	0	40	0	0	--
18.	OPK-01	390-2100	5	2	34	4	11.8	--
19.	OPK-07	350-1800	7	6	45	35	77.8	--
20.	OPS-05	300-1000	5	5	22	22	100	--
21.	OPT-17	300-2000	9	7	47	27	57.4	--
22.	OPZ-01	300-900	4	1	35	5	14.3	--
23.	OPZ-06	300-1200	7	6	31	21	67.7	--
24.	OPZ-10	350-2000	5	2	41	11	26.8	--
Total number of bands			142	86	974	404	41.5	

Table 15: Jaccard's similarity matrix of male and female *Givotia* plants revealed by RAPD analysis with 24 random primers

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

Table 16: ISSR data obtained with 12 random primers from genomic DNA of male and female *Givotia* plants

S. No.	Primer	Size range (bp)	Total loci	Total polymorphic loci	Total amplified bands	Total polymorphic bands	% polymorphism
1.	UBC-810	200-400	4	0	40	0	0
2.	UBC-812	200-800	8	3	68	18	26.5
3.	UBC-808	250-800	6	2	52	12	23.1
4.	UBC-815	300-1000	5	2	36	6	16.7
5.	UBC-826	350-1000	6	4	33	13	39.4
6.	UBC-823	200-1600	6	1	58	8	13.8
7.	UBC-824	300-2000	9	5	55	15	27.3
8.	UBC-834	280-1000	7	1	68	8	11.8
9.	UBC-835	300-2000	11	2	104	14	13.5
10.	UBC-846	300-1100	8	4	65	25	38.5
11.	UBC-880	320-1500	10	8	82	62	75.6
12.	UBC-899	300-2000	9	5	69	29	42.0
Total number of bands			89	37	730	210	28.8

Table 17: Jaccard's similarity matrix of male and female *Givotia* plants revealed by ISSR markers

	1F	2F	3F	4F	5F	1M	2M	3M	4M	5M
1F	1.000									
2F	0.886	1.000								
3F	0.868	0.882	1.000							
4F	0.899	0.864	0.846	1.000						
5F	0.873	0.863	0.868	0.923	1.000					
1M	0.864	0.900	0.835	0.843	0.841	1.000				
2M	0.886	0.899	0.882	0.864	0.886	0.900	1.000			
3M	0.845	0.835	0.795	0.847	0.845	0.859	0.880	1.000		
4M	0.844	0.833	0.813	0.823	0.868	0.813	0.882	0.795	1.000	
5M	0.744	0.671	0.711	0.725	0.744	0.655	0.691	0.682	0.781	1.000

Table 18: Jaccard's similarity matrix of male and female *Givotia* plants revealed by RAPD and ISSR markers

	1F	2F	3F	4F	5F	1M	2M	3M	4M	5M
1F	1.000									
2F	0.796	1.000								
3F	0.737	0.739	1.000							
4F	0.787	0.790	0.768	1.000						
5F	0.778	0.800	0.768	0.800	1.000					
1M	0.751	0.772	0.732	0.764	0.763	1.000				
2M	0.771	0.774	0.735	0.792	0.765	0.801	1.000			
3M	0.795	0.770	0.713	0.798	0.771	0.771	0.791	1.000		
4M	0.766	0.760	0.720	0.788	0.779	0.752	0.836	0.814	1.000	
5M	0.711	0.696	0.672	0.716	0.760	0.682	0.693	0.741	0.767	1.000

Table 19: Sequences of SCAR primers designed from cloned RAPD fragment generated with OPT-17 primer in female and male accessions of *Givotia*

S. No.	Primer Name	Primer Sequence 5' to 3'
1.	550 FP	CTA CCC AGC ATA CGC TCG AC
2.	550 RP	CCA ACG TCG TAG CAG AGG A
3.	550 FP	ACC CCT ACC GGT CAT TCT TC
4.	550 RP	AGG AAG AAT GAC CGG TAG GG

Table 20: RAPD analysis of micropropagated plants in comparison to donor plant

S. No.	Primer	Size range (bp)	Total loci	Total polymorphic loci	Total amplified bands	Total polymorphic bands	% polymorphism
1.	OPA-16	200-2800	9	0	90	0	0
2.	OPAB-06	300-1000	5	0	50	0	0
3.	OPAK-14	300-1600	5	0	50	0	0
4.	OPC-05	200-2000	6	0	60	0	0
5.	OPC-18	190-1900	7	0	70	0	0
6.	OPF-03	200-2200	6	1	54	4	7.4
7.	OPP-03	600-3000	4	0	40	0	0
8.	OPV-17	220-2900	11	0	110	0	0
9.	OPZ-01	300-1500	6	0	60	0	0
10.	OPZ-06	400-2200	8	1	74	4	5.4
Total			67	2	658	8	1.2

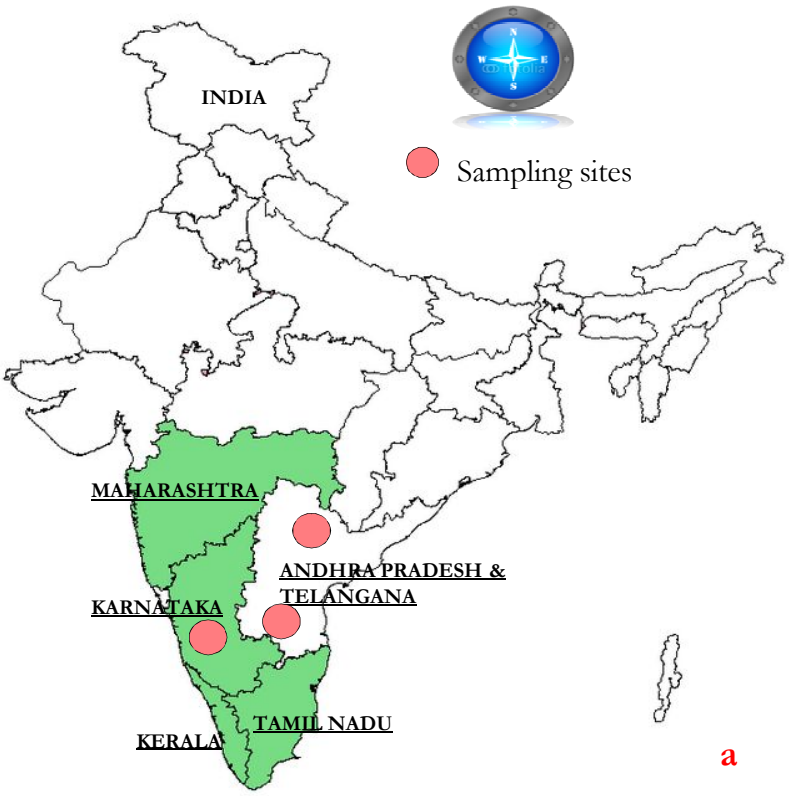
Table 21: Fatty acid composition of seed oil of *Givotia* compared with other seed oils.

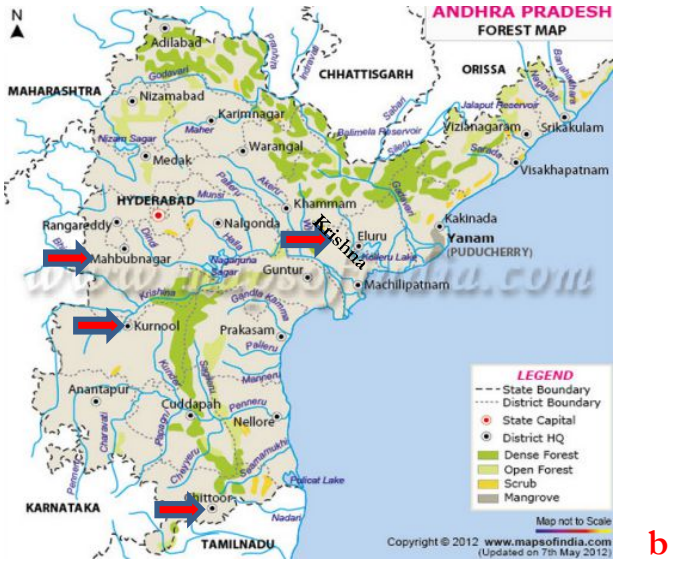
Fatty acid/lipid name	[1]*Givotia* seed oil (%)	**Jatropha curcas* seed oil (%)	**Terminalia belericaa* seed oil (%)	**Helianthus annus* seed oil (%)	***Vernicia fordii* seed oil (%)	****Hevea brasiliensis* seed oil (%)
Palmitic acid /16:0	8.21	14.20	11.60	6.80	2.30	8.56
Stearic acid/ 18:0	9.80	6.80	3.90	3.26	2.19	10.56
Oleic acid/ 18:1	10.43	43.10	61.50	16.93	6.40	22.95
Linoleic acid /18:2	25.14	34.30	18.50	73.73	7.23	37.28
α-eleostearic acid /18:3)	7.54	--	--	--	75.28	--
Other conjugated fatty acids	38.92	--	--	--	--	--

1- Present study; * Sarin *et al.* (2010); ** Zhang *et al.* (2014); *** Salimon *et al.* (2012)

Fig. 1a-f. Kondapalli toys made from softwood of *Givotia.*

Fig. 2a-f. Morphological characteristics of *Givotia*. (a) *Givotia* tree (b) Bark with blood red latex (c) Male and female twigs of *Givotia* (d) Male and female flowers of *Givotia* (e) Dried mature fruit with epicarp (EP), mesocarp (MP) and endocarp (EN) (f) *Givotia* plant with tuberous roots.





Source: http://envis.frlht.org/distrimaps/?plant_id=5651

Fig. 3a & b. Geo-distribution map of *Givotia*. (a) Distribution of *Givotia* trees in India (b) Sampling sites of *Givotia* trees in Telangana, Andhra Pradesh and Karnataka states.

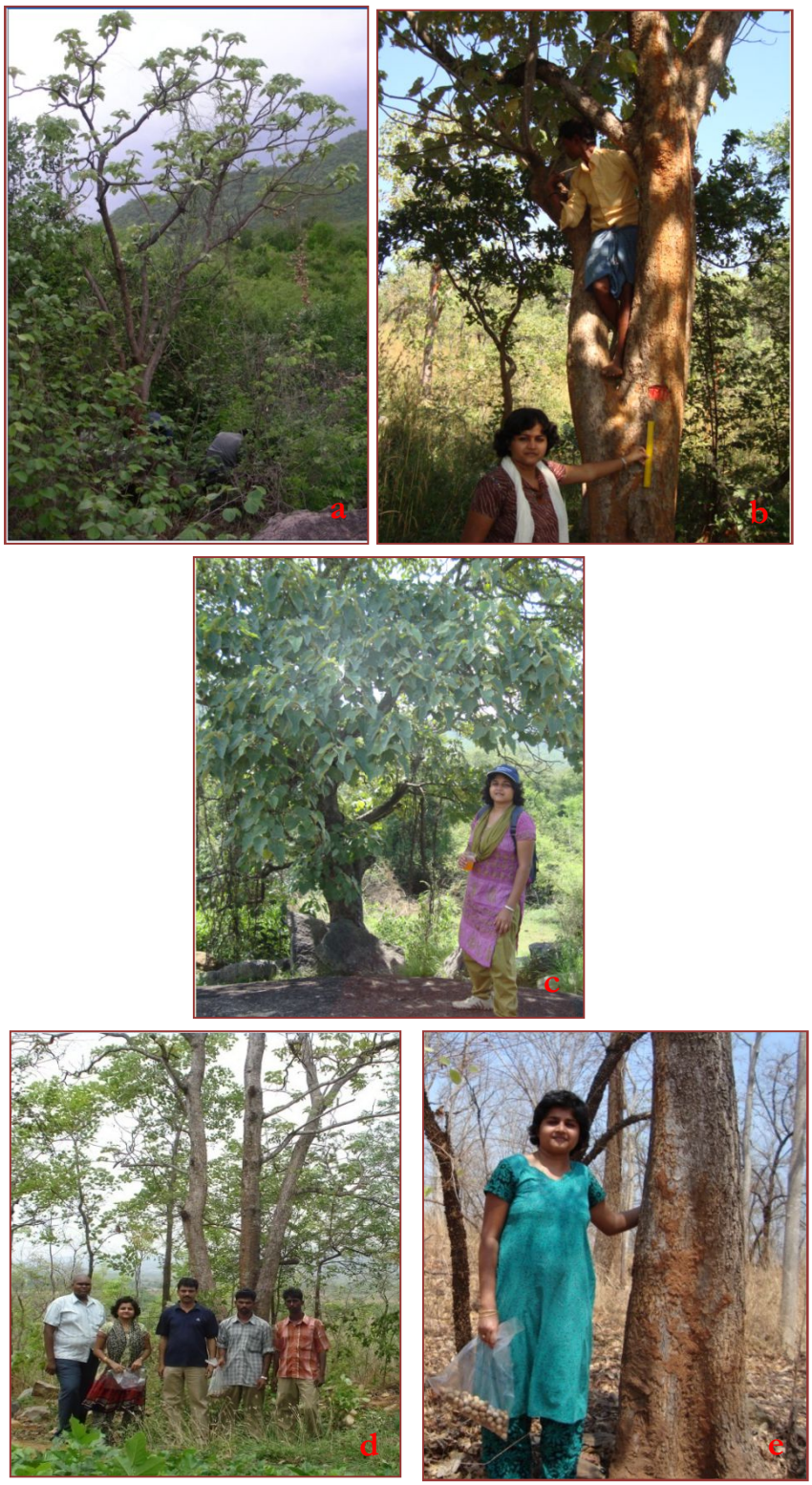


Fig. 4a-e. Collection of seeds and leaf samples from *Givotia* trees growing at different forest regions. (a) Kondapalli forest, Vijayawada, Krishna District, Andhra Pradesh (b) Nallamalla forest, Pacharla, Kurnool District, Andhra Pradesh (c) Devarayanadurga forest, Tumkur, Tumkur District, Karnataka (d) Nallamalla forest, Balapally, Chitoor District, Andhra Pradesh (e) Nallamalla forest, Achampet, Mahaboobnagar District, Telangana.

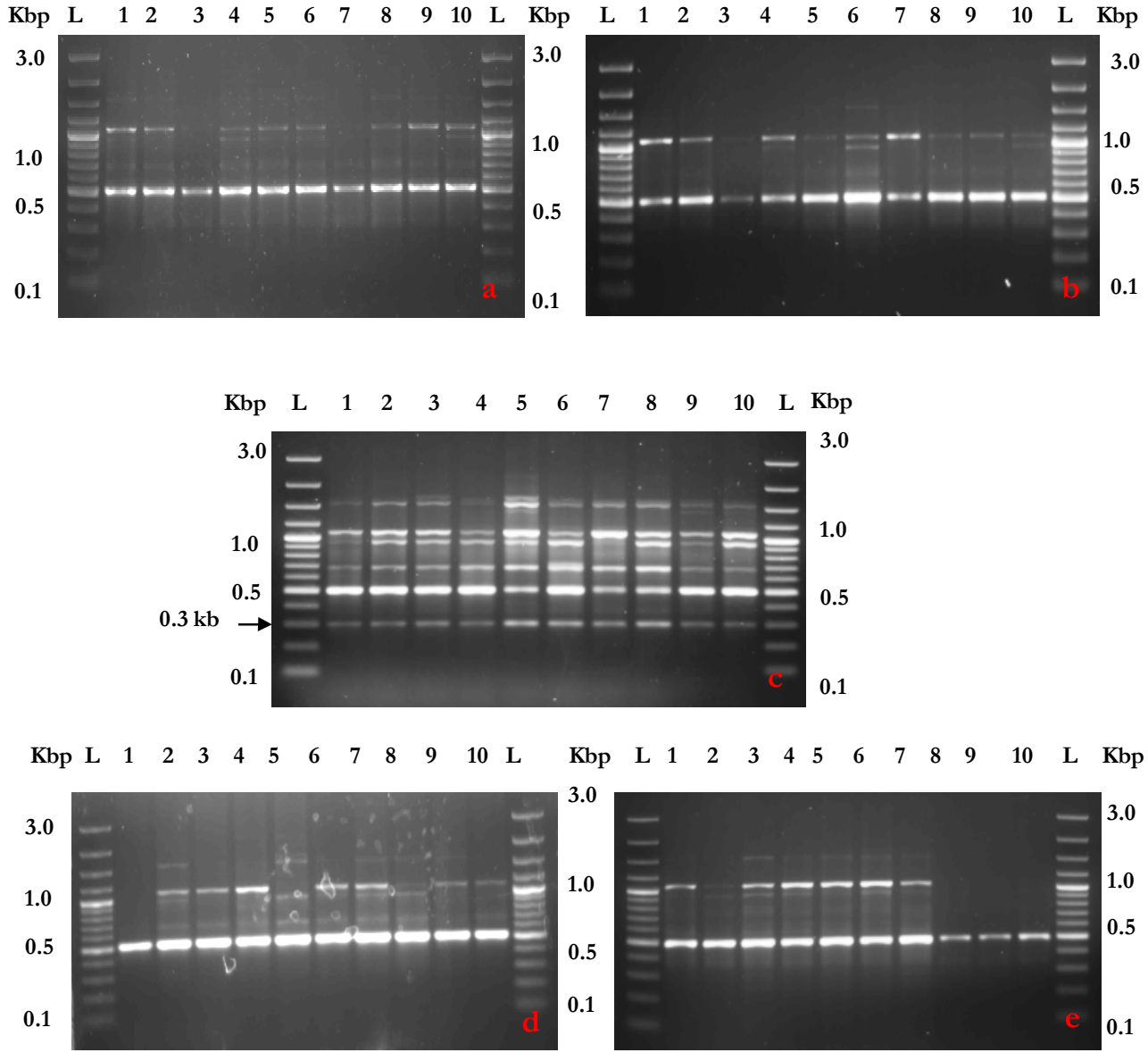


Fig. 5a-e. RAPD banding patterns of different accessions of *Givotia* with primer OPAK-14. (a) Achampet region (b) Kondapalli region (c) Devarayanadurga region (d) Balapally region (e) Pacharla region.

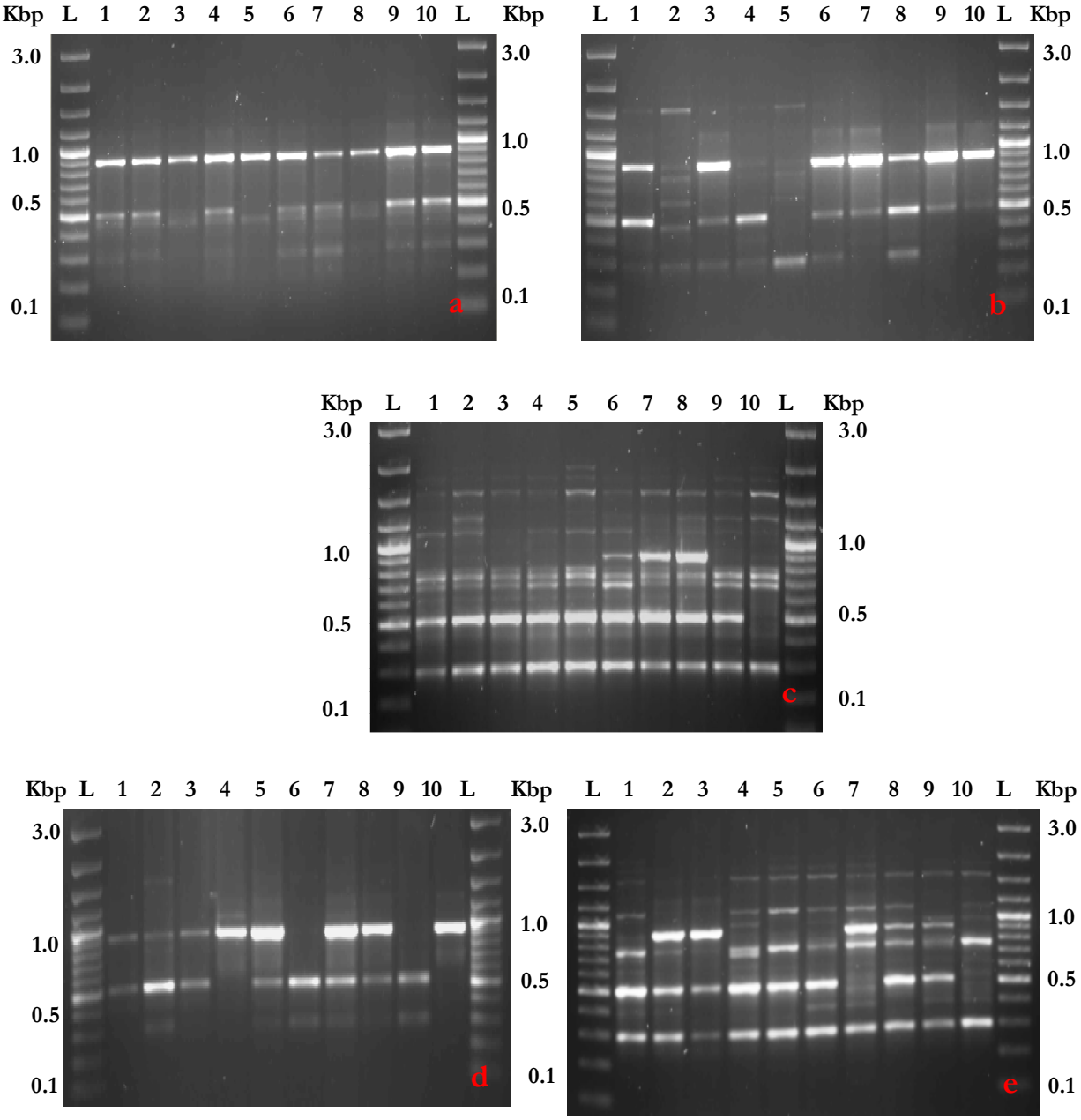


Fig. 6a-e. RAPD banding patterns of accessions of *Givotia* collected from different forest regions obtained with primer OPZ-01. (a) Achampet region (b) Kondapalli region (c) Devarayanadurga region (d) Balapally region (e) Pacharla region.

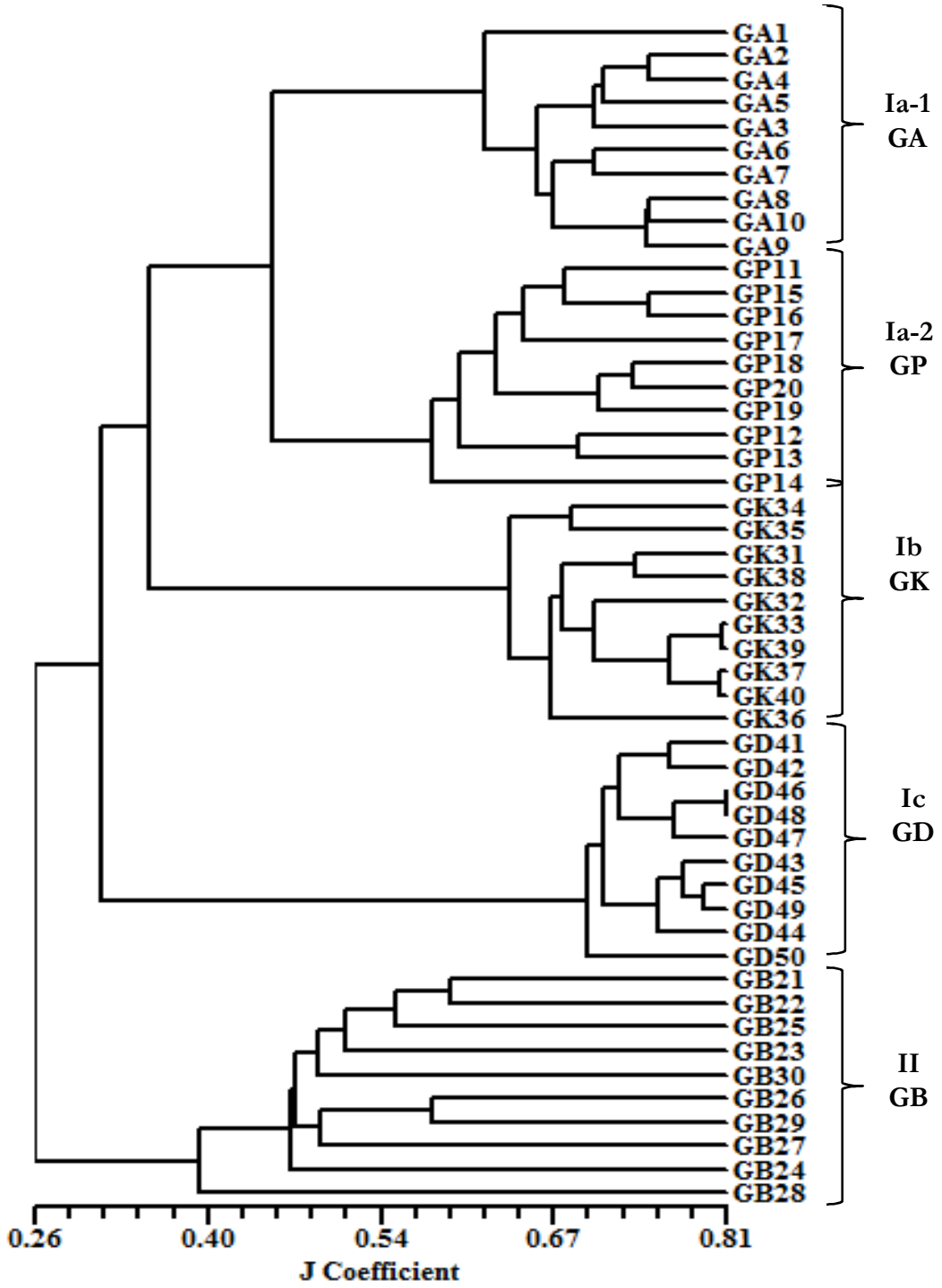


Fig. 7a. Dendrogram of *Givotia* accessions of different forest regions constructed from RAPD data based on Jaccard's similarity coefficient.

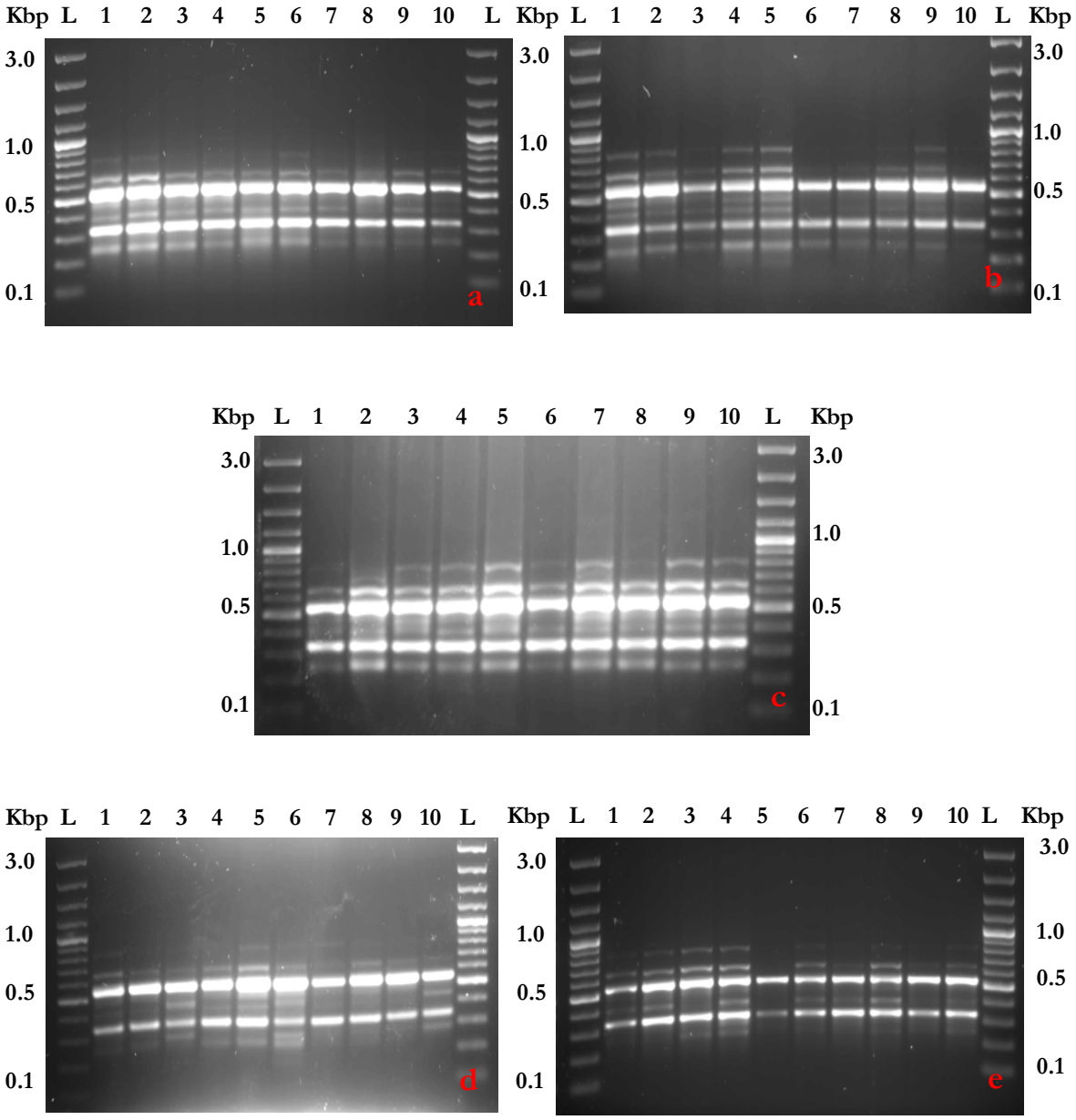


Fig. 8a-e. ISSR banding patterns of *Givotia* accessions collected from different forest regions with UBC-808 primer. (a) Achampet region (b) Kondapalli region (c) Devarayanadurga region (d) Balapally region (e) Pacharla region.

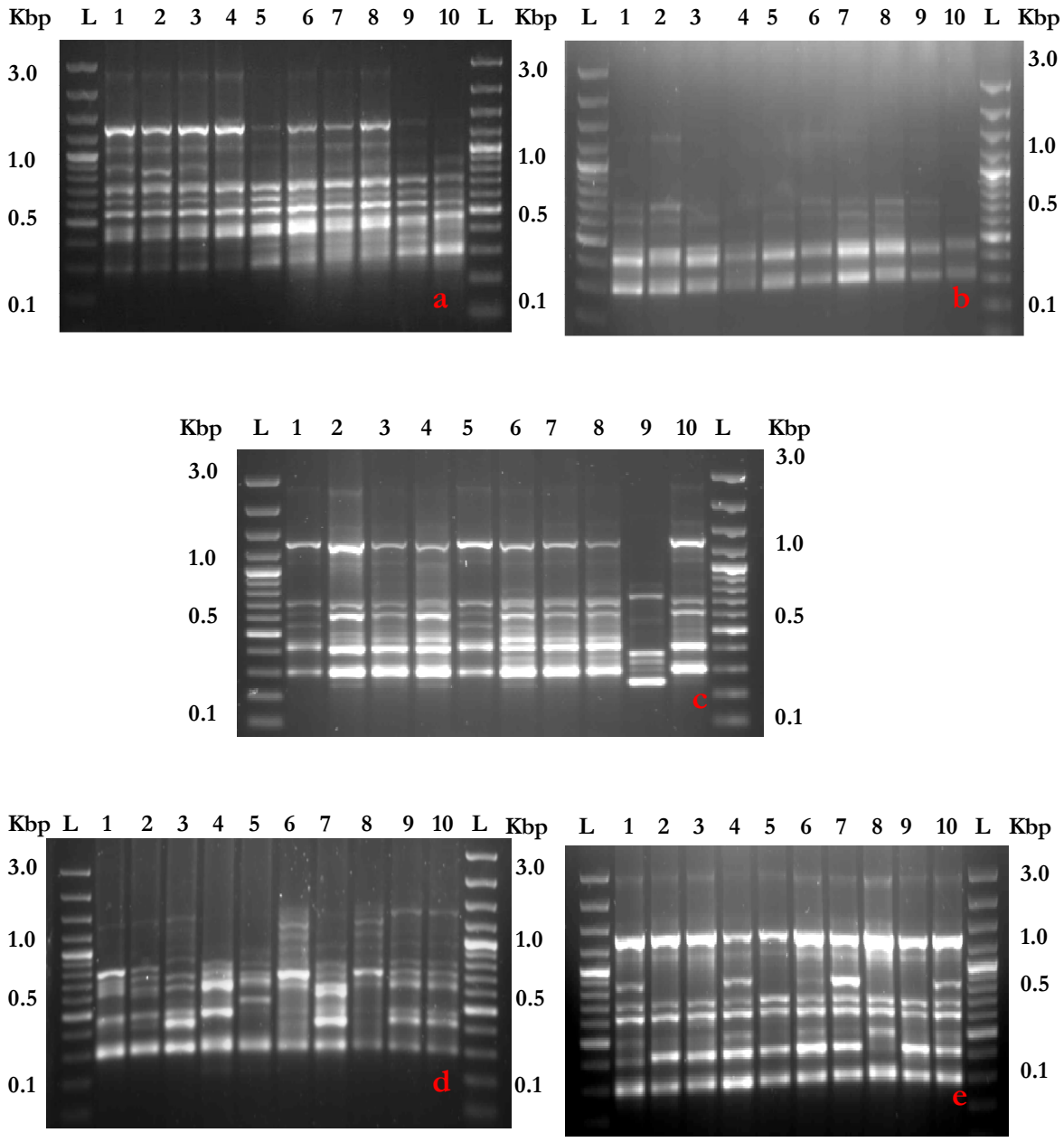


Fig. 9a-e. ISSR banding patterns of *Givotia* accessions collected from different regions with UBC-835 primer. (a) Achampet region (b) Kondapalli region (c) Devarayanadurga region (d) Balapally region (e) Pacharla region.

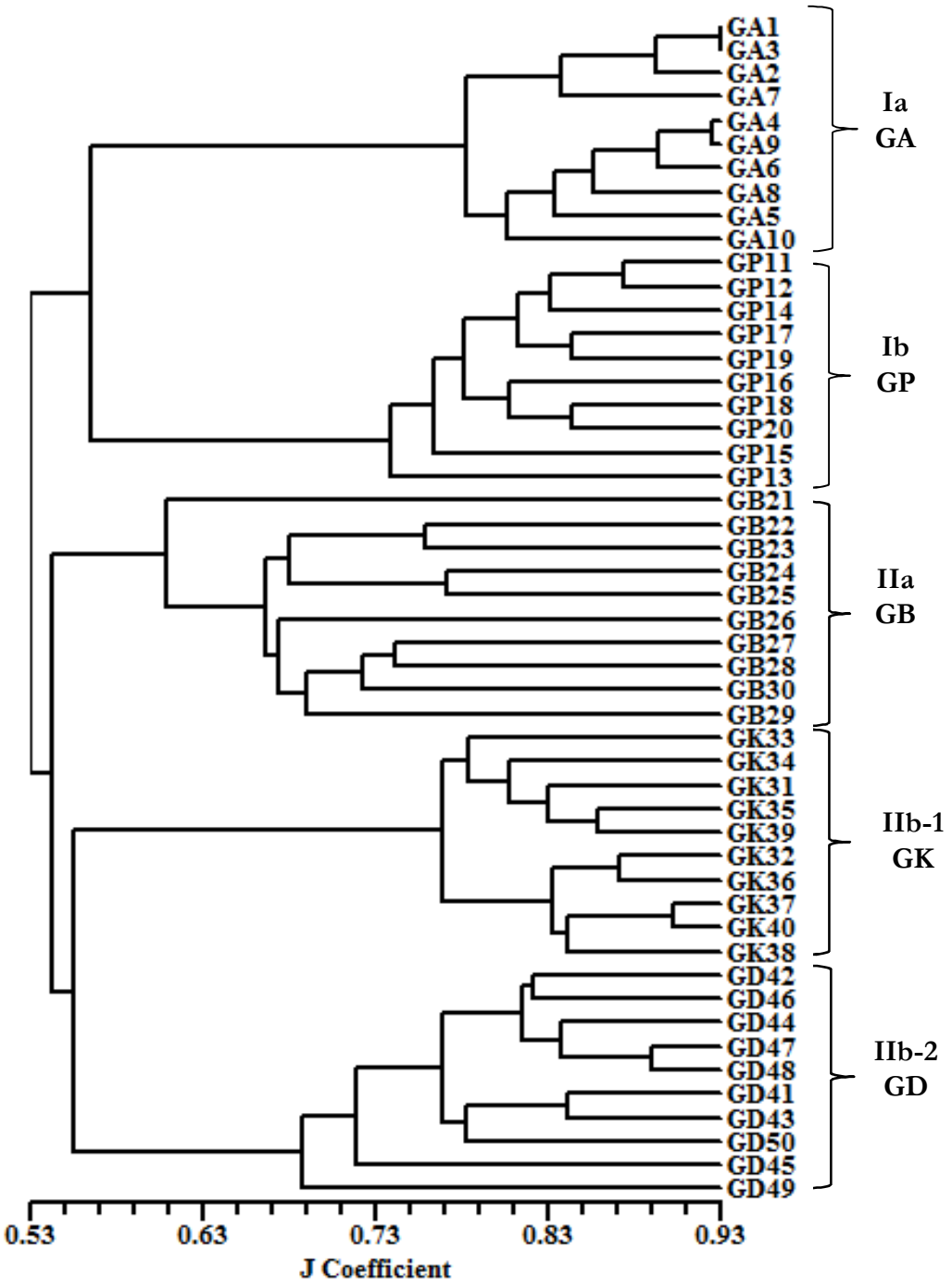


Fig. 10. Dendrogram of *Givotia* accessions growing at different regions constructed from ISSR data based on Jaccard's similarity coefficient .

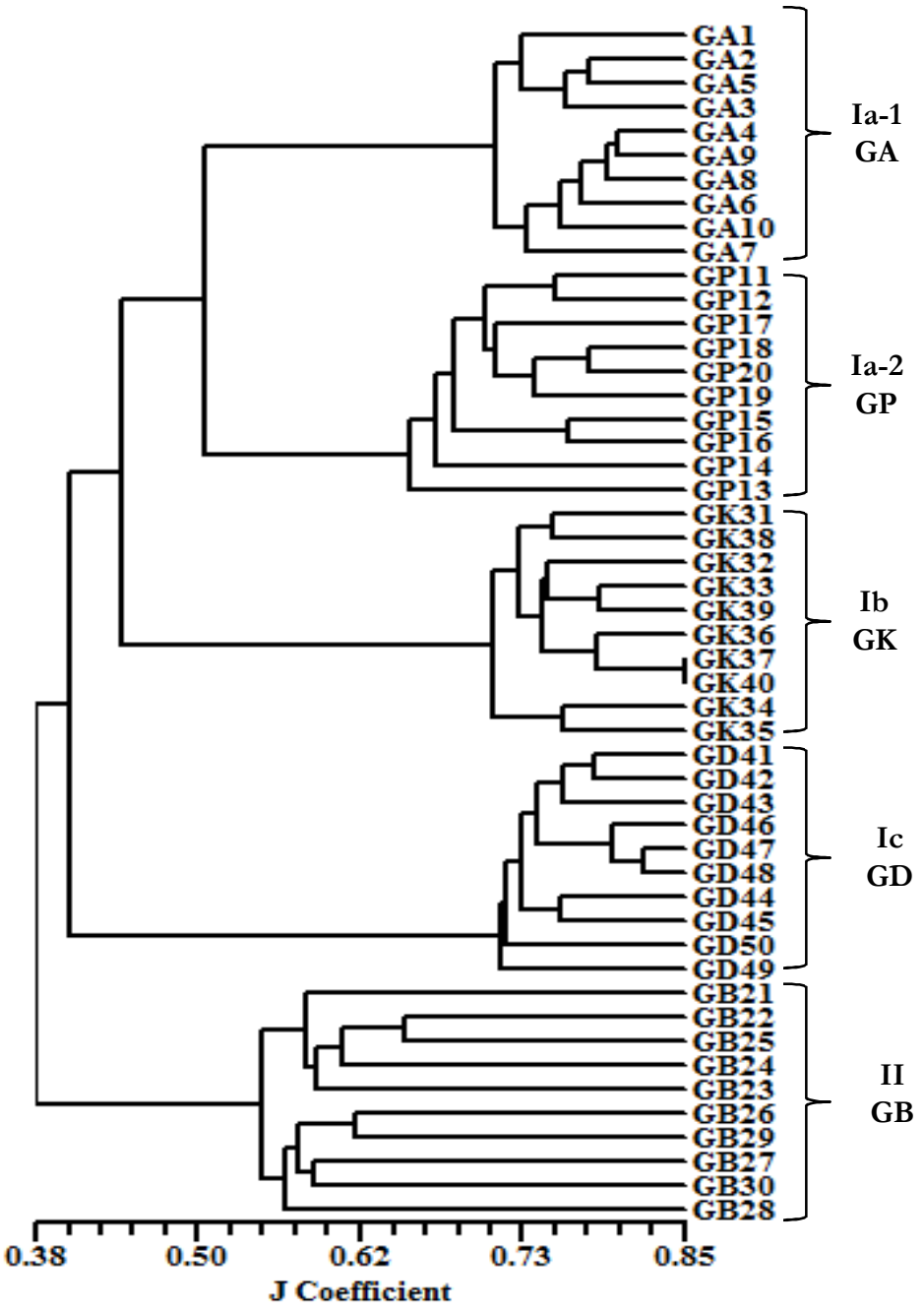


Fig. 11. Dendrogram of *Givotia* accessions of different forest regions constructed from RAPD and ISSR data based on Jaccard's similarity coefficient.

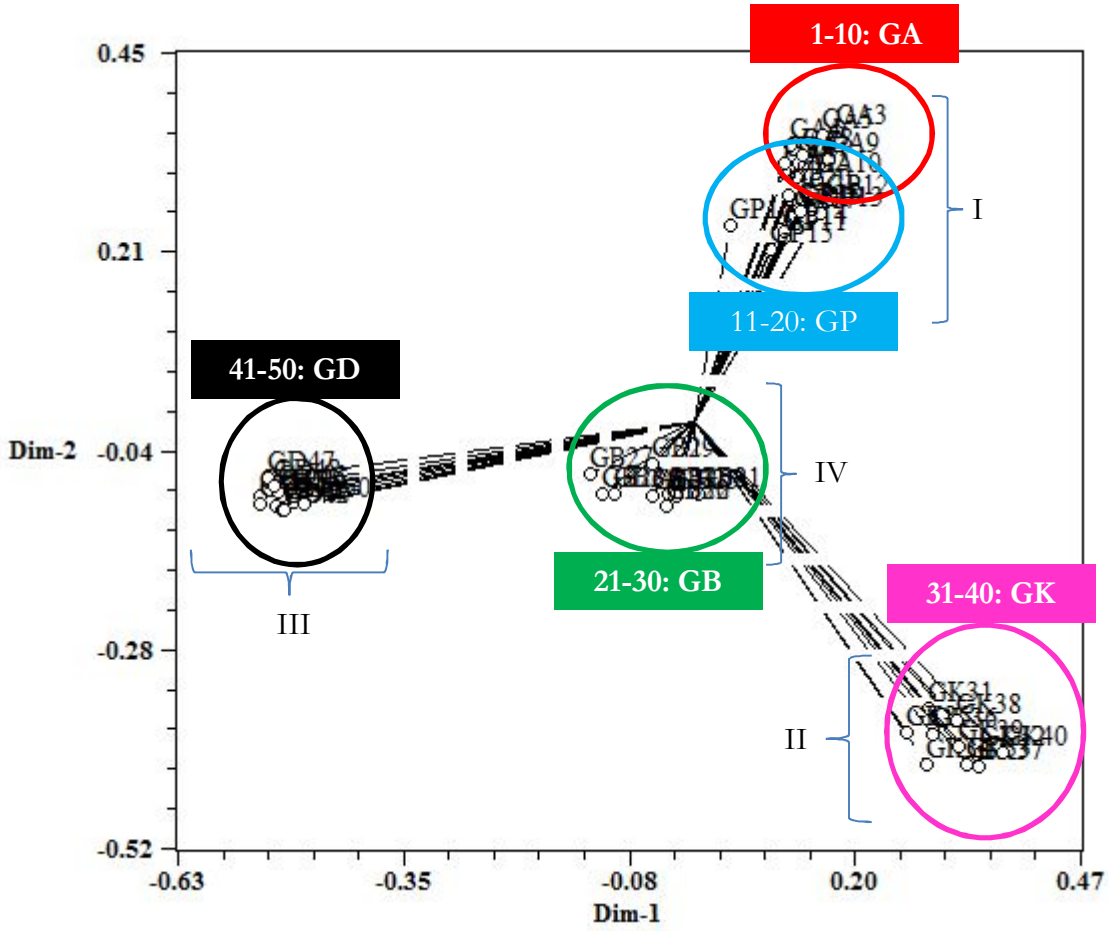


Fig. 12. Principal coordinate analysis of combined RAPD and ISSR data from 50 accessions of *Givotia*. PCA components (1 to 10 - GA , 11 to 20 - GP, 21 to 30 - GB, 31 to 40 - GK and 41 to 50 - GD).

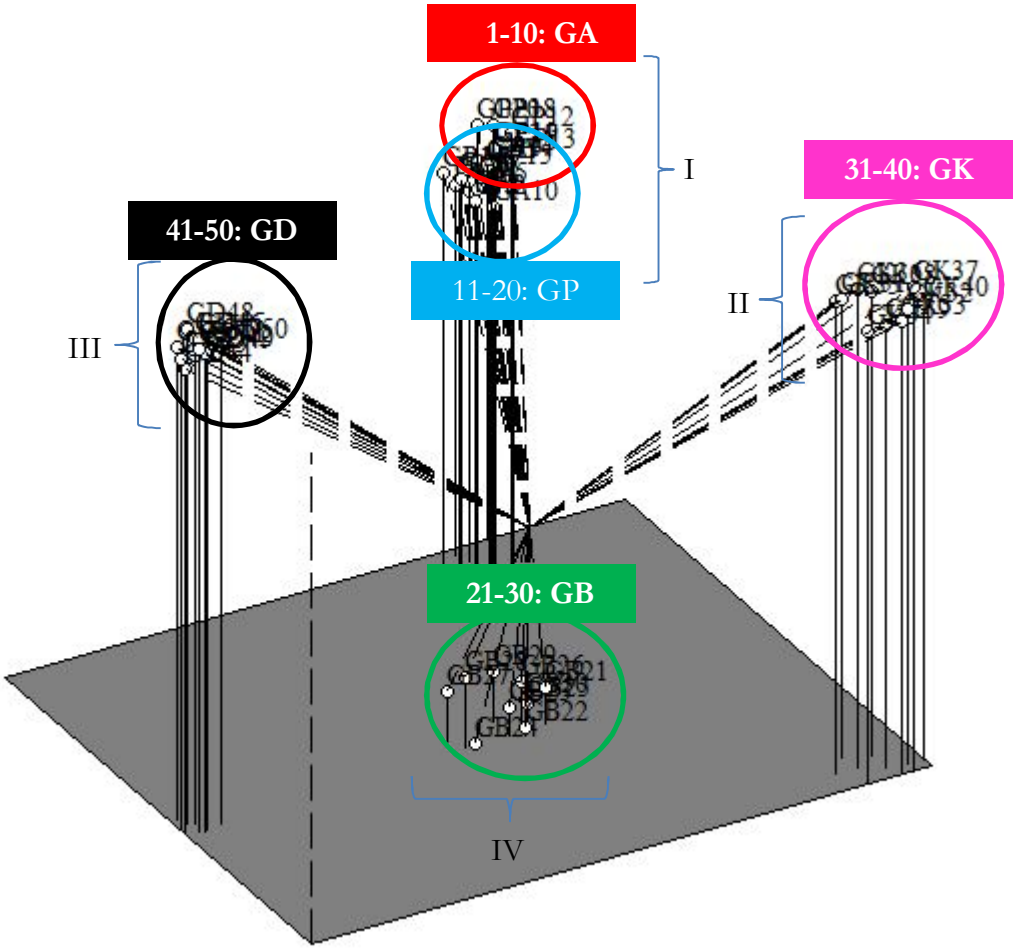


Fig. 13. Three dimensional plot showing relationships among 50 *Givotia* accessions using RAPDs and ISSRs. (1 to 10 - GA, 11 to 20 - GP, 21 to 30 - GB, 31 to 40 - GK and 41 to 50 - GD).

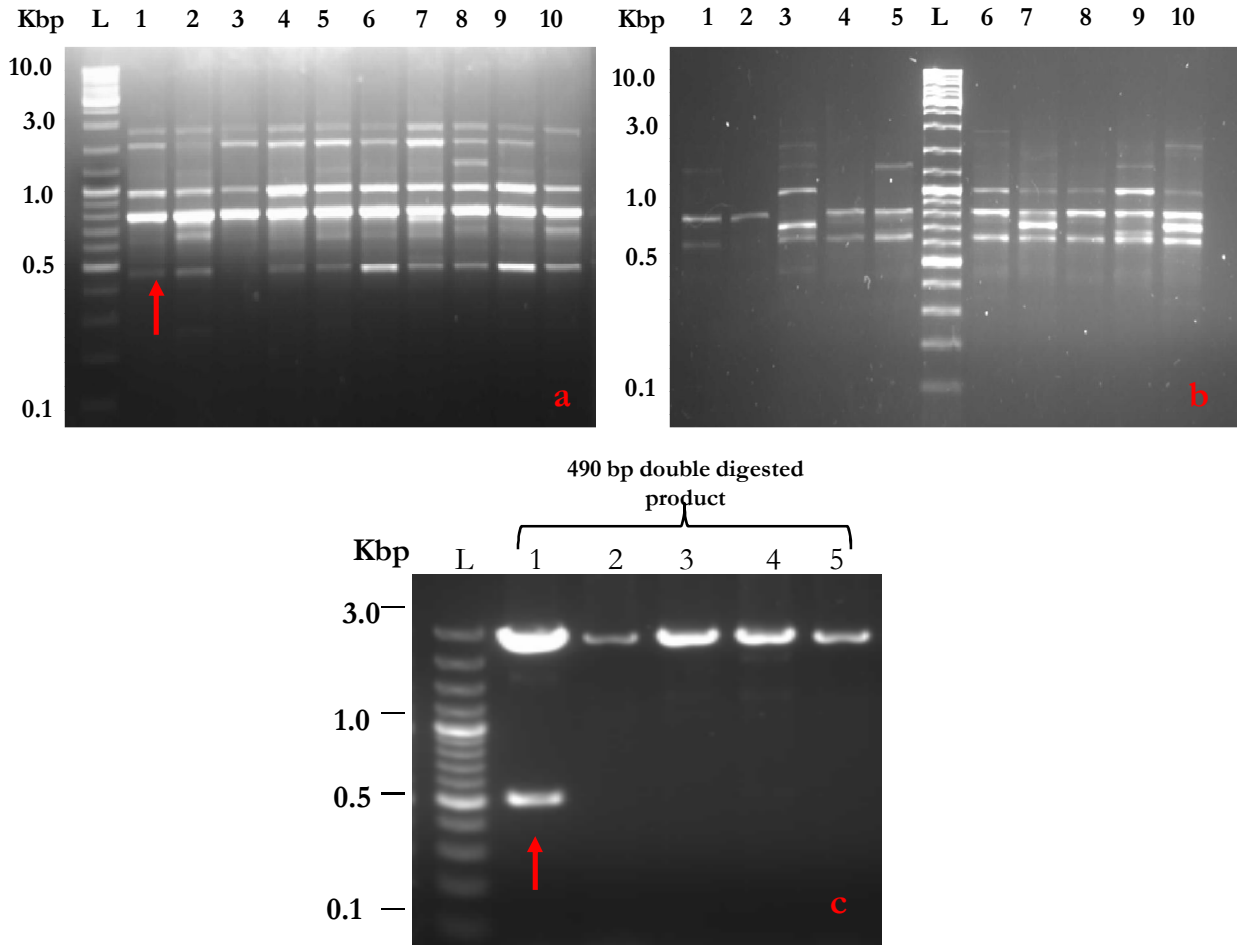


Fig 14d: *Givotia rottleriformis* nucleotide sequence of the amplified fragment with OPC-08 primer. GenBank: KF772203.1

TTGGACCGGTGTCTCAGGCGGGGCCTCACAACAGCAAAAAAAAAAAACC
CTCAACAGCGGCCGCCTGTCCTTCCTTCCTTCTTCTTCTTCTTCAACCTTTA
ATCAAACCCTCTTCACCATTACAGCCCTAAAAACACAGACACAGTAGCAAA
TCCAGCCGCCAGCCCCTATTTTCTTCTTCCTTTTCTTCTTTTCCCTTTCCCT
CCATCACCGACAGCAGCTGAATCACAGCTACCAGCTGCATCACTCCCCCTT
TGCTTCTTCTTCAACCAGCAACAGCCCCTTTCTTCTTCTTCAACCAATAGCA
CAGCCTCCATACAGCAGCTGTCTACTCCATTTTCTTCATTCATTTACAGCAG
ACGCCTCCACCATCACTAAAATCACCGATCCTCCATAACCACCGATCCTCCA
TCACCACCAGAATCCAAACACGGTCTTTCCCCATTTCTTTCTTCTCCCTTTC
ACCGGTCCAA

Fig. 14a-d. Cloning and sequencing of RAPD amplicon and confirmation by PCR amplification and double digestion of plasmid. (a) RAPD amplified fragment of 490 bp with OPC-08 primer in accessions of Achampet region (b) Banding pattern with OPC-08 primer showing lack of 490 bp PCR product in accessions of Devarayanadurga (c) Double digestion of recombinant pTZ57R/T plasmid yielded a 490 bp product (d) Nucleotide sequence of *Givotia* did not match with the nucleotide sequences in NCBI database.

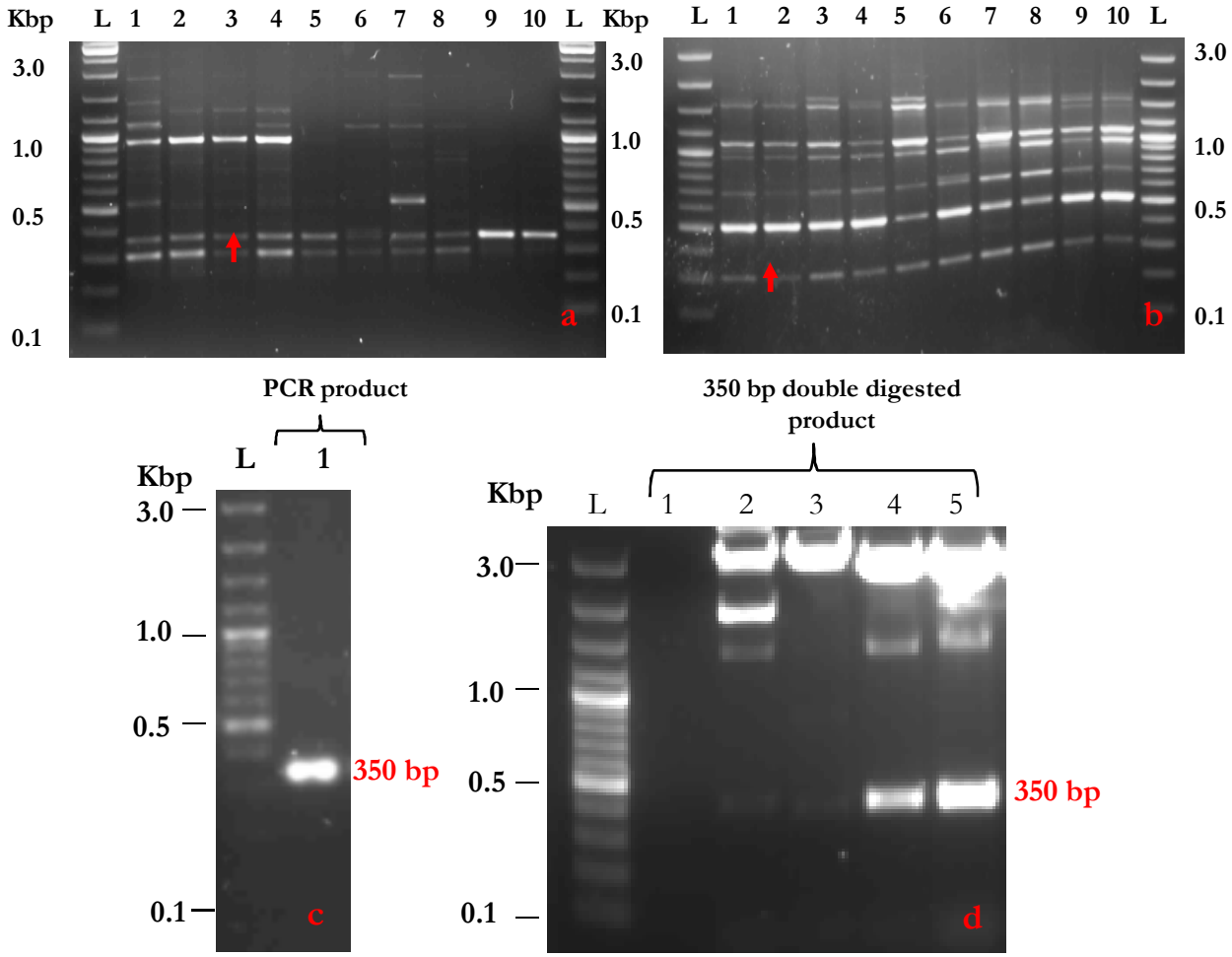


Fig 15e: *Givotia rottleriformis* clone 3 hypothetical protein gene, partial cds. of *Ricinus*. GenBank: JX026068.1

CCGATTCCAACGTCGTCCACCCCCATGAGAAATGCACAAGCTAGACAGTACTT
CAGCACTGCGAGTGCAGCCTTCAACCTTACACCTTTTCCCCCCACCATGTCTG
ATGCAAAGCCCCGACTTGCCTCGTGCAGCCTTGGTGCATCCATCTGCAAATC
CACATCTTCTGCCACCACCGTGTGCTATGCAAAAATCTGTCTTACCCTCAGCA
CTTTTAGTGCAGCCCAAGTGTTGACACCTCCTTCCTCCACCATGGGCCTTGCA
GTATGCAGTTCGGCTCTCAGCCCCTTTGTCACAACCTGGTTTGTGGCATCTC
TGTCCTCCCCCATGCCACGACGTTGG

Fig. 15a-e. Cloning and sequencing of RAPD amplicon and confirmation by PCR amplification and double digestion of plasmid. (a) RAPD amplified fragment of 350 bp with OPT-17 primer in accessions of Achampet region (b) Banding pattern with OPT-17 primer showing lack of 350 bp fragment in accessions of Devarayanadurga region (c) Lane 1 represents 350 bp PCR product of gel eluted band (d) Double digestion of recombinant pTZ57R/T plasmid yielded a 350 bp product (e) Nucleotide sequence of *Givotia* matched the hypothetical protein gene of *Ricinus*.

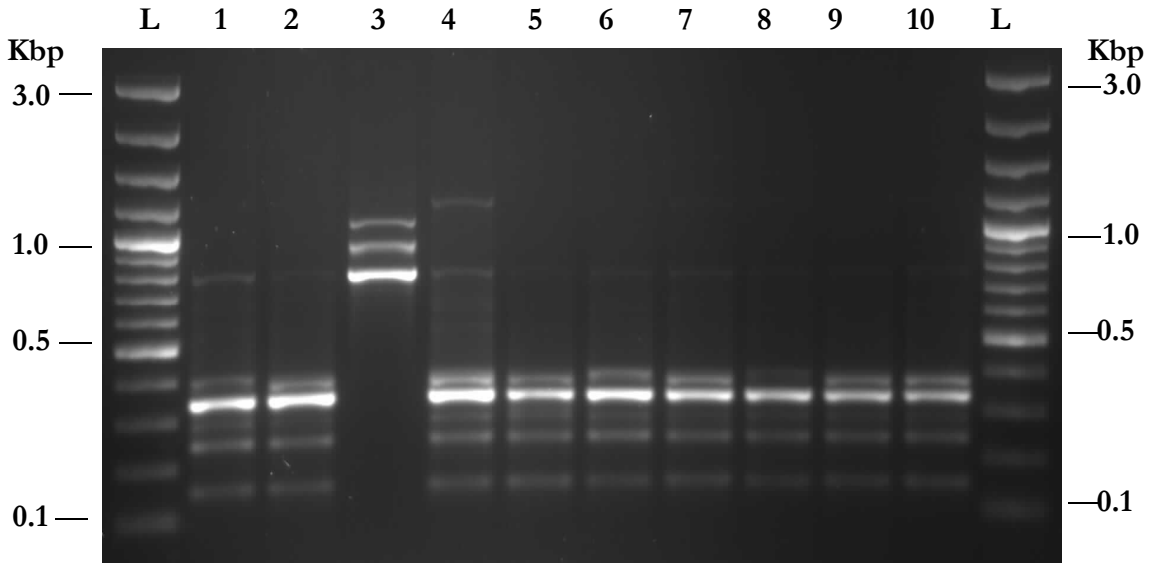


Fig. 16a. RAPD-SCAR banding patterns of *Givotia* accessions of Mulugu region with 350 FP and 350 RP. Lanes designated as L represent DNA ladder (100 - 3000 bp), lanes 1, 2, 3, 4, 5, 6, 7, 8, 9 & 10 represent individual plants of *Givotia*.

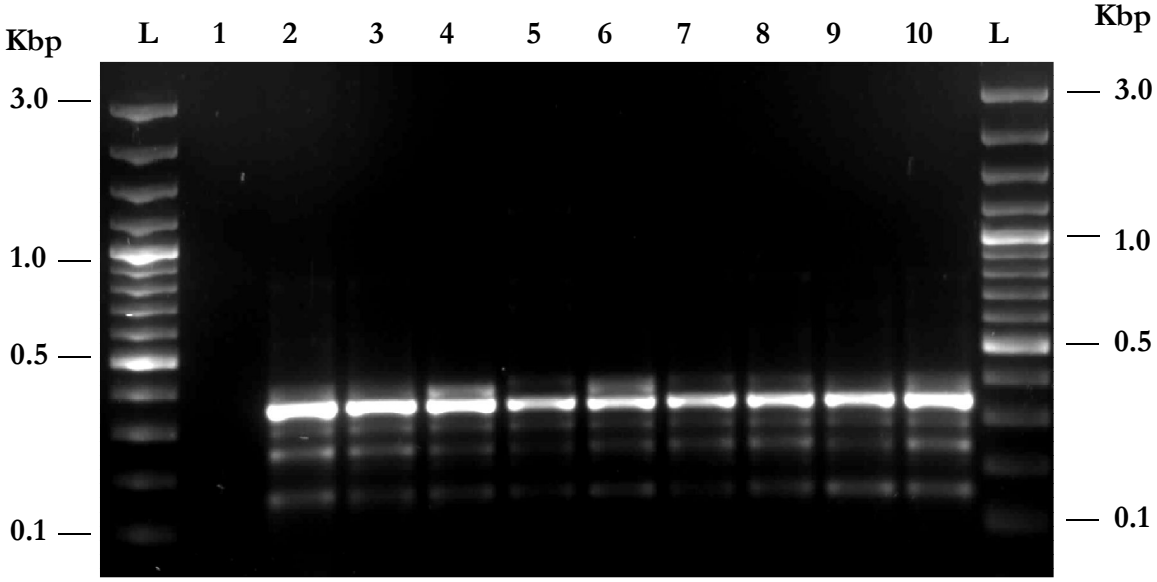
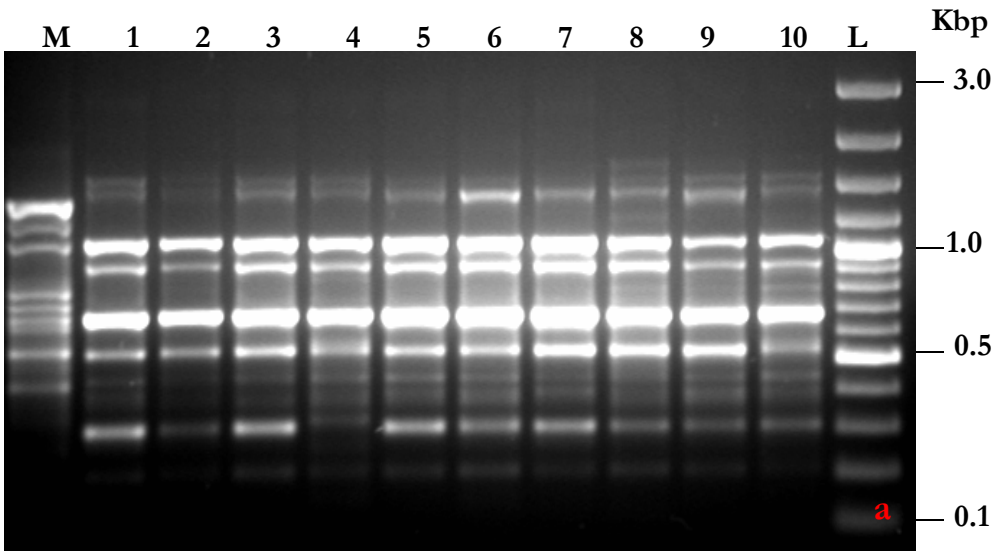


Fig. 16b. RAPD-SCAR banding patterns of *Givotia* accessions of Devarayanadurga region with 350 FP and 350 RP. Lanes designated as L represent DNA ladder (100 - 3000 bp), lanes 1, 2, 3, 4, 5, 6, 7, 8, 9 & 10 represent individual plants of *Givotia*.



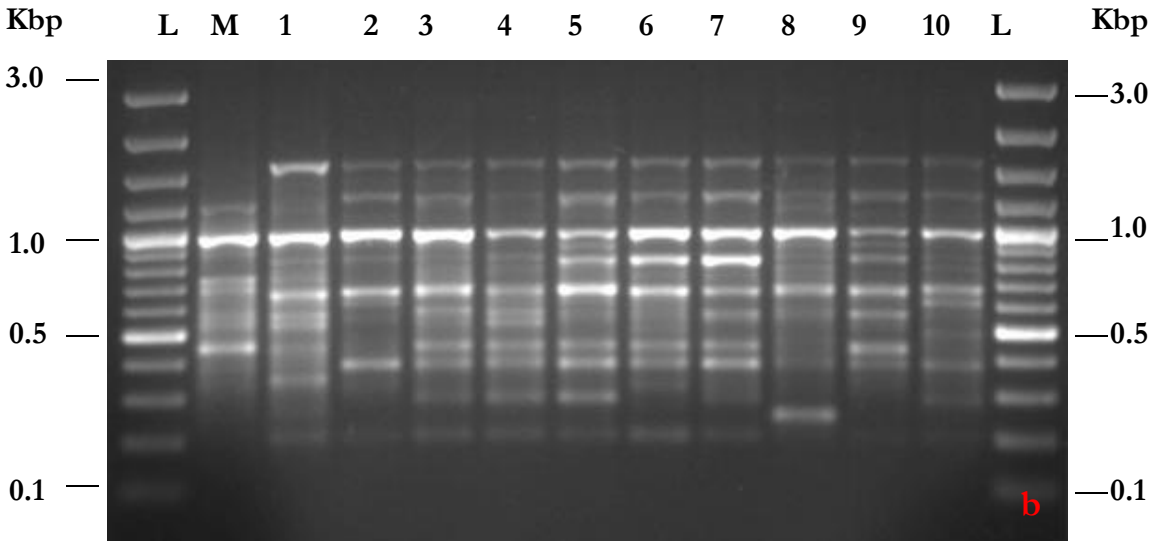


Fig. 17a & b. PCR amplification profiles of DNA samples of half-sib progeny of candidate plus tree produced with RAPD primers. (a) OPB-07 primer (b) OPAI-12 primer. Lanes designated as L represent DNA ladder (100 - 3000 bp), M represent maternal plant and lanes 1-10 represent individual half-sibs.

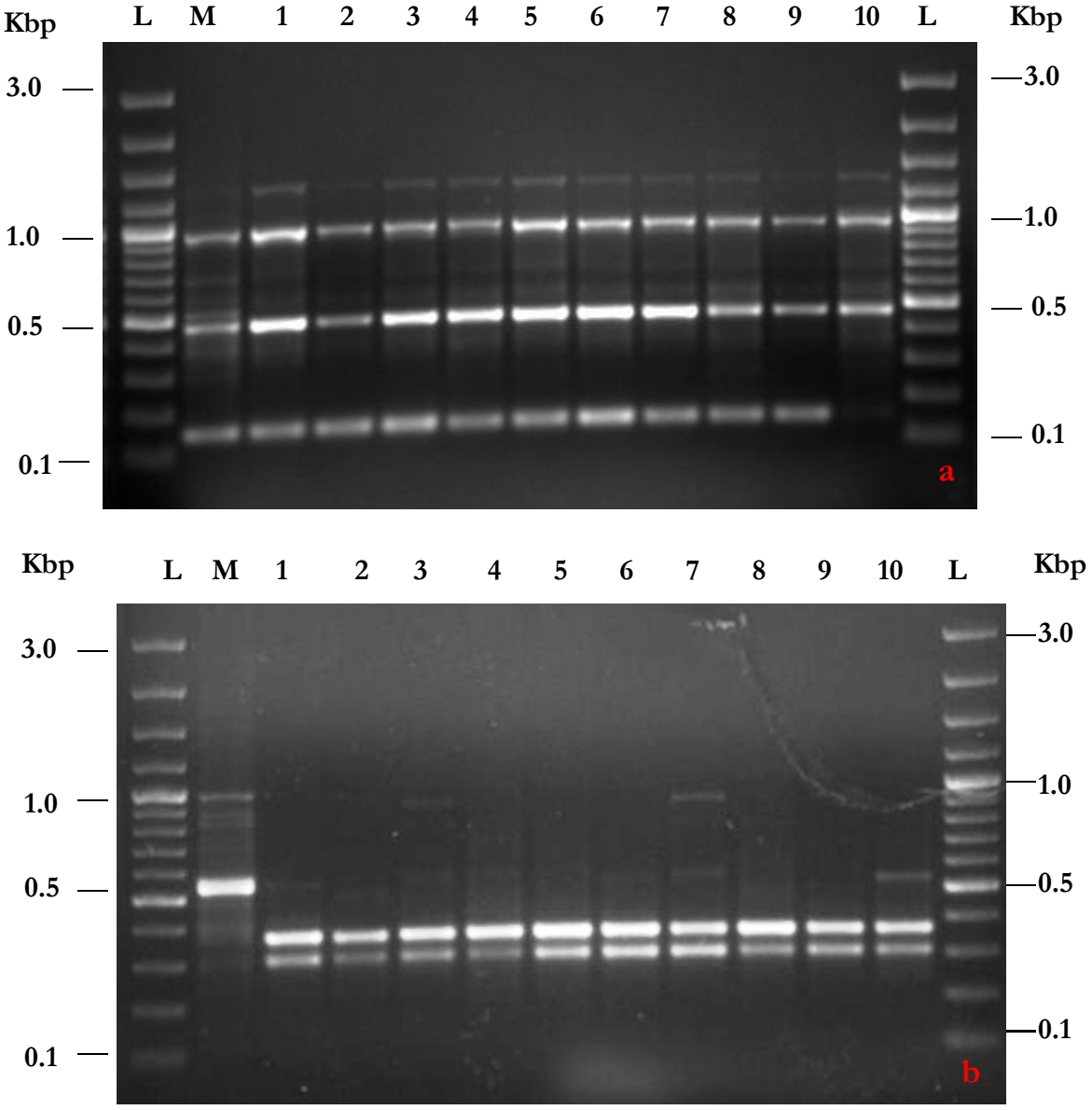


Fig. 18a & b. PCR amplification profiles of DNA samples of halfsib progeny of candidate plus tree produced with RAPD primers. (a) OPB-12 primer (b) OPT-17 primer. Lanes designated as L represent DNA ladder (100 - 3000 bp), M represent maternal plant and lanes 1-10 represent individual half-sibs.

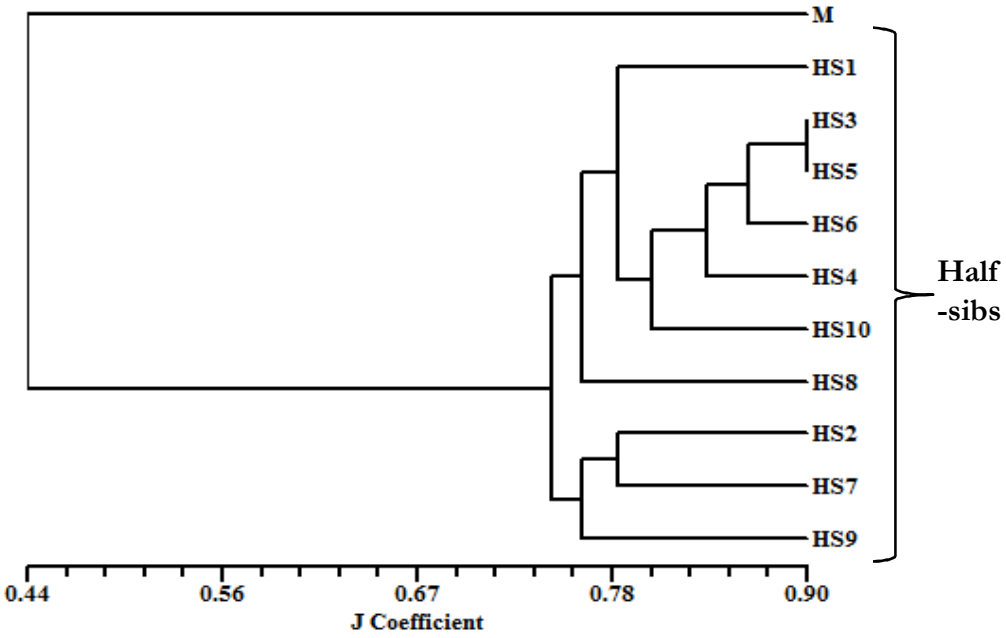


Fig. 19. Dendrogram of half-sib progeny of candidate plus tree of *Givotia* constructed from RAPD data based on Jaccard's similarity coefficient .

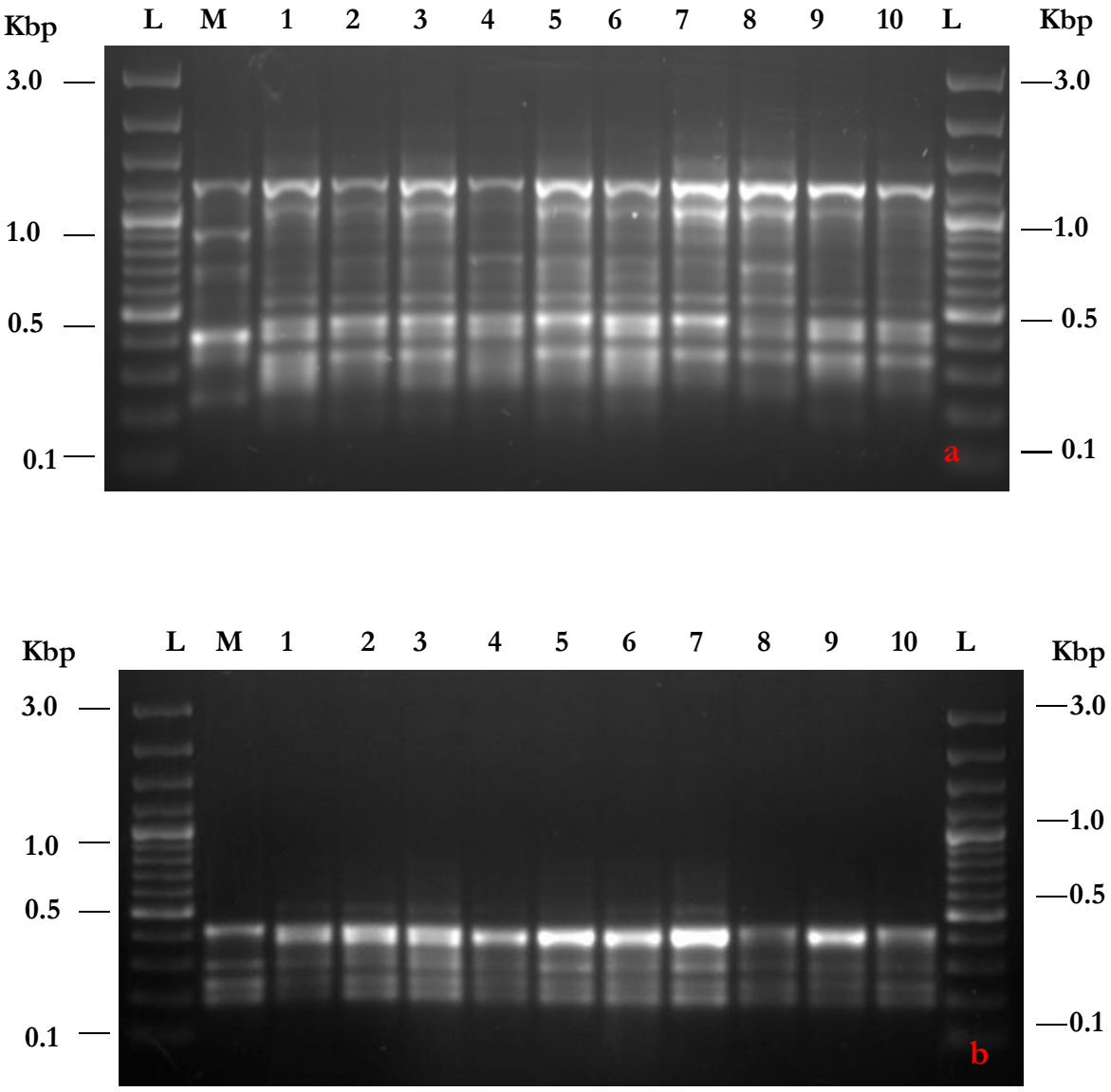


Fig. 20a & b. ISSR banding pattern in half-sib progeny of candidate plus tree. (a) UBC-815 primer (b) UBC-810 primer. Lanes designated as L represent DNA ladder (100 - 3000 bp), M represent maternal plant and lanes 1-10 represent individual half-sibs.

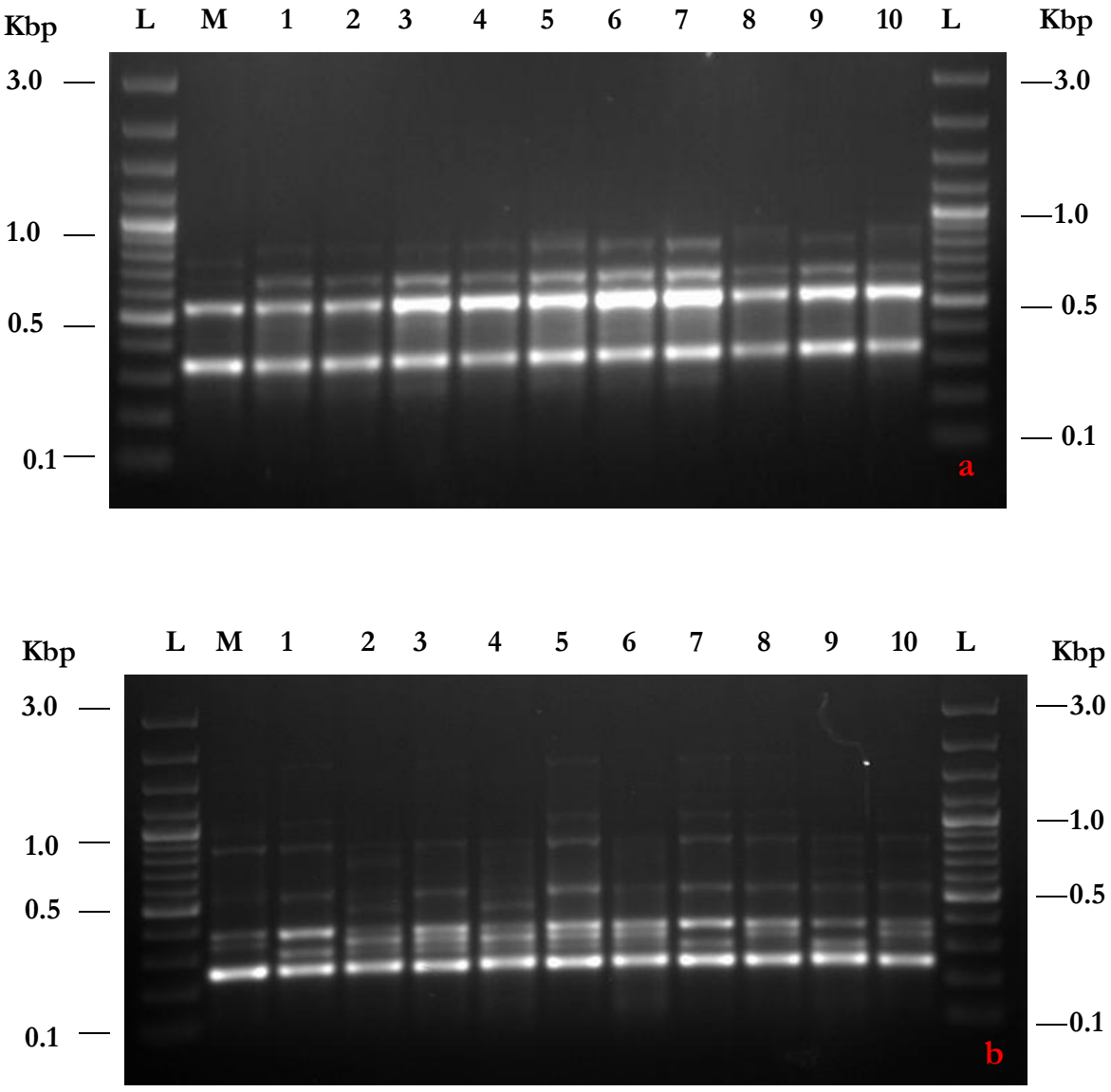


Fig. 21a & b. ISSR banding pattern in half-sib progeny of candidate plus tree. (a) UBC-880 primer (b) UBC-834 primer. Lanes designated as L represent DNA ladder (100 - 3000 bp), M represent maternal plant and lanes 1-10 represent individual half-sibs.

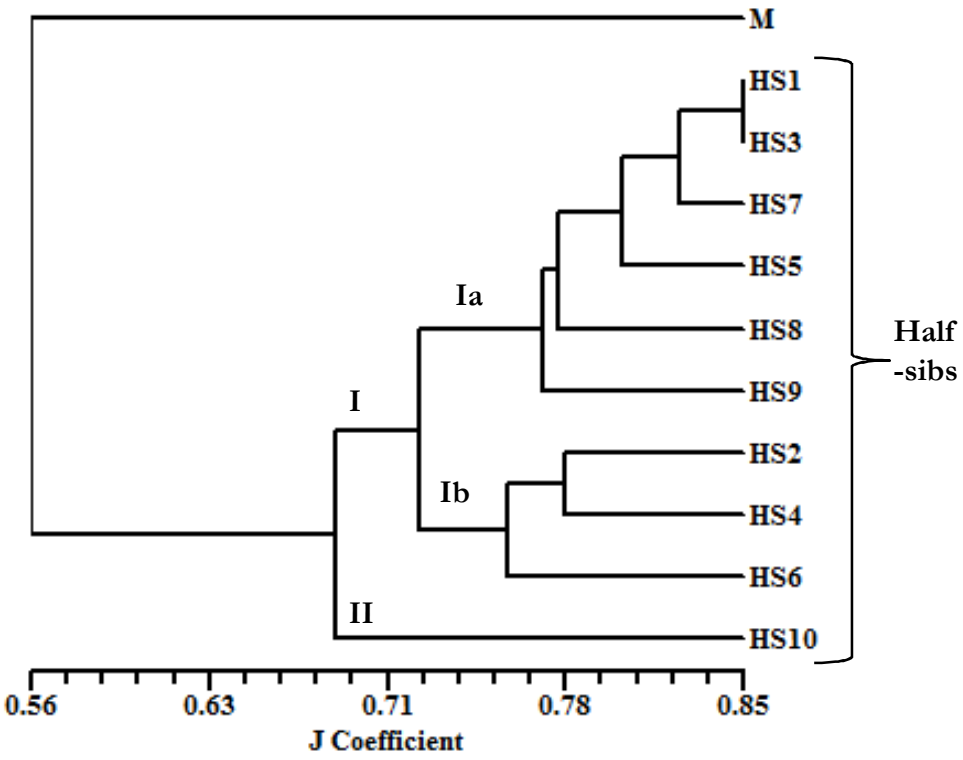


Fig. 22. Dendrogram of half-sib progeny of candidate plus tree of *Givotia* constructed from ISSR data based on Jaccard's similarity coefficient.

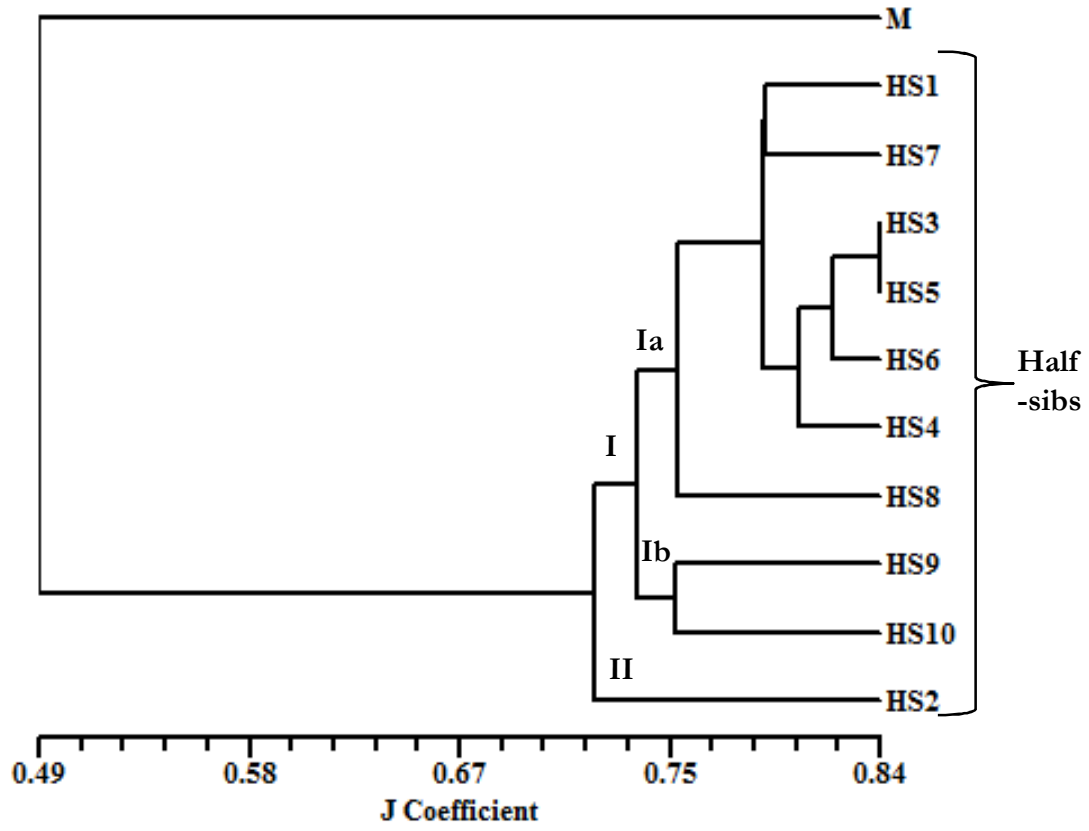


Fig. 23. Dendrogram of half-sib progeny of candidate plus tree of *Givotia* constructed from RAPD and ISSR data based on Jaccard's similarity coefficient.

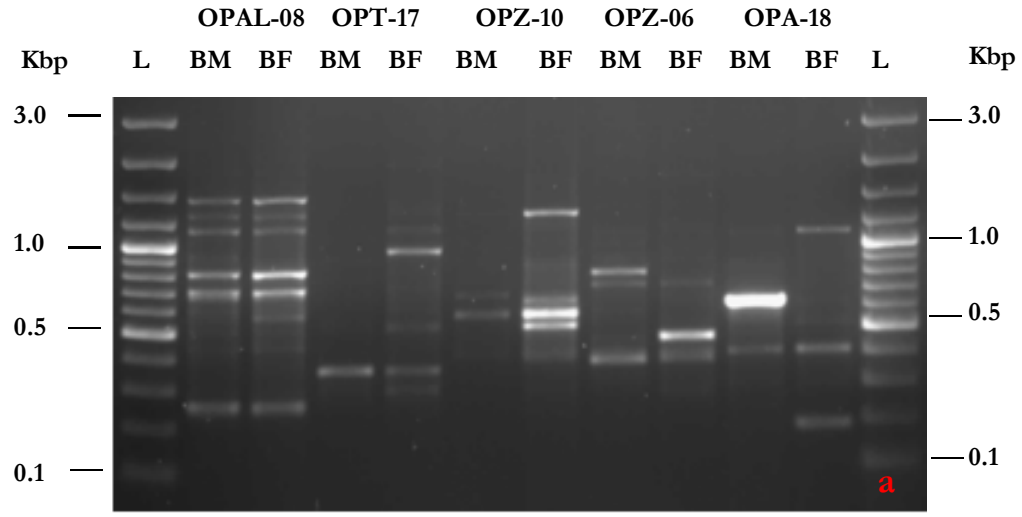


Fig. 24a. PCR-based DNA amplification profiles generated from different RAPD primers in bulk DNA of male and female *Givotia* plants. Lanes designated as L represent DNA ladder (100 - 3000 bp), BM represent bulk DNA samples of males, BF represent bulk DNA samples of females. The amplified product profiles 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).

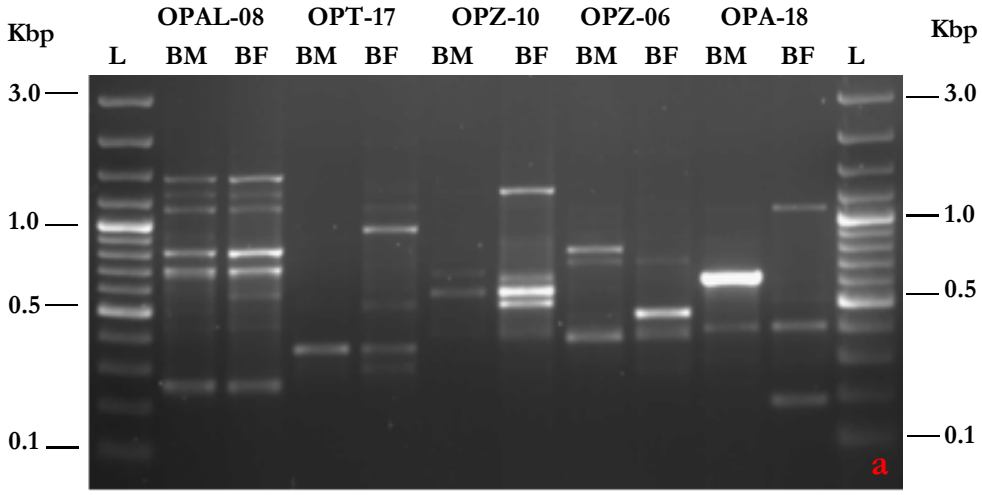


Fig. 24b. PCR-based DNA amplification profiles generated from different RAPD primers in bulk DNA of male and female *Givotia* plants. Lanes designated as L represent DNA ladder (100 - 3000 bp), BM represent bulk DNA samples of males, BF represent bulk DNA samples of females. The amplified product profiles 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).

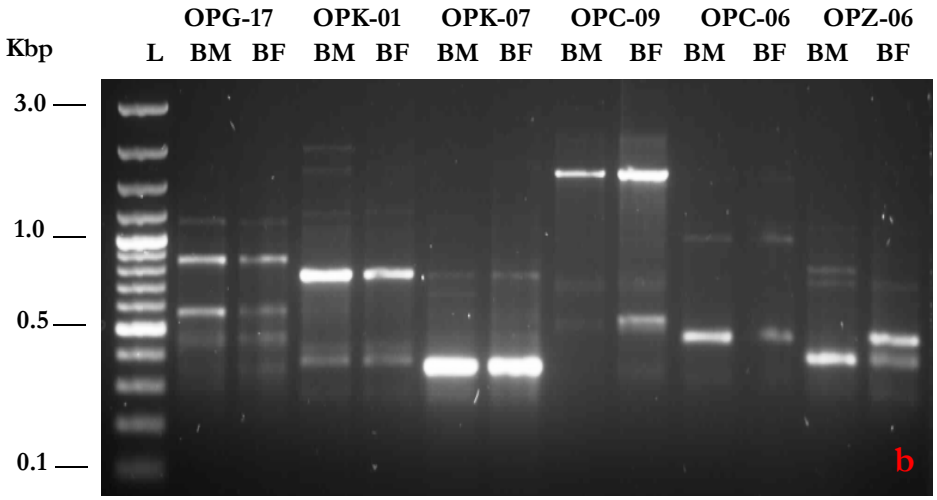


Fig. 25a. PCR-based DNA amplification profiles generated from different RAPD primers in bulk DNA of male and female *Givotia* plants. Lanes designated as L represent DNA ladder (100 – 3000 bp), BM represent bulk DNA samples of males, BF represent bulk DNA samples of females. The amplified product profiles were generated from the primers, OPG-17, OPK-01, OPK-07, OPC-09, OPC-06 and OPZ-06, respectively in bulk males (BM) and bulk females (BF).

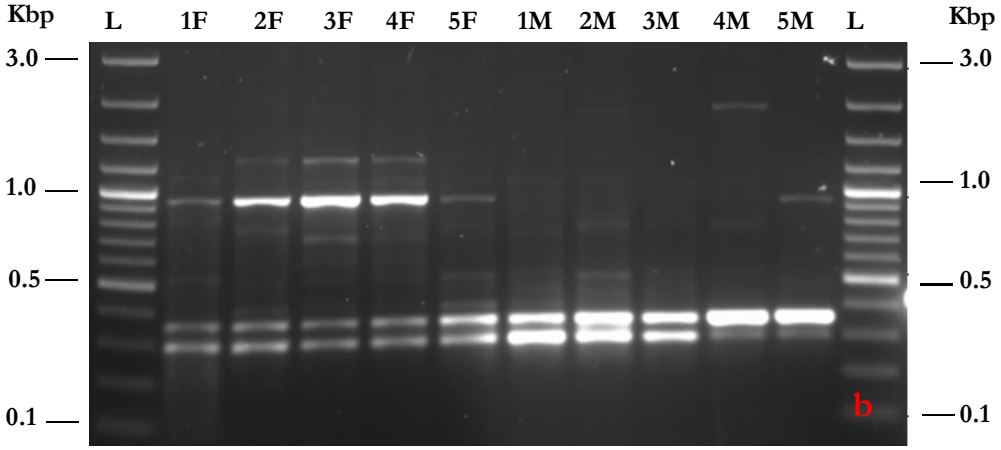


Fig. 25b. RAPD banding patterns of five individual female and male *Givotia* plants of Achampet region generated with the primer OPT-17. Lanes designated as L represent DNA ladder (100 - 3000 bp), lanes 1F, 2F, 3F, 4F & 5F represent the individual females and lanes 1M, 2M, 3M, 4M & 5M represent individual males.

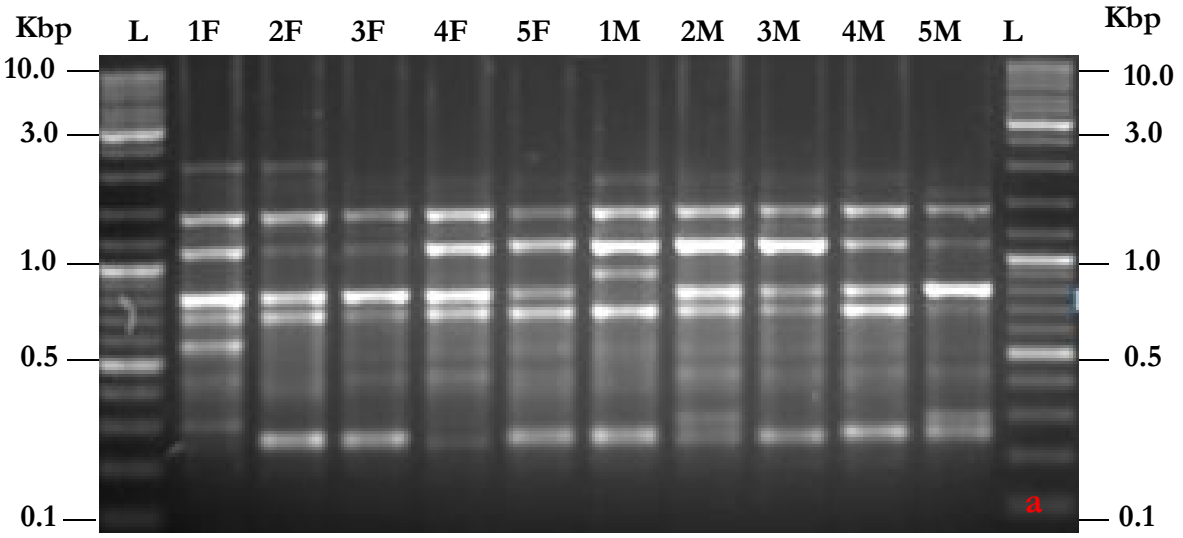


Fig. 26a. PCR amplification profiles of DNA samples of female and male *Givotia* plants produced with OPAL-08 primer. Lanes designated as L represent DNA ladder (100 - 10,000 bp), lanes 1F, 2F, 3F, 4F & 5F represent individual females, lanes 1M, 2M, 3M, 4M & 5M represent individual males.

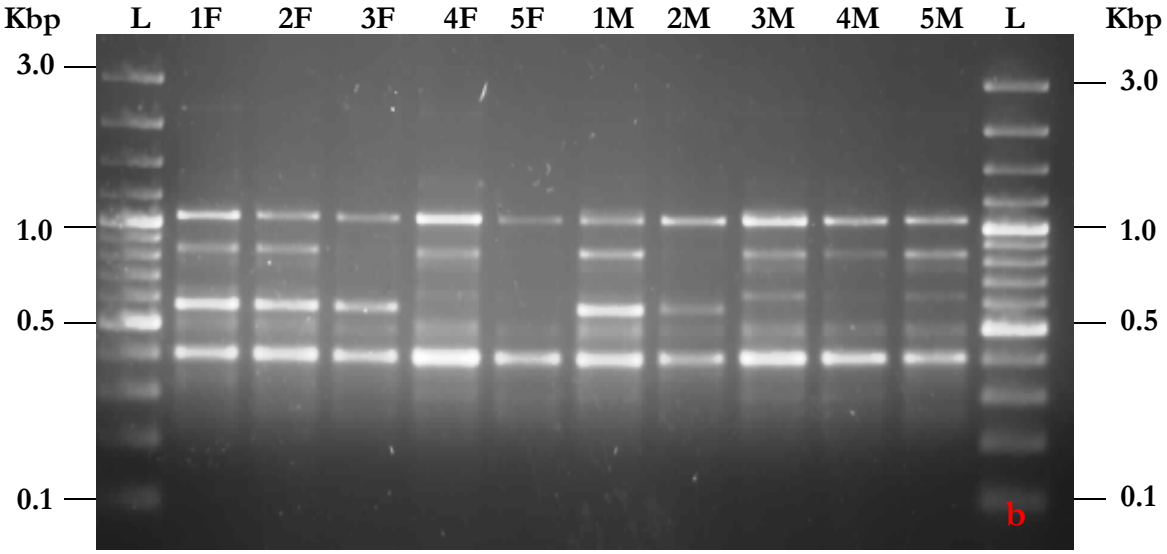


Fig. 26b. PCR amplification profiles of DNA samples of female and male *Givotia* plants produced with RAPD primer OPAB-06. Lanes designated as L represent DNA ladder (100 - 3000 bp), lanes 1F, 2F, 3F, 4F & 5F represent individual females, lanes 1M, 2M, 3M, 4M & 5M represent individual males.

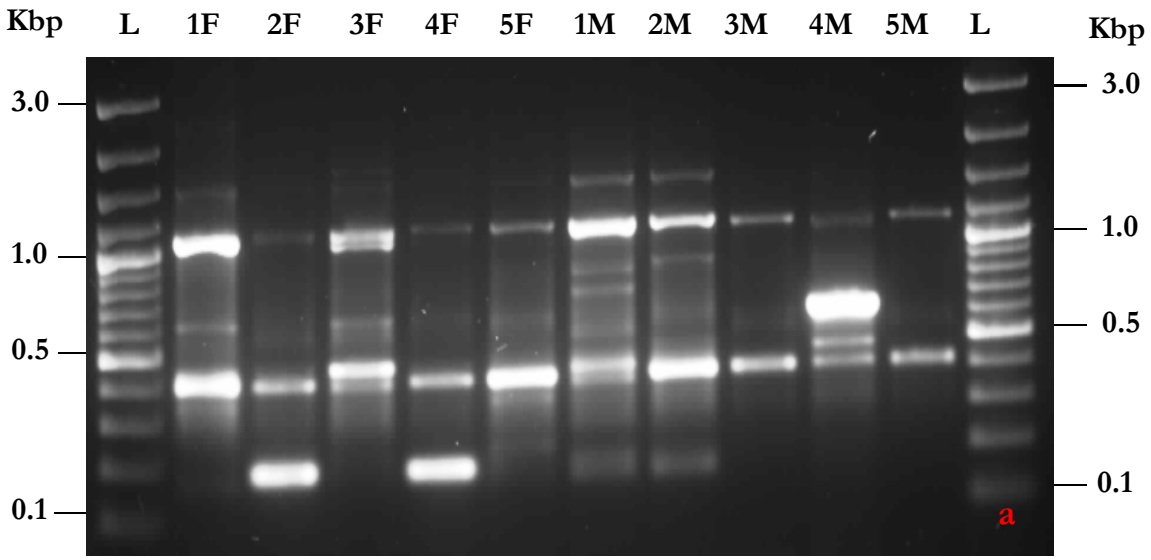


Fig. 27a. PCR amplification profiles of DNA samples of female and male *Givotia* plants produced with RAPD primer OPA-18. Lanes designated as L represent DNA ladder (100 - 3000 bp), lanes 1F, 2F, 3F, 4F & 5F represent individual females, lanes 1M, 2M, 3M, 4M & 5M represent individual males.

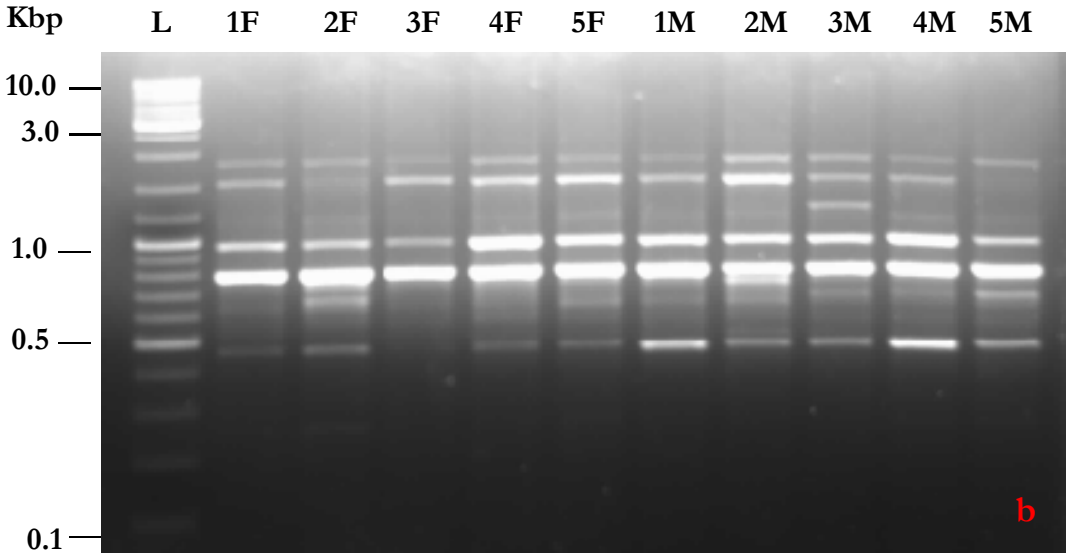


Fig. 27b. PCR amplification profiles of DNA samples of female and male *Givotia* plants produced with RAPD primer OPC-08. Lanes designated as L represent DNA ladder (100 - 10,000 bp), lanes 1F, 2F, 3F, 4F & 5F represent individual females, lanes 1M, 2M, 3M, 4M & 5M represent individual males.

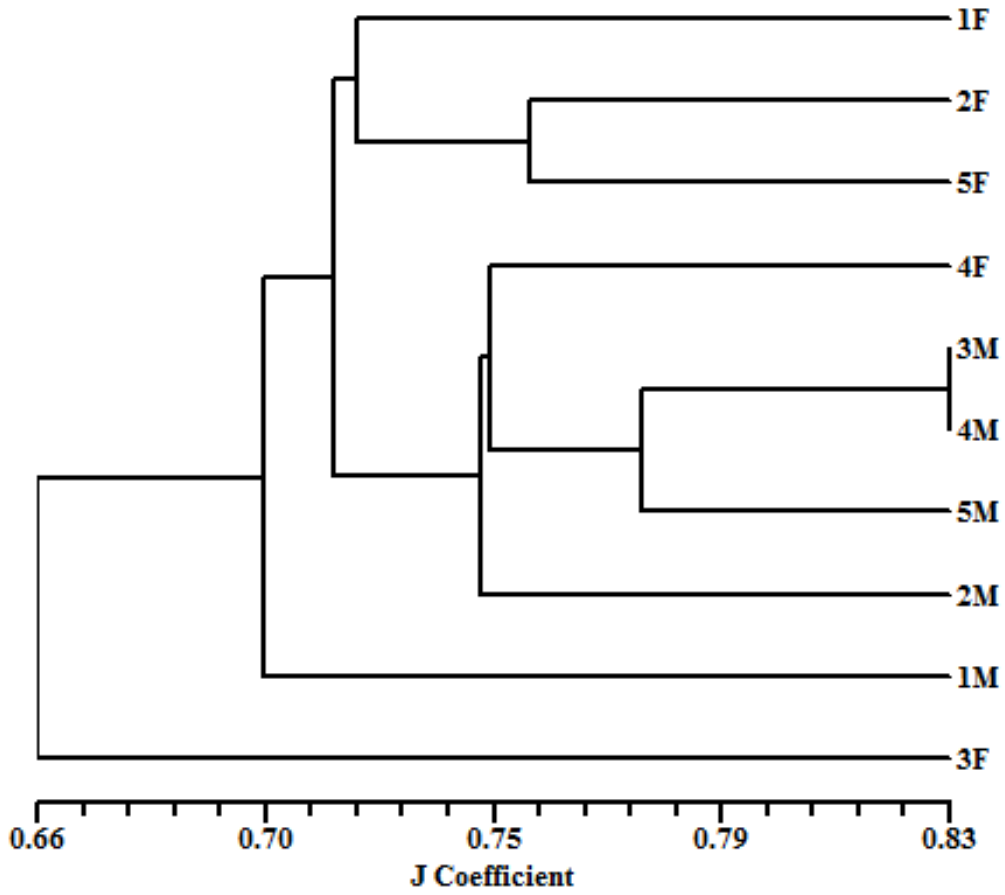


Fig. 28. Dendrogram of male and female plants of *Givotia* using RAPD data based on Jaccard's similarity coefficient (1M, 2M, 3M, 4M & 5M represent individual male plants and 1F, 2F, 3F, 4F & 5F represent individual female plants).

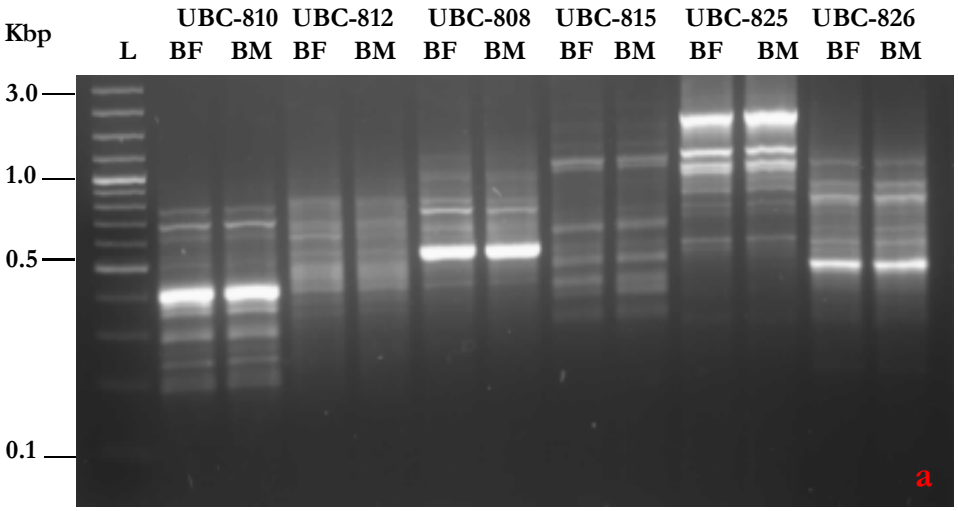


Fig. 29a. PCR-based DNA amplification profiles generated with different ISSR primers in bulk DNA of male and female *Givotia* plants. Lanes designated as L represent DNA ladder (100 - 3000 bp), BF represent bulk DNA samples of females, BM represent bulk DNA samples of males. The amplified product profiles were generated from the primers, UBC-810, UBC-812, UBC-808, UBC-815 & UBC-826, respectively in bulk males (BM) and bulk females (BF).

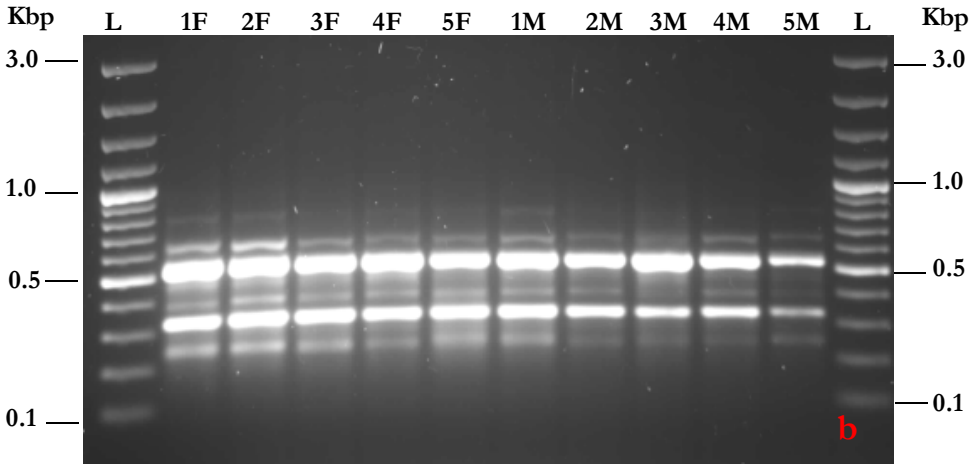


Fig. 29b. ISSR banding patterns of five individual female and male *Givotia* plants of Achampet region generated with the primer UBC-808. Lanes designated as L represent DNA ladder (100 - 3000 bp), lanes 1F, 2F, 3F, 4F & 5F represent the individual females and lanes 1M, 2M, 3M, 4M & 5M represent individual males.

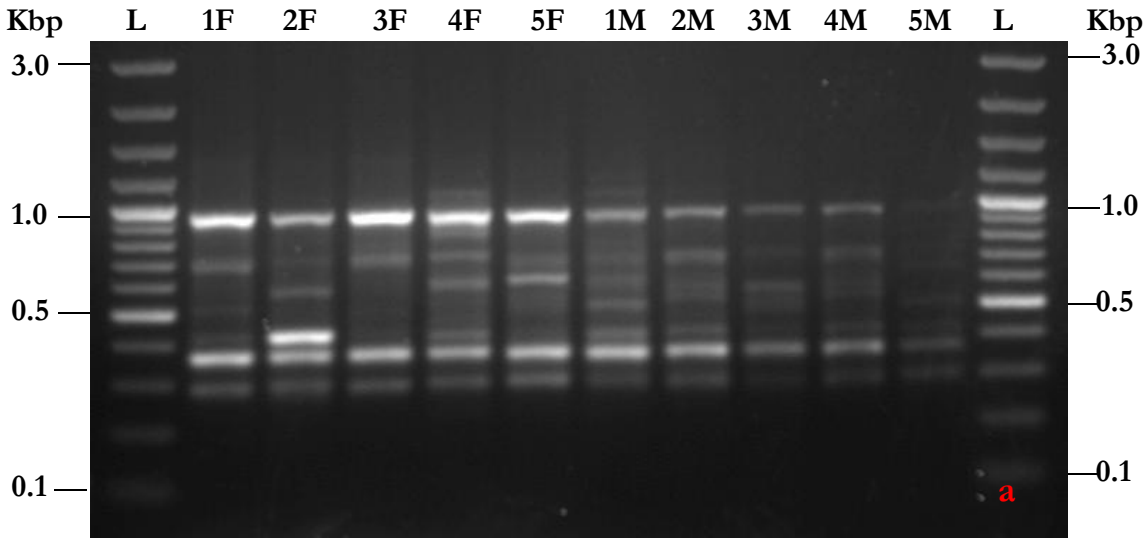


Fig. 30a. PCR amplification profiles of DNA samples of female and male plants of *Givotia* produced with ISSR primer UBC-846. Lanes designated as L represent DNA ladder (100 - 3000 bp), lanes 1F, 2F, 3F, 4F & 5F represent individual females, lanes 1M, 2M, 3M, 4M & 5M represent individual males.

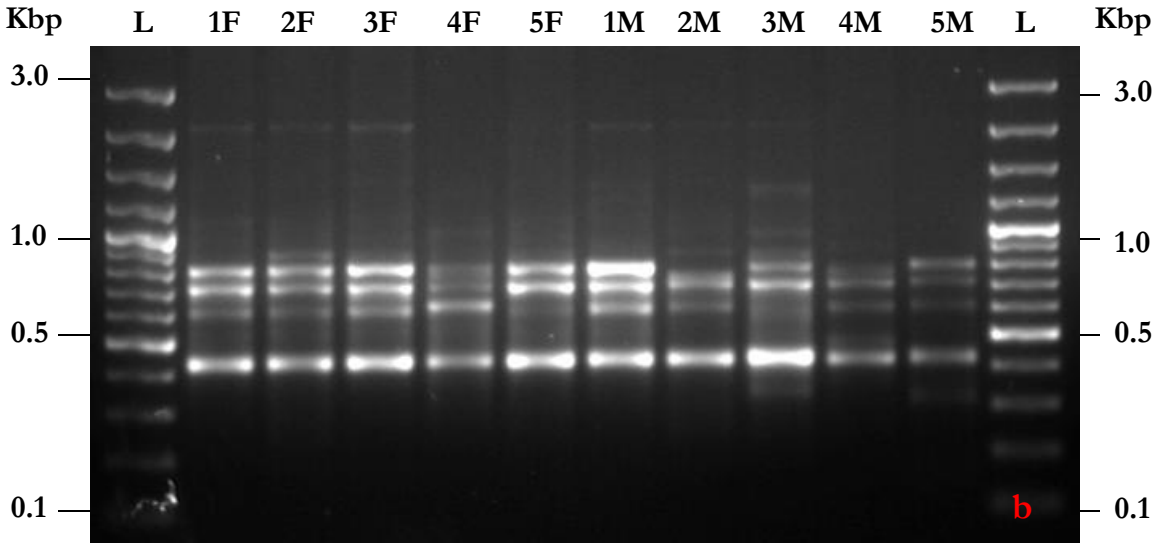


Fig. 30b. PCR amplification profiles of DNA samples of female and male *Givotia* plants produced with ISSR primer UBC-824. Lanes designated as L represent DNA ladder (100 - 3000 bp), lanes 1F, 2F, 3F, 4F & 5F represent individual females, lanes 1M, 2M, 3M, 4M & 5M represent individual males.

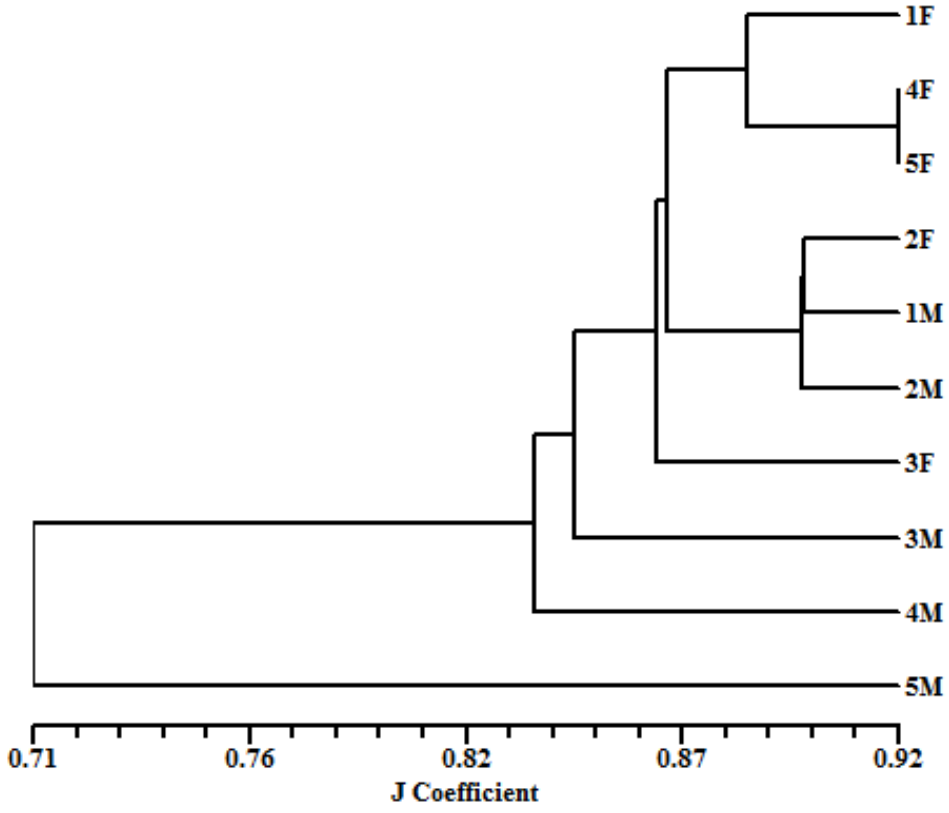


Fig. 31. Dendrogram of male and female plants of *Givotia* using ISSR data based on Jaccard's similarity coefficient (1M, 2M, 3M, 4M & 5M represent individual male plants and 1F, 2F, 3F, 4F & 5F represent individual female plants).

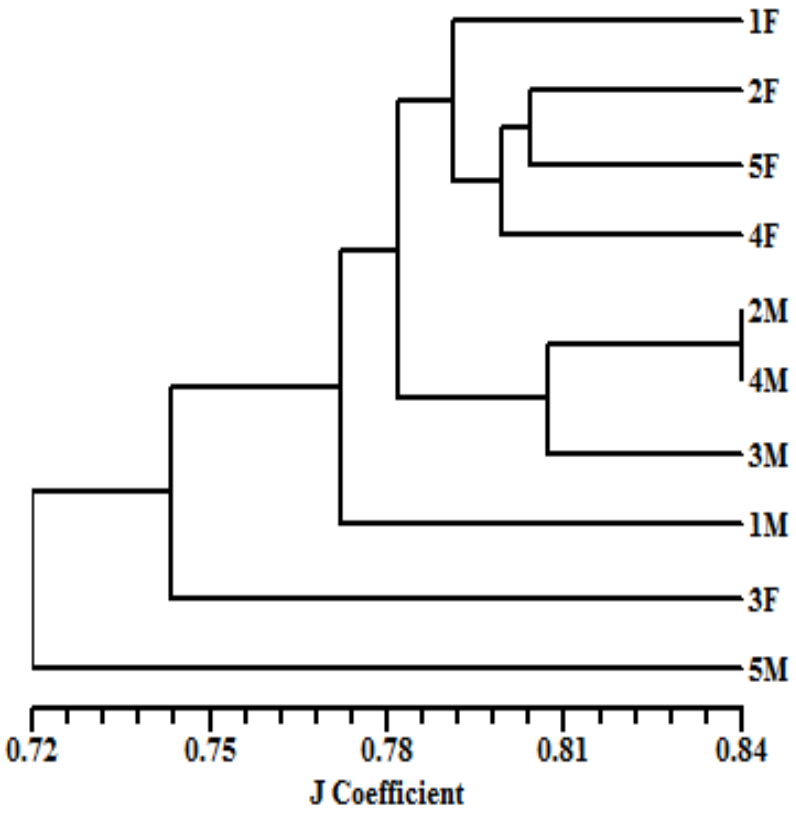


Fig. 32. Dendrogram of male and female plants of *Givotia* using RAPD and ISSR data based on Jaccard's similarity coefficient (1M, 2M, 3M, 4M & 5M represent individual male plants and 1F, 2F, 3F, 4F & 5F represent individual female plants).

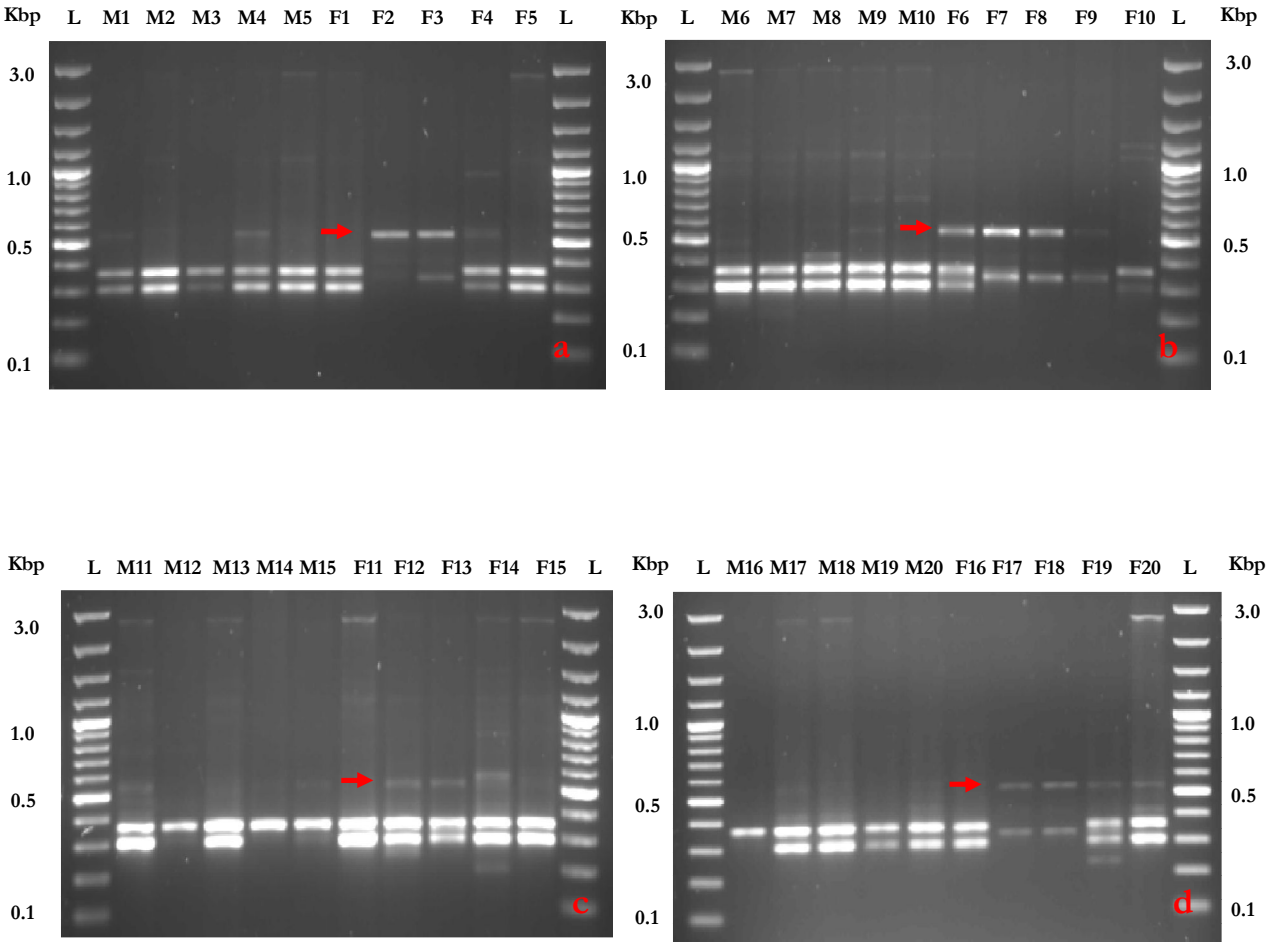


Fig. 33a-d. RAPD banding patterns of twenty individual female and male *Givotia* plants of Mulugu generated with the primer OPT-17. Lanes designated as L represent DNA ladder (100 - 3000 bp), lanes 1M - 20M represent individual males, lanes 1F - 20F represent the individual females.

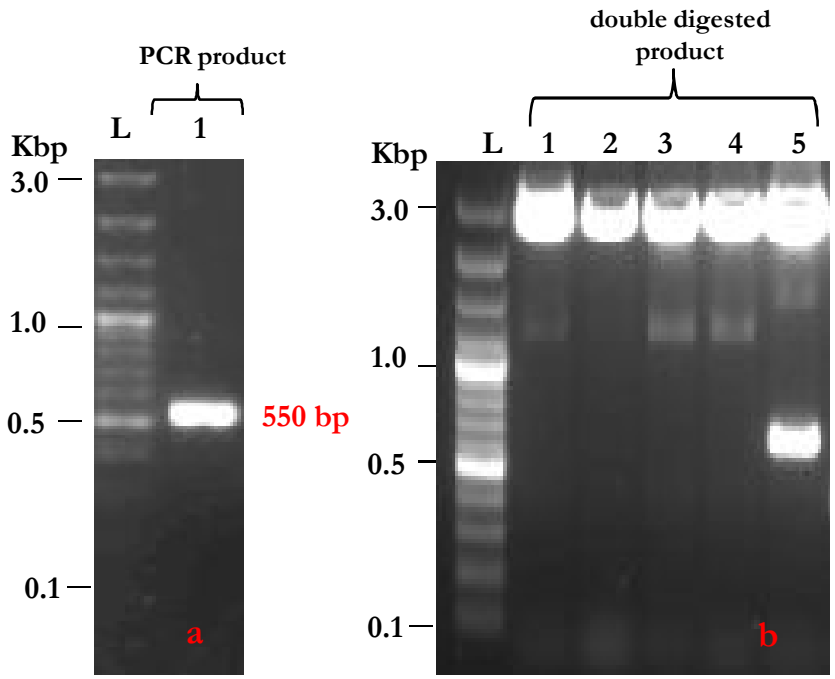


Fig 34c: Nucleotide sequence of the 550 bp amplified fragment with OPT-17 primer in *Givotia rottleriformis*.

TCCAACGTCGTCCAGCCCCGATGCGACACCGACCAACACTCTTTGTGTCTGTCACTGT
GCCGCCCATCAACAGCAGCAGCAGCAGCAAATGCGATCACTCCCGTACCCTTCGCGT
CAAGCCCCGCTCCCTCCTTCTACCCAGCATACGCTCGACACGCCGTTCACATCCCTGC
CCTCGGGTCATGTGCTCCCTCGTCTGCTCGCGTCCTCAGGCCCCTCCGTCTCCCATGC
GACGGCGCCATCCTCGCTCGCATCCACACCGAGTTCTGCGGCTCAAGCGGCAGCTCA
GCAGCAGCAGCAGAATCAGGGTACAGAAGTGATCACGCCGTCAAGCAGTACTTCAG
GCAAGTCGTTGCACCCCTACCGGTCATTCTTCCTCGCTGCCCATGGCATGGCCAGCT
CTGTGCCGCAGTCTGACTCATTCATGGCCACAACACCCAATGCCTCTGGCAGCGGTG
GCGGCAATGCAACGTTCTCCCACGCTCATGGGCGTCAGACTCCACATCCCGCGATCC
CTTCCTCTGCTACGACGTTGGTAATCTAATGCATTC

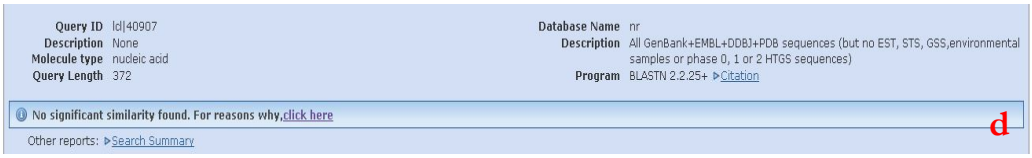


Fig. 34a-d. PCR confirmation, double digestion pattern of recombinant pTZ57R/T plasmid harbouring 550 bp insert and sequencing of amplified fragment generated with OPT-17 primer. Lane L represent DNA ladder 100 - 3000 bp. (a) Lane 1 represents 550 bp PCR product of gel eluted band (b) Lane 5 - Double digestion of plasmid yielded a 550 bp product. (c) Nucleotide sequence of the 550 bp amplfied fragment. (d) BLASTN report

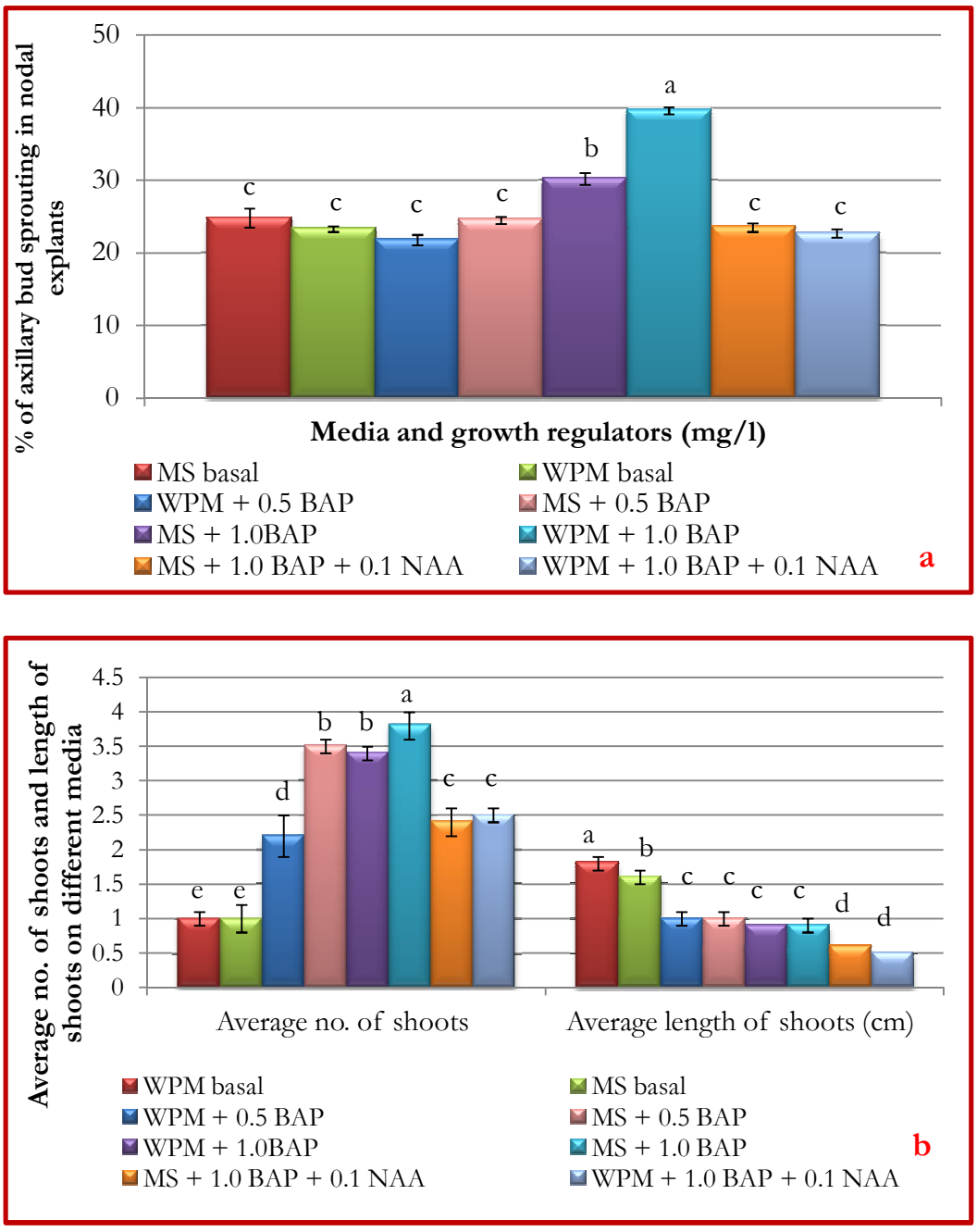


Fig. 35a & b. Axillary bud sprouting and shoot multiplication from nodal explants of mature trees of *Givotia*. (a) Percentage of axillary bud sprouting from nodal explants (b) Average number of shoots and length of shoots induced from the base of axillary buds of nodal explants after subculture on different media. Error bars indicate standard error. Significance of differences was analysed by one-way ANOVA and Newman-Kuel's multiple comparisons test. Means followed by the same letter are not significantly different ($p \leq 0.05$).

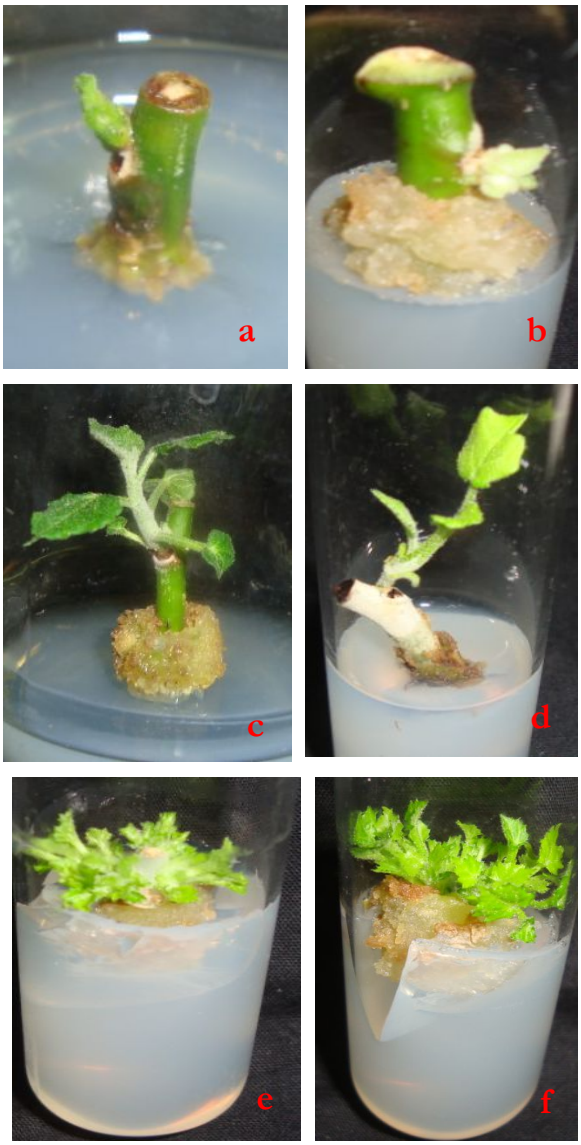
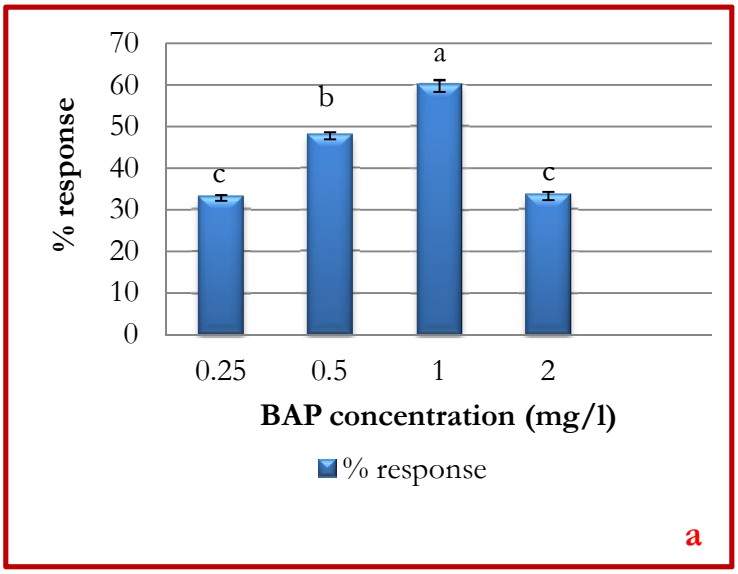
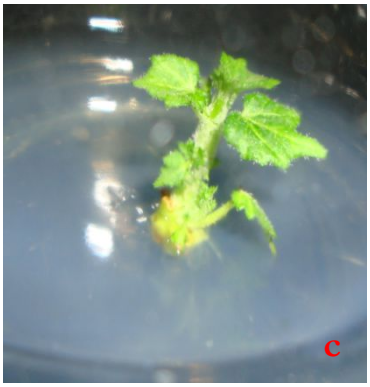


Fig. 36a-f. *In vitro* propagation from nodal explants of mature trees of *Givotia rottleriformis*. (a & b) Sprouting of axillary bud from nodal explant after 2 weeks of culture on MS and WPM basal medium, respectively, (c & d) Axillary shoots induced from nodal explant after 4 weeks of culture on MS medium with 1.0 mg/l BAP, and WPM medium with 1.0 mg/l BAP, respectively (e & f) Multiple shoots induced from the base of the axillary bud region of the nodal explant after subculture on MS medium with 1.0 mg/l BAP, and WPM medium with 1.0 mg/l BAP, respectively.





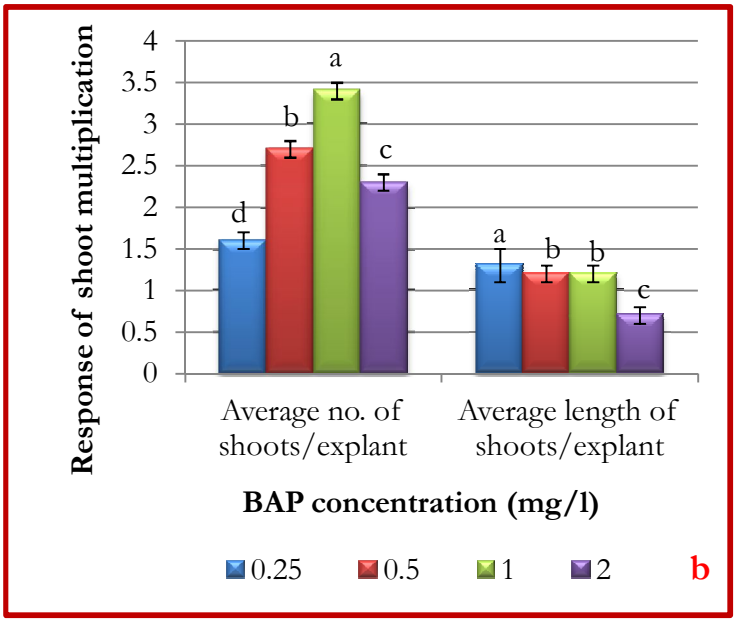
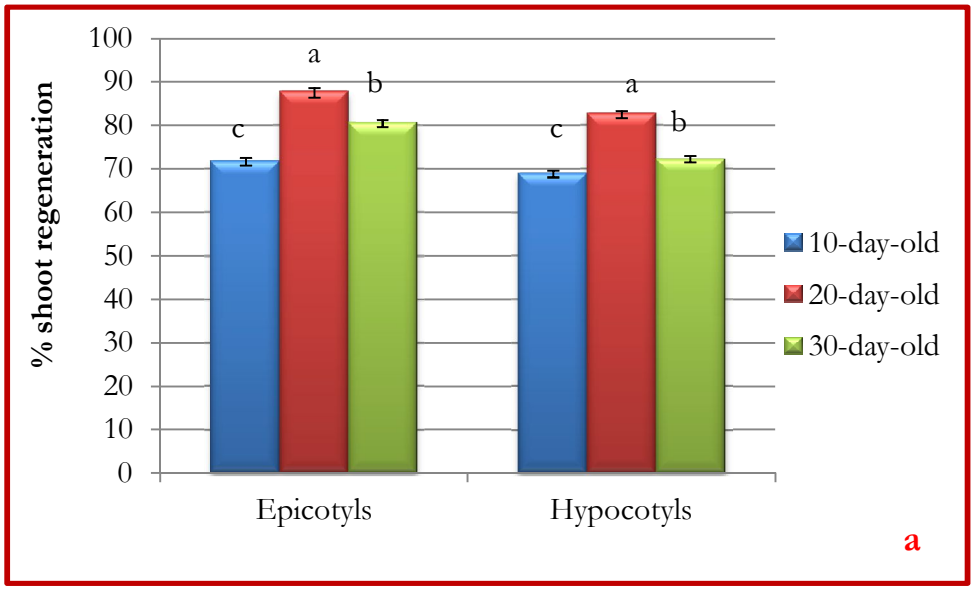


Fig. 37a-e. Shoot proliferation response from shoot tips of axillary shoots of *Givotia* cultured on medium with different concentrations of BAP (mg/l). (a) Percentage of explants showing multiple shoots (b) Multiple shoot induction and elongation on different media (c) Multiple shoot induction from shoot tips after 4 weeks of culture on MS medium 1 mg/l BAP (d & e) Multiple shoot induction and elongation after 2nd and 3rd subcultures, respectively on MS medium with 0.5 mg/l BAP.



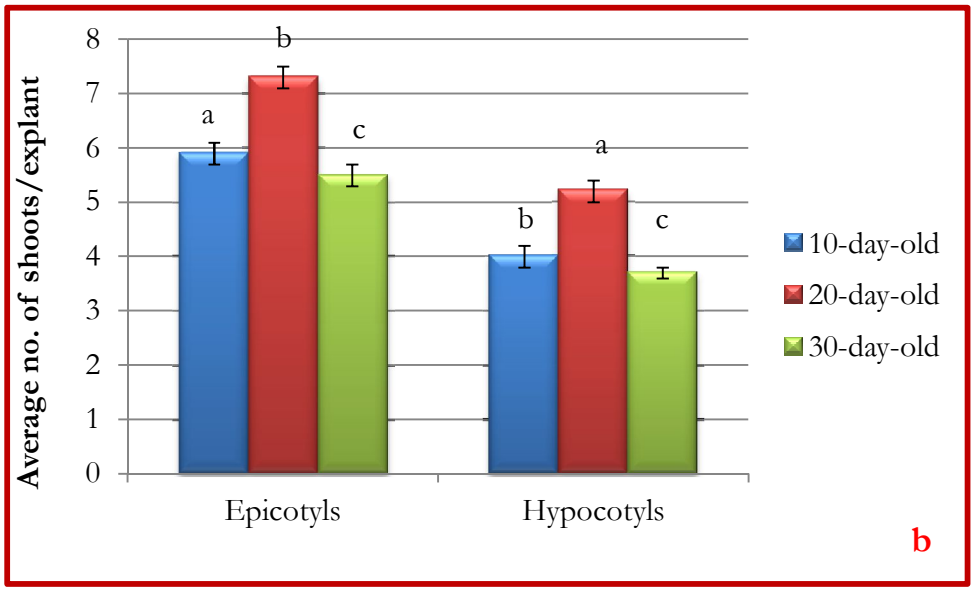


Fig. 38a & b. Effect of age of emblings on shoot regeneration from epicotyl and hypocotyl explants of *Givotia* cultured on MS medium with 1.0 mg/l BAP. (b) Percentage of shoot regeneration in epicotyl and hypocotyl explants (c) Average number of shoots per explant in epicotyl and hypocotyl explants. Error bars indicate standard error. Significance of differences was analysed by one-way ANOVA and Newman-Kuel's multiple comparisons test. Means followed by the same letter are not significantly different ($p \leq 0.05$) by Newman-Kuel's multiple comparisons test.

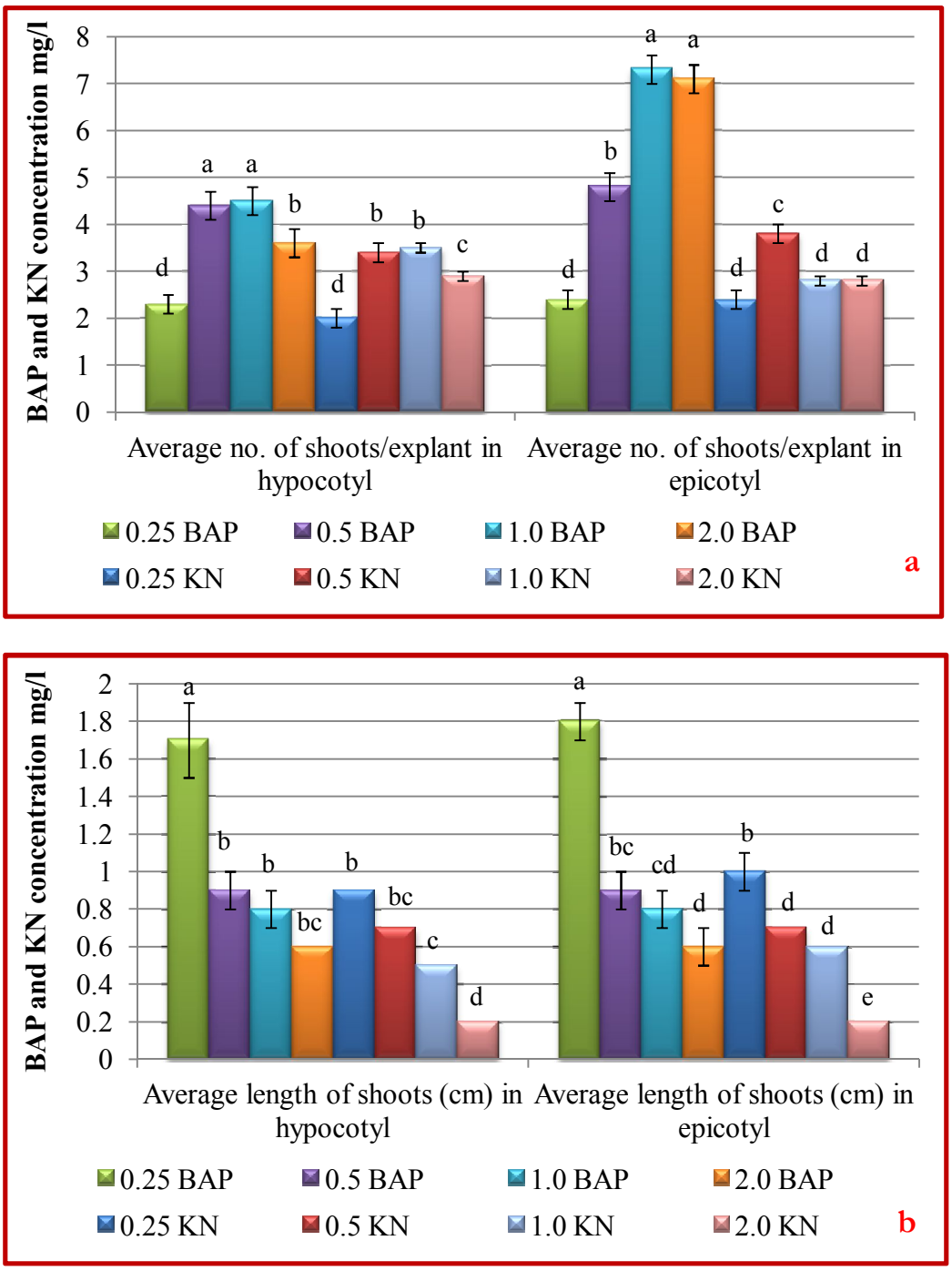


Fig. 39a & b. Effect of BAP and KN on shoot regeneration from hypocotyl and epicotyl explants of *Givotia*. (a) Average number of shoots in hypocotyl and epicotyl explants (b) Average length of shoots (cm) in epicotyl explants. Error bars indicate standard error. Significance of differences was analysed by one-way ANOVA and Newman-Kuel's multiple comparisons test. Means followed by the same letter are not significantly different ($p \leq 0.05$).

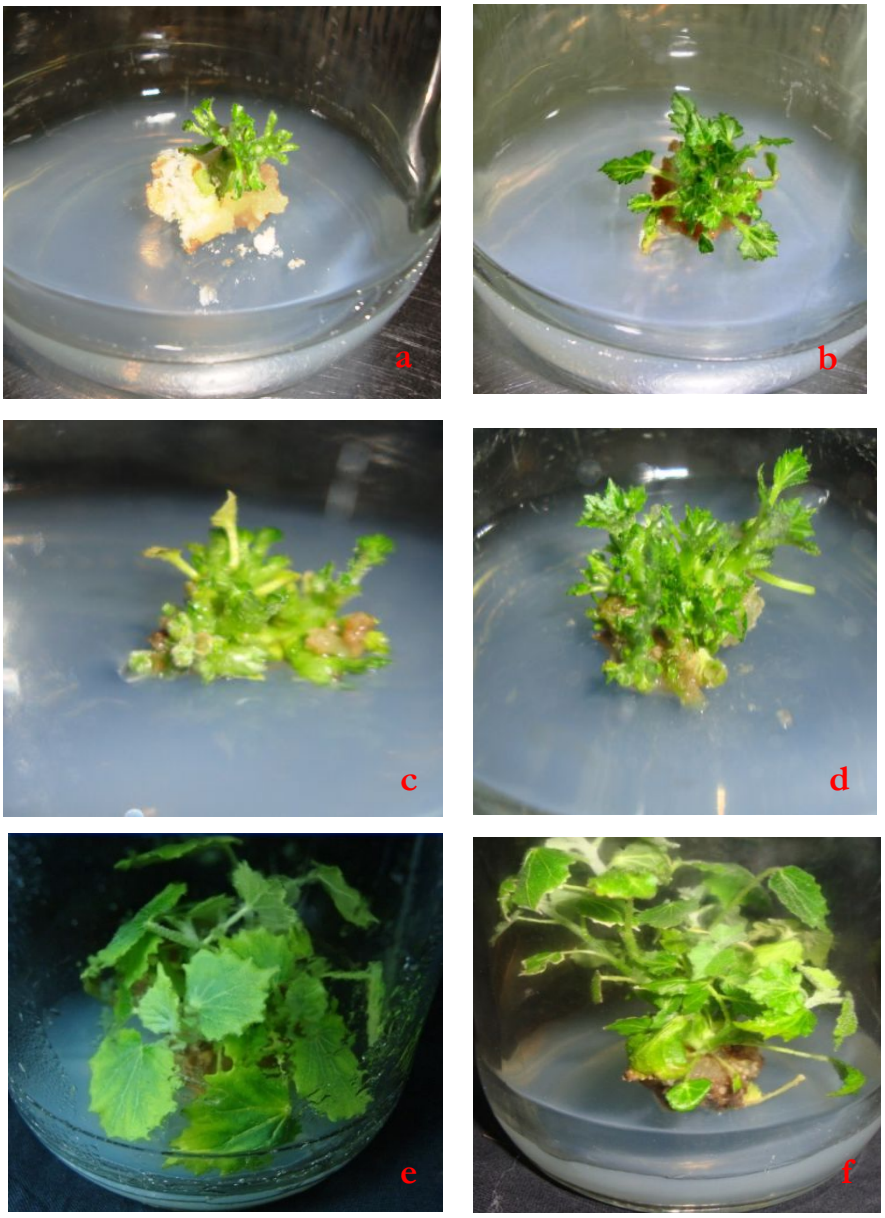
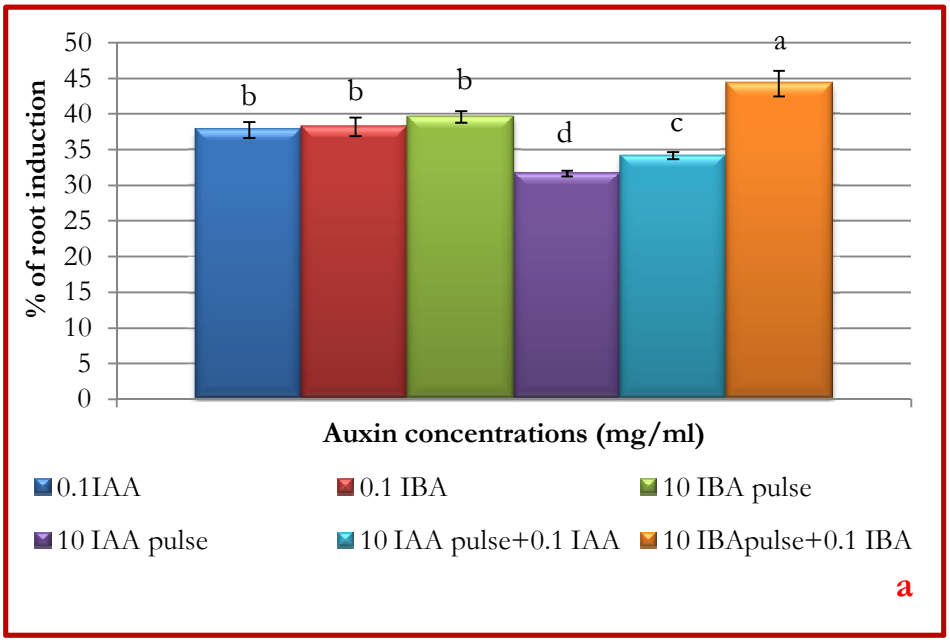


Fig. 40a-f. Direct shoot regeneration from epicotyl and hypocotyl explants of *Givotia* on MS medium with BAP. (a) Shoots regenerated from epicotyl explants on MS medium with 1.0 mg/l BAP (b) Shoots regenerated from hypocotyl explants on MS medium with 1.0 mg/l BAP (c) Proliferation of multiple shoots from epicotyl explants after subculture on MS medium with 1.0 mg/l BAP (d) Proliferation of multiple shoots from hypocotyl explants after subculture on MS medium with1.0 mg/l BAP (e) Elongation of shoots induced from epicotyl explants after subcultures on MS medium with 0.5 mg/l BAP (f) Elongation of shoots induced from hypocotyl explants after subcultures on medium with 0.5 mg/l BAP.



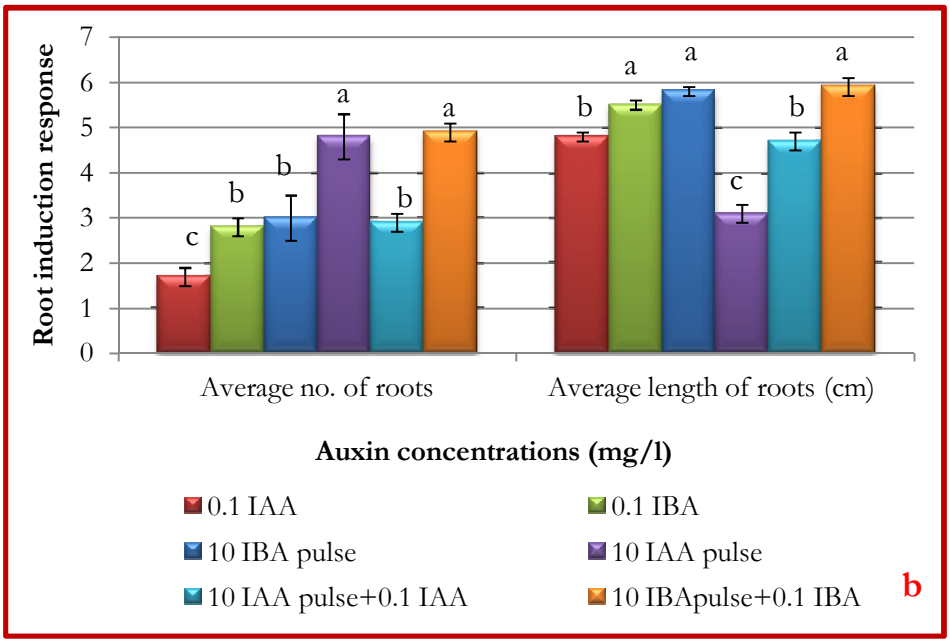


Fig. 41a & b. Effect of auxins on root induction from *in vitro* regenerated shoots of *Givotia*. (a) Root induction from shoots after pulse treatment with IBA and IAA (b) Average number of roots and root length (cm) per shoot. Error bars indicate standard error. Significance of differences was analysed by one-way ANOVA and Newman-Kuel's multiple comparisons test. Means followed by the same letter are not significantly different ($p \leq 0.05$) by Newman-Kuel's multiple comparisons test.

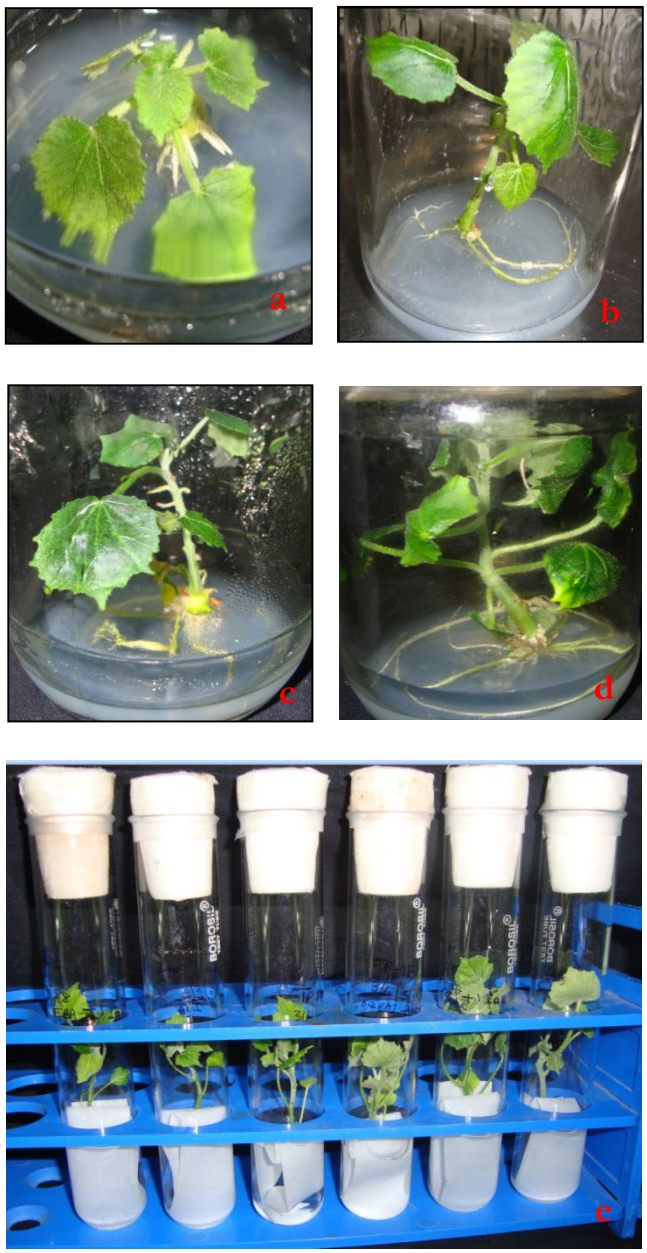


Fig. 42a-e. *In vitro* root induction from regnerated shoots. (a) Root induction from pulse treated shoots (10 mg/ml IBA) on MS medium with 0.1 mg/l IBA (b) Root induction from pulse treated shoots (10 mg/ml IAA) on MS basal medium (c) Root induction from pulse treated shoots obtained from nodal explants (10 mg/ml IBA) on MS medium with 0.1 mg/l IBA (d) Root induction from pulse treated shoots obtained from nodal explants (10 mg/ml IAA) on MS basal medium (e) *In vitro* regenerated shoots placed in liquid medium containing IBA with filter paper bridge.

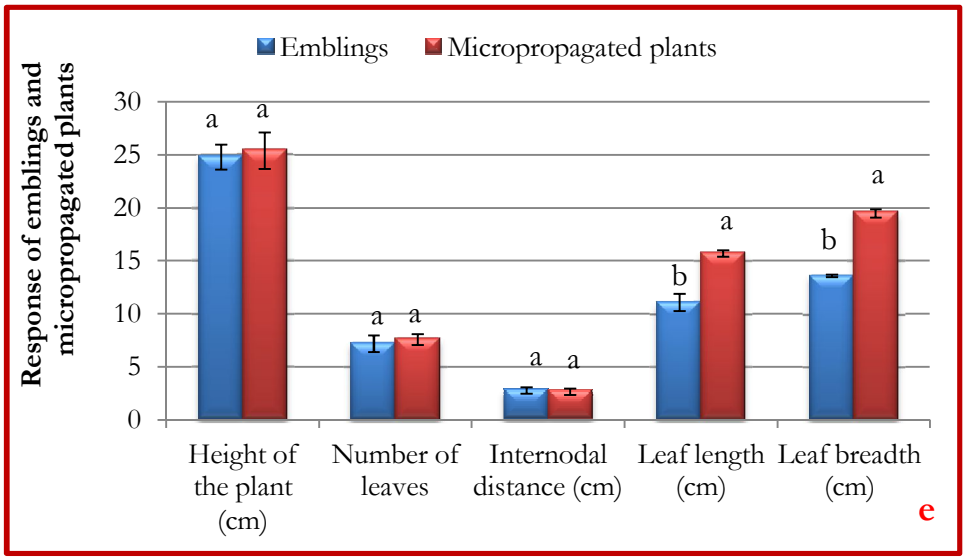


Fig. 43a-e. Acclimatization and establishment of *in vitro* regenerated plants in the field. (a) Acclimatization of *in vitro* regenerated plants in glasshouse (b) Acclimatized plants after transfer to pots in soil in glasshouse (c) Acclimatized plants established in pots (d) Plantlet established in field (e) Performance of micropropagated plants in comparison to the emblings in glasshouse.

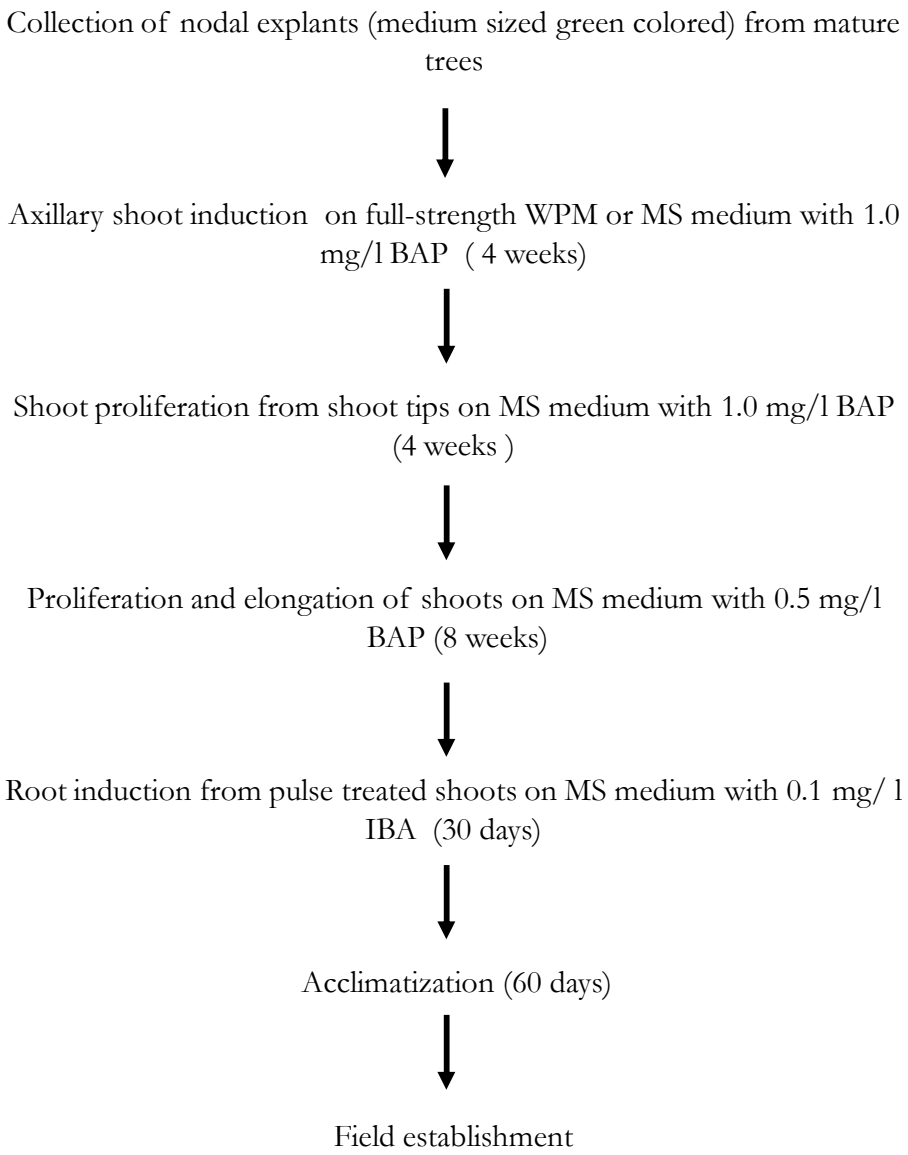


Fig. 44. Schematic representation for *in vitro* propagation from nodal explants collected from mature trees.

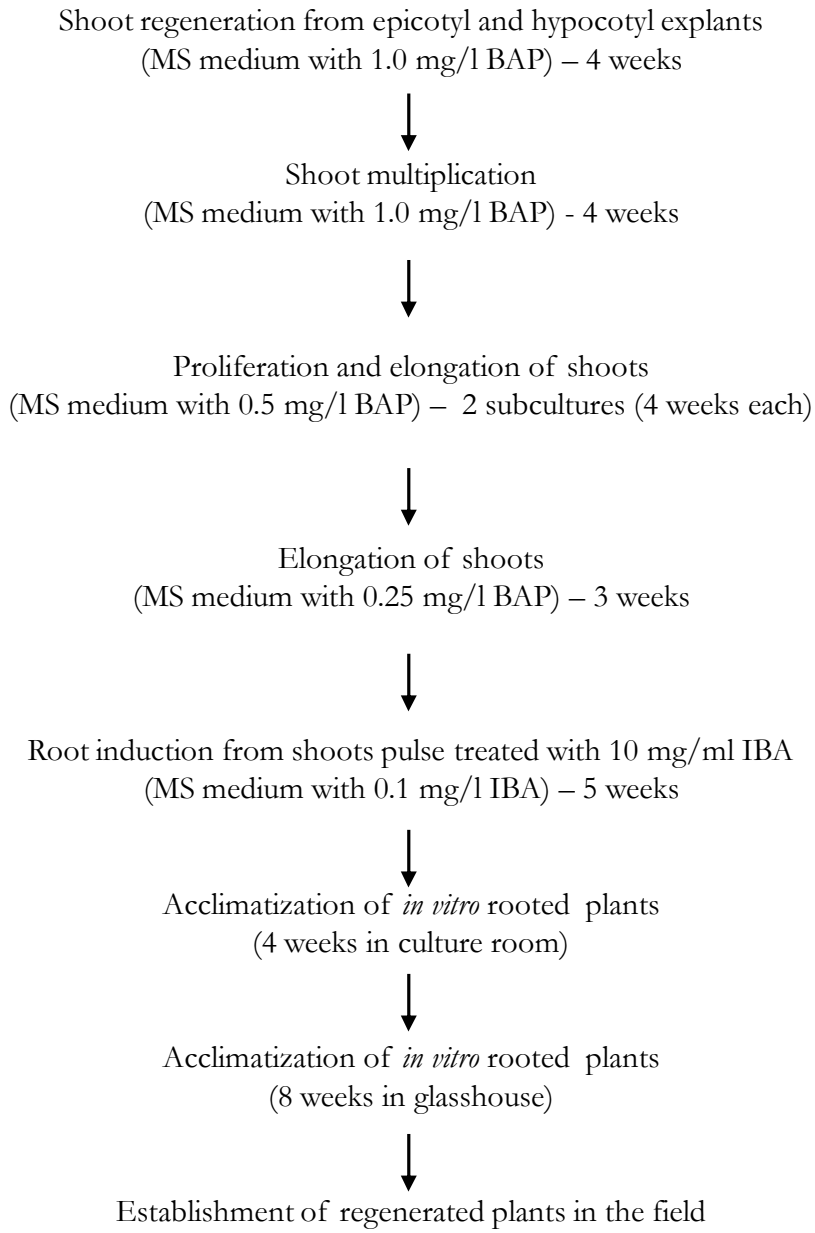


Fig. 45. Schematic representation of *in vitro* propagation from epicotyl and hypocotyl explants derived from *in vitro* germinated emblings of *Givotia* .

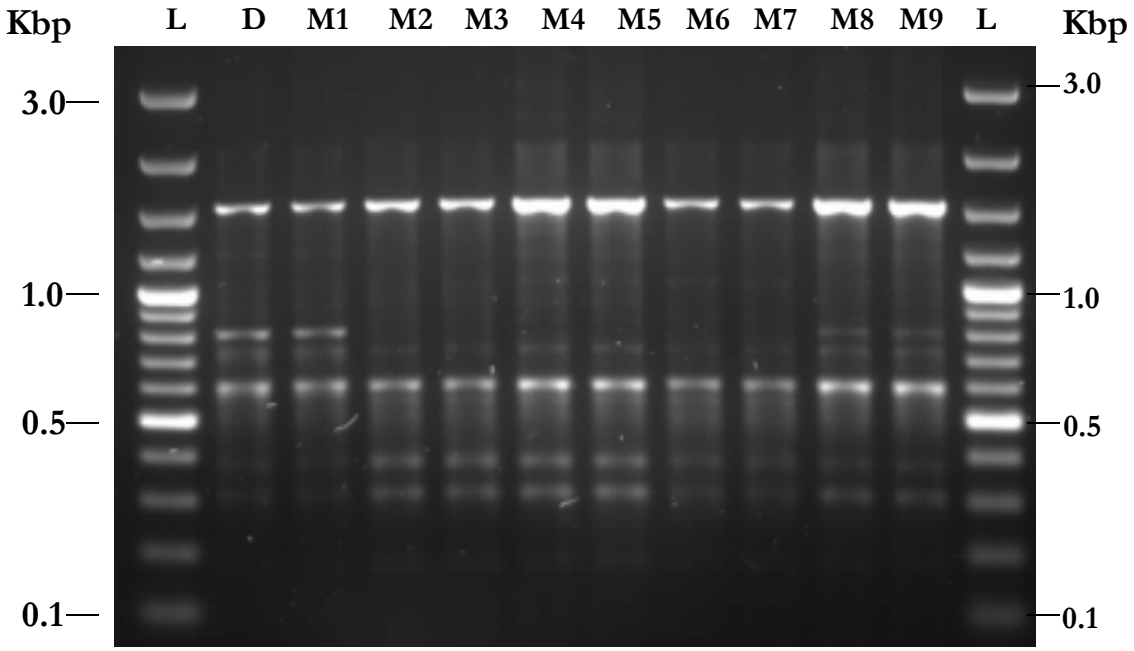


Fig. 46a. RAPD profiles of micropropagated plants of *Givotia* with OPF-03 primer. Lanes designated as L represent DNA ladder (100 - 3000 bp), lane D represents donor plant , lanes M1-M9 represent micropropagated plants.

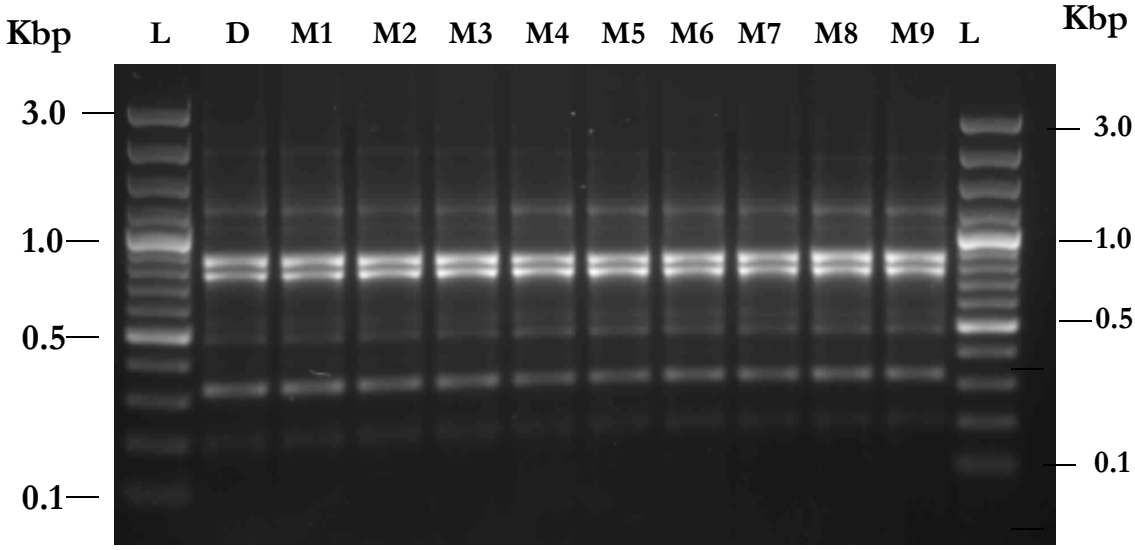


Fig. 46b. RAPD profiles of micropropagated plants of *Givotia* with OPC-05 primer. Lanes designated as L represent DNA ladder (100 - 3000 bp), lane D represents donor plant, lanes M1-M9 represent micropropagated plants.

Fig. 47a. Mature dry fruits collected from trees growing at five forest regions.

GA > GP > GK > GD > GB

Fig. 47b. Comparison of mature dry fruits of accessions of *Givotia* collected from different forest regions. GA- *Givotia* Achampet, GP- *Givotia* Pacharla, GK- *Givotia* Kondapalli, GD- *Givotia* Devarayanadurga. GB- *Givotia* Balapally.

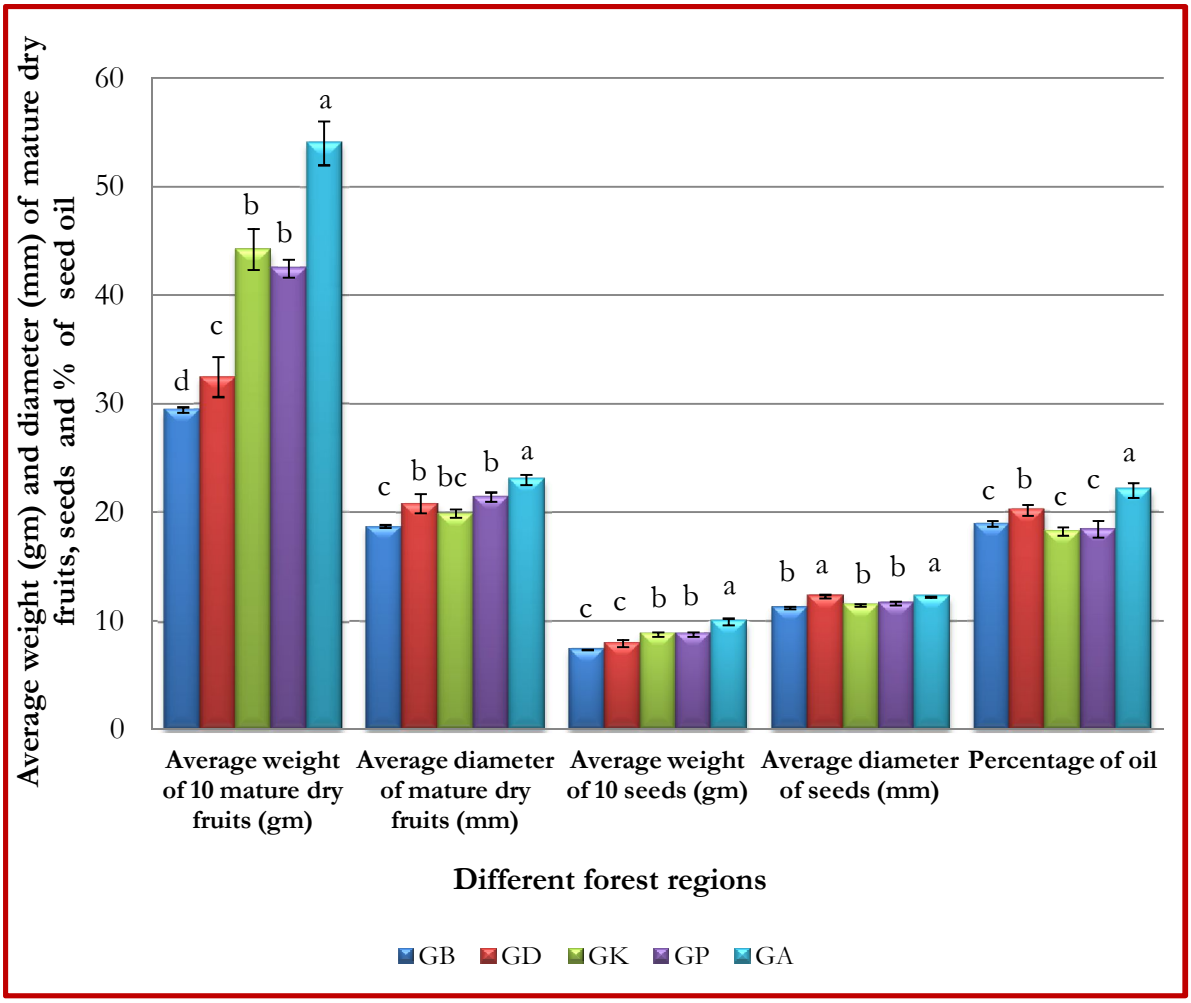


Fig. 48. Variation in weight and diameter of mature dry fruits, seeds and percentage of seed oil of *Givotia* accessions of different forest regions. GB- *Givotia* Balapally, GD- *Givotia* Devarayanadurga. GK- *Givotia* Kondapalli, GP- *Givotia* Pacharla, GA- *Givotia* Achampet. The average weight and diameter of 10 mature fruits and seeds of each accession was determined. The error bars indicate the average values of five accessions for each forest location. Means followed by the same letter are not significantly different ($p \leq 0.05$) by Newman-Kuel's multiple comparisons test.

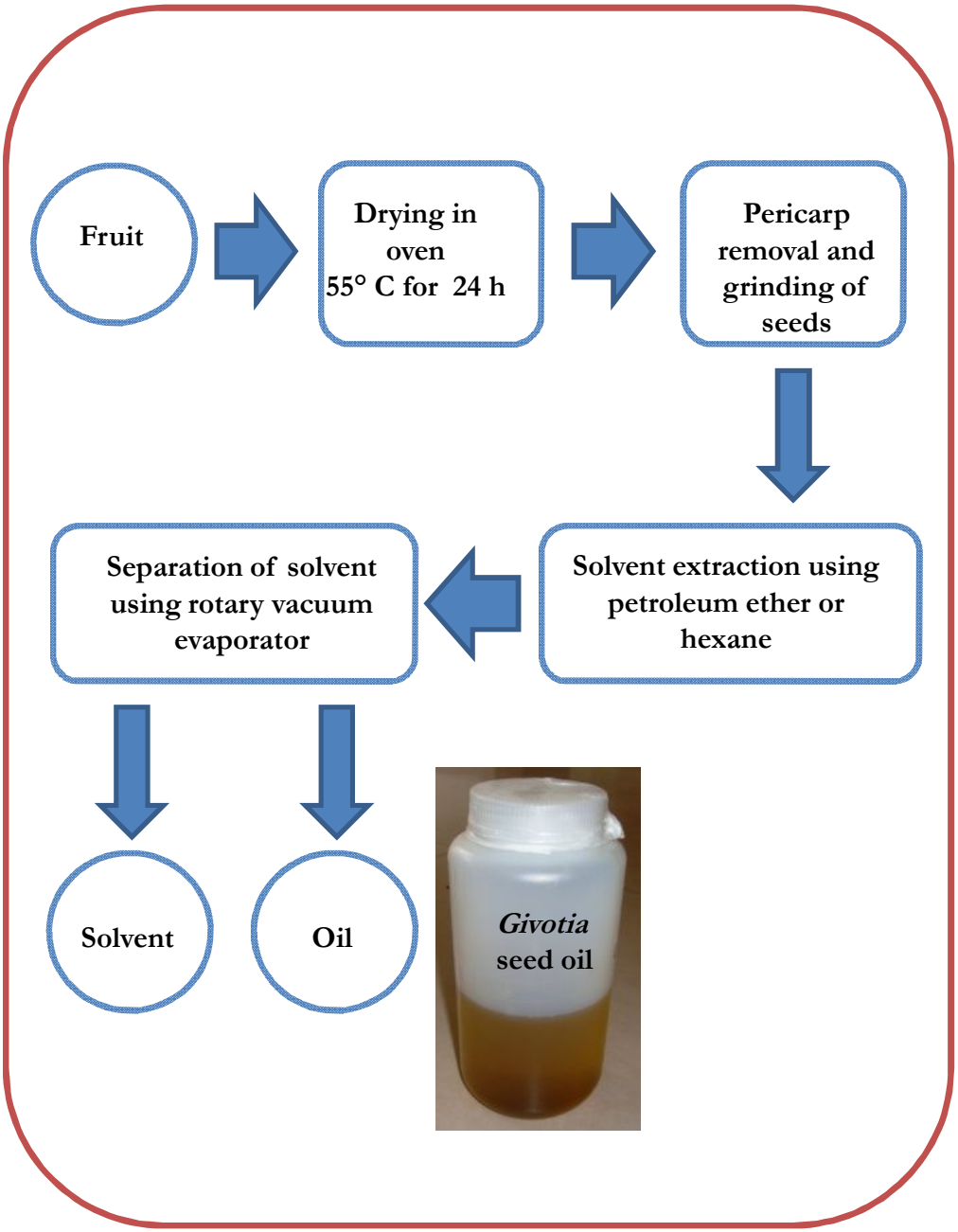


Fig. 49. Flow chart showing the process used for extraction of seed oil of *Givotia*.

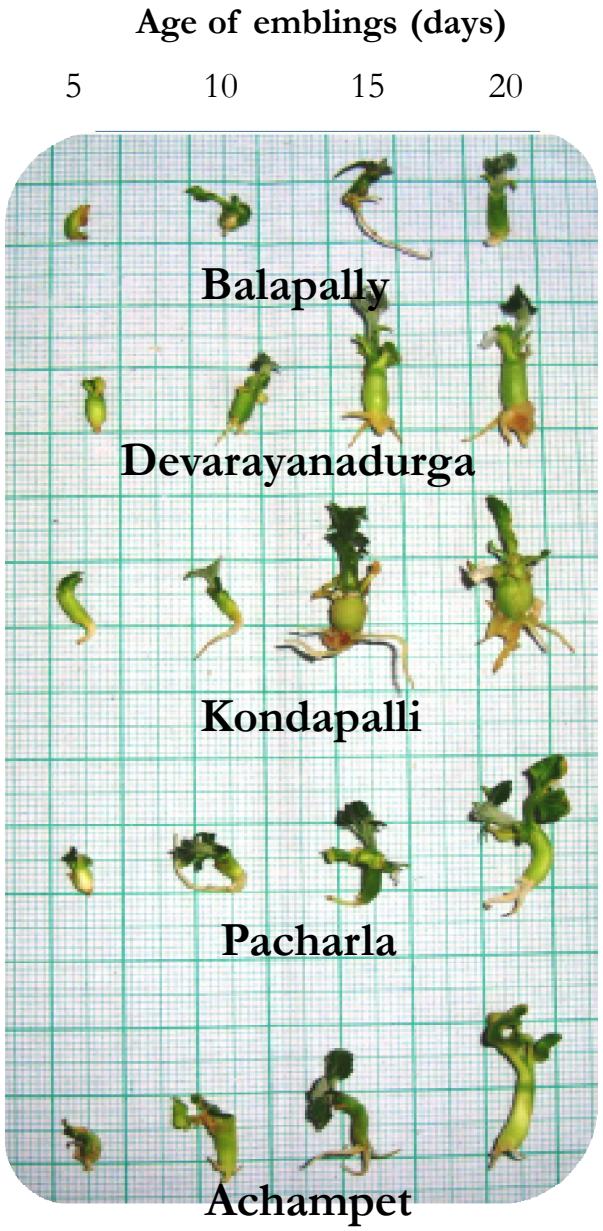


Fig. 50. Germination response from zygotic embryo axes of seeds of different accessions collected from five forest regions.

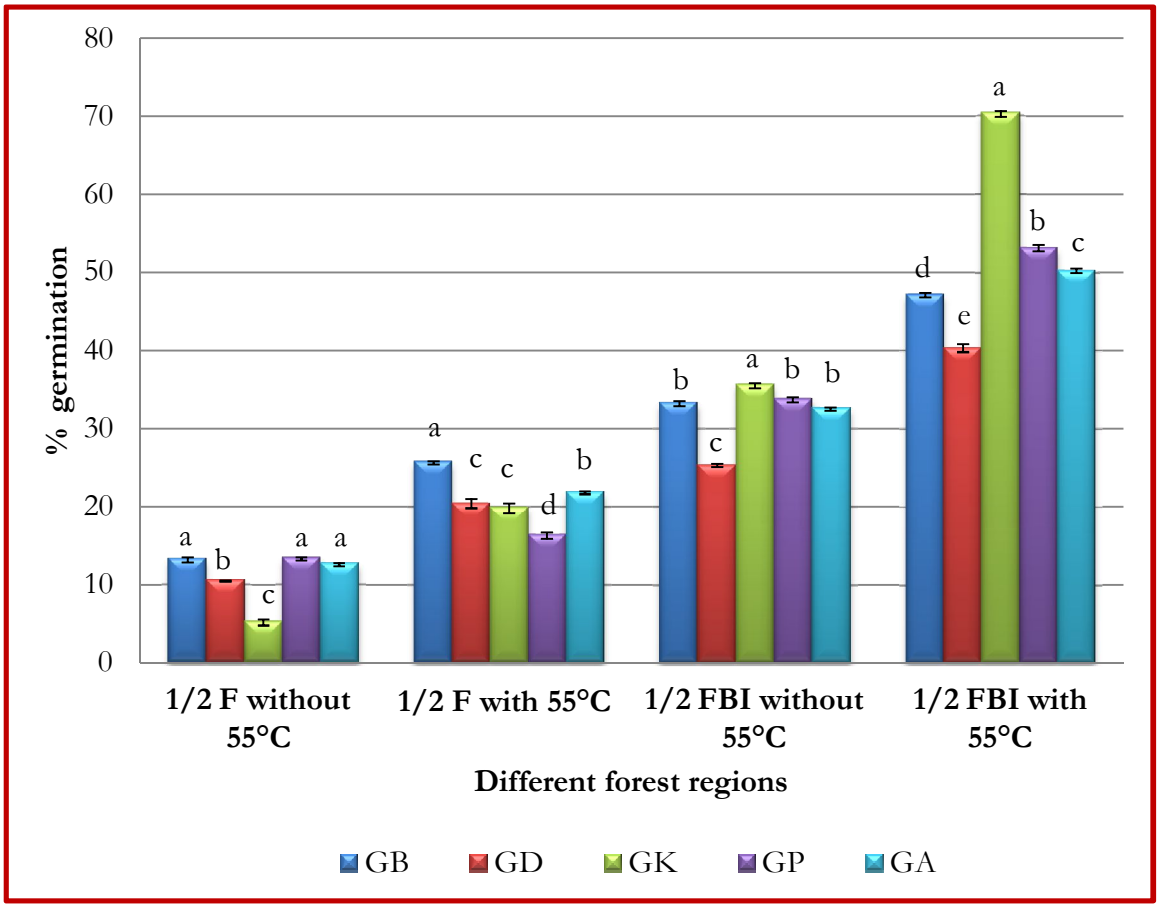
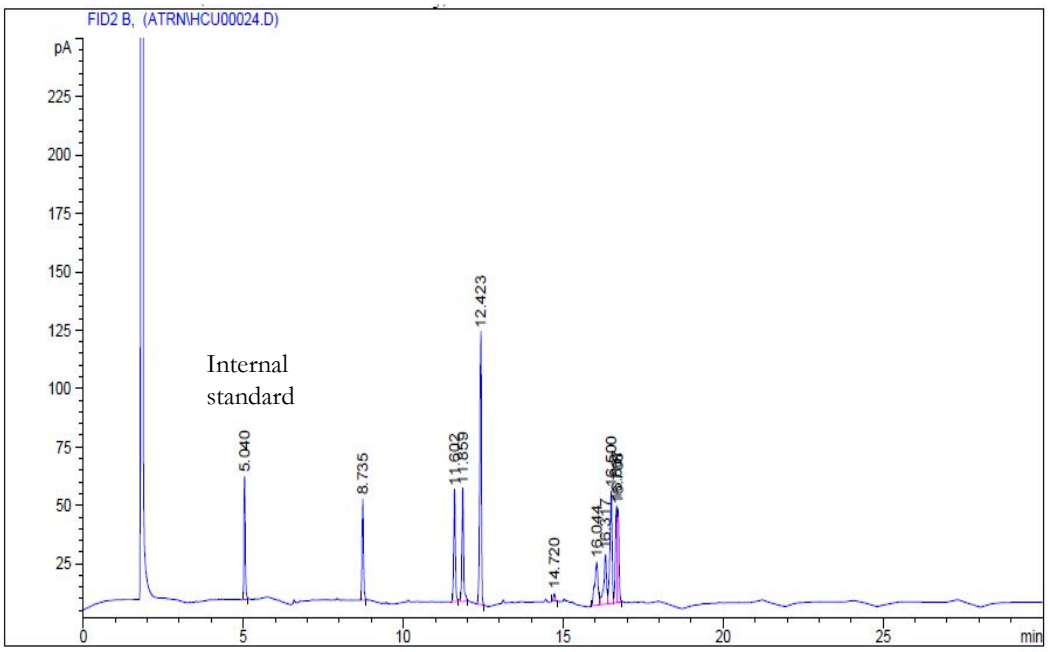


Fig. 51. Response of germination from zygotic embryo axes of seeds of different accessions of *Givotia* after different treatments. ½ F- MS basal with half-strength major salts, ½ FBI- MS basal with half-strength major salts containing 0.3 mg/l IBA and 0.1 mg/l KN. GB- *Givotia* Balapally, GD- *Givotia* Devarayanadurga. GK- *Givotia* Kondapalli, GP- *Givotia* Pacharla, GA- *Givotia* Achampeta. Error bars indicate standard error. Significance of differences was analysed by one-way ANOVA and Newman-Kuel's multiple comparisons test. Values are means ± SE. Means followed by the same letter are not significantly different ($p \leq 0.05$) by Newman-Kuel's multiple comparisons test.



S. No.	Retention Time	Width	Area	Area %	Height
1	8.735	0.0506	141.95676	8.215474	42.95632
2	11.602	0.0554	169.49205	9.809026	47.79455
3	11.859	0.0555	180.39157	10.43982	48.36290
4	12.423	0.0575	434.50595	25.1462	116.59696
5	14.720	0.0577	12.29351	0.711463	3.27921
6	16.044	0.1078	135.75781	7.856722	18.45312
7	16.317	0.0859	126.43511	7.317189	21.10245
8	16.500	0.0721	228.97818	13.25167	47.57915
9	16.667	0.0613	167.68315	9.70434	41.34811
10	16.708	0.0500	130.42511	7.548102	40.01350

Figure 52. Fatty acid composition of seed oil of *Givotia* collected from different forest regions. Peak 1 (RT = 8.735 min) is palmitic acid (8.215 %), peak 2 (RT = 11.602 min) is stearic acid (9.809%), peak 3 (RT = 11.859 min) is oleic acid (10.439 %), peak 4 (RT = 12.423 min) is linoleic acid (25.146%), peak 5-9 (RT = 14.720 min-16.667 min) isomers of conjugated fatty acids, peak 10 (RT = 16.708 min) is α-eleo stearic acid (7.548%).

5. DISCUSSION

The present study focused on characterization of genetic variability in *Givotia* accessions collected from five forest regions using RAPD and ISSR primers. Another point investigated in the study is the assessment of the molecular genetic variability in half-sib progeny of candidate plus tree as well as male and female trees growing at one forest region. The ability of RAPD and ISSR primers in generating bands specific to male and females was examined for determining molecular marker linked to sex. *In vitro* regeneration studies were carried out using nodal and shoot tip explants from mature trees and also from epicotyl and hypocotyl explants obtained from aseptically germinated emblings for optimizing methods for rapid propagation of *Givotia*. Further, the variability in fruit and seed traits, seed oil content and fatty acid composition of seed oil was determined for the first time in *Givotia*. The results obtained on the above mentioned aspects are discussed below.

5.1.1. Genetic diversity was revealed by RAPD and ISSR primers in *Givotia* accessions:

The effectiveness of tree improvement programmes depends upon the nature and magnitude of existing genetic variability and also on the degree of transmission of heritability of traits (Zobel and Talber, 1984; Rout *et al.,* 2009) because genetic variation is the fundamental requirement for maintenance and long-term stability of forest ecosystems. 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. A combination of diverse marker types are usually used for accurate assessment of the extent of intra- and inter-population genetic diversity of natural plant populations. There have been no studies conducted in *Givotia* to assess the variability growing in natural populations using morphological or molecular methods. The present study is the first attempt to assess the genetic variation in 50 *Givotia* accessions growing at 5 different forest regions using RAPD and ISSR methods. Several studies have shown that selection of primers is important for increasing the reliability of genetic diversity analysis in plants. RAPD markers were found to be more efficient in estimation of molecular genetic diversity in different accessions of *Givotia* than ISSR markers as evident from large number of polymorphic loci (12.1) detected in RAPDs as compared to ISSRs (9). Comparison of polymorphism percentages for two marker

systems indicated that the polymorphisms for RAPD primers was 92.2, while that of ISSR primers was 74.1 indicating better resolving power of RAPD markers over ISSRs in detecting genetic variability in *Givotia* accessions (Tables 2 & 3). The presence of high proportion of polymorphic loci suggests that there is high level of genetic diversity in its natural populations which is expected due to outcrossing behavior resulting from its dioecious nature. Similarly, Gajera *et al.* (2010) reported that RAPD markers were marginally more informative than ISSRs in the assessment of genetic diversity in castor.

Variations in DNA sequences lead to polymorphism and greater polymorphism is indicative of greater genetic diversity (Gajera *et al.*, 2010). The polymorphism of 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). The variation detected by ISSR primers could be the result of high rate of gaining and losing repeat units due to DNA slippage (Schlotterer, 1998). Chromosomal structural rearrangements have also been suggested as a source of ISSR variation (Wolfe and Liston, 1998). The possible explanation for the differences in polymorphism detected by RAPDs and ISSRs in the current study could be due to the existence of variation caused by the polymorphic loci, their frequency of distribution in a given population and also could be due to the fact that the two marker techniques target different portions of the same genome. Similar studies carried out by several researchers have demonstrated the effectiveness of different marker systems as well as low to moderate level of genetic diversity in *Jatropha curcas* (Reddy *et al.*, 2007; Basha and Sujatha, 2007; Gupta *et al.*, 2008).

In the present investigation, RAPD primers revealed relatively low level of polymorphism of 59.6% and 60.4% in the accessions of Devarayanadurga and Kondapalli, respectively whereas with ISSR primers the lowest level of polymorphism of 35.1% was observed in Achampet accessions (Tables 2 & 3). The differences in the number and nature of RAPD and ISSR primers used in the study might also account for the differences in the polymorphism detected among accessions of different regions.

One of the important concerns related to RAPD-generated phylogenies include homology of bands showing the same rate of migration, causes of variation in fragment mobility and origin of sequence in the genome (Stammers *et al.*, 1995). In spite of this limitation, RAPD markers had good capability to scan across all regions of the genome hence

highly used for phylogeny studies at species level (Wilikie *et al*., 1993; Demeke, 1992). In both RAPD and ISSR analysis in this study, the clustering pattern showed a clear grouping of accessions of specific region into clusters indicating greater similarities among accessions of a specific region as compared to accessions of other regions (Fig. 7 and 10). Further, the dendrogram constructed from RAPD and ISSR data was more or less comparable with respect to grouping of *Givotia* accessions collected from different regions although differences were noticed with respect to similarity coefficient values and positioning of *Givotia* accessions of different regions within clusters. The differences in the dendrograms obtained from RAPDs and ISSRs could be partially explained by the different number of PCR amplified products analyzed (4497 for RAPDs and 3864 for ISSRs) emphasizing the importance of the number of loci present and their coverage of the overall nucleotide genome for obtaining reliable estimation of genetic relationship among *Givotia* accessions. In a similar study using phylogenetic and PCA analysis, Gautam Murty *et al.* (2013) observed region-specific clustering patterns in *Jatropha* germplasm that revealed geographical variation, which was attributed to selection pressure exerted upon the accessions due to the differences in the environmental conditions. On the contrary, Gajera *et al.* (2010) reported that the dendrogram based on RAPD and ISSR primers did not show any clear pattern of clustering in castor genotypes according to the location indicating little or no location specificity among castor genotypes.

Hamrick and Loveless (1989) opined that the breeding system of a species is an important determinant of variability at both the species and population levels. In the present study, the phylogenetic analysis on the basis of RAPD and ISSR derived dendrogram separated the accessions into two main clusters at 38% similarity with first cluster comprising Achampet, Pacharla, Kondapalli, Devarayanadurga while the second cluster included Balapally region (Fig. 11). Thus the accessions from Balapally region resulted as more divergent population when compared to the accessions of other regions of Andhra Pradesh, Telangana and Karnataka. The high genetic diversity could be related to the population sizes which might have a positive effect on genetic variation within populations (Leimu *et al*., 2006). The densities of the *Givotia* plants in population in the regions investigated are expected to vary due to the cutting or removal of trees for its softwood or medicinal properties. The thinning of population due to human related activities might also lead to decrease in genetic variability. There have been instances where the diversity increased as a result of occurrence of the species in almost pure, high-density stands (Neale and Adams, 1985) or to the unintentional

retention of individuals with heterozygosity in the residual population (Buchert *et al*., 1997). In some case, partial removal of populations aimed at promoting natural regeneration resulted in increase in genetic diversity; however, there was a loss of rare alleles (Rajora *et al*., 2000).

In the present study, Mantel test showed a significant correlation (r = 0.523) between the two markers systems indicating that the polymorphism detected in different accessions by RAPD and ISSR markers had a similar pattern of distribution. The two and three dimensional PCA plot more or less corresponded with the UPGMA analysis based on RAPD and ISSR data which clearly differentiated the accessions of Balapally, Devarayanadurga and Kondapalli although minor differences were observed with respect to grouping of accessions of Achampet and Pacharla regions (Fig. 11, 12 & 13). Thus the molecular analysis employing RAPD and ISSR techniques of different geographically scattered populations of *Givotia* across Andhra Pradesh, Telangana and Karnataka showed the occurrence of genetic diversity among accessions. The genetic variation detected by RAPDs in *Givotia* accessions may be the combined result of its mating system, mutation, gene flow by means of pollen and seed, 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 abyssinca* (Asmare, 2005). Further studies with more number of primers and use of different molecular markers and inclusion of higher number of accessions across natural distribution range would provide a precise estimate of genetic diversity existing in the population. Similar findings also reported by several authors in *J. curcas* using RAPD and AFLP markers (Tatikonda *et al.,* 2009, Sudheer *et al*., 2009, Boora *et al*., 2009).

5.1.2. SCAR primers developed from RAPD amplicons failed to distinguish *Givotia* populations:

The reproducibility of RAPDs can be improved by converting RAPD amplified fragment into SCAR markers. SCAR markers are known to be specific, easy, reliable and reproducible and have been widely used in different applications such as species identification, marker-assisted screening and also map-based gene cloning experiments. In the present study, two amplified fragments of 490 bp and 350 bp were generated by OPC-08 and OPT-17 primers in only accessions of Andhra Pradesh and Telangana but missing in accessions of Karnataka State (Fig. 14a & b; Fig. 15 a & b). The amplified fragments were cloned and

sequenced for development of region-specific SCAR marker. The sequence of the cloned 490 bp fragment (Fig. 14d; GenBank: KF772203.1) when subjected to BLASTN analysis showed no homology with known nucleotide sequence at different sequence similarity levels. The nucleotide sequence of cloned 350 bp fragment (Fig. 15e) obtained with OPT-17 primer showed 91% similarity with *Jatropa curcas* uncharacterized (LOC105640258), mRNA (NCBI Accession No. - XM 012224569.1) and 86% similarity with clone-3 hypothetical protein gene of *Ricinus* (NCBI accession no - XM 002513664.1). The high degree of similarity of 350 bp fragment with *Jatropha curcas* and *Ricinus* is apparent as all these three species belong to the family Euphorbiaceae.

The SCAR primers designed from the sequence of 490 bp and 350 bp fragment when screened on 10 accessions each of Achampet and Devarayanadurga resulted in amplification in all the accessions analyzed indicating their inability to distinguish the accessions of Devarayanadurga from other accessions (Fig. 16a & b). The amplification of the bands in all accessions by SCAR primers indicates the presence of annealing site. The primer competition with the genomic DNA to anneal at specific sites, poor specificity of the short 10-mer primers might lead to differences in PCR amplication especially with RAPDs (Williams *et al*., 1990; Hernandez *et al*., 1999).

5.1.3. RAPD and ISSR primers revealed genetic variation in half-sib progeny of candidate plus tree:

The knowledge concerning the inheritance of selected traits and the estimation of variability in plus-trees and their progeny is important for accelerating the breeding process and for enhancing the efficiency of tree seed plantations (Efimov, 1991; Korshikov and Demkovich, 2010). The understanding of the relationship between level of genetic diversity in parental populations and their offsprings among tropical forest tree species is limited (Lyngdoh *et al*., 2013). It was of interest to analyze the molecular genetic variability in half-sib progenies of candidate plus tree of *Givotia* as there have been no studies conducted so far on these lines. The major idea behind the study has been to find the genetic contribution of plus tree to half-sib progenies by analyzing their similarities and differences and also understand the paternal contribution in this dioecious tree species. Such information would be useful in unravelling the exact pollination patterns in seed orchards (Moriguchi *et al*., 2004) and can

speed up the process of recognition and selection of valuable genotypes for the development of productive plantations from seed orchards.

The present study employing 12 RAPD primers and 10 ISSR primers revealed marginally higher polymorphism of 57.8% by RAPDs as compared to 51.4% by ISSR primers (Table 5 & 8). Seven RAPD primers and 6 ISSR primers can be considered as efficient primers as they generated polymorphism of ≥50% and can be used for comparative analysis of half-sib progenies with maternal tree. Similarly, De Greef *et al.* (1998) reported high level of polymorphism during RAPD and AFLP analysis in half-sibs of *Quercus petraea* (Matt.) Liebl. 'plus' trees depending on the primer or primer combination used. In a similar study, Asolkar *et al.* (2011) reported that 5 RAPD primers out of 10 tested were efficient in generated higher percentage of polymorphism in half-sib progeny in Cashew.

In the present study, the percentage of polymorphism varied and was not related to the total number of bands generated. Thus, OPAI-12 with 100 amplified bands resulted in 78.0% polymorphism as compared to OPV-08 primer with 86 bands that gave 87.2% polymorphism (Table 5). Similarly, ISSR primer UBC-825 with 60 bands generated 81.7% polymorphism than ISSR primers, UBC-808 and UBC-815 with the highest number of bands (93) generated 52.7% and 64.5% polymorphism, respectively (Table 8).

Investigations have been carried out to determine the relatedness in open-pollinated families with the help of DNA markers in different tree species (Dow and Ashley, 1996; Hansen and Nielsen, 2010; Asolkar *et al.*, 2011). Examination of the banding pattern generated by RAPD primers revealed that 4 primers generated 6 bands only in maternal tree and absent in all the 10 half-siblings analyzed (Table 7). None of the ISSR primers generated bands specifically present in maternal tree but 2 ISSR primers (UBC-815 & UBC-810) generated 3 and 1 band(s) each present in all half-siblings but missing in maternal tree (Table 10). The bands present in maternal tree and lacking in the half-siblings could be the result of the segregation of the corresponding amplified region in the progenies due to cross-pollination in this species. On the other hand, 2 RAPD primers (OPV-08 & OPAI-12) generated unique bands present only in half-siblings (HS9 & HS10) but absent in maternal tree (Table 7). The unique bands observed in half-siblings could be explained by contribution from different paternal parents or could be because of meiotic crossover leading to differences in primer annealing sites. Similarly the unique bands observed in the seedlings of argan were explained only by the input of dominant alleles from foreign pollen as it is cross-

pollinated species (Bani-Aameur and Benlahbil, 2004). According to paternal analysis made with microsatellite markers in experimental *Prunus avium* seed orchards, the calculation of the effective size of fathers per mother showed that few males contributed to the pollination of a given mother (Mariette *et al*., 2006) which might be because of a close neighbour which might dominate among fathers to a mother tree in a seed orchard (Vanden Broeck *et al*., 2006). Based on these results, Mariette *et al*. (2006) suggested that it is necessary to mix seeds harvested on different mothers in a seed orchard to make sure that resulting plantations will not be realized with a limited number of genotypes.

Eleven RAPD and all ISSR primers used in this study generated 30 and 25 monomorphic bands (Table 7 & 10), respectively present in all samples analyzed indicated that the genomic region amplified by these RAPD and ISSR primers is homologous in all individuals. Although few of the half-siblings exhibited similar banding pattern with some primers, they differed with respect to other primers used in the study. Overall, the profile generated with the use of RAPD and ISSR primers enabled the half-siblings to be distinguished easily from maternal tree. Rieseberg (1996) assumed that similarity of fragment size is an indicator of homology, which is approximately true for closely related populations or species. De Greef *et al*. (1998) inferred that the non-homology of co-migrating bands would introduce errors into the presence/absence data sets obtained after RAPD and AFLP analysis in half-sibs of 'plus' tree of *Quercus petraea* (Matt.), eventually leading to inaccurate estimates of genetic relationships.

The present study involving combined RAPD and ISSR markers showed that half-siblings displayed greater similarities among themselves ranging from 0.669 to 0.841 as compared to maternal trees where the similarity values ranged from 0.457 to 0.553 (Table 11). This is expected due to its dioecious nature where there is obligate necessity of cross pollination for seed formation. This is further evident by cluster analysis which grouped half-siblings into one cluster whereas the maternal tree formed an outlier (Fig. 19). The clustering pattern obtained with the combined data of RAPD and ISSR was more similar to that obtained with RAPD (Fig. 19 & 23). The average similarity coefficient value in half-siblings in comparison to the maternal tree was found to be higher (0.563) for ISSR markers as compared to RAPD which exhibited 0.443 similarity coefficient. In RAPD analysis, the HS5 displayed least genetic similarity of 0.385 to the maternal tree whereas with ISSR primers it was found to exhibit highest similarity (0.645). As the half-sibs are produced from the seeds

collected from the open-pollinated tree with different pollen donors, the paternal contribution would vary a lot as compared to contribution from a single maternal tree. This explains the differences that were observed with respect to least similarity coefficient values when RAPD and ISSR marker systems were employed. The observed differences with respect to RAPD and ISSR could also be explained by the different genomic regions targeted by the primers as well as the number of PCR products generated. It would be possible to obtain a more precise estimation on the nature and degree of genetic divergence among the half-siblings by employing more number of primers as well as using different marker systems. These results are in agreement with those reported by Bani-Aameur and Benlahbil (2004) who reported that RAPD fingerprinting even with eight primers and a limited population consisting of three parents and their open-pollinated offsprings (3 x 10) is an efficient tool for easing genetic variability of argan.

Overall the present results showed the potential of RAPD and ISSR primers in detecting the genetic variability in accessions collected from different regions and also in half-siblings which would provide a means of selection of highly diverse populations for plantations, breeding, efficient management, conservation and utilization of genetic resources of *G. rottleriformis*.

5.2.1. RAPD and ISSR primers revealed genetic variability in male and female plants of *Givotia*:

The knowledge related to the extent of genetic diversity present in male and female plants is important in *Givotia* as it might provide a means towards understanding sex determination mechanisms. This is the first report to utilize RAPD and ISSR primers for characterizing the genetic variability in different sexes in *Givotia* (Table 14 & 16). Twenty four primers generated 974 bands in 5 male and 5 female plants of which 404 bands were polymorphic accounting to 41.5% polymorphism. Of 730 bands produced by 12 ISSR primers, 210 were found to be polymorphic giving 28.8% polymorphism. Thus the level of polymorphism detected by RAPD primers was higher than that of ISSR primers. Interestingly, 5 primers generated unique amplified fragments in 4 male plants but were absent in other plants. None of the ISSR primers produced unique bands present in males or females. The present results suggest the occurrence of genetic changes in the genome of male

and female plants which is expected due to its out-breeding nature. Chromosomal crossing over during meiosis may result in a loss of primer attachment pair sites in the offsprings leading to novel RAPD patterns in the offsprings (Smith *et al*., 1996). The results of our study corroborate the fact that dioecious tree species, being long-lived, predominantly outcrossed, and often wind-pollinated and dispersed have usually a large proportion of their total neutral genetic variation within populations (Hiltfiker *et al*., 2004).

In the present study, the Jaccard's similarity coefficient values generated from RAPD and ISSR data revealed mean locus similarities of 0.753 between male and female plants. The grouping of male and female plants differed in RAPD and ISSR analysis. Cluster analysis revealed a lack of grouping of *Givotia* plants based on sex with the RAPD and ISSR 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.

5.2.2. RAPD and ISSR analysis of *Givotia* plants failed to identify a molecular marker 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. In the present study, bulk segregant analysis (BSA) in combination with RAPD data 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 total number of amplified loci by 24 RAPD primers in the bulk DNA samples of males and females was 100 which were less than the total number of amplified loci

in individual plants (142) which could be due to lesser template DNA of individual plants available for amplification in bulk DNA (Table 14). 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 different RAPD primers tested in the study, a higher number of bands (2) specific to males was generated by primers OPG-16 and OPK-01. The maximum number of bands (4) specific to females was produced by primer OPT-17 in bulked DNA samples. On the contrary, the ISSR primers tested resulted in monomorphic bands in bulked DNA samples although in individual analysis of male and female plants they could detect 28.8% polymorphism (Table 16). 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 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 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, twelve random primers that produced polymorphic bands in DNA bulks of males and females when screened on individual male and females failed to amplify bands specific to males and females. Only one primer, OPT-17 produced a 1000 bp amplified band present in all 5 females and 1 male and absent in 4 other males tested (Fig. 25b). However, OPT-17 primer failed to amplify 1000 bp fragment when screened on male and female plants of Mulugu location. Nevertheless, a 550 bp band was generated by the OPT-17 primer in 12 females out of 20 females and faintly present in one male out of 20 male analyzed. The existence of a high amount of variability in natural populations may be one of the reasons for the failure to identify markers tightly linked to loci. In a similar study, 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 *Asparagus* plants,

the band of 700 bp amplified by the 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 a single locus or several loci possibly interacting in an epistatic manner (Dellaporta and Calderon-Urrea, 1993). The sex-specific markers might represent either the amplified DNA from a sex chromosome or DNA polymorphisms (e.g. point mutations, deletions, transregions or inversions) that are linked to individual sex genes (Williams *et al.,* 1990; Ainsworth, 2000). Ling *et al.* (2003) found one male-associated band after screening 8372 RAPD bands of 1200 primers in *Ginkgo biloba.* The low frequency of sex-linked bands might indicate that the DNA segments involved in sex determination are very small and probably represents a single gene or very few genes (Hormaza, 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.

An effort has been made in the present study to clone and sequence the 550 bp amplified fragment generated by OPT-17 in many females to test its similarity with the nucleotide sequence present in the NCBI database and construct SCAR marker. The sequence of the 550 bp fragment did not show any homology with known sequences in the NCBI database. SCAR marker developed from the 550 bp fragment resulted in amplification in all male and female plants thus showing its inability to distinguish males and female plants. Joseph *et al.* (2014) reported that SCAR marker amplified a 556-bp band in male samples of *Garcinia gummi-gutta* but not in female plants although at low annealing temperatures amplification was observed in both the sexes. The lack of homology of sex-specific RAPD fragment in *Garcinia* to any sequence in the NCBI database has been suggested due to amplification in the non-coding region (Joseph *et al.*, 2014).

The use of different molecular marker systems with more primers and screening of a large number of males and females primers might help in identifying molecular marker(s) tightly linked to sex in *Givotia*. It would also facilitate in identification and isolation of the gene(s) involved in the process of sex determination.

5.3.1. *In vitro* propagation was achieved from nodal explants of *Givotia*:

Nodal meristems and shoot tip explants are generally preferred for micropropagation as plants raised from these are comparatively more resistant to genetic variation. However, *in vitro* recalcitrance, culture contamination, slow growth response and phenolic exudation have been the major obstacles for researchers dealing with woody trees (Rai *et al.* 2010). Despite these problems, a number of papers have also been published which reports the micropropagation of woody trees from explants derived from mature plants (Giri *et al.* 2004; Pandey *et al.* 2006; Phulwaria *et al.* 2011). The major difficulty faced in the present study in establishing aseptic cultures from nodal explants obtained from mature trees have been high contamination rates (60-80%) and browning of explants due to leaching of phenolic exudates into the medium. The browning problem was reduced by placing the nodal cuttings at room temperature for 2 days and treatment of explants for 5 minutes in antioxidant solution (100 mg/l each of ascorbic and citric acid). Similarly, Pankaj *et al.* (2014) reported that soaking of explants in an antioxidant solution containing ascorbic acid and citric acids eliminated browning from shoot tip explants of *Litchi* by reducing the polyphenol oxidase activity. Even though the contamination rates remained lower for shoot tip explants as compared to nodal explants they failed to develop into shoots and eventually turned brown on the media tested. On the contrary, shoot tips were found to be superior when compared to nodal segments for micropropagation of some woody species such as *Arbutus unedo* (Gomes and Canhoto, 2009).

In the present study, axillary bud sprouting was achieved at a higher frequency of 39.6% with induction of 1-3 axillary shoots on WPM medium containing 1.0 mg/l BAP as compared to other media tested (Fig. 35 & 36). The frequency of axillary bud sprouting (22.7-23.5%) as well as the shoot length decreased on MS or WPM medium containing 1.0 mg/l BAP and 0.1 mg/l NAA. The absence of synergistic influence of the cytokinin and auxin in certain plant species has been related to the endogenous auxin present in the explants (Julliard *et al.,* 1992). In contrast to shoot initiation during initial culture of explants, shoot multiplication was found to be superior with induction of 3-4 shoots per explant on MS medium with 1.0 mg/l BAP as compared to WPM with 1.0 mg/l BAP which induced lesser number of shoots with more callus formation from the base of the cultures.

5.3.2. Shoot regeneration from epicotyl and hypocotyl explants was influenced by the age of the emblings:

There have been no studies conducted to investigate the potential of epicotyl and hypocotyl explants derived from *in vitro* raised seedlings of *Givotia* for shoot regeneration. The easy availability of such explants, lack of requirement for sterilization, high potential for regeneration offer advantages for rapid propagation and transformation. Such explants can be used for plant regeneration when there is a need for maintaining the genetic diversity. In the present study, an attempt has been made to optimize the conditions for regeneration of plants from epicotyl and hypocotyl explants of *Givotia*. The shoot regeneration response from epicotyl and hypocotyl explants depended on the age of the seedlings, the position and orientation of explant, and the type of cytokinin used in the medium. The epicotyl and hypocotyl explants derived from 20-day-old emblings were more responsive for regeneration and produced higher number of shoots per explant (5.2-7.3) as compared to younger or older emblings (Fig. 38). Theoretically, the cells of the younger tissues have low degree of differentiation and are expected to exhibit higher morphogenesis. The lower shoot regeneration frequency from 10-day-old emblings as compared to 20-day-old emblings could be possibly due to low physiological competence of the cells for regeneration. Similar results in the morphogenic potential of explants of different ages derived from seedlings were reported in other species. Nayak *et al.* (2010) reported that epicotyl explants from 2-week-old seedlings were more responsive than those from 1-week-old seedlings in Indian gooseberry. In *Jatropha curcas*, explants from younger seedlings (<15 days) formed callus easily, whereas the regeneration response declined with increase in age of seedlings after 30 days (Sharma *et al.*, 2011). On the contrary, Shan *et al.* (2009) achieved higher shoot induction rate in the epicotyl explants from 2-day-old seedlings than from 4-d-old seedlings.

5.3.3. Position and orientation of explant affects the response of shoot regeneration from epicotyl and hypocotyl explants:

In this study, the ability of the epicotyl and hypocotyl explants to regenerate shoots on medium with 1.0 mg/l BAP varied depending upon their position on the seedlings. Higher shoot regeneration response with induction of 5-8 shoots per explant was observed from the

proximal region of epicotyl and hypocotyl explants close to the cotyledonary node as compared to 2-3 shoots per explant from distal region. The differences in regeneration potential observed from proximal and distal regions of epicotyl and hypocotyl explants could be due to the variation in the endogenous auxin concentrations. There have been conflicting reports with regard to the pattern of expression of morphogenesis along the epicotyl and hypocotyl explants in different species. Moreira-Dias *et al.* (2001) observed that the number of shoots formed from epicotyl segments of Troyer citrange decreased markedly with the distance of the cutting from the cotyledonary node. In contrast, Rasai *et al.* (1994) have reported that both distal (section near the root) and the middle part of atemoya hypocotyls produced more buds and shoots than proximal (section near cotyledons). Costa *et al.* (2004) observed a higher organogenic response in epicotyl explants as the distance from the cotyledonary node increased.

In the present study, the orientation of explants was found to be important with induction of more number of shoots from the explants placed vertically as compared to those placed horizontally on the medium containing 1.0 mg/l BAP. It is possible that higher uptake of BAP to the receiver cells is facilitated when the explants were orientated in the vertical position as suggested by Catapan *et al.* (2000). These results are in agreement with Saini and Jaiwal (2002) who reported higher shoot induction frequencies in epicotyl segments of blackgram when placed vertically in the medium in an upright position. Similarly, culturing of explants in the vertical position resulted in maximum shoot induction in *F. crassifolia* (Yang *et al.* 2006). These findings are in contrast with the results of Saini *et al.* (2010) who reported better response from the explants placed in horizontal orientation than vertical position in *Citrus jambhiri*.

5.3.4. BAP was more effective than KN in inducing shoot regeneration from epicotyl and hypocotyl explants:

Cytokinins are a critical factor for initiation of shoot primordia from cultured tissues. The present experiments revealed the superiority of BAP over KN on shoot regeneration from epicotyl and hypocotyl explants for the concentrations tested (Fig. 39). Similar results were obtained in castor in which BAP was found to be effective in inducing higher number of shoots than KN (Sujatha and Reddy 1998, Ahn and Chen 2008). Phillips and Hubstenburger

(1985) reported that in pepper tissue cultures, bud induction was strongly influenced by BAP in the medium whereas same success was not observed in the presence of KN (Phillips and Hubstenburger 1985). In the present study, the optimal shoot regeneration response in terms of number of shoots per explant was observed in the presence of 1.0 mg/l BAP whereas higher concentrations of BAP (2.0 mg/l) enhanced the callus formation from epicotyl and hypocotyl explants. Similarly, higher concentrations of BAP have been found to be unsuitable for propagation of other plant species (Sahoo and Chand 1998, Armstrong and Johnson 2001). In our experiments, the combination of 0.1 mg/l NAA with 1.0 mg/l BAP increased the callus formation and decreased the shoot growth. In contrast to the present result, Singh *et al.* (2002) showed that addition of NAA to cytokinin not only enhanced the shoot regeneration frequency but also reduced the days taken for shoot induction in hypocotyl segments of *Psidium guajava.* Nayak *et al.* (2010) reported that a combination of low concentration of auxin (1.4 mg/l IAA) along with BAP proved to be more effective than BAP alone for multiple adventitious shoot induction from epicotyl explants of Indian gooseberry (*Emblica officinalis* Gaertn).

In the present study, shoot multiplication was achieved at a higher rate after subculture on medium with 1.0 mg/l BAP with induction of 6-7 new shoots per explant but the shoots elongated slowly. Repeated subcultures on medium with 0.5 mg/l BAP facilitated shoot elongation along with induction of 3-4 new shoots at every subculture. These results are in accordance with the observations of Walia *et al.* (2007) in which BAP at 0.5 mg/l induced simultaneous elongation and proliferation of shoots from nodal explants of *Crataeva nurvala.*

5.3.5. Pulse treatment of the shoots with auxins improved the root induction from shoots:

Rooting is one of the crucial steps of *in vitro* multiplication of tree species. The *in vitro* regenerated shoots failed to root on MS basal medium without regulators which could be due to low levels of endogenous growth regulators which are insufficient for root induction. Pulse treatment with auxins has been used for enhancing the rooting percentage in different species such as *Photinia* (Ramirez-Malagon *et al.*, 1997) and chick pea (Anwar *et al.*, 2009). In the present study, the pulse treatment of the shoots with IBA induced better rooting response (44.3%) from the shoots when cultured on 0.1 mg/l IBA containing medium (Fig. 41). The

shoots that were pulse treated with IAA exhibited better rooting response (39.6%) on basal medium in comparison to those cultured on medium containing 0.1 mg/l IAA which exhibited greater callusing from the base with reduction in root growth. The shoots of *Givotia* exhibited rapid growth following rooting as reported earlier in *Juncus effuse* (Sarma and Rogers 2000). Anwar *et al.* (2009) achieved 100% success rate in root induction by pulse treatment for 10 sec with 100 mM IBA with development of strong rooting system within 14 days.

Root induction from the shoots was achieved with high success rate using liquid medium containing growth regulators in different species and this allowed the transfer of culture-grown plantlets to a glasshouse environment with minimal disturbance to the plant as a whole (Negi and Saxeni, 2011). Liquid medium is known to facilitate the easier uptake and better absorption of medium components as the growing shoots are in direct contact with the medium. Our results showed that liquid medium containing 0.1 mg/l IBA did not favor root induction from the pulse treated shoots but resulted in bulging of the base of the shoots which could be due to increased exposure to IBA and nutrients in liquid medium as compared to solid medium. Yepes and Aldwinekle (1994) studied the effect of indole-3-butyric acid (IBA) on various apple rootstocks and cultivars and have found that lower concentrations are necessary to induce rooting in liquid rather than in solid medium. Zheng *et al.* (2009) achieved root induction with a frequency of 80% from the regenerated shoots by dipping the shoots in IBA solution before placing on a filter-paper bridge that was maintained in ½ MS liquid medium supplemented with sucrose.

The success of *in vitro* propagation lies on the successful establishment of plants in the soil (Saxena and Dhawan, 1999). Hardening of plantlets *in vitro* has been suggested as one way of improving plant survival and for the speeding up of acclimatization (Pospisilova *et al.* 1999). In the present study, the micropropagated plants as well as emblings were initially acclimatizated for 4 weeks in culture room and maintained in glasshouse for at least 8 weeks before established in soil in field. In the present study, the emblings were successfully acclimatized and established in soil in greenhouse with a frequency of 80 and 70%, respectively. Comparison of the growth of micropropagated plants with that of emblings revealed their superiority with respect to leaf size whereas plant height, number of leaves per plant and intermodal distance did not differ significantly between the two types (Fig. 43). The acclimatized emblings and micropropagated plants were established with a high success rate of 90-95% during rainy season in the field. The possible reasons for the larger leaf size observed

in micropropagated plants as compared to emblings could be due to the true-to-type nature of micropropagated plants that were derived from nodal explants of selected mother tree in contrast to genetic heterogeneity existing in emblings of *Givotia* as a result of cross pollination. Similar observations have been made by Osorio *et al.* (2007) in Carob tree where micropropagated plantlets exhibited more vigour with the greatest number of branches and a large length increase in the main stem than seedlings. Dibi *et al.* (2010) observed a gain in dry rubber production per tree of *in vitro* micropropagated plants of *Hevea brasiliensis* in comparison with conventional mature budded clones during field trials. The results showed that the gain in girth and rubber production of *in vitro* plantlets has been influenced by the metabolic class and the physiological profile of the clone.

5.3.6. Micropropagated plants exhibited high degree of genetic stability:

DNA markers such as RAPDs have been widely used to detect somaclonal variations in tissue culture derived plants in several plant species. Out of 10 RAPD primers used to assess the genetic stability of the micropropagated plants of *Givotia*, 8 primers produced monomorphic bands while two primers, OPF-03 and OPZ-06 produced one polymorphic band each in the plants analyzed (Table 20; Fig. 46a). Three regenerated plants showed monomorphic bands with all the 10 RAPD primers tested. Overall, the micropropagated plants displayed high level of similarity to the donor plant indicating its suitability for mass multiplication. Similar observations have been recorded during the assessment of genetic stability in micropropagated apple rootstock plants using RAPD markers which revealed a good degree of clonal fidelity and low frequency of somaclonal variations (Gupta *et al.*, 2009). Some authors have reported that the time *in vitro* culture could promote somaclonal variation (Hartmann *et al.*, 1989; Nayak and Sen, 1991). Hammerschlag *et al.* (1987) suggested that the genotype and the nature of the explants could influence the phenotypic stability of the plants obtained in studies performed with micropropagated peach plantlets.

5.4.1. *Givotia* accessions displayed variation in fruit and seed traits and seed oil content:

The key for success of any genetic improvement programme lies in the availability of genetic variability for desired traits (Divakara *et al*., 2009). Estimation of variability for seed traits, seed oil content and its fatty acid composition is the first step towards exploiting any unconventional plant source for edible oil or industrial oil. The present study revealed differences with respect to fruit and seed diameter and weight among accessions of *Givotia* of different forest regions (Fig. 48). The average weight of 10 mature dry fruits ranged from 29.4 to 54.0 gm and seed weight ranged from 7.3 to 9.9 gm with the maximum values recorded for Achampet accessions. Similarly, the accessions of Achampet were characterized by larger fruit diameter (23 mm) than other accessions but the seed diameter (12.2 mm) did not differ from that of Devarayanadurga accessions. Similarly, intraspecific variations for different morphological traits such as seed weight, length and diameter among seed accessions from the same and different locations have been reported in many tree species (Singh, 2001; Kanmegne *et al*., 2010).

Genetic variation for seed traits and oil content has been estimated in many plant species as they are important components of yield (Kaushik *et al*., 2007; Kesari *et al*., 2008). In the present study, the oil content in the seeds of *Givotia* varied in 2 accessions with the highest (22%) in Achampet followed by Devarayanadurga (20%) whereas no significant differences were observed among other accessions (Fig. 48). Overall, the accessions of Achampet had larger fruit and seed weight and oil content as compared to other accessions. As per the literature, the seed oil content in commonly used edible oil sources ranged from 18-22% in soybean, 30-35% in safflower, 40-48% in rapeseed. Thus *Givotia* seed oil compared with that of soybean but lower than that of safflower and rapeseed. Although oil yield ranged from 18.2-22%, the difficulty in removal of the hard pericarp surrounding the *Givotia* seed would present problem in effective utilization of oil in this tree species. Economically feasible techniques for removal of hard pericarp will facilitate easy oil extraction from seeds this species, especially in rural areas. Raut *et al.* (2011) observed variation in seed, fruit and oil content in *Pongamia pinnata* collected from Konkan region of Maharashtra which were largely attributed to the heterogeneity of the genotypes and the genotype x environment interactions. On the contrary, Atangana *et al.* (2011) found no significant differences among sites for any

fruit and seed characteristics in trees of *Allanblackia floribunda* analyzed which was explained by the relative homogeneity of ecological conditions of the sites that were surveyed.

Seed germination greatly influences the success or failure of any afforestration programme. The ecological conditions in which the species is growing might influence the seed characters as well as the germination percentage. The evaluation of germination responses of different accessions of *Givotia* and their response to specific treatment might provide better understanding of the phenomenon of seed dormancy in this species and would help in enhancing the germination rate and selecting the best accessions for commercial plantations. The present investigations showed the inability of the whole seeds of all accessions to germinate in soil or *in vitro* whereas isolation of zygotic embryo axes followed by culture on MS medium induced germination. This observation emphasizes the need to conduct more studies for understanding seed dormancy as well as develop methods for inducing seed germination in soil. The present study showed the existence of seed dormancy as the germinate rate of the embryo axes remained low (5.2-13.3%) on MS medium in all the accessions. The use of IBA and KN in MS medium proved beneficial in increasing the germination rate (25.3-35.5%) from the zygotic embryo axes of all accessions (Fig. 50 & 51). Temperature treatment of the seeds followed by culture of the zygotic embryo axes on MS medium with IBA and KN induced maximum germination rate (40.3-70.3%) as well as enhanced the growth of the germinating embryos in terms of shoot and root formation as compared to untreated controls in all the accessions (Fig. 51). The improved germination rate from the zygotic embryo axes of temperature treated seeds on MS medium with IBA and KN in all accessions could be due to the neutralization of factors involved in maintenance of seed dormancy in this species. Irrespective of the treatment used, germination initiation as well as growth of the germinating embryo axes within the seeds collected from the same tree was not uniform which could be due to differences in maturity and physiological status of the embryos as suggested by Dosmann *et al.* (2000) during studies on seed germination of Katsura tree.

The germination rate of different accessions varied with the temperature of seeds inducing the highest response (70.3%) in accessions of Kondapalli upon culture on MS medium with IBA and KN as compared to other accessions tested. The superiority observed in Achampet accessions with respect to seed weight and oil content was not reflected in terms of germination. More detailed studies are required to find the correlation between seed traits,

oil content and germination. A significant tree to tree variation in response of *Garcinia kola* seeds to dormancy-breaking treatments has been reported and suspected to be due to the existence of variability within the species (Kanmegne and Omokolo, 2008).

Thus it is evident from the study that there is variability in seed sources with respect to the traits evaluated which can be further utilized as promising seed sources for mass multiplication of the species and selection of plus trees for plantation and conservation purposes in future.

5.4.2. *Givotia* seed oil is rich in polyunsaturated fatty acids:

The quality and utilization of any oil is determined by its fatty acid composition. The fatty acid composition which is the relative proportion of different fatty acids in the mixture of triglycerides is characteristic to each vegetable oil. The present results of the gas chromatographic analysis of the methyl esters of fatty acids in oil extracted from the seeds of *Givotia* revealed that the main fatty acids were linoleic acid, oleic acid, stearic acid, palmitic acid, α-eleostearic acid and other conjugated acids (Fig. 52). Thus polyunsaturated fatty acids including conjugated fatty acids comprised more than 70% of seed oil in *Givotia*.

Literature shows that α-eleostearic acid is uncommon in seed oils with tung seed oil being characterized by high levels of 75.28% of the total fatty acids whereas bitter gourd seed oil had 60% α-eleostearic acid. The high degree of unsaturation and drying property of tung seed oil has been shown to be due to the presence of α-eleostearic acid (Burr *et al.,* 1932). The increased life expectancy in people inhabiting Okinawa, a region in Japan has been attributed to the consumption of bitter gourds where the seed oil contained 60% α-eleostearic acid (Tsuzuki *et al*., 2004). In rats, α-eleostearic acid is converted to a conjugated linoleic acid in the liver and plasma (Tsuzuki *et al.,* 2006). Conjugated linoleic acids have been previously reported to have anticancer (Parodi, 1999), antiatherogenic (Arbones-Mainar *et al*., 2006) and antidiabetic effects (Peck *et al*., 1998) and atheroprotective properties (Toomey *et al*., 2003).

In the present study, linolenic acid was not present in seed oil of *Givotia*. Similarly, Zhang *et al*. (2014) reported low amount (less than 0.5%) of linolenic acid in tung seed oil and was undetectable in oils from several tung tree accessions. In contrast to tung seed oil which contained large amount of conjugated linolenic acid (C18:3c; α-eleostearic acid and no

linolenic acid (C18:3), the main fatty acid of linseed oil was linolenic acid with no conjugated linolenic acid being present (Schonemann *et al*., 2006).

The high proportion of polyunsaturated fatty acid, comprising linoleic acid, α-eleostearic acid along with other unidentified conjugated fatty acids makes the seed oil of *Givotia* as a new and valuable source of edible oil. Similarly, rubber seed oil contained significant amounts of linoleic acid (42.13%) and oleic acid (38.96%) which did not show marked differences from two different sources (Kittigowittana *et al*., 2013). The highly consumed sunflower and safflower oils contain 88-90% unsaturated fatty acids comprising oleic and linoleic acid (Mandal *et al*., 2006). The high level of linoleic acid in the oil was found to reduce the blood cholesterol level and played a key role in preventing atherosclerosis (Ghafoornuissa and Krishnaswamy, 1994). Linoleic acid has been reported to have anti-inflammatory, acne reductive, and moisture retentive properties when applied topically on the skin (Darmstadt *et al.,* 2002). A diet only deficient in linoleate causes mild skin scaling, hair loss (Cunnane and Anderson, 1997), and poor wound healing in rats (Ruthig and Meckling, 1999).

The increase in stability over oxidation of vegetable oil is attributed to oleic acid (Abdulkarim *et al*., 2007). The percentage of oleic acid in *Givotia* seed oil was 10.4% which was higher than tung seed oil but lower that of rubber seed oil (Table 21). Stearic acid is more abundant and present up to 30% in animal fat than vegetable fat typically <5% with important exceptions being cocoa butter and shea butter where the stearic acid content was 28-45% (Rogers *et al*., 2001). It has been widely used in the production of detergents, soaps, and cosmetics such as shampoos and shaving cream products. The percentage stearic acid in seed oil of *Givotia* was almost similar to that of reported by Salimon *et al*. (2012) in *Hevea brasiliensis*. It is possible that the reported lubrication properties of seed oil could be due to the presence of stearic acid and α-eleostearic acid.

It is well known from literature that the bark and seed powder of *Givotia* are used in the treatment of rheumatism and psorosis. It is speculated that the medicinal properties found in *Givotia* could be due to the presence of high levels of polyunsaturated fatty acids including conjugated fatty acids as there is scientific evidence showing they have important roles in curing skin disorders, decreasing rheumatoid arthritis, maintaining good skin health and reducing inflammation. Based on the oil content and fatty acid profile it can be

concluded that that the seeds of *Givotia* can become valuable resource for use as functional foods and for production of high value essential oil products in addition to industrial uses. Further investigations on physicochemical properties of seed oil, fatty acid composition in seed oil of different sources and toxicological studies are required for determining the quality of oil for exploiting it as nutritional dietary component commercially.

6. SUMMARY AND CONCLUSIONS

In the present study, the genetic variation in *Givotia* accessions collected from 5 forest regions was characterized for the first time using RAPD and ISSR primers. The genetic similarities and variation in half-sib progenies of candidate plus tree were assessed by RAPD and ISSR analysis. In addition, the potential of RAPD and ISSR markers in detecting the genetic variability in male and female trees and identification of sex was determined. Studies were carried out to develop methods for rapid *in vitro* propagation of *Givotia* using explants obtained from mature trees and *in vitro* germinated emblings for overcoming the difficulties associated in propagation of these plants through seeds and vegetative cuttings. The variability in fruit and seed diameter, weight, germination percentage, oil content in seeds of different accessions was evaluated. The fatty acid composition of seed oil of *Givotia* was determined. The major findings that emerged from the work are presented below:

- RAPD primers were found to be more efficient in detection of polymorphism (92.2%) as compared to ISSR primers (74.1%) indicating that the genomic regions amplified by the RAPDs have greater variability.
- Irrespective of RAPD, ISSR or combined RAPD and ISSR methods, the accessions within a region formed a cluster indicating region specificity among *Givotia* accessions.
- Both ISSR and RAPD markers revealed that accessions of Balapally region were genetically more divergent as compared to accessions of other forest regions.
- RAPD and ISSR analysis revealed differences with respect to grouping of *Givotia* accessions in clusters and positioning of accessions in the clusters which could be explained by the fact that RAPD and ISSR markers target different regions of the genome.
- Two amplified fragments of 490 bp and 350 bp produced by OPC-08 and OPT-17 in all accessions of Andhra Pradesh and Telangana were cloned, sequenced and deposited in Genbank of NCBI database (KF772203.1 and JX026068.1, respectively). The sequence of the 350 bp matched exhibited 91% similarity with uncharacterized m-RNA sequence of *Jatropha curcus* and 86% similarity with hypothetic protein gene of *Ricinus* whereas that of 490 bp did not show any sequence similarity with the nucleotide sequences in NCBI database.

- Molecular analysis of half-siblings showed 45.7-55.3% genetic similarity to their maternal parent and the half-sibs formed a separate major cluster in the dendrogram constructed from combined RAPD and ISSR data which is expected due to dioecious and highly heterozygous nature of this tree species.
- Two RAPD primers *viz*., OPV-08 and OPAI-12 amplified unique bands in half-sibs HS9 and HS10, respectively which could be the result of meiotic crossover leading to differences in primer annealing sites or gametic contribution by the paternal parent.
- The extent of genetic variation in male and female trees of *Givotia* was determined for the first time using molecular markers. RAPD markers revealed higher polymorphism (41.5%) followed by ISSR (28.8%) markers.
- Twenty four RAPD primers and 12 ISSR primers used for analysis of male and female trees failed to amplify bands specific to males or females demonstrating that the amplified genomic regions are not associated with sex.
- A 550 bp amplified product of OPT-17 produced in many females was cloned and sequenced. The sequence of the cloned fragment did not match with any sequence in the NCBI database.
- *In vitro* propagation was achieved from nodal explants of mature trees and also explants derived from *in vitro* germinated emblings.
- Desiccation of the nodal explants at room temperature for 2 days reduced the browning and resulted in successful establishment of cultures.
- Axillary bud sprouting was achieved with a frequency of 39.6% from nodal explants of mature trees on Woody Plant medium with 1.0 mg/l BAP with induction of 1-3 shoots per explant.
- Direct shoot regeneration was achieved at a frequency of 87.6% and 82.6% from epicotyl and hypocotyl explants derived from the *in vitro* germinated emblings on MS medium with 1.0 mg/l BAP.
- Shoot multiplication and elongation was successfully achieved by subculturing the shoot clumps on medium with 1.0 mg/l BAP followed by two subcultures on medium containing 0.5 mg/l BAP.
- The regenerated shoots were successfully rooted at a frequency of 44.3% by pulse treatment with 10 mg/ml IBA followed by culture on MS with 0.1 mg/l IBA.

- Ten RAPD primers were used to assess the genetic stability of micropropagated plants which revealed high percentage (98.8%) of similarity among micropropagated plants.
- The micropropagated plants were established in soil and had larger size as compared to emblings.
- High frequency germination from zygotic embryo axes of temperature-treated (55°C for 24 h) seeds was observed after culture on MS medium with 0.3 mg/l IBA and 0.1 mg/l KN. Highest germination percentage of 70.3% from zygotic embryo axes was recorded in accessions of Kondapalli whereas relatively low germination percentage (40.3%) was observed in Devarayanadurga accessions.
- Significant variations were noticed with respect to fruit weight, fruit diameter, seed weight and seed diameter of different accessions with the maximum values recorded for Achampet accessions as compared to other accessions.
- The oil content in seeds of different accessions collected from different forest regions varied from 18.2-22.0%.
- The variability observed with respect to fruit and seed traits, germination, seed oil content and fatty acid composition in seed oil could be due to both genetic and environmental effects.
- The main fatty acids identified in seed oil of *Givotia* were palmitic acid, stearic acid, oleic acid, linoleic and α-eleostearic acid with oleic, linoleic and α-eleostearic acid comprising 43% of total fatty acids.

In conclusion, the present study for the first time demonstrated the potential of RAPD and ISSR primers in detecting genetic diversity in *Givotia* accessions growing at different forest regions. The information on polymorphism detected by different RAPD and ISSR primers and genetic diversity in *Givotia* accessions growing at different forest regions will have important implications on breeding, management and conservation of genetic variability in this important species.

The study also led to the development of methods for *in vitro* propagation using nodal explants derived from mature trees and also explants derived from *in vitro* germinated emblings. Thus the objective to micropropagate the plants using explants from mature trees and *in vitro* germinated plantlets has been achieved and the regenerated plants have been successfully established in soil. The genetic fidelity of micropropagated plants was assessed

using RAPD method which revealed high degree of similarity in comparison to the donor plant. The *in vit*ro methods developed in the study would help in overcoming plant propagation problem due to prolonged seed dormancy in *Givotia* and generate plantlets within short time frame which can be used to raise plantations in target areas to ensure the continuous supply of softwood for toy making and other commercial uses such as boxes and catamarans. *Givotia* seed was found to be a good source of monounsaturated fatty acid (oleic acid), polyunsaturated fatty acid (linoleic acid), conjugated linolenic acid (α-eleostearic acid) along with unidentified conjugated fatty acids and the presence of these fatty acids enhances its commercial value for therapeutic, pharmaceutical and other industrial applications.

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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	

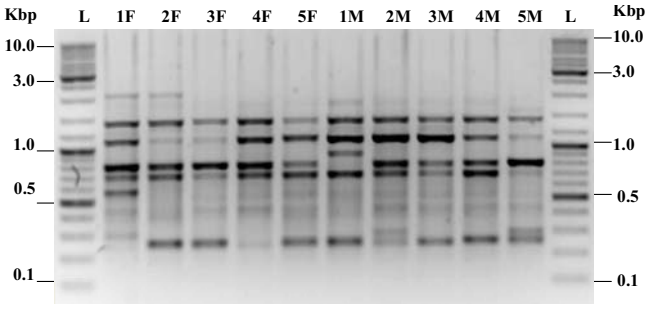

Fig. 1 PCR amplification profiles of DNA samples of female and male *Givotia* plants produced with RAPD primer OPAL-08. Lanes designated as L represent DNA ladder (100 – 10,000 bp), lanes 1F, 2F, 3F, 4F and 5F represent individual females, lanes 1M, 2M, 3M, 4M and 5M represent individual males.

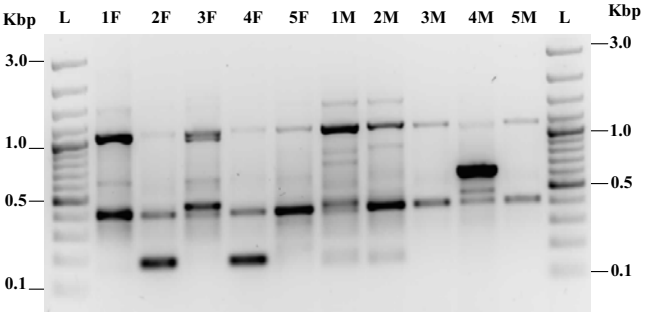

Fig. 2 PCR amplification profiles of DNA samples of female and male *Givotia* plants produced with primer OPA-18. Lanes designated as L represent DNA ladder (100 – 3000 bp), lanes 1F, 2F, 3F, 4F & 5F represent individual females, lanes 1M, 2M, 3M, 4M & 5M represent individual males.

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.

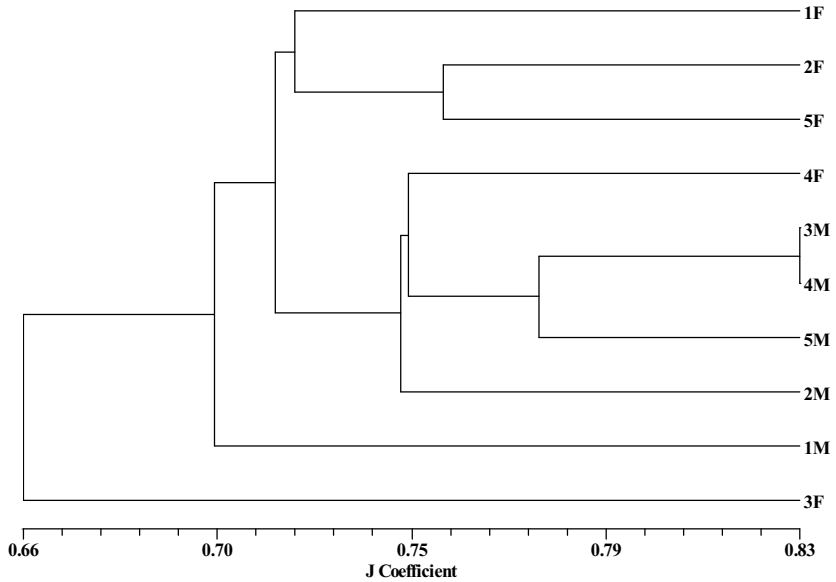

Fig. 3 Dendrogram of male and female plants of *Givotia* based on Jaccard's similarity coefficient (1M, 2M, 3M, 4M and 5M represent individual male plants and 1F, 2F, 3F, 4F and 5F represent individual female plants).

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

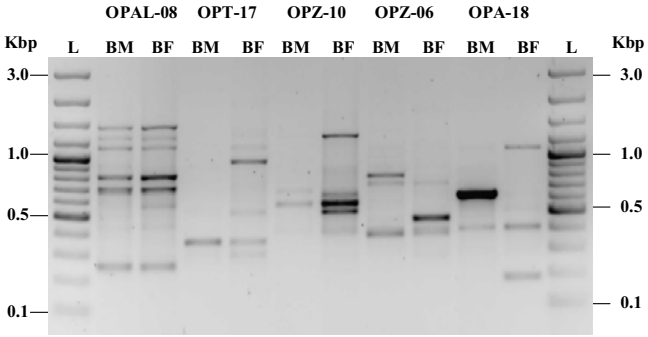

Fig. 4 PCR-based DNA amplification profiles generated from different RAPD primers in bulk DNA of male and female *Givotia* plants. Lanes designated as L represent DNA ladder (100 – 3000 bp), BM represent bulk DNA samples of males, BF represent bulk DNA samples of females. The amplified product profiles 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).

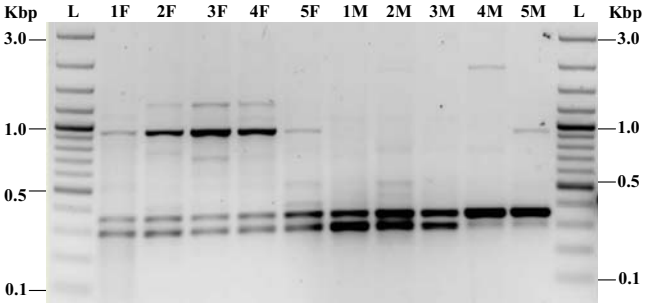

Fig. 5 RAPD band patterns of five individual female and male *Givotia* plants generated from the primer OPT-17. Lanes designated as L represent DNA ladder (100 – 3000 bp), lanes 1F, 2F, 3F, 4F and 5F represent the individual females and lanes 1M, 2M, 3M, 4M and 5M represent individual males.

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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