GENETIC EVALUATION OF ELITE SANDALWOOD (*Santalum album* L.) CLONES USING RAPD MARKERS

G. SHASHIDHARA

DIVISION OF HORTICULTURE
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE
2002

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G. SHASHIDHARA

Thesis submitted to the

University of Agricultural Sciences, Bangalore

in partial fulfillment of the requirements

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Affectionately Dedicated

To My

Beloved Parents,

&

My Mentor

Late. Dr. Suresh N Sondur

A tribute to my Mentor

DR. SURESH N SONDUR

(1955-2001)
A personification of honesty, dedication and humility

Dr. Suresh was born in 1955 in Hyderabad to Mr. Narayan Rao K, Sondur and Mrs. Lakshmi Bai N Sondur. His schooling years were all over Karnataka and his degree was from the University of Agricultural Sciences, Bangalore where he earned a Bachelor's degree in Horticulture and later a Master's degree specializing in Plantation Crops. He joined the same institution as a Research Associate in 1978 and as Assistant Professor in 1986 and later proceeded to the University of Hawaii, USA for a Doctoral degree with a Full bright scholarship in 1987. While in Hawaii, Dr. Suresh worked on molecular genetics in papaya and he was the first to have ever mapped the papaya genome and located the sex loci with molecular markers. He married Sandhya in 1988 and their son Manu was born while they were still in Hawaii. He returned to India in 1994 and served as an Associate Professor of Horticulture, later his daughter Shachi was born. He was so proud of his family and loved them dearly.

Dr. Suresh had been a unique person. On the social level, he was a personification of child at heart. He loved and respected every one of us and always wanted us to be happier and content. He was an outstanding officer and also a human being of perfect qualities. To find a person of his virtues as a "professional" as well as a "person" is indeed a rare occurrence. I remember one of my early encounters with him, which was during my colloquium for my Master's thesis. I was nervous to defend my research by myself. To my surprise, Dr. Suresh came to my rescue and greatly appreciated my work. Later I realized that he did not hesitate to show his appreciation for anyone's efforts. This quality of him always made me feel good about me being a student and a student worthy of my own.

As a researcher and teacher he has made tremendous contributions in the field of Horticulture. He was responsible for starting the Plant Molecular Biology laboratory in the Department, which presented a window to so many students to study molecular biology techniques. I was fortunate to have been associated with him. He was exceedingly knowledgeable, enthusiastic, witty and yet very humble. He always gave me unstinting and constructive support in everything I endeavored to do and never ridiculed my ideas or ignorance. In my view this is the truest sign of a great scholar and teacher and above all gentleman. Dr. Suresh wanted to establish a strong molecular genetics research and teaching base at the Division of Horticulture, UAS, Bangalore and laid the foundation. However, destiny was cruel and death snatched him at a very early age before all his dreams were put to action. With demise of Dr. Suresh, it is true that the biggest looser is the University, especially the student community who needed him to be the guiding light.

He was a living example of something I had read in my earlier years as a hallmark of a noble person: "Having themselves done a good deed, they are silent; but when another does a good deed, they extol it in an assembly. "Though his life was short, it had a powerful impact on many lives and he will live on in the hearts of those who he had touched. May his soul rest in peace.

DIVISION OF HORTICULTURE
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE

CERTIFICATE

*This is to certify that the thesis entitled "*GENETIC EVALUATION OF ELITE SANDALWOOD (***Santalum album*** L.) CLONES USING **RAPD** MARKERS*" submitted in partial fulfillment of the requirements for the degree of* **DOCTOR OF PHILOSOPHY in HORTICULTURE** *to the University of Agricultural Sciences, Bangalore, is a record of research work carried out by* **Mr. G. SHASHIDHARA,** *under my guidance and supervision and that no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or other similar titles.*

Bangalore
20th May, 2002

(A. A. FAROOQI)
Professor and Head
Department of Horticulture

APPROVED BY :

Chairman : (A. A. FAROOQI)

Members : (S. JAGANATH)

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(D. THEERTHA PRASAD)

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G. Shashidhara

May, 2002
Bangalore

(***G. Shashidhara***)

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INTRODUCTION

I. INTRODUCTION

Among the various tropical tree species, sandalwood is one of the most economically important species acknowledged as "Royal Tree" in the Indian sub continent (Jeeva *et al.*, 1997). In India, the genus is represented by *Santalum album* Linn. belonging to the family Santalaceae and is generally accepted to be indigenous to Peninsular India as its history of occurrence dates back to at least 2500 years (Gode, 1961). Its wood commercially known as "East Indian Sandalwood" and the essential oil from it as "East Indian Sandalwood Oil" are among the oldest known perfumery material.

In India, sandalwood is found distributed all over the country (9600 km^2) with over 90 per cent of the area in Karnataka (5245 km^2) and Tamil Nadu (3040 km^2). The trees are distributed between 30^0 N and 40^0 S latitude (Sprague and Summerhayes, 1927) with more than 90 per cent of the population occurring in the dry deciduous forests of Deccan Plateau covering 8300 km^2. Other states where sandalwood trees are found distributed are Andhra Pradesh, Kerala, Maharashtra, Orissa, Rajasthan, Uttar Pradesh, Bihar, Gujarat and Manipur.

Sandalwood (*Santalum album*) is hemi-root parasitic, small to medium sized evergreen tree, attaining a height of 15-18 m and a girth of 1-2.4 m with slender drooping as well as erect branching. The tree flourishes well from sea level up to 1800 m altitudes in different types of soils like sandy, clayey, red, laterite loam and even in black cotton soils. However, the formation of heartwood seems to be the best between 600 m and 900 m above MSL. Generally this species is located in the undulating terrains and survives well under moderate rainfall of 600-1600 mm in cool climate with long periods of dry weather, but adapts well to different climatic conditions excepting water logged or very cold places.

Sandalwood has been known to be used for more than twenty-five centuries and has immense potential for earning foreign exchange, thereby popularly acknowledged as "Vegetable Gold" (Srimathi and Kulkarni, 1995). It has many traditional uses, most of them related to its prestigious fragrance. The trees are harvested mainly for their fragrant heartwood and oil (90% santalols). Both heartwood and oil are used in handicrafts, cosmetics, and pharmacopoeia, and are the most valuable perfumery material in the world from the olden days to modern times. The wood is also quite appreciated for sculpting liturgical objects, cremation and incense. The oil is famous for its antiseptic, soothing, diaphoretic and diuretic properties and holds the most prominent place in the country's export trade in essential oils. Annual world requirement for sandalwood oil is about 200 tonnes, which corresponds to 10,000 tonnes of heartwood. India produces nearly 140 tonnes, about 60 per cent of which is exported earning a foreign exchange of about 2.5 crores every year (Tandon, 1995). The market requirement is big sized logs without cracks as it fetches a good price and better foreign exchange.

Due to large domestic and international demand coupled with inadequate state regulation and control, sandalwood is indiscriminately exploited both legally and by illicit felling. Consequently, the area under sandalwood has been rapidly dwindling and the natural forests have few mature trees with a diameter (dbh) of more than 30 cm (Swaminathan *et al.*, 1997). Most of the growing populations are not dense and are devoid of larger girth classes, not only due to illicit felling, but also due to grazing, hacking, recurrent fires and other biotic/abiotic interferences. Cyclic epidemics of spike disease have also contributed to the depletion of the growing stock both in area and density. Hence, it is feared that there is a continuous loss of genetic resources, which may have far reaching consequences on the genetic improvement of sandalwood for its heartwood and oil content.

Whenever the tree species are severely endangered and threatened, preservation of genetic diversity has been a priority in many conservation programmes (Templeton, 1995). This has advantages both as short term adaptation to environmental change and long term impact on species and communities. Conservation of sandalwood genetic resources is mainly concerned with maintaining all the genetic variation contained within and among carefully selected target species. It places the maintenance of intraspecific genetic diversity as a central building block in the conservation effort (Rogers and Ledig, 1996). It not only involves preventing extinction of genetic resources but also ensures their availability for future use (Namkoong, 1997). Therefore, assessment of genetic diversity is vital in developing effective conservation strategies (Loveless, 1992) and sustainable management guidelines. Unfortunately, the sustained management of sandalwood genetic resources has been constrained due to lack of base line information on the levels and structure of genetic diversity. A systematic study is thus, essential in *Santalum album*, in order to examine the existing genetic diversity among populations and also to gain knowledge on the impact of disturbance on genetic variability of the species.

Morphological characters can be used for the evaluation of diversity. But most often, the desired phenotypic characters are phylogenetically inherited and are highly influenced by environmental conditions. Hence, the data obtained by such evaluations are not easily understood at genetic level, often resulting in taxonomic confusion and in maintenance of duplicate accessions. Moreover, in perennials it is required to grow for many years to observe growth and reproductive parameters, which is economically not feasible. In this regard, the advent of molecular markers has revolutionized the entire scenario of plant sciences. Ever since their development, they are constantly being modified to enhance their utility in the process of genome analysis (Swati *et al.*, 1999). DNA based markers *viz.*, RAPD, RFLP, AFLP, SCAR and SSRs provide excellent tools for studying diversity at the DNA

level, which assists in elimination of duplicates in germplasm, discriminating cultivars and elucidating misnaming, genetic purity analysis, development of marker based gene tags, map based cloning of agronomically important genes, variability studies and phylogenetic analysis and are seldom influenced by environmental conditions (Swati *et al.*, 1999). Thus, giving new dimensions to breeding and marker aided selection of desirable genotypes, which can reduce the time span of developing new and better varieties.

With this background, the present investigation was planned with the following objectives;

1. Standardization of DNA extraction and RAPD analysis protocol in sandalwood,
2. Fingerprinting and evaluation of genetic diversity and relatedness among the clones of sandalwood by using RAPD marker and
3. Identifying a core collection in the population studied.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

In this chapter an effort has been made to review the existing literature pertaining to the importance, origin and distribution, taxonomy and cytology of sandalwood. Since no work has been reported on the use of DNA markers in sandalwood in India or elsewhere, exhaustive literature pertaining to DNA extraction protocols, polymerase chain reaction, DNA markers and its applications *viz.*, Genetic diversity analysis, Fingerprinting, Cultivar identification, Mapping etc in other horticultural crops have been reviewed and presented here under.

2.1 Importance

Throughout the ages, *S. album* has been acknowledged as "Vegetable Gold" highly valued in perfumery, cosmetic, medicine and handicraft industries (Srimathi and Kulkarni, 1995). The tree is harvested for its heartwood, which is rich in fragrant oil. Medicinally it has astringent and antipyretic properties. The wood is quite appreciated for carving, sculpting liturgical objects, cremation and incense as it has uniform straight close wood grain.

Oil extracted from sandalwood (East Indian Sandalwood Oil) is highly priced as a key ingredient in the cosmetics and perfumes and holds prominent place in the country's export trade in essential oils. It has a characteristic sweet, woody odour and has excellent blending properties due to the presence of santalols (90%), which makes it valuable as a fixative for other fragrances. The fragrant oil is also famous for its antiseptic, soothing, diaphoretic and diuretic properties. In India, it is used in the manufacture of traditional attars, scented tobacco (zarada), joss stick, soaps and detergents.

India is the largest exporter of sandalwood oil supplying around 75 per cent of the world demand. The predominant export markets for Indian

sandalwood oil are USA, UK, France, China and the Middle East. Annual world requirement for sandalwood oil is about 200 tonnes for the production of cosmetics, perfumery and medicines, which is equivalent to 10,000 tonnes of heartwood. India produces nearly 140 tonnes, about 60 per cent of which is exported, earning a foreign exchange of about 2.5 crores every year (Tandon, 1995). Hence, it is necessary to get good quality sandalwood i.e., big sized logs without cracks as it fetches better foreign exchange.

2.2 Origin and distribution

There are approximately 16 species of sandalwood (*Santalum album, S. spicatum, S. austrocaledonicum, S. yasi, S. lanceolatum, S. ellipticum, S. macgregorii, S. insulare*) occurring naturally throughout Australia, India, Indonesia, Papua New Guinea and the islands of the South Pacific. The most important commercial species today is *Santalum album* L. and is generally accepted that it is indigenous to Peninsular India (Gode, 1961). The occurrence of *S. album* in India has raised doubts about its nativity. Some are of the view that it was introduced to India from Timor in Indonesia (Fischer 1938 ; Thirawat, 1955). Fischer (1938) considered Timor island of East Indian Archipelago as the original home of *S. album* and from there it has been introduced to India some time in the remote past. Thirawat (1955) endorses Fischer's theory that sandal could be exotic to India as the same is substantiated by the natural occurrence of many species of *Santalum* in Malaysian islands and Australian mainland and availability of only one species in India. Boyce (1959) and Rajgopalshetty (1977) corroborate the above statement, and gave the following two evidences in support of the theory (a)- Sandal has been found to invade into all the eco-climatic zones in India (b)- Occurrence of spike disease in India which is absent in Timor island (Indonesia) indicate that the immigrant species is adopting to the new ecosystem. However, sandal has been found to exist in India for more than 2500 years (Gode, 1961).

A diphyletic origin of sandal in India and Indonesia (Timor) has also been suggested by Majumdar (1941). While putting forward the theory of diphyletic origin, he justifies its possibility by citing the present distribution of the genus *Mida*, one species of which grows in New Zealand and other in Juan Fernandez Islands, which are separated by a distance of nearly 5000 miles.

Santalum album is tropical in distribution and is distributed in the south eastern region of the Southern hemisphere between 30^0 N and 40^0 S latitudes from Indonesia in the West to Juan Fernandez islands in the East and from Hawaiian Archipelago in the North to New Zealand in the South (Fig. 1). In India, it is found distributed all over the country (9600 km^2) with over 90 per cent of the area in Karnataka and Tamil Nadu (Fig. 2). The rest is distributed in other states like Andhra Pradesh, Kerala, Madhya Pradesh, Orissa, Maharashtra, Rajasthan, Uttar Pradesh, Bihar and Manipur (Venkatesan and Srimathi, 1981; Srinivasan *et al.*, 1992).

In Karnataka, it is spread over 5245 km^2 mainly in southern parts of the state and sparsely in the north accounting for more than 50 per cent of the total area in the country. It is most plentiful in Shimoga, Chickamagalur, Coorg, Hassan, Mysore, Dharwad, Kolar, Bangalore, Belgaum, Uttar Kannada, Dakshina Kannada, Bellary and Tumkur. Natural regeneration in most of the populations is poor due to biotic and abiotic interference. Most of the growing populations are devoid of larger girth classes due not only to illicit felling, but also to grazing, browsing, hacking and other biotic interference (Swaminathan *et al.*, 1997).

The sandalwood population in Tamil Nadu is distributed over 3040 km^2 mainly in North Arcot (Javadi's and Yelagiri hills), Salem, Periyar, Coimbatore and Vellore districts and sparsely in Nilgiris, Madurai and Trichy. About 175 km^2 of area is covered by sandalwood trees in Andhra

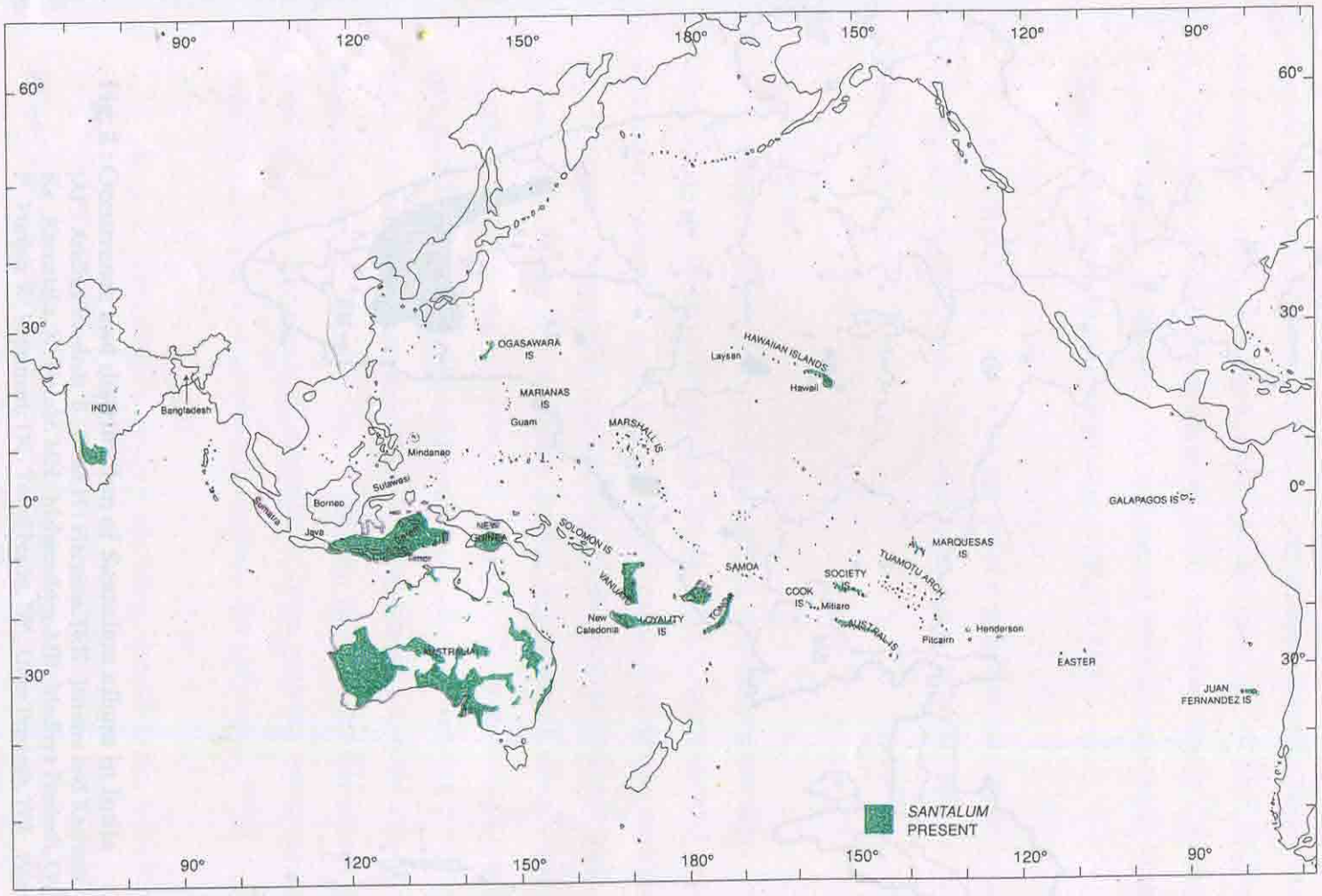

Fig. 1 : Distribution of genus *Santalum* in the world. [Complied from two maps : 1- Distribution of sandalwood in India from an article by S.N. Rai in USDA Forest Service General Technical Report PSW-122 (1990) : 66; and 2- Distribution of *Santalum* in the pacific Region from an article by P. Brennan and M. Merlin in ACIAR Procedings No. 49 (1993) : 31]

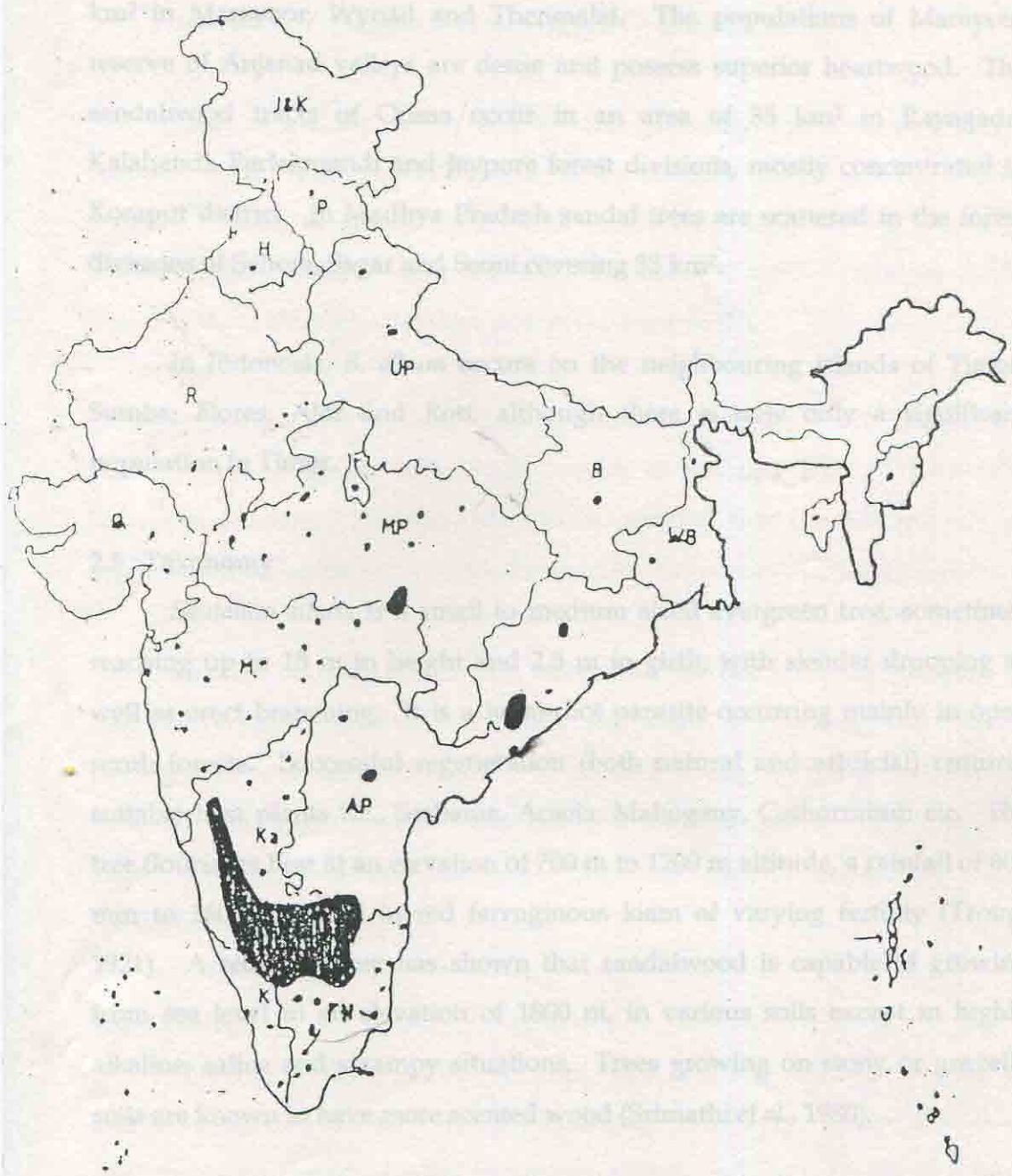

Fig. 2 : Occurrence and distribution of *Santalum album* in India

(AP : Andhra Pradesh, B : Bihar,H : Haryana, J&K : Jammu and Kashmir, Ka : Karnataka, K : Kerala, MH : Maharashtra, MP : Madhya Pradesh, O : Orissa, P : Punjab, R : Rajasthan, TN : Tamil Nadu, UP : Uttar Pradesh, WB : West Bengal

Pradesh and are mainly distributed in Chittoor, Kadapa, Tirumala hills, Hyderabad, Kurnool and Arakku valley. In Kerala, it is distributed over 15 km^2 in Marayoor, Wynad and Thenmalai. The populations of Marayoor reserve of Anjanad valleys are dense and possess superior heartwood. The sandalwood tracts of Orissa occur in an area of 35 km^2 in Rayagada, Kalahandi, Parlekmandi and Jeypore forest divisions, mostly concentrated in Koraput district. In Madhya Pradesh sandal trees are scattered in the forest divisions of Sehora, Sagar and Seoni covering 33 km^2.

In Indonesia, *S. album* occurs on the neighbouring islands of Timor, Sumba, Flores, Alor and Roti, although there is now only a significant population in Timor.

2.3 Taxonomy

Santalum album is a small to medium sized evergreen tree, sometimes reaching up to 18 m in height and 2.5 m in girth, with slender drooping as well as erect branching. It is a hemi-root parasite occurring mainly in open scrub forests. Successful regeneration (both natural and artificial) requires suitable host plants *viz.*, Sesbania, Acacia, Mahogany, Cathormium etc. The tree flourishes best at an elevation of 700 m to 1200 m altitude, a rainfall of 600 mm to 1600 mm and in red ferruginous loam of varying fertility (Troup, 1921). A recent survey has shown that sandalwood is capable of growing from sea level to an elevation of 1800 m, in various soils except in highly alkaline, saline and swampy situations. Trees growing on stony or gravelly soils are known to have more scented wood (Srimathi *et al.*, 1980).

It is a polymorphic species showing considerable variation in leaf pattern (Ramaswamy, 1972). Leaves are opposite and decussate, sometimes show whorled arrangement. Kulkarni and Srimathi (1980) reported the occurrence of six morphological types like ovate, lanceolate, linear, elliptical, big and small types. Trees having ovate leaves occur most predominantly in

nature, while those with lanceolate leaves occur to a small extent and those with elliptic or linear leaves occur at a low frequency. The colour of leaves varies from bluish/greenish yellow to green (Srimathi *et al.*, 1983).

Stem is initially green and tender, gradually turns brownish and becomes hard. The bark is reddish brown or dark brown and red inside. Stem is smooth in young trees, turns rough with deep vertical cracks in mature trees. Wood is hard and oily. Sapwood is white, scentless, while heartwood is yellowish to brown and strongly scented.

Root is moderately long, delicate and contains scented oil. It develops nodular growth at an early stage, which is the first sign of haustoria. However, Nagaveni and Srimathi (1985) reported that a small number of sandalwood plants do exist without haustorial nodules even up to two years.

Flowers are purplish brown, unscented and are borne in auxiliary or terminal cymose panicles. Flowers are tetra to pentamerous, rarely hexamerous and hermophrodite. Fruit is a drupe, purplish when fully matured and single seeded. The shape of the fruit varies from round to oblong and sometimes show tapering ends (Nagaveni and Ananthapadmanabha, 1986).

2.4 Cytology

Cytological analysis of root tips indicated the diploid number of chromosomes (2n=20) in *S. album* (Darlington and Wylie, 1955). However, drift in chromosomal number in various tissues and parts of the tree have been reported. In the haustorium up to 40 chromosomes have been reported. A two to five fold increase in size of the chromosome was observed in many reports. This has been attributed to the phenomenon of endopolyploidy and chromosomal degeneracy (Srimathi and Srinivasayya, 1962).

2.5 Genetic erosion and conserving genetic diversity

Tropical forests, which harbor a significant proportion of the global biological diversity, are also the most vulnerable to anthropogenic pressures. Tropical forests covered 16 per cent of earth's surface originally, is now reduced to only 7 per cent (Gentry, 1990). Therefore, genetic erosion of forest resources, as the loss of genetic variation within species is now a central problem. In the conservation of forest genetic resources, priority should be given to both the present rate of extinction as well as the need to ensure the availability of the resources for future use and adaptability to changing environment. Therefore, comparative genetic diversity studies are needed with the objective of saving gene pools by preventing loss of genotypes, genes and gene complexes. Such studies are particularly needed in tropical tree species like sandalwood where ruthless destruction of natural stands is taking place (Gill and Singhal, 1997; Guarino and Rao, 1995).

Genetic diversity within and among populations is the backbone of conservation of forest genetic resources in tropical and temperate species, both for present as well as for future development (Namkoong, 1997). Unlike most agricultural species that can generate crop varieties with multiple breeding cycles over a few years, forest tree breeders cannot rapidly produce new varieties nor can they quickly breed for new variations among populations. Therefore, the existing genetic diversity among sandalwood populations is fundamental and serves as a backbone of the genetic resources to conserve for both their local survival as well as for future development. These resources also form the basis for advanced breeding programmes.

Although, traditional practices of silviculture and tree breeding remain significant in current sandalwood improvement activities, existing conventional breeding methods are limited by the long growth cycles and the inability of the breeder to always distinguish between genotypic expression and environmental effects. Further, sustained management of existing genetic

resources has been constrained due to lack of base line information on the levels and structure of genetic diversity. Therefore, assessment of genetic diversity is vital in developing effective conservation strategies and sustainable management guidelines (Loveless, 1992). In this regard, genetic markers can provide potential tools in addressing these issues in sandalwood improvement (Watt, 1997).

2.6 Genomic DNA Isolation

The first step for the application of a molecular biology to any crop species is to develop techniques to extract high quality DNA. Extraction of highly purified genomic DNA from plant tissues is a difficult task due in part to their rigid cell wall composed of large amounts of complex carbohydrates (Hattori *et al.*, 1987). Contamination by polysaccharides has been reported as the most common problem affecting plant DNA purity (Demeke and Adams, 1992; Murray and Thompson, 1980). Therefore, purification of high molecular weight DNA from plant tissues is essential for many procedures used in molecular genetics (Boiteux *et al.*, 1999).

Polymerase Chain Reaction (PCR) based methods are most versatile tools for genetic diversity and evolutionary studies. PCR is based on the efficient action of a thermostable polymerase such as *Thermus acquaticus* (*Taq*) DNA polymerase (Arnheim and Erlich, 1992). Several factors present in plant DNA preparations inhibit *Taq* polymerase activity (Gelfand and White, 1990). Some classes of polysaccharides reduce the activity of polymerases, ligases and restriction endonucleases (Do and Adams, 1991). As a result of contamination by polysaccharides and/or other DNA binding substances, false negative polymorphic bands have been observed in PCR based fingerprinting, which may confound the interpretation of genetic differences between individuals (Gelfand and White, 1990).

Negative consequences of unsuitable DNA purification protocols have been described in plants by Rogers and Bendich (1994). The major adverse effects are 1) relatively low DNA yields; 2) co-isolation of DNA serving quinonic compounds (brown pigments) associated with the activity of polyphenol oxidases; 3) low DNA utility due to contaminants (polyphenol and other secondary compounds) which affects restriction endonucleases, polymerase and/or ligase activity, and 4) premature DNA degradation.

With the wide spread use of PCR in molecular genetics, several methods have been developed for extraction of DNA from plant materials (herbaceous plants and trees), which allows for recovery of small amounts of DNA of sufficient purity for PCR amplification (Doyle and Doyle, 1987). It can be achieved using organic (Schulz *et al.*, 1994) or inorganic solvents. However, inhibitions of PCR by traces of organic solvents (Gelfand and White, 1990) as well as their toxicity (Chaves *et al.*, 1995) are the drawbacks of protocols involving organic solvents.

A critical factor in the isolation of plant DNA is the efficient disruption of the plant cell wall and the separation of DNA from other cell components without affecting the integrity of DNA. Unfortunately, many techniques for breaking open cells also shear DNA and thus any method must be a compromise between DNA lengths and yield (Tapan *et al.*, 2000). Moreover, the use of liquid nitrogen or lyophilization for grinding the tissue as well as phenol, protease and RNAse treatment, CsCl purification and column chromatography for removing the impurities increase the cost of the procedures

2.6.1 DNA isolation protocols

One of the most widely followed extraction procedure involves the use of a nonionic detergent cetyltrimethyl ammonium bromide (CTAB) which complexes with carbohydrates and can be phenol extracted (Murray and

Thompson, 1980). It is a relatively simple procedure for the rapid isolation of high molecular weight DNA (50kb), which is free of contaminants, needed for various applications such as PCR, cloning etc. It can be used for crops, which are rich in polysaccharides, phenols and other secondary compounds. In this method nucleic acids can be selectively precipitated with CTAB. RNA and DNA are soluble in CTAB and NaCl (0.7 M), but precipitate when the salt is reduced below 0.4M. However, many polysaccharides are insoluble over this salt range and are thus not solubilized. The CTAB precipitated DNA was again purified with CsCl (Cesium chloride of different density). This approach has been used to isolate DNA from carrot, wheat, oats, tobacco, mung bean, and peas. Results indicated that dry tissue can be effectively disrupted while the DNA is unhydrated and thus less susceptible to shear. The protocol yielded 20-70 μg of DNA per 100 mg of dry tissue.

Dellaporta *et al.* (1983) reported a rapid micro scale method for isolation of plant DNA without the use of ultra centrifugation with CsCl. This is a mini-preparation procedure that can be followed for crops less in polyphenols, polysaccharides, and other secondary compounds. In this method SDS is used to remove proteins and has been successfully used on *Nicotiana tobacum, Lycopersicon* sp, *Amaranthus* sp, *Petunia hybrida* and *Glycine max*. The DNA produced is of moderately high molecular weight (>50kb) and serves as a satisfactory substrate for most restriction enzymes and is suitable for genome blot analysis. DNA yields from leaf tissues of most species tested with this protocol were 50 to 100 μg per gram of fresh tissue and remarkably uniform from sample to sample.

Early protocols for isolation of plant DNA called for the use of liquid nitrogen to assist in the grinding of plant material. Although these protocols yielded good quantities of high quality DNA, the use of liquid nitrogen presented some problems. Once exposed to liquid nitrogen, it was imperative that the tissue not be allowed to thaw before extraction. Again if large

number of samples were to be extracted, then processing of the tissue to extractable form and storage is another bottleneck in DNA isolation. To circumvent these problems, Tai and Tanksley (1990) have developed an inexpensive method for isolation of DNA from dehydrated leaves of tomato by following the protocol outlined by Dellaporta *et al.* (1983). Tissue is dried at 45° C to 55° C for 12 to 24 hours in a food dehydrator and subsequently powdered using paint mixer. The DNA obtained is of high molecular weight (>50kb) and is comparable to the one obtained from lyophilized and fresh frozen tissue with respect to both quality and quantity. The advantages of this procedure is, it is fast, does not requires expensive equipment (e.g. lyophilizer) and can be used in situations where large number of samples must be extracted.

Polysaccharide contamination is the most common problem affecting plant DNA purity. For example DNA isolated from muskmelon, cucumber, potato and geranium crops were often contaminated with large amount of polysaccharides. Fang *et al.* (1992) demonstrated a quick and inexpensive method to remove polysaccharide contamination from plant DNA. The contamination was removed by re-dissolving the DNA in TE (10mM Tris HCl with pH 7.4 and 1mM EDTA) with 0.5 to 3.0 M NaCl, and then precipitated with two volumes of ethanol. Most of the polysaccharides were removed effectively in a single high salt precipitation at 1.0 to 2.5 M NaCl. At 3.0 M NaCl, the salt precipitated out of solution. Purified DNA was easily digested by restriction enzymes and was satisfactory as a template for PCR indicating that high salt precipitate effectively removed polysaccharides and their inhibitory effects on restriction enzymes and *Taq* polymerase activity.

A relatively quick, inexpensive and consistent protocol for extraction of DNA from expanded leaf material containing large amounts of polysaccharides, tannins and polyphenols was described by Porebski *et al.* (1997). Mature strawberry leaves, which contain high levels of these

secondary compounds, were used as study group. This method involves a modified CTAB extraction, involving high salt concentrations to remove polysaccharides, the use of PVP to remove polyphenols, an extended RNAse treatment and a phenol-chloroform extraction. Average DNA yield from this protocol ranged from 20 to 84 μg per gram mature leaf tissue for both octaploid and diploid *Fragaria* species. The resulted DNA was PCR amplifiable before and after extended storage.

Purifying DNA from mature leaves at the end of the season is difficult because of their thick cell wall and high content of polysaccharides and phenolic compounds. A simple, fast and efficient method for DNA purification from mature leaf samples of four hard wood tree species (*Acer*, *Fraxinus*, *Prunus* and *Quercus*) was described by Lefort and Douglas (1999). The protocol is a modified CTAB method, involves a combination of β-mercaptoethanol, PVP, SDS and lithium chloride, including a short centrifugation runs. It is very efficient yielding up to 950 μg per gram of fresh weight, even when very matured leaves were processed. It is more efficient compared to four commercially available kits and two other published CTAB protocols.

2.6.2 Protocols for problematic crops

The main obstacle in isolating quality DNA from the genus *Abelmoschus* is the stickiness of solution after grinding the leaves in liquid nitrogen because of large amounts of polysaccharides produced during photosynthesis. Kochko and Hamon (1990) used cotyledons of dark grown seedling to overcome this problem. About one gram of seven days old unopened cotyledons yielded 100 μg of DNA. The quality of DNA was confirmed by digesting it with several restriction endonucleases. The use of cotyledons to isolate DNA has the disadvantage of causing destruction of the analyzed plants, and that it cannot be used for intravarietal variability studies.

Isolation of highly purified DNA is difficult particularly from plants rich in polyphenolic compounds because damage to the leaves causes significant buildup of polyphenolic complexes. Jennifer and Paul (1990) developed a protocol for isolation of DNA from plants like cocoa high in polyphenols. This method relies on both concentrating the nuclei away from cytoplasmic components prior to lysis and strongly inhibiting the formation of oxidized polyphenolic compounds in the extraction solution. The DNA obtained was relatively pure, yielding 20 µg of DNA per gram of starting material and no visible RNA contamination was observed when electrophoresed.

Crops like sweet potato and its related species of *Convolvulaceae* are characterized by several secondary metabolites including iridoid compounds and flavonoids in addition to latex/mucilaginous secretions. These secondary compounds forms a complex with nucleic acids resulting in a sticky gelatinous matrix and thus isolation of restrictable DNA is problematic. Varadarajan and Prakash (1991), followed SDS method of minipreparation outlined by Dellaporta *et al.* (1983) with three main departures; pretreatment of tissue prior to cell disruption, recovery of DNA from the crude insoluble gelatinous mass by processing it as the second fraction, and additional periods of centrifugation and ethanol washes of DNA pellets. The pretreatment steps increased the amount of DNA in the gelatinous fraction and at the same time, reduced the amount of the insoluble material. The additional periods of centrifugation helped in the purification and concentration of DNA and made possible an average recovery of up to 150 µg of DNA per gram of leaf tissue from gelatinous suspension.

Extraction of high quality DNA from plant species like *Opuntia* and other cacti is difficult. These plants contain high levels of mucilages and complex polysaccharides that bind water present in the extraction buffer producing a gel like mixture, thus preventing isolation of quality DNA.

Jacobo *et al.* (2000) developed a modified CTAB method for extraction of enzyme restrictable DNA from four *Opuntia* sp. The method involves adjusting the amount of tissue used according to species and age, followed by processing in an extraction buffer to separate coarse material. Extended centrifugation and digestion time in a separation buffer with 2 per cent CTAB was found to maintain polysaccharides in solution and allowed easier recovery of the aqueous phase containing DNA. This method was successful in extracting DNA from tender tissue of other cacti species like *Stenocereus* sp., *Cleistocactus* sp., and *Echinocereus* sp. The DNA obtained was suitable for PCR amplification, producing clear, distinctive and reproducible banding patterns useful for a variety of applications.

2.6.3. Effect of tissue and method of isolation on PCR

Rogers *et al.* (1996) evaluated six methods of DNA extraction on PCR amplification, using leaf discs and other plant tissues such as seed, root and tubers from six plant species (sugar beet, sea beet, *Brassica oleracea*, rapeseed, potato and maize). Comparison using leaf material indicated differences among species in the PCR success rate and reliability of the tested methods. However, method two was found to be applicable to all the species tested, while other methods are found effective only with a particular species. Further, DNA extracted from non-leaf tissues (root, seed and tubers) showed low success rates of PCR amplification.

Seven plant genomic DNA purification protocols were evaluated for genetic fingerprinting analysis using six tissues obtained from inbred carrot lines. Evaluations included DNA yield, purity, DNA cleavage with *Hind*III, DNA integrity and DNA suitability for RAPD. Significant differences were observed among tissues and purification methods for the total amount of DNA. An extraction method using CTAB buffer + organic solvents gave the best results in DNA yield, purity and *Hind*III cleavage when compared with other six non-organic extraction methods. Of the tissues examined, flowers

yielded the most DNA, followed by seeds, lyophilized leaves, calli and taproots. Differences in RAPD band intensity and number were observed across tissues and DNA extraction methods using identical PCR conditions for RAPD. Callus was the best type of tissue for RAPD based fingerprinting producing a consistently higher number of more intense amplicons compared to other tissues. Judicious and uniform selection of DNA purification method as well as tissue source for DNA extraction are, therefore, important for reliable RAPD based DNA fingerprinting analysis in carrot or any other horticultural crops (Boiteux *et al.*, 1999).

2.6.4 DNA quantification and purity assessment

Yield of DNA can be quantified by flurometry, spectrometry and agarose gel electrophoresis by comparing with standard DNA concentrations (Boiteux *et al.*, 1999). While, the purity is assessed based on digestion with restriction endonucleases (*Pst*I, *Eco*RI, *Hind*III, *Bam*HI etc.) and spectrophotometer readings. Quality refers to purity of DNA and to what extent it is free from secondary metabolites and other contaminants, which hinders its use in molecular genetics. A good quality DNA generally exhibits the following spectral properties; A_{230}, A_{230}/A_{260}, A_{280}/A_{260} or A_{260}/A_{280} ratios of less than 0.10, less than 0.45, less than 1.65 or more than 1.80, respectively (Shantha *et al.*, 1998). If A_{260}/A_{280} ratio is more than 1.80, then it indicates RNA contamination and if the ratio is less than 1.65, indicates the presence of contaminants such as proteins (Sambrook *et al.*, 1989).

2.7 Polymerase Chain Reaction (PCR)

PCR is an enzymatic method of making multiple copies of a pre-selected segment of DNA. This method was invented by Kary-Mullis *et al.* (1986) and described by Saiki *et al.* (1988). It is carried out in three steps at discrete temperatures; heat denaturation (94-98°C), annealing of synthetic primers to template DNA (35-55°C), followed by primer extension (72°C) at their 3'ends. Since the 3'ends of the primers point towards each other,

repeated cycles of heating and cooling lead to chain reaction resulting in the exponential synthesis of many copies of the specific segment bounded by the primers. The exponential amplification is because of the products synthesized in cycle "n" function as a template for the other primer in cycle "n+1" cycle. The length of the product generated during the PCR is equal to the sum of the length of the two primers plus the distance in the target sequence (Erlich *et al.*, 1991).

2.7.1 Steps involved in PCR

2.7.1.1 Denaturation of template DNA

PCR commences with the denaturation step, which ensures complete strand separation of the template DNA. Typically denaturation conditions are 95° C for 30 seconds, or 97° C for 15 seconds. However, higher temperatures may be appropriate, especially for G+C rich templates. It takes a few seconds to denature DNA at its strand separation temperature. Incomplete denaturation allows the DNA strands to "snap back " and thus, reduces the product yield. In contrast, denaturation steps that are too high and/or too long lead to unnecessary loss of enzyme activity. Therefore, it is better to perform PCR with temperature range of 94-97° C for denaturation in order to avoid mispriming, misincorporation of incorrect nucleotides (Innis and Gelfand, 1990).

2.7.1.2 Primer Annealing

Annealing refers to hybridization of the primer to single stranded DNA. The temperature and length of time required for primer annealing depend upon the base composition, length and concentration of the amplification primers. An applicable annealing temperature (T_m) is 5° C below the T_m of the amplification primers. At typical primer concentrations (0.2 μM), annealing will require only a few seconds (Innis *et al.*, 1988).

Kim and Smithies (1988) reported that increasing the annealing temperature especially during the first several cycles will increase specificity of priming and reduces incorrect priming as well as misextension of incorrect nucleotides at the 3′ end of primers. For maximum specificity in the initial cycle, *Taq* DNA polymerase should be added after the first denaturation step during primer annealing. Some investigators have reported that for better results PCR should be carried out using longer primers at 55° C to 75° C for annealing and extension, and 94° C to 97° C for denaturation.

2.7.1.3 Primer extension

Primer extensions are traditionally performed at 72° C because this temperature was optimal for extending the primers. Low extension temperature together with high dNTPs concentrations favours misextension of primers. Therefore, it is better to perform PCR at high temperature for primer extension (Kim and Smithies, 1988). Innis *et al.* (1988) reported that extension time depends upon the length and concentration of target sequence and upon temperature.

An extension time of one minute at 72° C is considered sufficient for products up to 2 kb in length. However, longer extension times may be helpful in early cycles if the substrate concentration is very low, and at late cycles when product concentration exceeds enzyme concentration. The rate of nucleotide incorporation at 72° C varies from 35 to 100 nucleotides per second, depending upon the buffer, pH, salt concentration and nature of the DNA template (Saiki and Gelfand 1989).

2.7.1.4 Number of cycles

The optimum number of cycles will depend mainly upon the starting concentration of target DNA, when other parameters are optimized. A common mistake is to execute too many cycles. Too many cycles can increase the amount and complexity of nonspecific background products. In contrast,

too few cycles give a low product yield. Innins and Gelfand (1990) reported that when the number of target molecules is $3x10^5$, $1.5x10^4$, $1x10^3$ and 50, then number of cycles to be performed is 25-30, 30-35, 35-40 and 40-45 respectively.

2.7.2 Reaction components of PCR

2.7.2.1 Template DNA

Template DNA should be stored in TE or water. PCR does not require highly purified template DNA, even small amounts of crude DNA preparations are adequate. Slightly degraded or sheared DNA may amplify more readily because it denatures more easily, allowing better primer access. Low concentrations of DNA are recommended because, an overabundance of template DNA will favour annealing of the two strands of the template sequence, rather than their annealing to the primer pair, and will also increase the chances of forming non specific products. A single copy gene can be amplified sufficiently in 30 cycles from less than 0.2 μg of DNA. Higher copy number genes will therefore require smaller quantities of template DNA for optimal amplification (Baumforth *et al.*, 1999).

2.7.2.2 Magnesium ion

The magnesium may affect primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer-dimer artifacts, enzyme activity and fidelity. Therefore, it is beneficial to optimize the magnesium ion concentration. *Taq* DNA polymerase requires free magnesium on top of that bound by template DNA, primers and dNTPs. Accordingly, PCR should contain 0.5 to 2.5 mM magnesium over the total dNTPs concentration. The presence of EDTA or other chelators in the primer stocks or template DNA may disturb the apparent magnesium optimum (Innis and Gelfand, 1990).

2.7.2.3 Primers

The primer concentration should not be more than 1 μM (Baumforth *et al.*, 1999). Primer concentrations between 0.1 to 0.5 μM are generally optimal (Innis and Gelfand, 1990). Higher concentrations promote mispriming, formation of primer dimers, or the generation of non-specific products (Baumforth *et al.*, 1999). Non specific products and primer dimers are themselves substrates for PCR and compete with the desired product for enzyme, dNTPs and primers resulting in a lower yield of desired product (Innis and Gelfand, 1990).

Arbitrary primers used in RAPD will be usually of 9-10 base pair long with a GC content of 50-80 per cent and do not have palindromic sequences. The number of DNA fragments that are amplified depends on the length of time and genomic DNA used (Williams *et al.*, 1990). Kang *et al.* (1998) reported that, GC content of the primers affected their sensitivity to the length of time allowed for annealing. RAPD patterns obtained from primers with high GC content (70-80%) were affected by short annealing time, where as those obtained from primers with 50-60 per cent GC content were reduced in intensity even with 30 seconds of annealing time.

2.7.2.4 Deoxynucleotide Triphosphates (dNTPs)

The stability of dNTPs during repeated cycles of PCR is such that approximately 50 per cent remains as dNTPs after 50 cycles (Innis and Gelfand, 1990). Usually, each dNTP is used at a concentration between 50 μM and 200 μM, which results in the optimal balance among yield, specificity, and fidelity. Higher concentrations encourage misincorporation by the DNA polymerase (Baumforth *et al.*, 1999). The four dNTPs should be used at equivalent concentrations to minimize misincorporation errors. Low dNTP concentrations minimize mispriming at non-target sites and reduce the likelihood of extending misincorporated nucleotides. Therefore, one should decide on the lowest dNTP concentration appropriate for the length and

composition of target sequences (Innis *et al.*, 1988). Concentrations of 50 μM and 200 μM of each dNTP are sufficient to synthesize 6.5 μg and 25 μg of DNA respectively.

2.7.2.5 DNA polymerase

A variety of polymerases could be used for chain reactions. However, the most commonly used DNA polymerase is *Taq* polymerase, which is isolated from a bacterium found in hot springs known as *Thermus aquaticus*. *Taq* polymerase works optimally at 72^0 C and is also heat stable, allowing the enzyme to withstand repeated denaturation cycles (Baumforth *et al.*, 1999). Therefore, it need not be added at each cycle, which greatly simplifies the automation of PCR. One drawback of *Taq* polymerase is, it lacks 3′ to 5′ exonuclease (proof reading) activity, which can lead to misincorporation of nucleotides (Gelfand, 1992; Eckert and Kunkel, 1992). Enzyme activity is also sensitive to the concentrations of magnesium and other monovalent ions.

Recently new DNA polymerase has been isolated from the thermophilic bacteria (*Thermococus litoralis*) called vent DNA polymerase. It has 3′ to 5′ exonuclease activity and may therefore have lower misincorporation rate. However, the capacity of this to degrade single stranded molecules (like oligonucleotide primers or PCR product prior to primer annealing) will pose problem for PCR amplification (Erlich *et al.*, 1991).

A recommended concentration range for *Taq* DNA polymerase is between 1 and 2.5 units per 100 μl reaction when other parameters are optimum. However, enzyme requirements may vary with respect to individual target templates or primers. When optimizing a PCR, it is recommended, testing the enzyme concentration from 0.5 to 5 units per 100 μl and assaying the results by gel electrophoresis. If enzyme concentration is too high, nonspecific background products may accumulate, and if too low an insufficient amount of desired product is made. *Taq* polymerase from

different suppliers may behave differently because of different formulations, assay conditions and or unit definitions (Innis and Gelfand, 1990).

2.7.2.6 Reaction Buffer

The buffer most often used in the PCR is 10 mM Tris buffer, with a pH range between 8.5 and 9.0 at 25° C. Because the pH of Tris buffers decreases by 0.3 units for each 10° C rise in temperature, a buffer made to pH 8.8 at 25° C is only pH 7.4 at 72° C. This value is optimal for the activity of *Taq* polymerase, since *Taq* appears to have a pH optimum of 7.0-7.5 at 72° C (Baumforth *et al.*, 1999).

2.7.2.7 Detergents

Taq is highly hydrophobic protein and tends to precipitate from aqueous solution. The addition of non- ionic detergents (Triton X-100, NP-40, or Tween 20 at a final concentration of 0.01%) helps to maintain full activity, both in storage solutions and in amplification reaction. Highly purified (low peroxide) preparations of detergents should be used (Baumforth *et al.*, 1999).

2.7.3 Problems associated with PCR

Though PCR is a powerful technique, it has certain limitations that should be kept in mind while designing experimental strategies. Although the high processivity of *Taq* makes production of long PCR products conceivable and some 10 kb products have been detected by southern blotting, PCR is most useful for the amplification of DNA segments less than 2 kb in length. Longer products are likely to be out competed in the reaction by products from mis-primed sites, either internal to the amplified product or from elsewhere in the genome. Alternative priming makes it more difficult to amplify long products from complex genome (Baumforth *et al.*, 1999).

With the repeated cycles of denaturation and annealing used in PCR, the possibility exists for products only partially extended during one cycle to

reanneal to a different template in a later cycle. If two alleles or related genes are present, it is possible to create "shuffle clones" which are recombinant products of the two sequences (McBride *et al.*, 1989). Shuffle clones may be more frequent in amplifications from highly damaged DNA. If the template DNA consists of fragments shorter than the desired amplification product, the fragments from different alleles may be assembled randomly by PCR (Paabo *et al.*, 1990).

Since, PCR can generate millions of copies of DNA from a template sequence, contamination of the amplification reaction with products of previous PCR reaction (product carry over), exogenous DNA and other cellular materials, can create problems both in research and diagnostic application. To minimize the risk of contamination, it is advisable to irradiate the reaction mixture at 254 nm before adding the template DNA. This serves to nick and cross-link any contaminating sequences and therefore makes them unamplifiable (Baumforth *et al.*, 1999). Further, all reagents and consumables should be autoclaved before use. Operator contamination can also be reduced by wearing suitable protective clothing (Kwok and Higuchi, 1990).

2.8 Genetic markers and their types

A marker is usually considered as a constituent that determines the function of a construction. Genetic marker can be defined as any stable and inherited variation that can be measured or detected by a suitable method, and can be used subsequently to detect the presence of a specific genotype or phenotype, which otherwise is very difficult to detect (Mitra *et al.*, 1999). Such variations occurring at different levels, i.e., at the morphological, chromosomal, biochemical or DNA level can serve as the genetic markers.

2.8.1 Morphological markers

Phenotypic traits are the oldest and widely used genetic markers that can be used for the evaluation of diversity. They are very informative in

germplasm management where the cultivars can be identified based on morphological traits *viz.*, seedling and leaf characters, flower types, fruit size, shape, colour etc. Morphological markers are simple and inexpensive which can be studied even from herbarium specimen or other dead tissues. But the disadvantage of this marker is, phenotypic characters are highly influenced by environmental conditions. Hence, the data obtained by such evaluations are not easily understood at genetic level, often resulting in maintenance of duplicate accessions. Moreover, in perennial crops it is required to grow for many years to observe growth and reproductive parameters, which is economically not feasible. For meaningful assessment of genetic diversity a large number of polymorphic markers are required; this limits the use of morphological markers that reveals a low degree of polymorphism (Mitra *et al.*, 1999). The actual identity of some cultivars is still in question, because similar cultivars grown in different regions often have different names (Pathak and Ojha, 1993) because of the differences in the manifestation of morphological traits (Lakshminarayana, 1980).

Cruz *et al.* (1999) estimated the genetic diversity among 326 accessions of 3 species of *Dioscorea viz., D. alta* (202), *D. esculenta* (91) and *D. hispida* (33) with 113 agromorpological characters from different regions of Philippines. Multivariate analysis was carried out and the PCA and dendrogram revealed the relationship between the three species. The PCA and dendrogram revealed distinct and tight clusters. Eight accessions clustered next to the cluster of *D. alta* and were reclassified as *D. bulbifera.* The lowest genetic diversity was observed in *D. esculentum* and highest in *D. hispida.*

Morphological characterization of 35 accessions of *Arachis* comprising of 13 species was carried out with 32 qualitative (20 vegetative and 12 reproductive) and 33 quantitative (22 vegetative and 11 reproductive) characters (Chandran and Pandya, 2000). Thirteen clusters were formed on the dendrogram four of which were accessions from *A. duranensis.* The three

perennial species *viz., A. cardenasi, A. correntina* and *A. kempff-mercadoi,* showed lot of similarity and clustered together. Some *A. kempff-mercadoi* accessions clustered with *A. duranensis. A. hypogaea* and *A. batizocoi* clustered together. It was earlier reported that *A. batizocoi* was an intermediate between *A. duranensis* and *A. monticola,* but the present study revealed that there exists quite a variation between these species.

2.8.2 Molecular markers

Recent developments in DNA technologies have made it possible to uncover a large number of genetic polymorphisms at the DNA level, and to use them as markers for evaluation of the genetic basis for the observed phenotypic variability. These markers revealing variations at the DNA level is referred to as molecular markers (Mitra *et al.,* 1999). Ever since their development, they are constantly being modified to enhance their utility and to bring about automation in the process of genome analysis (Swati *et al.,* 1999).

Molecular markers include biochemical constituents (e.g. secondary metabolites in plants) and macromolecules *viz.,* proteins and deoxyribonucleic acids (DNA). However, analysis of secondary metabolites is restricted to those plants that produce a suitable range of metabolites which can be easily analyzed and distinguish between varieties. These metabolites, which are being used as markers, should be neutral to environmental effects or management practices. Hence, amongst the molecular markers used, DNA markers are more suitable and ubiquitous to most of the living organisms (Swati *et al.,* 1999).

2.8.2.1 Isoenzymes / Biochemical markers

Isoenzymes, also called as isozymes are multiple molecular forms of enzyme occurring within the same organism, having identical catalytic activities and separable by electrophoretic procedure (Feret and Bergmann,

1976). The isoenzyme technique can be used for identification of clones, hybrids or varieties of tree species, for early prediction of quality characters of forest species, genetic analysis in breeding studies (Parthasarathi and Angadi, 1984) and to study gene flow in stands and seed orchards (Rudin and Lindgren, 1977). Typically isoenzymes exhibit simple inheritance, codominance, complete penetrance and consistency of expression under wide range of environmental conditions (Weeden and Windel, 1989).

2.8.2.1.1 Isoenzyme analysis in sandalwood research

Among the electrophoretic techniques, isoenzyme analysis is widely used for its efficiency in intra specific variability studies. Phenotypic characterization of sandalwood trees at cell level by Parthasarathi *et al.* (1985) revealed characteristic differences between leaf types, both at vegetative and flowering stages in their pattern of isoenzymes (POD-peroxidase and MDH-malate dehydrogenase). It is also helpful in diagnosing the deficiency symptoms of a particular element and restoring normalcy by providing that trace element at the proper time (Kamala *et al.*, 1986). Apart from this, change in the pattern of POD and MDH in the diseased plant could be used to confirm the presence of spike disease in doubtful cases and even diagnosing early stage of onset of disease (Angadi and Ananthapadmanabha, 1988).

Isoenzyme analysis is of great use in identifying provenances (Egerton-Warburton, 1990), estimating genetic distance between sandal plants with in the same population and between different population (Brand, 1994) and also in genetic resource mapping of highly distributed *S. album* population (Venu *et al.*, 1997). Recent developments in isoenzyme analysis have made it possible to develop a biochemical marker for oil-bearing capacity, which can be put to use in forecasting oil bearing potential, even at an young age (Parthasarathi *et al.*, 1986). This will facilitate in rapid screening of sandal plants, raised by hybridization for their oil bearing potential.

2.8.2.2 DNA markers

Study of DNA variation in forest genetics is not a new phenomenon, but the recent approach is to develop appropriate system of markers to examine the diversity, genetic differentiation and gene flow in fragmented populations. Information emerging from such studies is useful in evolutionary studies, linkage mapping, in identifying genes responsible for disease resistance, map based cloning and formulating proper conservation strategies.

The DNA based markers are powerful tools in detecting polymorphism and are used extensively because of the following advantages; (1)-Potentially a large number of polymorphism can be detected in any taxa, (2)-It allows investigation of both coding and non-coding regions of the whole genome, (3)-Both mendelian and non mendelian markers can be identified as DNA is found in nucleus, chloroplast and mitochondria, (4)-It is independent of the growth and developmental stages of the plant and the tissue type sampled, and (5)-are seldom influenced by environment. Because of these unique advantages, they are gaining enormous popularity day by day. In this review, an attempt has been made to explain some of the widely used DNA markers in genetic diversity analysis.

There are different types of DNA markers and are generally classified as hybridization based markers (e.g. RFLP, microsatellites and minisatellites) and PCR based markers (e.g. RAPD, AFLP, SCARs and STS). In the former, DNA profiles are visualized by hybridizing the restriction enzyme digested DNA to a labeled probe, which is a DNA fragment of known sequence. The latter involves *in vitro* amplification of particular DNA sequences or loci, with the help of specific or arbitrary primers and a thermostable DNA polymerase enzyme. The amplified fragments are separated electrophoretically and banding patterns are detected by different methods such as staining and autoradiography (Swati *et al.*, 1999). The various types of markers and their

application in horticulture, particularly perennial crops has been reviewed and presented.

2.8.2.2.1 Restriction Fragment Length Polymorphism (RFLP)

RFLP technique involves digestion of genomic DNA with specific restriction endonucleases that cleave the DNA at specific and repeatable recognition sites. The resulting fragments are then fractionated by electrophoresis and specific fragments are visualized by hybridizing them with known base sequences (probes). Polymorphism generated by variation in restriction sites is detected in the form of distinct bands after suitably labeling the probes with radioactive material.

Grodzicker *et al.* (1974) first used this technique in retrovirus and later found various applications in many crop improvement programmes. Nuclear RFLPs are now available in many tree species e.g. *Pinus* (Sitbon and Gustafsson, 1988), *Acacia mangium* (Butcher *et al.*, 1998), *Eucalyptus nitens* (Byrne *et al.*, 1998). Xiao and Zhang (1995) used RFLPs for assessing genetic differences between *Citrus reticulata, C. ichangensis* and their F_1 hybrid H10, *C. medica* and *Fortunella crassifolia.* RFLPs have been used efficiently to study the taxonomic relationships in eucalyptus at various levels i.e., individual trees to provenance level (Maunders, 1992). The amount, distribution and nature of cp-DNA polymorphisms were studied via RFLPs in Douglus fir (Ponoy *et al.*, 1994) and in three closely related species of conifers - *P. attenuata, P. muricata* and *P. radiata* (Hong *et al.*, 1993). Byrne (1999) used nuclear RFLP loci to estimate genetic diversity and phylogenetic relationships among the three eucalyptus species *E. kochii, E. plenissima* and *E. horistes.* The populations showed little differentiation, low genetic distance and high gene flow between them.

RFLPs are codominant markers and can detect whether a linked trait is present in homozygous or heterozygous state in an individual, information

highly desirable for recessive trait. Hence, they are reliable markers in mapping studies. RFLPs have been used to construct genetic linkage maps in tomato, pepper (Tanksley *et al.*, 1988), and Pine (Costa *et al.*, 2000). However, their utility is hampered due to the large amount of DNA required for restriction digestion and southern blotting. The requirement of radioactive isotopes makes it relatively expensive and hazardous. Further, the assay is time consuming and only one out of several markers may be polymorphic, which is highly inconvenient especially for crosses between closely related species.

2.8.2.2.2 Microsatellites and Minisatellite markers

The term microsatellite (SSRs - Simple Sequence Repeats) was coined by Litt and Luty (1989), while the term minisatellite (VNTRS - Variable Number of Tandem Repeats) was coined by Jeffreys *et al.* (1985). Both are multilocus probes creating complex banding patterns and are usually non-species specific occurring ubiquitously. They form an ideal marker system for DNA fingerprinting in forensic studies as well as in genome mapping (Jeffreys *et al.*, 1991). Fingerprints generated by these probes are also known as oligonucleotide fingerprints. The methodology has been derived from RFLP and the specific fragments are visualized by hybridization with a labeled micro/minisatellite probes.

The DNA content in higher plants is highly variable. Arumuganathan and Earle (1991) estimated the DNA content in over 100 important crop species. DNA content varied from 0.30 picogram (pg) $2C^{-1}$ nuclei or 145 million base pairs (mbp) in *Arabidopsis* to over 50 pg or 24,255 mbp in leek. On our request, the genomic DNA content of sandalwood (*Santalum album* L.) was estimated by Flow Cytometry by using leaf tissue by Dr. Arumuganathan, University of Nebraska, Lincoln, USA to be 0.45 pg $2C^{-1}$. Higher plants have a considerable portion of DNA as repetitive non-coding DNA that is not transcribed. Species with larger genomes normally have

more repeated DNA and a higher proportion of repeated DNA to single copy DNA (Tanksley and Pichersky, 1988). Thus, only a small fraction of the total genetic variation at a DNA nucleotide sequence level reveals itself as a distinct trait, showing Mendelian inheritance. The degeneracy of the genetic code ensures that about one in three nucleotide changes will not affect the amino acid sequence of the protein produced. Thus, the great bulk of genetic variation at the nucleotide level may not have any detectable expression at phenotypic level. SSRs are used to detect polymorphisms in these tandem repeats. They can be hybridized based, or PCR based where the primer(s) used flank-repeated sequences. SSRs are co-dominant and relatively easy to perform and are extensively used in marker studies.

2.8.2.2.3 Sequence Tagged Sites (STSs)

RFLP probes specifically linked to a desirable trait can be converted into PCR based STS markers, based on nucleotide sequence of the probe giving polymorphic banding pattern to obtain specific amplicons. This technique overcomes the tedious hybridization procedure involved in RFLP analysis and is extremely useful for studying the relationship between different species (Bustos *et al.*, 1999). When these markers are linked to some specific traits like stem rust resistance gene in barley, they can be easily integrated into plant breeding programmes for marker assisted selection of the trait of interest.

2.8.2.2.4 Randomly amplified polymorphic DNA (RAPD)

With the advent of PCR machine, a new type of DNA based marker has been introduced, which has revolutionized the entire scenario of molecular biology. In 1990, two groups independently described a new PCR based genetic assay namely randomly amplified polymorphic DNA (Welsh and McClelland, 1990; Williams *et al.*, 1990). This technique is now being used extensively to complement RFLP.

The RAPD amplification reaction is preformed on a genomic DNA template and primed by an oligonucleotide primer resulting in amplification of several discrete DNA products. Each amplified product is derived from a region of the genome that contains two short segments which share sequence similarity to the single primer and which are on opposite strands and sufficiently close together for amplification to occur. Polymorphism in these products is generated due to primer initiation at many sites, probably small inverted repeats of DNA, which may often be imperfectly complementary to the primer (Williams *et al.*, 1990). The discrete products, separated on agarose gels are visualized by ethidium bromide staining and the polymorphism is detected as the presence or absence of a particular band in a specific locus.

RAPDs are relatively quick, no prior sequence information of the target genome is necessary, can detect good number of polymorphisms and the process can be automated (Williams *et al.*, 1990). They are dominant markers, therefore impossible to determine directly whether an individual is homozygous or heterozygous for any particular RAPD locus. Hence, have limitations in their use as markers for mapping, which can be overcome to some extent by selecting those markers that are linked in coupling (Williams *et al.*, 1993).

Since the introduction of RAPD markers in 1990 (Williams *et al.*, 1990) their use in genetic analysis has increased in an exponential manner, due to the easiness of the procedure and the very low amount of DNA required for analysis (10-30 ng/reaction). RAPD analysis has been used for genotype characterization (Lu *et al.*, 1996), to assess intra or interpopulation genetic variability (Huff *et al*, 1993; Alberto *et al.*, 1997), for genetic mapping (Chaparro *et al.*, 1994) and to identify molecular markers linked to genes of interest (Nair *et al.*, 1996). It has been also used for hybrid identification in several plant species including sandalwood (McComb and Jones, 1997), *Malus* (Harada *et al.*, 1993), peach (Pooler and Scorza, 1995), *Prunus* (Ozaki *et al.*,

1995) and *Cyrtranda* (Smith *et al.*, 1996). In *Citrus*, RAPD analysis has been used mostly for genetic mapping (Cai *et al.*, 1994) and to study genetic relationships between species and cultivars (Machado *et al.*, 1996).

2.8.2.2.5 Amplified Fragment Length Polymorphism (AFLP)

Vos *et al.* invented AFLP in 1995. This method combines the reliability of RFLP with the power of PCR technique. It uses restriction enzyme digested genomic DNA as the template for a PCR reaction with primers that contain the restriction enzyme recognition site as well as a number of arbitrary nucleotides. The amplified products are resolved by gel electrophoresis and then visualized after exposure to X-ray film as the primers are labeled with radioactive p32. Recently, the technique has been automated using fluorescent labeled primers. This has tremendously reduced the risk of continuous exposure to carcinogenic radiation.

AFLPs are dominant markers and are extremely useful in detection of polymorphism between closely related genotypes. Unique fingerprints are produced regardless of the origin and complexity of the genome. Most AFLP fragments correspond to unique positions on the genome and hence can be exploited as landmarks in genetic and physical mapping (Vos *et al.*, 1995). AFLPs are extremely useful as tools to provide informative and reproducible fingerprints of peach, eucalyptus, oak, poplar and loblolly pine (Cervera *et al.*, 2000) and also for cloning and mapping of variety specific genomic DNA sequence.

AFLP amplification of genomic DNA has been used to estimate genetic diversity and relatedness in eucalyptus (Yang and Li, 2000), *Azadirachta indica* (Singh *et al.*, 1999), *Pinus sylvestris* (Lerceteau and Szmidt, 1999) and gooseberry (Lanham and Brennan, 1999). Thus AFLPs provides a newly developed, important tool for a variety of applications.

2.8.2.2.6 Other types of markers

In recent years, various types of DNA markers are being developed with a variety of application in crop improvement. More sophisticated markers like Expressed Sequence Tags (ESTs), Single Strand Conformation Polymorphism (SSCP), Inter Simple Sequence Repeat Markers (ISSR), Restriction Landmark Genomic Scanning (RLGS) and Allele Specific Associated Primers (ASAPs) are now being utilized for variability analysis and crop improvement.

2.8.2.2.7 Application of RAPD markers in the improvement of horticultural crops

2.8.2.2.7.1 Genetic diversity analysis

Following domestication, genetic variation in crop plants has continued to narrow due to continuous selection pressure for specific traits. Hence, much emphasis is now being placed upon the need to carryout germplasm analysis, in order to study genetic diversity and relatedness. This information has potential in strategic planning of future breeding programmes. RAPDs have been used by Crawford *et al.* (1994) to study the genetic diversity in endangered plants e.g. *Lactoris fernandeziana.* It was used to confirm low levels of genetic variation in red pine (Mosseler *et al.*, 1992) and to study genetic variation in oil palm germplasm collected from Africa (Shah *et al.*, 1994).

The diversity was assessed among 300 clones of *Theobroma cocoa* from different parts of the world with RAPD and RFLP markers. The clones formed 2 distinct clusters in the dendrogram, one comprising of the Criollo types and the other Forestero types. The genetic diversity among the Criollo types (cultivated types) was more than that of the Forestero types (Laurent *et al.*, 1994; N'Goran *et al.*, 1994). Later, Motamayor *et al.* (1997) arrived at the same conclusion and reported a narrow genetic diversity among the Criollo types, which were also quite similar to the Trinitario types. The Forestero

types were more diverse than the other types and were not commercially cultivated.

The potential use of RAPD marker for taxonomic studies was investigated in *Malus* using 20 wild species and 27 apple cultivars (Dunemann *et al.*, 1994). DNA fingerprints generated with pre-selected random primers were analyzed for polymorphic amplification fragments and coefficients estimating genetic similarity were calculated on the basis of about 50 polymorphic RAPD loci in each set of genotypes. Cluster analysis by UPGMA revealed that, the molecular classification of cultivars was in good agreement with the known lineage. Closely related species from section I (*Malus*) were clearly distinguishable from those of section II (*Eriobolus*) and IV (*Chloromeles*). A high degree of genetic diversity was found at the molecular level among the different apple cultivars and wild species of the genus *Malus*. The results provided an additional evidence for the hypothesis that *M. primila* and *M. sylvestris* were involved in the origin of the cultivated apples. In another study, Yae *et al.* (1995) classified *Malus domestica* cultivars using RAPD markers. Of the 139 bands generated using 16 random primers, 106 were polymorphic. Cluster analysis based on these polymorphisms showed six groups; Group I contained 'Ralli Janet', 'Fuji' and the bud mutations of 'Fuji'; Group II contained 'Sikaiichi', 'Earliblaze', 'Delicious' and its bud mutant; Group III contained 'Indo', 'Gala', 'Master', 'Jonagold', and their strains formed group IV; 'Jonathan', 'Jonared', 'Kogitsu' and 'Mollies Delicious' formed group V; Group VI contained only 'Supergold Delicious'.

RAPD markers were used to assess the genetic similarity between 13 different *Rubus* species from the important subgenera *Idaebats, Eubats* and *Anoplobats* (Graham and McNicol, 1995). All the ten primers revealed scorable polymorphisms within both the closely related and genetically diverse individuals. Estimates of similarity, dendrogram and principle coordinate analysis were calculated, with the results generally in agreement

with previous classification of the species studied, confirming the validity and usefulness of the RAPD assay. Among the species studied, *R. macraei* (subgenera *Idaebats*) proved more diverse and grouped in with both the subgenera *Idaebats* and *Eubats* at only 26 per cent similarity.

Karihaloo *et al.* (1995) reported RAPD variation in the egg plant using 130 fragments generated by 22 primers. *Solanum melongena* exhibited 117 fragments, which were also present in *S. incanum.* *S. incanum* showed an additional 13 fragments, which were absent in *S. melongena.* Overall, the *S. incanum* accessions were more diverse compared to *S. melongena.* Genetic similarity between them was 0.95 and the RAPD analysis were closely concordant with the results of previously published isoenzyme analysis performed on the same accessions. The results indicated that, even though both are morphologically highly diverse, it is no longer appropriated to distinguish them taxonomically.

One hundred and eleven RAPD fragments generated by 21 random primers were used to assess the genetic relationships among 39 Mediterranean mandarins (*Citrus deliciosa* Tenmore). UPGMA clustering revealed a low level of genetic variation between accessions of Mediterranean mandarins, whereas their hybrids with other citrus species showed greater genetic dissimilarity. Twenty accessions produced similar patterns indicating that, they could be a single clone. Certain genotype specific markers were identified mainly in known hybrids. The large number of hybrids and the low level of polymorphism between the accessions strengthened the earlier hypothesis that Mediterranean mandarins are all true hybrids of common mandarins (Machado *et al.*, 1996).

RAPD analysis was carried out on 16 grape cultivars that were morphologically distinct types *viz.*, subgenera *Euvitis* (bunch grapes) and *Muscadinia* (muscadine grapes) (Qu *et al.*, 1996). One hundred and fifty six

bands were generated from 19 primers ranging from 100 bp to 1600 bp. Out of the 156 bands, 11 were monomorphic and 145 polymorphic. One primer UBC-237 produced 2 unique bands that were able to distinguish the *Euvitis* and *Muscadinia* types. Genetic distances were estimated which revealed that a small genetic variation exists among the muscadine cultivars and a high variation between the muscadine and bunch grape cultivars. The two grape types clustered separately in the dendrogram and the variety 'Miss Blanc' clustered separately in the bunch grape cluster probably because it was a descendant of *V. bourquiniana* unlike the other cultivars in this group. In another study, Wang *et al.* (1999) used RAPD markers to assess inter and intra specific variation among 42 accessions of *Vitis*, representing 13 species. Principal Component Analysis (PCA) revealed that *V. rotundifolia* was different from other bunch grapes and the American and Asian grapes appear to be more closely related to muscadines than *V. vinefera*.

Graham *et al.* (1996) estimated genetic diversity in eight strawberry cultivars using RAPD markers. Ten random primers successfully amplified DNA fragments from each cultivar and specific fingerprints were generated from the molecular marker data. The cultivars were traced back to founding clones and the relationships between the cultivars were examined from both the molecular and the pedigree data.

Huang *et al.* (1997) studied the variability among provenances of *Acacia mearnsii*. The genomes of eleven provenances of *Acacia mearnsii* were tested by RAPD analysis using 22 primers. Dendrogram established based on the DNA bands of the amplified products showed diversity among the different provenances. There was no significant correlation between provenance hardiness and longitude, latitude or altitude of origin.

Mneney *et al.* (1997) used RAPD markers to study the genetic differences among twenty Tanzanian cashew accessions and between

individual cashew genotypes from Tanzania, Mozambique, Guinea-Bissau, and Brazil. Out of six Operon 10 mer primers studied, three of them *viz.*, OPF 2, OPF 3 and OPF 5 reveled maximum polymorphism. The results revealed that there was considerable similarity among the twenty Tanzanian genotypes and differences between the genotypes from different geographical regions. The importance of broadening the present narrow genetic base of Tanzanian cashew by including exotic germplasm been emphasized. In another study, RAPD markers were used to study the genetic relatedness among accessions from Ceylon, India, Mozambique, Tanzania, Brazil and Cook Island. Results revealed a close relation among the accessions from India, Mozambique and Tanzania and a unique fragment was identified for the accessions from Cook Island (Mneney *et al.*, 2001).

Genetic variation among forty-three date palm (*Phoenix dactylifera* L.) accessions, including thirty-seven accessions from Morocco and six cultivars from Iraq and Tunisian was analyzed with RAPD markers (Sedra *et al.*, 1998). Out of 123 primers screened, 19 were selected which revealed polymorphism among all the genotypes analyzed. Cluster analysis clearly separated the Iranian and Tunisia cultivars from the Moroccan accessions. There was low polymorphism among the accessions from Morocco, which could be owed to the mode of introduction and maintenance of germplasm. The genetic diversity of date palm was reported to be narrow and the need for broadening the genetic base was emphasized.

RAPD markers were used to analyze the molecular diversity among the different species of citrus and related genera (Sawazaki *et al.*, 1997). Dendrogram established revealed that the sweet orange cluster was distinct from tangerine group, and sour orange fell between the two. Another cluster observed was Palestine lime, West Indian lime, Rangpur lime and Citron, confirming their close genetic relationships and the theory that Citron is derived from the lemon and lime parent. Among the species compared,

trifoliate oranges was the most distantly related of all the species. RAPD results were in agreement with the existing traditional taxonomic and morphological approach to citrus classification system. In another study, Coletta *et al.* (1998) evaluated genetic similarity among 35 mandarin accessions, including 10 species and 7 hybrids using RAPD markers. Jaccard's coefficient revealed that the genetic similarity with in the mandarin group was high and the similarity of mandarins to other citrus species was low. Results indicated that cultivated mandarin group is a single species (*C. reticulata*) rather than composed of a large number of species as proposed by some taxonomic studies and have a narrow genetic base.

Kim *et al.* (1998) used RAPD markers to assess the genetic diversity in East-Asian *Pinus* species. The cluster analysis indicated that East-Asian pines had higher levels of genetic diversity than pine trees from Europe and North America. Among the different species studied, Diploxylon pines (natural hybrid of *P. tabulaeformis* and *P. yunnanensis*) showed higher levels of genetic diversity than Haploxylon pines (*P. pumila, P. sibirica and P. koraiensis*). Genetic variation at the polymorphic loci was partitioned such that 96 per cent of the diversity was found within the population, while 4 per cent accounted for population differentiation, indirectly indicating that gene flow among Asian pine tree populations is high enough to counteract the effect of genetic drift.

Neem is an evergreen, multipurpose tree of the tropics and is believed to be highly cross-pollinated. RAPD markers were used to study inter-provenance variation among 34 accessions of neem. Similarity matrix computed based on UPGMA method revealed that the similarities in RAPD profile amongst the different accessions was more than that expected due to the out crossing nature of neem. The results indicated that neem has a narrow genetic base and further improvement of this tree species may require

the introduction of additional germplasm into breeding programmes (Nuzhat *et al.*, 1998).

RAPD markers were used to determine intraspecific variability among 15 accessions of *Andrographis paniculata* (a popular antipyretic and hepatoprotective drug) collected from India and south-east Asia (Padmesh *et al.*, 1999). Molecular analysis revealed a moderate level of variation within the species. Similarity measurement using UPGMA followed by cluster analysis resulted in five major groups based on geographical distribution that generally reflected expected trends between the genotypes. There were also important exceptions like accession AP-48 from Thailand showing close resemblance to AP-38 from Tamil Nadu and AP-29 from Assam significantly diverse from the rest of the native genotypes.

Stevens *et al.* (1999) assessed variation in genetic diversity among 23 local populations of populus seedlings with respect to altitude, substrate, plant competition and geographical location. One hundred and thirty two RAPD markers based on six primers were used to assess genetic relationships within and among the population. The results indicated that most of the variation occurred with in the population. Even genetically distant populations shared nearly 90 per cent of the same markers and there was no geographical differentiation among the population.

Thirty-four released cashew varieties and hybrids from India and a clone resistant to tea mosquito have been fingerprinted by using RAPD markers. One hundred and fifty seven amplified products generated by ten random primers were used to estimate genetic relationship and the results indicated that the genetic base of the cashew cultivars is not narrow as reported earlier, but is moderate. The clustering of the cultivars was more or less similar to their geographical origin. The cultivars from Kerala were the

most diverse when compared to the cultivars from other geographical regions (Murali, 1999).

Genetic diversity among 12 genotypes of gooseberry (*Ribes grossularia*) was evaluated by using RAPD, ISSR and AFLP markers (Lanham and Brennan, 1999). Similarity matrices and dendrograms constructed revealed the genetic similarity between the different genotypes. Clustering varied with the type of marker used. The genetic base of the European cultivated gooseberry was reported to be narrow and the need for introduction of additional germplasm into breeding programmes was emphasized.

Lim *et al.* (1999) performed RAPD analysis to evaluate genetic relatedness among 12 species of *Vanda*. One hundred and fifty eight fragments generated by eight primers were used for statistical analysis. The dendrogram constructed by UPGMA analysis formed two major clusters. *V. teres and V. hookeriana* formed one of the clusters while the other 10 species formed another cluster. The genus *Ascocentrum* appears to be closely related to the *Vanda* species.

Saikia *et al.* (2000) performed RAPD analysis to estimate genetic diversity between 24 yew trees (has anti tumor and anti cancer properties) from the Himalayan region. Forty-eight polymorphic bands generated by 13 primers were used for RAPD analysis. In the dendrogram the accessions AP 151 and WB 214 clustered separately and were distinct from the others. The accessions from Arunachal Pradesh area were more diverse than the accessions from Darjeeling and West Bengal. Even though many of the accessions could be differentiated based on their RAPD profiles, the accessions assessed are believed to have a narrow genetic base.

Yang and Li (2000) used RAPD markers to estimate genetic variability in *Eucalyptus microtheca*, a native Australian species grown in the arid and

semiarid zones. One hundred and two polymorphic bands generated by 18 primers were used to analyze twelve natural populations from widely separated locations. Gene diversity values for each population ranged between 0.176 (the population from southeastern Australia) and 0.232 (the population from western Australia) with an average of 0.200. Total gene diversity for this species was 0.240, where 83.3 per cent of the variation was found within populations and 16.7 per cent between populations.

RAPD markers were used to evaluate genetic diversity and to identify interspecific hybrids among citrus germplasm collections from Embrapa-Mandioca. Thirty accessions were characterized by using 20 random primers and a fingerprint of each of the cultivars was obtained. Cluster analysis showed five distinct groups of genotypes, which agreed with the data on their origin and taxonomic classification. In the hybrid analysis, six primers were available which showed polymorphism between Volkamer and Rangpur (Vilarinhos *et al.*, 2000).

Gemas *et al.* (2000) performed RAPD analysis to characterize and to estimate genetic diversity among the three commercial varieties of *Olea europaea* L. *viz.*, 'Galega Vulgar' (GV), 'Cordovil de Serpa' (CS), and 'Verdeal Alentejana' (VA). One hundred and fifty six amplified products generated by 20 decamer primer were used to assess diversity among 28 trees of CS, 28 trees of GV and 22 VA trees. The dendrogram constructed clearly formed three distinct clusters for CS, GV and VA types.

Ravishankar *et al.* (2000) assessed the genetic relatedness among 18 commercial mango cultivars from India using RAPD markers. Out of 30 primers screened 19 of them produced a total of 178 bands (130 polymorphic and 48 monomorphic), which were used to study the genetic relatedness. The cultivars from western, northern and eastern India clustered together, while the south Indian cultivars clustered separately. In another study, fifty

commercial mango cultivars from different parts of India were fingerprinted by using 139 RAPD markers generated by 10 decamer primers and the genetic distance among the cultivars was found to be moderate to high. The mango hybrids with a common parent clustered together while the regular and irregular bearers clustered separately. The variety 'Mulgoa' was found to be distinct from all the cultivars studied and the south Indian cultivars were more diverse than the other cultivars (Kumar *et al.*, 2001).

Apple Simple Sequence Repeats (SSRs) were intergenerically applied to the characterization of 36 pear accessions, including 19 Japanese pear (*Pyrus pyrifolia*), 7 Chinese pear (*P. bretschneideri, P. ussuriensis*), 5 European pear (*P. communis*), 3 wild relatives (*P. calleryana*) and 2 hybrids. All the tested SSRs from apple produced discrete amplified products in pear that could be used to differentiate the accession and to estimate the diversity (Yamamoto *et al.*, 2001).

Kafkas and Perl-Treves (2001) studied the taxonomic relationships and genetic variation between *Pistacia vera* and wild species, *P. atlantic, P. terebinthus P. eurycarpa,* and *P. khinjuk* with RAPD markers. Forty wild *Pistacia* genotypes and two *P. vera* varieties generated 138 bands with 10 arbitrary primers. The dendrogram revealed that *P. terebinthus* was the most divergent species and clustered separately, while *P. vera* and *P. eurycarpa* were closely related. Species-specific bands were identified for each of the four species. From their results they suggested that *P. eurycarpa* may be a hybrid between *P. khinjuki* and *P. atlantica,* and the close relationship of *P. vera* and *P. khinjuk* confirmed the research findings of the earlier workers who suggested that they are one species based on the analysis of chloroplast DNA.

Pillay *et al.* (2001) evaluated genetic diversity and phylogenetic relationships of 29 East African highland banana (*Musa* spp.) cultivars and 2 out group taxa, *M. accuminata* Calcutta-4 and Agbagba using RAPD markers.

RAPDs revealed a narrow genetic base in highland germplasm of East Africa though a high level of morphological variability exists. This discordance was attributed to the fact that the RAPD primers used did not anneal to areas of the genome responsible for morphological variation. The major cluster in the dendrogram consisted of all the AAA types with the two beer varieties (Isha and Ikigeregere) and two cooking varieties (Igisahira and Kibungo). The cultivars 'Calcutta 4' (AA), 'Kamaramas' (AB) and 'Kisubi' (AB) clustered separately from all the AAA types.

2.8.2.2.7.2 Mapping of genetic traits

RAPD markers are suitable for mapping genetic traits. Martin *et al.* (1991) used RAPD analysis on a pair of near isogenic tomato lines, differing in resistance to *Pseudomonas syringae pv. tomato*, to identify RAPD bands coming from an introgressed region of chromosome 5 containing the *Pto* resistance gene. One hundred and forty four RAPD primers were used to produce 625 amplified bands, seven of which were amplified in one near isogenic line but not in the other. In the subsequent experiment, three of these RAPDs were used as RFLP probes to show that they cosegregated with the *Pto* resistance gene.

Hemmat *et al.* (1994) constructed a linkage maps for two apple clones Rome Beauty and White Angel by using RAPD and isozyme markers, with the objective to associate a marker for powdery mildew resistance. The mapping population consisted of 56 individuals, which were obtained by crossing commercial variety Rome Beauty and White Angel. The maps produced had 17 linkage groups and the gene responsible for imparting resistance to powdery mildew (Pl_w) in White Angel was located on the linkage group 7 near the isozyme markers *Acp-3* and *Aat-p*.

Apple scab caused by *Venturia inaequalis* is one of the most damaging diseases causing considerable reduction in yield in the US. Manganaris *et al.*

(1994) had associated an isozyme marker *Pgm-I* with the resistant gene, but though the gene was found in some of the F_1s the marker was absent and it does not always segregate in the progenies segregating for V_f gene. Koller *et al.* (1994) associated a RAPD marker, OPU 01 more closely linked to the gene for scab resistance. Gianfranceschi *et al.* (1996) sequenced the band produced by OPU 01 and converted it to a STS (Sequence Tagged Site). Hemmat *et al.* (1998) constructed a genetic linkage map using 73 F_1s obtained from a cross between Golden Delicious (susceptible to scab) and Prima (resistant to scab) with RAPD markers. They identified a RAPD marker, S_5 to be associated with resistance to apple scab. This marker was located immediately adjacent to V_f gene on opposite side to OPU-1 marker. These two markers (OPU-1 and S_5) should be an excellent set of flanking markers for marker assisted selection.

The sensitivity of many eucalyptus species to very low temperature is a major constraint for their use in cold temperature regions of the world. Therefore, identification of regions of genome influencing frost tolerance will lead to a greater understanding of the trait and enable further selection and manipulation of the trait in breeding programs. Byrne *et al.* (1997) mapped, QTLs influencing frost tolerance in an outbred family of *E. nitens* and identified two regions of the genome having an effect on frost tolerance. These two regions were located on the same linkage group (6) and they were 40 cM apart.

Debener and Mattiesch (1999) mapped the genome of two accessions of *Rosa multiflora* based on RAPD and AFLP markers. Map was constructed using F_1 population of 60 individuals obtained from a cross between diploid genotypes, 93/1-117 (accession with single pink flowers with 5 petals) and 93/1-119 (double flowers pink in colour with more than 15 petals). Seven linkage groups were obtained in both the parents and the gene responsible for double flower type (*Blfo*) was located on linkage group 3 on the 93/1-119

parent and the gene responsible for pink colour (*Blfa*) was located on the second linkage group of both the parents. Both the traits are important ornamental characters for both cut and garden roses.

Citrus nematode (*Tylenchulus semipenetrans*) is one of the major parasitic pests of the plant rhizosphere that causes serious damage to citrus roots and long-term production loss throughout the citrus growing regions of the world. Ling *et al.* (2000) studied the inheritance of citrus nematode resistance in an intergeneric backcross family [Citrus X (*Citrus* X *Poncirus*)] and its linkage with molecular markers. They identified eleven RAPD markers linked to a gene conferring resistance to citrus nematodes in an intergeneric backcross family. The individual hybrids were also screened with a newly developed RGC marker $Pt8a_{440}$ and SCAR markers $SCA07_{650}$ and $SCAD08_{1100}$. The results indicated that the gene responsible for nematode resistance *Tyr 1* are most likely located in the 3.3 cM interval defined by RGC marker $Pt8a_{440}$ and co-segregating markers $OPO07_{650}$ and $SCA07_{650}$.

The watermelon strain of papaya ringspot virus (PRSV-W) and zucchini yellow mosaic virus (ZYMV) are potyviruses that cause significant losses in cucumber. To select more efficiently for virus resistances, it is necessary to identify molecular markers tightly linked to PRSV-W and ZYMV resistances in cucumber. Young *et al.* (2000) generated 49 F_6 RILs from a cross between Straight 8 (susceptible to both virus) and a line from Taichung Mou Gua (TMG1, resistant to both the viruses) and studied the segregations of AFLPs, RAPDs, RFLPs and resistances to PRSV-W and ZYMV. A 353-point map of cucumber was generated, which had 12 linkage groups and the resistances to PRSV-W and ZYMV were tightly linked (2.2 cM) and mapped to the end of linkage group Q. This information will be useful as indirect selection tool for potyvirus resistance in cucumber.

Shell thickness is an important trait in oil palm breeding, which is controlled by single locus with 2 alleles (sh^+ and *sh*) showing co-dominant expression. Based on this, there are three types of oil palms namely, Dura, Tenera and Pisifera. Dura is homozygous dominant for shell thickness (sh^+ sh^+), characterized by the production of large fruits with thick shell and very poor oil content. Pisifera is homozygous recessive for shell thickness (sh sh), characterized by papery shell and is female sterile. While Tenera is a natural hybrid between Dura and Pisifera and is heterozygous dominant for shell thickness (sh^+ sh), characterized by the production of smaller fruits with medium thick shell and a larger proportion of oil bearing mesocarp. Moretzsohn *et al.* (2000) used a F_1 population of 95 individuals from a cross between Tenera and Pisifera for mapping. Two RAPD markers, R11-1282 and T19-1046 were identified to be linked on both the sides of the *sh*+ locus on linkage group 4 and they were 17.5 cM and 23.9 cM respectively. This information is useful in MAS for desirable fruit types in oil palm, where desirable types can be selected at nursery stage itself.

2.8.2.2.7.3 Genetic purity and Paternity analysis

RAPD markers were used to assess the genetic relationship among species, cultivars and hybrids of lilac. Thirteen random primers were used to examine 87-130 bands per cultivar/hybrid. The percentage band sharing among *Syringae* x *chinensis* cultivars ('Alba', 'Saugaena' and 'Red Rothomagensis') and *Syringa* x *persica* was surprisingly high. It was felt that RAPD markers were very useful for assessing the origin of cultivars and hybrids of many plant species (Marsolais *et al.*, 1993).

A study was conducted to determine the genetic purity of hybrid seed in watermelon (*Citrullus lantus)* and tomato (*Lycopersicon esculentum)* using RAPD analysis (Hashizume *et al.*, 1993). Fifty-nine oligonucleotides were screened in inbred parental lines (Ha, HB) currently used for commercial seed production in watermelon. The PCR using nine primers resulted in

polymorphism and the primer RAPD-12-12 (5'-ACCACCTGGCTC-3') generated a fragment specific to the F_1 and male parent (HB), and thus enabling the female parent (HA) to be discriminated from the hybrid.

Rom *et al.* (1995) used RAPD technology to test purity control of commercial F_1 hybrid tomato. DNA from these F_1 hybrids ('Naama', 'TY20' and '5692') and their parental lines were subjected to RAPD analysis. Polymorphism between the parents generated paternal specific RAPD markers, enabling a clear distinction to be made between hybrids and their maternal parents. In addition, combination of the polymorphic DNA products generated by these primers exhibited hybrid-specific patterns, enabling each cultivar to be identified. In another study, RAPD analysis was performed to determine genetic purity of F_1 hybrid seeds of *Cichorium intybus* L. by Bellamy *et al.* (1996). Comparison of PCR products obtained by using 100 arbitrary primers (10 bp) allowed identification of all the lines tested. Several primers produced line-specific RAPD markers, and the differences between the lines were confirmed both on individual heads and young seedlings.

Walker and Werner (1997) attempted to analyze the 'Cherokee' rose for its putative hybrids 'Silver Moon' and 'Anemone'. RAPD analysis carried out with sixteen primers produced forty reproducible polymorphic bands. On analyzing the shared bands it was evident that 'Anemone' shared 69.4 per cent of its bands with the 'Cherokee' rose types while 'Silver Moon' shared only 22 per cent of its bands. The results indicated that 'Anemone' is likely to be a progeny of 'Cherokee' rose type.

Since its discovery in 1987, it was not known whether *Eucalyptus graniticola* is a relict species or a rare hybrid. The similarity of features such as leaf, bud and fruit morphology, to those of *E. rudis*, a common tree found in the vicinity suggested that *E. graniticola* is a hybrid. Rossetto *et al.* (1997) used

RAPDs to determine the putative parents of *E. graniticola*. The results revealed that there was an additive inheritance of RAPD markers from *E. rudis* and *E. drummondii*, the putative parent species in *E. graniticola*. All the markers detected for *E.* graniticola using nine primers were shared with either *E. rudis* (40%), *E. drummondii* (35%) or both parent species (25%), strongly suggesting that *E. graniticola* is a rare hybrid between *E. rudis* and *E. drummondii*.

Padgett *et al.*, (1998) investigated the plants intermediate in appearance between *Nuphar microphylla* and *N. variegata* (Nymphaeaceae) that have been assumed to be the result of hybridization. They employed morphological and RAPD markers to test the hypothesis that *N. rubrodisca* represents a natural interspecific hybrid between *N. microphylla* and *N. variegata*. Examination of 15 morphological characters demonstrated the intermediacy of *N. rubrodisca* between *N. microphylla* and *N. variegata*. Eight 10-mer primers produced 13 species-specific RAPD markers for *N. microphylla* and nine for *N. variegata*, with all 22 markers present in *N. rubrodisca*. The RAPD results were concordant with morphological data in implicating that *N. microphylla* and *N. variegata* are the putative parents of *N. rubrodisca*.

Heinkel *et al.* (2000) performed a parental analysis on the plum cultivars 'Cacaks Beauty', 'Cacaks Best', 'Cacaks Early' and 'Cacaks Fruitful' with RAPD markers. It has been earlier reported that cultivars 'Cacaks Beauty', 'Cacaks Best' and 'Cacaks Early' are from 'Wangenheim' x 'Pozegaca' and 'Cacaks Fruitful' from 'Stanley' x 'Pozegaca'. Twenty six selected primers amplified 158, 144 and 160 fragments in 'Wangenheim', 'Pozegaca' and 'Stanley' respectively. Out of these 102 were monomorphic and 18 specific to 'Wangenheim', 12 specific to 'Pozegaca' and 37 to 'Stanley'. On studying the inheritance of these markers it was evident that 'Stanley' and 'Wangenhein' showed high conformity with 'Cacaks Beauty', 'Cacaks Best' and 'Cacaks Early' contrary to the earlier report on their origin. The RAPD results for the

variety 'Cacaks Fruitful' was in accordance with the earlier report where 'Pozegaca' and 'Stanley' are the parents.

2.8.2.2.7.4 Cultivar identification

Identification of avocado cultivars with three RAPD primers homologous to regions of the chromosomal DNA was reported by Lewis (1992). Variety 'Fuerte' and 'Edranol' were closely related compared to 'Haas'. In another study, Parent *et al.* (1993) used RAPD markers generated by the combination of three primers to characterize various cultivars of Quebec raspberry.

Identification of cultivars and validation of genetic relationships in *Mangifera indica* L. was reported using RAPD markers (Schnell *et al.*, 1995). Twenty-five accessions of mango were examined for genetic markers with 11 primers that are reproducible. No primer generated unique banding patterns for each of the 25 accessions. However, ten different combinations of two primer banding patterns produced unique fingerprints for each accession.

Rong *et al.* (1995) studied the effectiveness of RAPD markers to identify persimmon (*Diospyros kaki*) cultivars. The fifteen cultivars analyzed were completely distinguishable from each other using RAPD profiles generated by the primer OPA-6 or OPA-8. Further, two bud mutants of cv. 'Hiratenenashi' (i.e. 'Tonewase' and 'Sugitawase') showed different banding patterns with a few additional bands with the primer OPA-6. In addition, polymorphisms among 11 *Diospyros* species were observed by RAPD, using OPA-10 primer. The same method revealed little polymorphism among intraspecific levels of *D. kaki* or *D. lotus*.

Iqbal *et al.* (1995) showed that RAPD markers have the potential to reveal genetic similarity among the plants. Profiles of thirteen rhododendron hybrids, species and cultivars were analyzed to study their genetic

relationships. The cluster analysis grouped together varieties and/or hybrids in accordance with their known genetic relationship. For instance, two varieties of *Rhododendron yakushimanum,* (Mist Maiden and Ken Janec) clustered together while variety 'Pink Parasol' clustered with a hybrid of 'Pink Parasol' × *Rhododendron smirnowii.* These three varieties and the hybrid then clustered with *R. smirnowii.* Similarly, the other hybrids with common parents showed a closer genetic relatedness with each other than with other rhododendrons. The genetic relationship revealed from cluster analysis on the basis of RAPD profiles was similar to their known genetic makeup.

Matsumoto and Fukui (1996) reported that, RAPD markers were successfully tested for the identification of nine rose cultivars and three clonal plants. Using only three primers identified all the cultivars. Moreover, unique RAPD bands also distinguished individual cultivars. In another study, Gallego and Martinez (1996) studied molecular typing of twenty five rose cultivars using RAPD techniques with twenty 10-mer primers. The data obtained revealed high degree of variation between cultivars but no variability within cultivars. The authors also reported that the banding patterns obtained with two of the primers were able to unequivocally identify all the rose cultivars.

Damasco *et al.* (1996) detected dwarf off-types in micropropagated Cavendish (*Musa* spp. AAA) bananas using RAPD markers. A RAPD marker specific to the dwarf off-type of Cavendish cultivars 'New Guinea Cavendish' and 'Williams' was identified following an analysis of 57 normal and 59 dwarf plants generated from several micropropagation events. Of the 66 random primers used in the initial screening, 28.8 per cent revealed polymorphisms between normal and dwarf plants. Primer OPJ-4 amplified a 1.5 kb fragment that was consistently present in normal but absent in all dwarf plants of both cultivars. Reliable detection of dwarf plants was

reported using the RAPD marker, providing a suitable means of *in vitro* detection.

Wiesman *et al.* (1998) performed RAPD analysis to identify and characterize two groups of olive varieties *viz.*, 'Nabali' and 'Souri' in West Bank of Israel. Genetic similarity was evaluated among the eight 'Nabali' variants collected from different villages in Israel using 14 random primers. Accessions from the place Salfit exhibited a high level of similarity suggesting a same mother source. On the other hand, RAPD profiles of the 'Souri' variants also revealed a high degree of similarity, indicating that these variants represent environmental phenotypes of the same genome. RAPD profiles of the four other variants *viz.*, 'Buchibinder', 'Amka', 'Lud' and 'Latrun' were identical for all 20 primers screened. They all appeared to be the same variety known by different names because of the slight differences in their morphological characters.

RAPD data from 11 primers in *Heliconia* species, cultivars and hybrids suggested that 10-mer primer (OPA 18) could produce distinct RAPD profiles for 16 cultivars of *H. psittacorum*. The phylogenetic tree derived from RAPD data showed that all the 16 cultivars evaluated are closely related to each other, providing the first genetic evidence that this large group of cultivars has a common background. Also, two triploid cultivars of *H. psittacorum* (Iris and Petra) showed identical banding patterns with 10 different primers, suggesting that they are of the same genotype (Kumar *et al.*, 1998).

Genetic variation among California Almond cultivars and breeding lines was studied by RAPD analysis (Bartolozzi *et al.*, 1998). The genetic relatedness among 17 almond genotypes and one peach genotype was estimated using 37 RAPD markers. Despite its need for obligate out-crossing, diversity within almond cultivars was reported to be limited. A similarity index based on the proportion of shared fragments showed relatively high levels of similarity (0.75 or greater) within the almond germplasm. The level

of similarity between almond and peach was 0.424, supporting the value of peach germplasm to future almond genetic improvement.

'Albarino' is one of the most important grapevine (*Vitus vinefera)* grown in Spain for its wine, however ampelographic data often fails to identify 'Albarino' from the other closely related clones. The clone also appears different when cultivated in different geographical regions, hence misnaming and identification is a problem. Sixteen accessions of 'Albarino' collected from different parts of Spain and some related cultivars (which were thought to be 'Albarino') were analyzed with RAPD and microsatellite markers. Both techniques revealed the authenticity of the 16 'Albarino' clones and separated them from the other clones. No polymorphism was observed between the clones of 'Albarino' but was observed between the other accessions (Loureiro *et al.*, 1998).

Lim *et al.* (1999) reported on the genetic closeness of various species of *Vanda* using RAPD markers. Strap leaved *Vanda* species (including *Vanda sanderiana*) and *Aseocentura miniatum* were more closely related to each other than to the terete-leaved *Vanda* species studied. RAPD analysis supported the suggestions that terete-leaved *Vanda terete* and V*anda hookeriana* be classified in the separate genus *Papilionathe* and that V*anda sanhoriana* should remain in the genus *Vanda.* In another study, Starman *et al.* (1999) investigated the DNA amplification fingerprinting to evaluate the genetic relationship among 11 cultivars of poinsettia. Thirty one per cent of the bands were polymorphic and distinguished among cultivars.

2.8.2.2.7.5 DNA Fingerprinting

RAPDs are useful for DNA fingerprinting where there is a need to identify varieties of a crop or to determine parentage within breeding material. Wilde *et al.* (1992) reported that RAPD analysis has resulted in reliable discrimination both between and with in groups of cocoa genotypes.

In another study, Julie *et al.* (1994) reported the use of PCR for fingerprinting of ten red raspberry cultivars. Nine out of the ten random primers used, successfully generated specific fingerprints for all the raspberry cultivars that could be used for cultivar identification.

RAPD fingerprinting of four dwarfed clones of cashew (*Anacardium occidentale* L.) was reported by Neto *et al.* (1995). Twenty-seven primers generated amplified products in the range of 240-1780 bp few of which were found suitable to distinguish the four seedlings. The four dwarfed clones; CP 06, CP 09, CP 76 and CP 1001 could be most effectively differentiated from each other by using OPA 8, OPA 16 and OPB 15. The amplified products of six selected primers were used to study the similarity among the four clones. CP 06 and CP 76 were found to be most similar (77.8 %) followed by CP 06 and CP 1001, which were 66.7 per cent similar, and least similarity was reported between CP 76 and CP 1001 (59.3%). Similarly, Karihaloo *et al.* (2000) reported fingerprinting of 19 cashew accessions using decamer primer S-11.

In another investigation, Ye *et al.* (1998) reported on DNA fingerprinting utilizing RAPD polymorphisms to determine the relationship among 16 grapevine cultivars and sports thought to have arisen from these cultivars. From 53 primers, a total of 464 bands were generated, of which 29 per cent were common to all the genotypes tested. Cluster analysis classified all tested cultivars into two main groups (*Vitis vinifera* L. and *Vitis* x *labrusca* Bailey) as expected. No polymorphism was detected among known clones of 'Chardonnay' or 'Pinot Noir'.

Tyson *et al.* (1998) employed RAPD markers to determine if a hybrid copse between *Eucalyptus risdonii* and *E. amygdalina* was clonal. Based on lignotuber morphology this copse was composed of approximately 20 separate individuals. No variation in RAPD was observed for 67 bands

scored from nine primers among the 20 individuals. In contrast, variation was observed between individuals sampled from outside the copse (average 28.6 bands differences between any two individuals) and within full sibling families (average 12.9 band differences between individuals). On this basis, the copse was considered to be clonal, originating from lignotuber fragmentation, and measured 5.5 x 3.5 m.

Molecular fingerprinting has great potential for quality control in tree breeding. Vaillancourt *et al.* (1998) have reported an example of this application in Eucalyptus. During the grafting stage of *E. nitens* breeding programme, the labels identifying 37 different ramets were mistakenly removed. The authors used RAPD markers to correct this mistake. Based on the RAPD fingerprinting data, 34 ramets were successfully assigned to an ortet (scion source for grafting), while three of the ramets could not be assigned. These probably represent errors during the collection of scion material or samples for DNA analysis. In another study, Gan-SiMing *et al.* (1999) developed DNA fingerprints of 13 clones of five provenances of *E. urophylla* and nine clones of four provenances of *E. tereticornis* with RAPD markers. The construction of such fingerprints could be of great importance for the identification of clones, clarification of genetic relationship between clones, and selection of parental combinations in hybridization of the two species.

2.8.2.2.7.6 Sex determination

One of the most recent applications of RAPDs has been shown in sex identification of dioecious plants, where male and female plants do not show any sex specific morphological difference until flowering. Hormaza *et al.* (1994) performed Bulked Segregant Analysis (BSA) to identify a marker associated with sex in *Pistacia vera* using RAPD technique. Two crosses were used in the study and the DNA of the parents were bulked and screened with 700 decamer primers. The primer OPA-8 produced a 945 bp fragment that

was present only in the female parents of both the crosses and in the female F_1s. To test the usefulness of this marker it was tried on a population of 94 seedlings and the marker was consistently segregated in the expected ratio of 1:1. This 945 bp RAPD marker could be used to screen the gender of *Pistachio* plants before they reach reproductive maturity, resulting in considerable saving of time and economic resources. Similarly, Sakamoto *et al.* (1995) and Mandolino *et al.* (2000) associated a 750 bp and 400 bp RAPD fragment with maleness in dioecious *Cannabis sativa* L.

A genetic linkage map of papaya was constructed by Sondur *et al.* (1996) based on RAPD markers and F_2 population derived from a cross between UH 356 x 'Sunrise'. A total of 96 polymorphic bands were detected and 62 of them were mapped to 11 linkage groups comprising 999.3 cM. The sex locus 'SEX1' was located on the first linkage group and was flanked by T1C and T12 RAPD markers. These two flanking regions can serve as the excellent set of markers in identifying the sex of papaya seedlings.

Atriplex is a diploid dioecious plant belonging to the family Chenopodiaceae. Claudete *et al.* (1998) reported the discovery of a male specific DNA fragment in *A. garretti* by following BSA. A total of 158 decamer primers were tested and a 2075 bp male specific fragment produced by the primer OPAF-14 was identified. When 125 male, 126 female and one hermaphrodite plant were tested individually, the male specific band was present in the hermaphrodite and all but one of the male plant and was absent in all the female plants. In another study, Rapaport *et al.* (1998) identified a RAPD marker $UBC354_{560}$ associated with femaleness in *Salix viminalis* L.

Kiwifruit (*Actinidia*) is dioecious with a juvenile phase of 3-5 years. Harvey *et al.* (1997) used BSA in two families of *Actinidia* and associated an 800 bp (SmY) fragment with the maleness and an 850bp (SmX) fragment with the female sex. In another study, two RAPD markers associated with sex

were converted into SCARs and the SCAR marker linked to the male sex (SmY) was reported to be more useful in screening a populations of *A. chinensis* and *A. delicos* (Gill *et al.*, 1998).

Shibu (1998) identified two RAPD markers associated with female nutmeg plant from Thrissur region of Kerala and Koppa region of Karnataka. RAPD analysis with the primer OPE-11 generated two female specific bands; a 504 bp and 416 bp fragment in plants from Koppa and Thrissur area respectively. The author reported that these bands did not appear in any of the male plants. In another investigation, Banerjee *et al.* (1999) identified two male specific RAPD markers (908 bp and 757 bp fragment generated with the random primers OPA-10 and OPAC-12 respectively) in *Piper longum.* The RAPD results were confirmed by screening 25 plants collected from different parts of the country.

2.8.2.2.7.7 Other applications

RAPDS have been used by many workers to study phylogenetic relationships in various economically important crops like mango (Schnell *et al.*, 1993), *Citrus* (Federici *et al.*, 1998; Nicolosi *et al.*, 2000), chicory (Koch and Jung, 1997), *Digitalis* (Nebauer *et al.*, 2000), peach (Warburton *et al.*, 1996), and timber yielding trees like *Shorea* (Rath *et al.*, 1998). In addition to phylogenetic analysis, RAPDs finds application in identification of somatic hybrids (Xu *et al.*, 1993) and somaclonal variants in various plant species *viz.*, micropropagated clones of *Populus deltoides* (Rani *et al.*, 1995), peach (Hashmi *et al.*, 1997) and *Pinus* (Goto *et al.*, 1998).

2.8.2.2.8 Genetic variation revealed by morphological and molecular markers

Hoey *et al.* (1996) performed phylogenetic analysis of 17 wild and cultivated pea taxa using nine morphological characters, seven allozyme and 38 RAPD markers. Morphological and allozymes were analyzed together

and RAPDs independently. Cladograms developed confirmed the close relationships among the wild species and cultivars of *Pisum*.

Limited interspecific introgression has been achieved in the breeding of the two primary cultivated species of cotton (*Gossypium hirsutum* and *G. barbadense*). Genetic diversity of 16 near homozygous elite cotton genotypes derived from interspecific hybridization was assessed at the DNA level with 135 RAPDs and at the phenotypic level with 19 stable and highly heritable characters. Dendrograms generated produced two clusters with one resembling *G. hirsutum* and one *G. barbadense*. Classification of all genotypes based on the two methods gave similar results with a correlation of 0.63 between the genetic and taxonomic distance. Several genotypes were identified that were genetically and phenotypically distant from typical *G. hirsutum* and *G. barbadense*. The level of polymorphism exhibited by these genotypes could be exploited in genetic mapping to tag economically important traits, such as fiber quality (Tatineni *et al.*, 1996).

Genetic diversity was assessed among 60 Ecuadorian cocoa accessions based on morphological, RAPD and RFLP analysis (Lerceteau *et al.*, 1997). Dynamic clustering analyses classified the genotypes into two or three groups depending on the markers used. The dendrogram obtained by the molecular data was unable to differentiate eight accessions, but the dendrogram of the phenotypic data was able to differentiate all the accessions. Though the phenotypic data was able to differentiate all the accessions it was not preferred since there was some discrepancies in the clustering of accession based on the filament colour. Even though phenotypic characters are useful for making crude differentiation in cocoa it is not considered reliable. Harrison *et al.* (1997) studied the relationships among 37 North American octoploid strawberry populations by evaluating 44 morphological traits and 38 RAPD markers obtained from 7 primers. Morphological data defined five groups; east of the Missouri River, the Black Hills, from the eastern Cascades

to eastern Rocky mountains, the western Cascades and Olympic Peninsula and the Pacific coast. Canonical analysis also revealed the same populations into these provenances suggesting that these groups are morphologically distinct from each other. RAPD data defined only three groups that were quite distinct from each other. Morphological and RAPD data were discordant, but both the data agreed with the sub species relationship between *F. virginiana* ssp. *virginian* and ssp. *glauca.* All octoploid North American strawberries have likely derived from a common ancestor and have differentiated into *F. chiloensis* and *F. virginiana* by adapting to moister and drier environments respectively.

Dillmann *et al.* (1997) differentiated 145 maize inbred lines, adapted to various climatic conditions in France, using 220 RFLP markers and ten quantitative traits. Molecular and morphological divergence was estimated and correlated, which revealed that the relationship between the two data is triangular and not linear. Because, all the morphological traits used were QTLs, and different genes are responsible to bring about the same effect generating a triangular relationship.

RAPD markers and agronomic traits were used to determine the genetic relationships among 32 breeding lines of melon belonging to seven varietal types. One hundred and fifteen polymorphic RAPD bands generated from 43 primers and 24 agronomic traits were scored for genetic distance and cluster analysis, which showed a high concordance between RAPDs and agronomic traits (0.79). RAPDs were found effective in assessing the genetic relationship among the breeding lines, since they cover a large portion of the genome, including the coding and noncoding regions of DNA. The resulting dendrogram showed two main groups. The first group included 'Piel de Sapo' lines together with the Yellow and Rochet representatives. The second one comprised 'Galia' lines together with the Japanese, American and Charentais representatives. Similar conclusions were also drawn from

Principal Component Analysis. In fact the agronomic traits revealed that the 'Piel de Sapo' types seem to be more dispersed than revealed by RAPDs (Garcia *et al.*, 1998).

Finnish tansy (*Tanacetum vulgare* L.) is an aromatic, herbaceous perennial plant producing bioactive compounds that have pesticidal, antibacterial and antifungal properties. Genetic and morphological diversity was estimated among 20 tansy genotypes from different geographical locations with 92 RAPD markers and morphological traits. The RAPD and morphological data were subjected to complete linkage analysis and grouped the genotypes into two groups. The same grouping was supported by the principal component analysis. Both the data grouped the genotypes into the same groups, which also was correlated with the geographical regions (Keskitalo *et al.*, 1998).

RAPD analysis of phenotypic diversity was performed on twenty-one accessions of *Allium sativum* L. (Garlic) from different geographical regions of India and two accessions from Argentina. In the cluster analysis, all Indian accessions grouped together, out-grouping the two Argentinean accessions AS03 and AS23 from the rest. The Argentinean accession AS03 was more similar to the Indian accessions than the other Argentinean accession AS23 though morphologically both are similar. Morphologically Indian accession AS13 clustered with the Argentinean accessions AS23 and AS03, which are distinctly different from the Indian accessions. RAPD analysis could distinguish a diversity of about 60 per cent, where as morphologically a diversity of only 20 per cent was observed (Shasany *et al.*, 2000). Twelve morphological traits and 47 RAPD markers were used to discriminate 12 rhubarb (*Rheum* spp.) cultivars and to estimate their genetic relatedness (Persson *et al.*, 2000). Considerable variability was found among the cultivars analyzed. Clustering and multidimensional scaling analyses revealed a partly overlapping pattern of relatedness ($r = 0.41$) among the cultivars.

Kafkas and Perl-Treves (2001) evaluated the taxonomic relationships among 40 *Pistacia* germplasm from Turkey using morphological and RAPD data, in order to understand the genetic variation that would facilitate their use in rootstock breeding. Cluster analysis based on morphological data revealed that the closest species to *P. vera* (domesticated species) is *P. eurycarpa,* followed by *P. atlantica* and *P. terebinthus.* One hundred and thirty eight RAPD markers produced from 10 primers were used for DNA fingerprinting of these genotypes. The resulting molecular phylogeny revealed that all the four *Pistacia* species are clearly separated from each other and *P. terebinthus* was the most divergent of all the species, while *P. atlantica* and *P. eurycarpa* were the closest pair of species.

The genetic distances were evaluated between 47 pepper (*Capsicum annuum*) inbred lines belonging to five varietal types using phenotypic (41 traits), AFLP and RAPD markers. Cluster analyses showed that small-fruited pepper inbred lines are very divergent from ancestral and modern large fruited inbred lines. The resulting dendrogram split pepper large fruited inbred lines into two main groups; the first group comprised of all blocky types and the second group comprised of long types. The half-long types split up into two groups. A regression analysis indicated a high correlation (r = 0.62) between molecular and phenotypic data in their ability to detect genetic relationships between pepper inbred lines and the relationship between the molecular and phenotypic distances is not linear but triangular owing to the QTLs used in the study (Lefebvre *et al.,* 2001).

2.9 Core collection utilization and development

A core collection is a limited set of accessions representing, with a minimum of repetitiveness, the genetic diversity of a crop species and its wild relatives (Frankel, 1984). It should include as much as possible of its genetic diversity (Brown, 1995). Core collection offers a way to improve access to

germplasm collections by providing a highly diverse, representative sub-sample of the total collection.

2.9.1 Identifying the material to be represented and the size of the core

Identification of the material that is to be represented by the core depends on the availability of material and the objectives behind the establishment of a core sample. Most often a core collection will aim to represent all material of a crop in a genebank. Such cores have been developed in perennial *Glycine* (Brown *et al.*, 1987), peanut (Holbrook *et al.* 1993), Peruvian quinoa (Ortiz *et al.*, 1998). In some cases, cores have been developed to represent only a part of a collection as in sesame (Bisht *et al.*, 1998), lentil (Erskine and Muehlbauer, 1991) and *Medicago* (Diwan *et al.*, 1994).

The size of the core will always be substantially smaller than the collection from which it is formed. Brown (1989b) suggested that it should not be more than 10 per cent of the whole collection and always less than 2000 entries. In practice, most core sample is between 5 per cent and 20 per cent of the collections from which they were established and the largest to date is 2000 accessions.

The size of the international barley core consists of 1600 accessions and is less than 0.3 per cent of the world barley holdings. ICRISAT (International Crop Research Institute for the Semi Arid Tropics) has a sorghum core sample of 600 accessions developed from a collection of 40,000 (1.5%) accessions. A multispecies core of wild and cultivated *Solanum* species was 31 per cent of the collection (Hintum *et al.*, 2000). Yonezawa *et al.* (1995) reported that the optimum sample size of the core largely depends on the degree of genetic redundancy among the accessions, resources available for maintenance of the core and the frequency of regeneration of the entries. Bisht *et al.* (1998) reported that a core consisting of 5-10 per cent of the original populations were optimal to capture 75-90 per cent of the genetic diversity. In contrast,

Noirot *et al.* (1996) have suggested that higher percentages (20-30%) are needed particularly where the objective is to capture genetic diversity of quantitatively inherited characters.

2.9.2 Development of a core sample

For effective representation of the genetic diversity of the whole collection in a core, it has to be first divided into meaningful groups, referred to as stratification. The group should be constructed so as to maximize variation between groups and minimize variation with in groups.

There are different approaches and a stepwise hierarchical procedure of first making major groups, and subsequently splitting these major groups into smaller groups is usually followed (Hintum, 1994). Grouping can also be based on taxonomy or ecogeographic groups. Examples of this type of classification include spring and winter snow barley (Knupffer and Hintum, 1995) and sesame from different Chinese production regions (Zhang *et al.*, 2000).

An alternative approach in creating groups of similar accessions is using multivariate analysis (Crossa *et al.*, 1995) based on agro-morphological or marker data. To quote a few examples, Diwan *et al.* (1994, 1995) used agronomic data to cluster *Medicago* accessions, Peters and Martinelli (1989) in barley, Spagnoletti and Qualset (1993) in durum wheat, Charmet and Balfourier (1995) in perennial rye grass and Hintum *et al.* (2000) in *Solanaceae.* Another approach to cluster individuals is based on marker and morphological data. Johnson *et al.* (1999) used 14 morphological characters and 68 RAPD bands generated by six selected primers to develop a core collection in *Poa pratensis* L. from 228 accessions. The core developed represented only 10 per cent of the total population.

A representative core subset consisting of 45 accessions has been established for the Uruguayan barley collection, simplifying access to its variability (Malosetti *et al.*, 2000). Dhanaraj *et al.* (2002) used 123 RAPD markers generated from seven arbitrary primers to estimate the diversity in 90 accessions of cashew. Clustering was based on Ward's method and distance matrix by Squared Euclidean distance. A core collection consisting of 54 accessions were identified from the 90, which represented the same diversity of the entire population.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

The major emphasis of this study was to fingerprint and assess the genetic diversity among the sandalwood clones. The study specifically involved,

1) Standardization of DNA extraction and RAPD analysis protocol in sandalwood
2) Fingerprinting and evaluation of genetic diversity and relatedness among the clones of sandalwood by using RAPD marker, and
3) Identifying a core collection in the population studied.

The details of various materials used and methodologies followed to accomplish the above mentioned objectives have been described below. All the laboratory investigations were made at the Plant Molecular Biology Laboratory, Division of Horticulture, Gandhi Krishi Vignana Kendra, Bangalore.

3.1 Plant material used for fingerprinting and diversity analysis

The plant material belonging to *Santalum* album was obtained from germplasm block maintained by Institute of Wood Science and Technology (IWST), Bangalore, where sandalwood genotypes procured from different geographical regions of India are conserved. The present study was carried out on a set of 54 clones *viz.*, 51 *S. album* clones collected from different states namely, Karnataka (24), Tamil Nadu (24), Kerala (2), Andhra Pradesh (1) and 3 exotic *S. spicatum* genotypes obtained from W. Australia. The clonal variants of sandalwood analyzed in this study are depicted in Table 1.

3.2 Isolation of genomic DNA

3.2.1 Sample preparation

The leaf sample for DNA extraction was prepared by following Tai and Tanksley (1990) method with minor modifications. Recently matured leaves

Table 1 : Sandalwood germplasm analyzed for RAPDs and their place of collection

Sl. No.	Genotype	Place of collection	Origin
1.	T4	Vellore, Tamil Nadu	India
2.	KL1	Munnar, Kerala	India
3.	K35	Kushalnagar, Madikeri, Karnataka	India
4.	K10	Bangalore, Karnataka	India
5.	K29	Hassan, Karnataka	India
6.	K37	Mudigere, Karnataka	India
7.	K19	Siddapura, Sagar, Karnataka	India
8.	T20	Tirunelveli, Tamil Nadu	India
9.	T22	Dharmpuri, Tamil Nadu	India
10.	T3	Hosur, Tamil Nadu	India
11.	T24	Dharmpuri, Tamil Nadu	India
12.	K23	Bhadravathi, Karnataka	India
13.	K28	Hassan, Karnataka	India
14.	T26	Dharmpuri, Tamil Nadu	India
15.	K16	Chamarajnagar, Karnataka	India
16.	K8	Bangalore, Karnataka	India
17.	K4	Chitradurga, Karnataka	India
18.	T30	Hosur, Tamil Nadu	India
19.	T11	Salem, Tamil Nadu	India
20.	K13	Chamarajnagar, Karnataka	India
21.	K2	Bangalore, Karnataka	India
22.	KL3	Munnar, Kerala	India
23.	K27	Shikaripura, Sagar, Karnataka	India
24.	T2	Coimbatore, Tamil Nadu	India
25.	AP4	Hyderabad, Andhra Pradesh	India
26.	K14	Kolar, Karnataka	India
27.	T8	Kumbakonam, Tamil Nadu	India
28.	T5	Thirupathur, Tamil Nadu	India
29.	T13	Salem, Tamil Nadu	India
30.	K30	Chikkamangalur, Karnataka	India
31.	K31	Kadur, Karnataka	India
32.	T7	Vellore, Tamil Nadu	India
33.	K6	Bangalore, Karnataka	India
34.	T19	Tirunelveli, Tamil Nadu	India
35.	T27	Dharmpuri, Tamil Nadu	India

36.	K9	Bangalore, Karnataka	India
37.	T14	Salem, Tamil Nadu	India
38.	T12	Salem, Tamil Nadu	India
39.	T6	Vellore, Tamil Nadu	India
40.	K11	Chamarajnagar, Karnataka	India
41.	T9	Salem, Tamil Nadu	India
42.	K34	Somwarpet, Madikeri, Karnataka	India
43.	T28	Hanur, Tamil Nadu	India
44.	K5	Bangalore, Karnataka	India
45.	T23	Dharmpuri, Tamil Nadu	India
46.	T1	Kumbakonam, Tamil Nadu	India
47.	T29	Dharmpuri, Tamil Nadu	India
48.	T21	Tirunelveli, Tamil Nadu	India
49.	K32	Kadur, Karnataka	India
50.	K36	Hunsur, Karnataka	India
51.	K7	Bangalore, Karnataka	India
52.	*S. spicatum* 1	Western Australia	Australia
53.	*S. spicatum* 2	Western Australia	Australia
54.	*S. spicatum* 3	Western Australia	Australia

were harvested and brought to the laboratory. They were wiped and dried at 40^0 C for 24 hours. The dried leaves were then sealed in plastic bags and used later.

3.2.2 Processing of samples

Approximately 6-8 g of dried leaves were powdered by using "Remi Mixer" for 45-60 seconds and sieved through a 60-mesh sieve to obtain a fine powder. The required quantity of powdered leaf sample was weighed and used for DNA extraction.

3.3 Standardization of protocol for DNA isolation and purification

Stock solutions used

CTAB	:	6 per cent
NaCl	:	5 M
Tris-Base	:	1 M
EDTA	:	0.5 M
Chloroform/Isoamylalcohol	:	24 : 1 (v/v)
Poly vinyl pyrolidone (PVP)	:	2 per cent
β-Mercaptoethanol	:	1 per cent
Sodium acetate	:	3 M
Tris-EDTA (TE) buffer	:	10 mM Tris-Hcl and 1 mM EDTA, pH 8.0
Isopropanol		
Ethanol	:	70 %

3.3.1 DNA extraction protocols

Sandalwood leaves are rich in polysaccharides and phenolics, which interferes with the isolation and purification of DNA samples. The polysaccharide contamination can also cause down stream problems in PCR by inhibiting many of the enzymes. Hence, four different genomic DNA purification protocols outlined by different workers with minor modifications

were evaluated to isolate high molecular weight DNA from sandalwood leaves. These four methods are described below.

Method 1 - is a modification of the original CTAB method outlined by Doyle and Doyle (1987). Five hundred milligram of leaf powder was transferred to a sterile centrifuge tube containing 15 ml of extraction buffer (50 mM Tris-Hcl pH 8.0, 10 mM EDTA pH 8.0, 0.7 M NaCl, 0.4 M LiCl, 1 % w/v CTAB, 1 % w/v PVP and 0.25 % β-Mercaptoethanol) preheated at 65^0 C. The contents were then incubated in a water bath at 65^0 C for 30 minutes with intermittent shaking. After incubation, the contents were brought to room temperature and 10 ml of chloroform : isoamylalcohol (24:1) was added to the tube, the mixture was agitated thoroughly and centrifuged at 6000 rpm for 5 minutes. The aqueous phase was transferred to a new tube and centrifuged for 5 minutes at 6000 rpm in order to pellet possible debris. The supernatant was then transferred to a new tube and an equivalent volume of isopropanol was added to the aqueous solution. The tube was swirled gently to precipitate the DNA. The tube was then centrifuged for 5 minutes at 6000 rpm and the supernatant was withdrawn. The DNA pellet was washed with 70 per cent ethanol and was air-dried for 10 minutes. DNA pellets were resuspended in 500 µl of Tris-EDTA buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). As the solution contained RNA and other impurities, the extract was subjected to further purification.

Method 2 - The protocol outlined by Porebski *et al.* (1997) was followed with minor alterations. 500 mg of the leaf powder with 20 ml extraction buffer (20 mM Ethylene Diamine Tetra Acetic acid pH 8.0, 100 mM Tris-Base pH 8.0, 1.4 M NaCl, 3 % CTAB and 2% Poly Vinyl Pyrolidone with 1 % β-mercaptoethanol) was incubated on a water bath at 65° C for one hour with intermittent shaking. The mixture was then cooled on ice bath and 10 ml of cold chloroform : isoamylalcohol (24:1 v/v) was added and the contents were mixed well by inverting the tube and spun at 9000 rpm for 20 minutes.

The supernatant was transferred to a fresh tube and this step was repeated for four times till clear supernatant was obtained. To the aqueous phase, half the volume of 5 M NaCl was added and gently mixed followed by addition of one volume of cold propanol to precipitate the DNA. The solution was kept at 4° C over night to accentuate the precipitation of DNA. The mixture was spun at 10,000 rpm for 25 minutes to pellet the DNA. The pellet was washed with 70 per cent ethanol and dried in a vacuum drier for one hour. The pellet was resuspended in 300 µl of TE buffer (10 mM Tris HCl and 1 mM EDTA pH 8.0) and the samples were stored in eppendorf tube at -40^0 C until further analysis.

Method 3 - was that described by Edwards *et al.* (1991) with minor modifications. The powdered leaf sample (500 mg) was added into 10 ml of extraction buffer [100 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% w/v sodium dodecyl sulphate (SDS)], vortexed briefly, and centrifuged at 8000 rpm for 10 minutes at room temperature. The aqueous phase was removed, and then one volume of isopropanol (at room temperature) was added and mixed by gently inversion. After 20 minutes (at room temperature) the solution was centrifuged at 8000 rpm for 20 minutes and the DNA pellet collected was rinsed with 70 per cent ethanol and then air dried for 20-25 minutes. Then the pellets were resuspended in 500 µl of TE buffer at 4^0C overnight. Samples were stored in eppendorf tube at -40^0 C until analysis.

Method 4 - consisted of a combination of two genomic DNA purification methodologies (Dellaporta *et al.*, 1983; Do and Adams, 1991) with an extraction solution of high buffering capacity and alkaline pH. Five hundred milligram of powdered leaf samples were dispersed in 10 ml of extraction buffer (200 mM Tris pH 8.0, 250 mM NaCl, 25 mM EDTA, 0.5% w/v SDS, 1% β-Mercaptoethanol) preheated to 65^0 C. Solution was mixed by several tube inversions and incubated for 15 minutes at 65^0 C. The solution

was then allowed to cool at room temperature and the extract was emulsified in 1/3 (3.3 ml) volume of potassium acetate 5M by gentle inversion and incubated on ice for 20 minutes. After centrifugation (8000 rpm for 20 minutes at 4^0 C), the aqueous phase was removed and one volume of isopropanol (at room temperature) was added and mixed by gentle inversion. After 15 minutes the solution was centrifuged at 8000 rpm for 20 minutes and the resulting pellet was treated essentially as described in method 3.

3.3.2 DNA purification

Since the DNA isolated by the above methods may contain considerable amount of RNA, polysaccharides, phenolics and other impurities, the extract was subjected to further purification as follows (common to all the methods),

To remove RNA, each sample was incubated with 3 µg/ml RNAse on a water bath at 37° C for over night. The proteins were removed by mixing the solution with equal volume of phenol, then with phenol : chloroform (1 :1 v/v) and finally with chloroform (spun at 10,000 rpm for 10-15 minutes). Two volume of cold ethanol was added to precipitate DNA. The precipitation was improved by keeping at -20° C for 1 hour. Then spun at 12,000 rpm for 20 minutes to pellet the DNA. The pellet was washed with 70 per cent ethanol and dried in a vacuum drier for one hour. The pellet was dissolved in 300 µl of TE buffer and to it $1/10^{th}$ volume of 3 M sodium acetate was added and left overnight. The supernatant was collected after 24 hours by spinning at 12,000 rpm for 20 minutes. The DNA was quantified by using "Hoefer's Dyna Quant" and verified by electrophoresis on 0.8 % agarose gel (GIBCO BRL).

3.3.3 Evaluation of DNA extraction protocols

Different genomic DNA isolation protocols were evaluated for RAPD analysis based on DNA yield, DNA purity and DNA cleavage with restriction enzymes.

3.3.3.1 DNA yield/ quantification

Reagents used –

H33258	: 10 µl
10X TNE buffer	: 10 ml
DD filtered water	: 90 ml
Assay solution contained	: 0.1 µg/ml H33258 in 1X TNE (0.2 M NaCl, 10 mM Tris-Hcl, 1 mM EDTA pH 7.4)

The DNA yields for all the samples were quantified by using Hoefer's DyNA quant (Hoefer Scientific Instruments, Calif.) using bis-benzimidazole dye (Hoechst 33258). Values were transformed to µg g^{-1} of DNA on dry tissue basis. Using these values a working stock of 500 µl containing 6 ng μl^{-1} of DNA was prepared for further use in PCR reactions.

3.3.3.2 DNA purity assessment

The purity of sandalwood genomic DNA was evaluated by measuring absorbance (A260nm/A280nm ratio) with a UV spectrophotometer. Pure DNA preparations show a ratio between 1.8 to 2.0.

3.3.3.3 DNA cleavage with restriction enzymes

Reagents used

0.8 per cent Agarose

1x Tris-Borate EDTA (TBE) running buffer

Ethidium bromide (10 mg/ml stock)

Loading buffer (0.25% Bromophenol blue, 0.25% Xylene cyanol, 40% sucrose w/v)

The size, purity and integrity of DNA isolated were determined by agarose gel (0.8%) electrophoresis using λ phage DNA cleaved with *Hind*III as a size standard. The quality of DNA obtained by different methods was compared by restricting with 2 units of *Hind*III restriction enzyme per microgram of DNA. Further, the purity of DNA was assessed by digesting

with 2 units of restriction endonucleases (*Bam*HI, *Hind*III, *Pst*I, *Hae*III, *Eco*RI) per microgram of DNA in a 20 µl reaction. Reactions were incubated at 37^0 C for overnight and then subjected to agarose gel electrophoresis. Electrophoresis was carried out in a horizontal gel system on 0.8 per cent agarose in 1x TBE buffer at constant voltage of 60 V for 4 to 6 hours and visualized by staining the gel with ethidium bromide (0.5 µg ml^{-1}).

3.4 PCR based Randomly Amplified Polymorphic DNA analysis

3.4.1 PCR amplification

Reagents used

Template DNA	: 6 ng/µl
$MgCl_2$	: 25 mM stock
DNTPs	: 1.25 mM (10 mM each, Finzymes)
Taq polymerase	: 3 u/µl (Bangalore Genei)
Random primer	: 10 pico moles (Operon Technologies Ltd.)
PCR buffer	: 10x stock (50 mM KCl, 10 mM Tris-Hcl pH 9.0, 1.5 mM $MgCl_2$, 0.1% Gelatin, 0.05% Triton-X 100 and 0.05% NP40)
Mineral oil	: 1 drop (Sigma)

The genomic DNA of sandalwood clone KL1 was used as a template DNA for optimization of PCR amplification. Amplification was achieved by following the procedure outlined by Williams *et al.* (1990) with slight modifications. A single decamer primer was used in each reaction. PCR conditions were optimized to achieve informative and reproducible fingerprinting profiles using different levels of template DNA (10-15 ng, 25-30 ng, 40-50 ng), $MgCl_2$ (1.0 mM, 1.5 mM, 2.0 mM and 2.5 mM) and dNTPs (150 µM, 200 µM, 215 µM and 225 µM).

The PCR reactions were carried out in a final volume of 25 µl reaction mixture containing 25-30 ng template DNA, 2.5 µl 10x buffer, 215 µM dNTPs (4.3 µl of 1.25 mM), 1 unit of Taq DNA polymerase and 5 pico moles primer.

The PCR mixture was overlaid with a drop of mineral oil. Amplification was achieved in a MJ Research Thermalcycler (PTC-100) programmed for initial denaturation at 95^0 C for four minutes, followed by 45 cycles; each cycle consisting of denaturation at 94^0 C for one minute, primer annealing at 35^0 C for 2 minutes, primer extension at 72^0 C for 2 minutes, and a final extension of 10 minutes at 72^0 C. The PCR reactions were repeated three times, using the same conditions to check the repeatability of amplification products both within and between reactions.

3.4.2 Primer selection

To select primers that can amplify informative RAPD fragments, PCR was carried out to screen 220 random primers of arbitrary sequence (Operon Technologies Inc.). Of the 220 primers screened (viz., A, B, C, D, E, F, G, H, I, J and K series), 98 primers produced atleast one band. During preliminary selections, 30 out of 98 primers yielding more than 6 bands were selected. Finally, eleven (out of 30) primers producing strong, intense and unambiguous bands were selected for fingerprinting and estimating genetic diversity among the sandalwood clones. Reproducibility of the selected primers was tested by repeating the PCR amplification for atleast three times under the same amplification conditions.

3.4.3 Electrophoresis in agarose gel

Reagents used

Agarose	: 1.2 per cent (GIBCO BRL)
Running buffer	: 1x TBE buffer (54 g Tris, 27.5 g Boric acid, 20 ml EDTA 0.5 M pH 8.0 in 1 liter double distilled water)
Ethidium bromide	: 0.5 $\mu g\ ml^{-1}$ (10 mg/ml stock)
Loading buffer	: 5 µl (0.25% Bromophenol blue, 0.25% Xylene cyanol, in 40% w/v sucrose)

Amplification products were resolved by electrophoresis in 1.2 per cent agarose gel containing ethidium bromide (0.5µg/ml) using 1x TBE buffer (Sambrook *et al.*, 1989). Wells were loaded with 25 µl of reaction mixture mixed with 5 µl of loading buffer. Electrophoresis was run at a constant voltage of 60 V for 5-6 hours in 1x TBE buffer. The gels were stained with 0.5 µg/ml of ethidium bromide and photographed under UV light by using Hero Lab Gel Documentation System (Fig. 3).

3.5 Genetic diversity analysis

3.5.1 Statistical analysis for fingerprinting and estimation of genetic diversity using RAPD markers

3.5.1.1 Data analysis

Each reproducible band was visually scored for the presence (1) or absence (0) for all the sandalwood clones studied and the binary data were used for statistical analysis. Only clear and unambiguous bands were taken in to account and the bands were not scored if they were faint or diffuse, as such fragments possess poor reproducibility. The band sizes were determined by comparing with the 100 bp DNA ladder, which was run along with the amplified products.

3.5.1.2 Cluster analysis and estimating genetic distances

The presence or absence data from each study were analyzed with the computer package "STATISTICA". The dissimilarity matrix was developed using Squared Euclidean Distance (SED) that estimated all pair wise differences in the amplification product (Sokal and Sneath, 1973). The genetic distance is computed as,

$\Sigma^{n}_{j=1}$ dj^2 where $dj = (X_{ik} - X_{jk})$

Where X_{ik} refers to binary code of i^{th} tree for allele "k" and X_{jk} refers to the binary code of the j^{th} tree for allele "k".

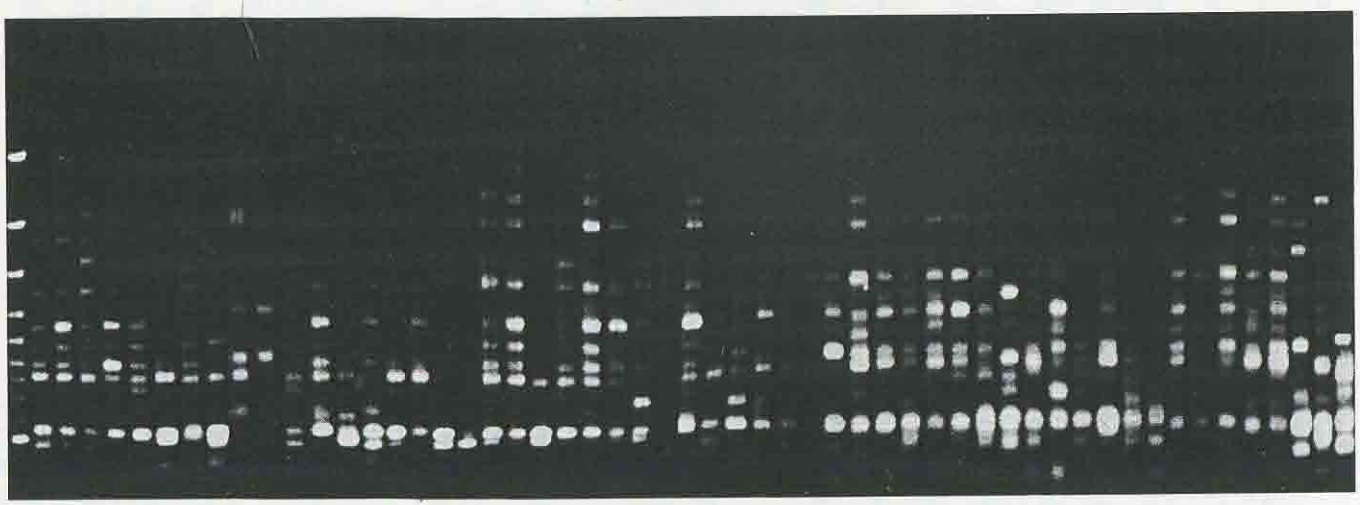

Fig. 3 : RAPD gel profile of sandalwood as photographed by Herolab Gel Doc System

A dendrogram was computed based on Ward's method of clustering, using minimum variance algorithm (Ward, 1963).

3.5.1.3 Principal Component Analysis (PCA)

This technique helps in converting a set of variables in to a few dimensions using which the genotypes/clones under study can be depicted in a two or three dimensional space (Ludwig and Reynolds, 1988). Thus, the variations of several variables will be condensed in to a set of limited axes. Such a graphical analysis helps in identifying the individuals that tends to cluster together. The genetic relationships between the different genotypes were estimated with the PCA developed from dissimilarity matrix. The genotypes were clustered on the first three axes and the pattern of clustering or separation of genotypes from one another was also analyzed.

3.5.2 Statistical analysis for the estimation of diversity by using morphological markers

Six morphological characters data based at Institute of Wood Science and Technology, Bangalore, was used in this study to estimate the genetic diversity among the 51 Indian genotypes (Table 2).

Morphological traits were standardized by subtracting the character mean from each value and then dividing by the character standard deviation. The standardized morphological characters were then used for developing a Squared Euclidean Distance matrix. Cluster analysis was based on Ward's method for the construction of a dendrogram. Principal Component Analysis was also constructed which revealed the relationship among the individuals in the third dimension. The dissimilarity matrix generated by Squared Euclidean Distance was further used for the correlation studies.

Table 2 : Morphological characters of the Indian sandalwood genotypes utilized in the investigation

Sl. No.	Clone/ Genotype	Girth (cm)	Leaf area (cm^2)	Leaf shape	Leaf margin	Leaf tip	Leaf colour	Oil# (%)	SW# (cm)	HW# (cm)
1.	T4	47.5	3035.9	EL	Wavy	Acute	Green			
2.	KL1	50.0	2488.4	L	Entire	Acute	Dark green	1.97	4.0	2.0
3.	K35	25.0	1898.6	EL	Entire	Acute	Greenish yellow			
4.	K10	46.0	3264.5	EL	Entire	Acuminate	Dark green			
5.	K29	38.0	2067.5	L	Wavy	Mucronate	Greenish yellow	2.64	8.0	2.5
6.	K37	58.0	1118.5	L	Wavy	Acumiante	Green	2.14	7.0	1.5
7.	K19	41.0	1247.6	EL	Entire	Acute	Greenish yellow			
8.	T20	53.6	2090.7	E	Wavy	Obtuse	Greenish yellow	2.14	5.0	2.0
9.	T22	34.9	2107.3	E	Entire	Acute	Greenish yellow			
10.	T3	38.0	2092.2	EL	Entire	Mucronate	Greenish yellow	2.21	7.0	1.0
11.	T24	22.0	2084.7	O	Wavy	Acute	Dark green			
12.	K23	69.0	3492.8	E	Entire	Mucronate	Greenish yellow			
13.	K28	32.0	2010.6	E	Entire	Acute	Greenish yellow	1.68	6.0	2.0
14.	T26	29.5	2342.5	L	Entire	Acuminate	Greenish yellow	1.49	5.0	1.0
15.	K16	42.3	1549.3	EL	Entire	Acute	Greenish yellow			
16.	K8	28.0	1950.6	EL	Entire	Mucronate	Greenish yellow			
17.	K4	29.5	4538.2	E(b)	Wavy	Acute	Green			
18.	T30	52.4	2475.5	EL	Entire	Acute	Green	1.49	6.8	2.0
19.	T11	57.0	2153.7	EL	Entire	Acuminate	Dark green	1.85	7.0	2.5
20.	K13	62.5	1354.5	L	Entire	Acute	Green	1.93	6.0	2.0
21.	K2	33.5	1915.0	EL	Entire	Acute	Light green			
22.	KL3	39.0	2489.5	L	Entire	Acute	Dark green	3.14	5.0	2.5
23.	K27	61.5	1786.4	OL	Entire	Mucronate	Greenish yellow	2.24	6.0	2.5
24.	T2	37.5	2542.3	OBE	Entire	Acuminate	Dark green			
25.	AP4	25.5	2549.7	E	Entire	Acuminate	Greenish yellow	3.45	6.0	1.0

26.	K14	42.5	2135.8	OL	Entire	Acute	Green			
27.	T8	43.5	1998.2	EL	Entire	Acute	Dark green			
28.	T5	40.5	2905.3	EL	Entire	Acute	Greenish yellow			
29.	T13	36.0	1855.1	E	Entire	Acute	Yellowish green			
30.	K30	31.5	2040.2	OL	Entire	Mucronate	Greenish yellow			
31.	K31	54.5	1418.8	O	Wavy	Mucronate	Dark green			
32.	T7	37.5	3034.5	OL	Wavy	Acuminate	Dark green			
33.	K6	50.0	3593.5	EL	Wavy	Acuminate	Dark green			
34.	T19	40.0	3977.2	OL	Wavy	Acute	Dark green	1.86	8.0	1.0
35.	T27	66.0	3663.3	L	Entire	Acute	Dark green			
36.	K9	44.0	2415.7	OL	Wavy	Acuminate	Dark green			
37.	T14	26.0	2256.8	OL	Entire	Acute	Greenish yellow			
38.	T12	37.0	1485.1	EL	Entire	Acute				
39.	T6	25.5	3090.2	EL	Entire	Acute	Dark green			
40.	K11	41.0	1967.9	EL	Wavy	Acute	Dark green			
41.	T9	32.0	1730.2	A	Entire	Acute	Greenish yellow			
42.	K34	33.0	1993.5	E	Entire	Mucronate	Dark green	1.84	6.0	1.0
43.	T28	31.5	2581.8	E	Entire	Obtuse	Dark green			
44.	K5	42.0	3305.9	OL	Entire	Acute	Light green			
45.	T23	45.5	4094.4	OL	Entire	Acute	Dark green			
46.	T1	41.0	3239.0	E	Entire	Obtuse to mucronate	Dark green	4.34	7.0	1.0
47.	T29	54.7	2545.0	EL	Wavy	Obtuse	Greenish yellow	1.09	8.0	2.0
48.	T21	52.5	2454.3	OL	Entire	Acute	Greenish yellow	0.58	7.0	2.0
49.	K32	55.0	3194.8	A	Entire	Acuminate	Dark green	2.34	9.0	3.5
50.	K36	47.0	1972.7	L	Wavy	Mucronate	Greenish yellow	1.56	6.0	1.5
51.	K7	35.5	1291.3	EL	Wavy	Mucronate	Greenish yellow			

E – Elliptic L – Lanceolate EL – Elliptic-Lanceolate O – Ovate A – Asymmetric
E(b) – Elliptic broad OBE – Obovate-Elliptic OL – Ovate-Lanceolate SW – Sapwood HW – Heartwood
- Not included in the present study

3.5.3. Correlating the genetic distances estimated by RAPDs and morphological markers

Out of the 54 genotypes used in the present study, the morphological characters for 51 of them are available. The distance matrices developed for these 51 genotypes from RAPD and morphological markers, was further correlated by using the Excel software from Windows. The 'r' values were estimated in order to determine the correlation between the sets of data studied.

3.6 Identifying a core collection from the genotypes studied using RAPD markers

A core collection was identified among the 54 genotypes by eliminating the overlapping individuals from the PCA that was computed using RAPD data. The dendrogram of the 54 genotypes was also used to identify closely related (duplicate) individuals, which were eliminated from the core sample.

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

The results of the present investigation entitled "Genetic evaluation of elite sandalwood (*Santalum album* L.) clones using RAPD markers" have been presented in this chapter.

4.1 Standardization of protocol for DNA isolation

4.1.1 Preparation and processing of leaf samples

Recently matured leaves were collected for DNA extraction from all the genotypes. Very young leaves were not preferred since they burnt on drying and mature leaves were also avoided as they are rich in phenols, secondary metabolites and polysaccharides. The leaf samples were collected from the new flushes before the onset of reproductive phase, as the flowering phase is associated with high phenol content.

Leaf samples were packed in brown paper covers and brought to the laboratory. They were wiped with the tissue paper and evenly spread in the hot air oven and dried at a temperature of 40^0 C for 24 hours. Prolonged drying of samples for more than 24 hours and higher temperature (> 45^0 C) resulted in poor DNA yields. The dried leaves could be stored in sealed polythene bags, while the leaf powder could not be stored for a longer time because of its hygroscopic property. Powdering was done using a 'Remi Mixer'. About 500 mg of fine powder obtained after passing through 60-mesh sieve was used for DNA extraction.

4.1.2 DNA isolation and purification

Isolation of DNA from dried leaf tissue was tried by following four methods (Methods 1 and 2 using CTAB, Methods 3 and 4 using SDS) outlined in Material and Methods (3.3.1). Comparison between the different DNA extraction protocols is given in Table 3. Among the four methods evaluated, method 2 yielded maximum DNA with an overall mean of 116.7 µg/g dry

tissue, followed by method 1 (46.2 μg/g dry tissue), while the method 3 gave minimum DNA yield of 7.3 μg/g dry tissue (Table 3 and Fig. 4a). Further the quality of DNA was evaluated by digesting with *Hind*III restriction enzyme. The quality of DNA isolated by method 2 was found good and complete cleavage of DNA by *Hind*III was observed, where as digestion of DNA was incomplete / partial for other methods (Fig. 4b). The OD 260/280 ratios ranged from 1.40 to 1.95 (Table 3). The success of different protocols was evaluated based on the usefulness of DNA isolated for PCR amplification. The results of PCR showed good amplification for the DNA isolated from method 2, while no amplification was observed for DNA extracted from method 3. DNA extracted from methods 1 and 4 showed poor amplification (Table 3 and Fig. 5).

Extraction of DNA from different amounts of leaf powder (300 mg, 400 mg and 500 mg), with different volumes of extraction buffer (10 ml, 15 ml and 20 ml) were tried along with different concentrations of CTAB (1%, 2%, 3% and 4%) and PVP (1%, 2%, 3% and 4%) by following method 2 out lined in Material and Methods (3.3.1). For sandalwood, 400 mg of leaf powder using 20 ml extraction buffer, 3 per cent CTAB and 2 per cent PVP was found to be optimal resulting in good quality, high molecular weight DNA which was confirmed by agarose gel electrophoresis (Fig. 6a & 6b).

4.1.3 DNA yield and purity assessment

The protocol (method 2) resulted in white translucent DNA pellets easily solubilized in TE buffer. The DNA resulted by this protocol was homogeneous, not degraded with good quality sized about 21 kb (Fig. 6a). Further, the quality of DNA was assessed by digestion with restriction enzymes *viz.*, *Bam*HI, *Hind*III, *Pst*I, *Hae*III and *Eco*RI. Complete cleavage of DNA was observed by restriction endonucleases. This was confirmed by electrophoresis on 0.8 per cent agarose gel using λ-phage DNA cleaved with *Hind*III as a size standard (Fig. 6b).

Table 3 : Comparison of DNA yields from sandalwood leaf tissue using different methods of DNA isolation

Sl. No.	Purification Method	Dry weight of leaf tissue (mg)	DNA yield (µg/g dry tissue)*	OD ratio 260/280	PCR amplification
1.	CTAB	500	46.2	1.95	+
2.	CTAB	500	116.7	1.82	++
3.	SDS	500	7.3	1.40	-
4.	SDS	500	25.4	1.65	+

* Average of 5 replications

\+ Poor amplification

++ Good amplification

\- No amplification

Fig. 4a : Agarose (0.8%) gel electrophoresis of sandalwood genomic DNA isolated by different methods

Lanes 1&2 : DNA obtained by method 1 (Doyle and Doyle, 1987)
Lanes 3&4 : DNA obtained by method 2 (Porebski *et al.*, 1997)
Lanes 5&6 : DNA obtained by method 3 (Edwards *et al.*, 1991)
Lanes 7&8 : DNA obtained by method 4 (Do and Adams, 1991)

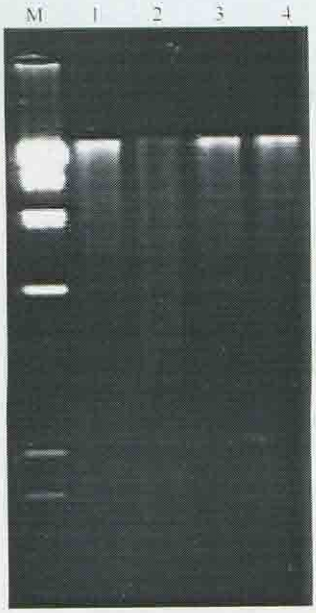

Fig. 4b : Agarose (0.8%) gel showing *Hind*III digestion of sandalwood genomic DNA isolated by different methods (method 2 presented complete DNA cleavage, whereas digestion was incomplete for methods 1, 3 & 4)

Lane M : λ DNA/*Hind*III digest marker
Lanes 1 : DNA obtained by method 1 (Doyle and Doyle, 1987)
Lanes 2 : DNA obtained by method 2 (Porebski *et al.*, 1997)
Lanes 3 : DNA obtained by method 3 (Edwards *et al.*, 1991)
Lanes 4 : DNA obtained by method 4 (Do and Adams, 1991)

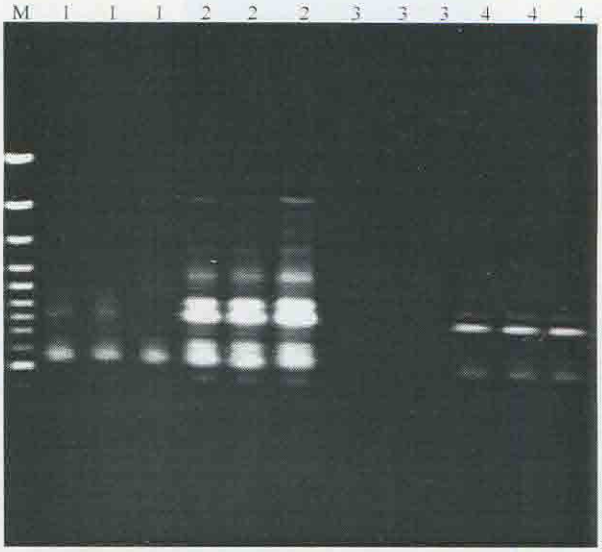

Fig. 5 : Comparison of PCR amplification of sandalwood genomic DNA isolated by different methods with the primer 5′-CCGAATTCCC

(Each sample was amplified in triplicate to assess the reproducibility of the reaction. Method 2 showed good amplification, Method 1 and 4 showed poor amplification, where as Method 3 showed no amplification)

Lane M : 100 bp DNA Ladder

Lanes 1 : DNA obtained by method 1 (Doyle and Doyle, 1987)

Lanes 2 : DNA obtained by method 2 (Porebski *et al.*, 1997)

Lanes 3 : DNA obtained by method 3 (Edwards *et al.*, 1991)

Lanes 4 : DNA obtained by method 4 (Do and Adams, 1991)

Fig. 6a : Agarose (0.8%) gel showing high molecular weight sandalwood genomic DNA purified by modified CTAB method (Method 2)

Lane M : λ DNA/*Hind*III digest marker ; Lanes 1, 2, 3, 4 and 5 DNA isolated from sandalwood clones KL1, K35, K37, T2 & AP4 respectively

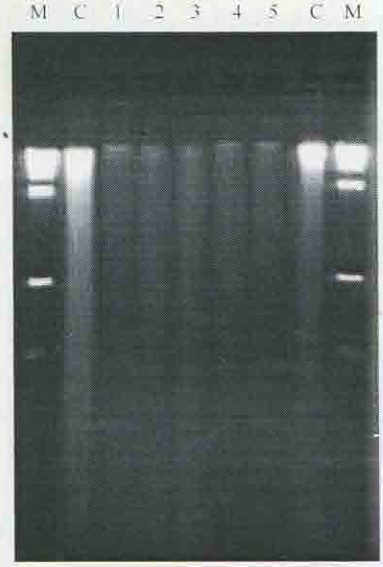

Fig. 6b : Restriction enzyme digestion of sandalwood genomic DNA purified by modified CTAB method (Method 2)

Lane M : λ DNA/*Hind*III digest marker; Lane C : Uncut/undigested DNA
Lanes 1, 2, 3, 4, 5 DNA digested with restriction enzymes *Bam*HI, *Hind*III, *Pst*I, *Hae*IIII and *Eco*RI respectively.

Genomic DNA was extracted from 51 clones of *S. album* and 3 genotypes of *S. spicatum*. For *S. album*, the yield of DNA varies between 58 µg (T20) to 126 µg (KL1) per gram dry leaf tissue (approximately corresponding to 3-4 g of fresh leaf tissue), where as for *S. spicatum*, the DNA yield ranged from 67 µg to 96 µg/g tissue (Table 4). The purity of DNA as determined by A_{260}/A_{280} ratio varied from 1.70 to 2.01 (Table 4) indicating that the DNA was relatively of high purity and was suitable for PCR amplification.

4.2 Standardization of protocol for PCR based RAPD analysis

4.2.1 Amplification conditions

For fingerprinting and diversity analysis, PCR amplification conditions were optimized based on the protocol outlined by Williams *et al.* (1990) and Welsh and McClelland (1990) with minor modifications.

In order to obtain high amplification rate and reproducible banding patterns, different durations for hot start, denaturation, primer annealing and primer extension was tried. The PCR reaction was evaluated for 30, 40 and 45 cycles using standard buffer outlined in Material and Methods (3.4.1). From the results, the optimum condition for each cycle of PCR was developed for obtaining high amplification levels (Table 5). The optimum PCR condition consisted of the following steps, which were repeated for 45 times.

Initial strand separation or hot start at 95^0 C for four minutes followed by, 45 cycles of,

(i) Denaturation at 94^0 C for 1 minute

(ii) Primer annealing at 35^0 C for two minutes

(iii) Primer extension at 72^0 C for two minutes and

A final extension period of 10 minutes at 72^0 C.

Table 4 : Yield and absorbance ratios of DNA from 54 sandalwood genotypes obtained after purification

Sl. No.	Clone/ genotype	DNA yield (µg/g dry wt.)	OD ratio (260 : 280)	Sl. No.	Clone/ Genotype	DNA yield (µg/g dry wt.)	OD ratio (260 : 280)
1	T4	64	1.81	28	T5	96	1.75
2	KL1	126	1.80	29	T13	85	1.90
3	K35	96	1.87	30	K30	118	1.82
4	K10	59	1.80	31	K31	110	1.81
5	K29	96	1.80	32	T7	96	1.89
6	K37	83	1.90	33	K6	74	1.81
7	K19	119	1.82	34	T19	120	1.90
8	T20	58	1.70	35	T27	84	2.00
9	T22	84	2.00	36	K9	63	1.92
10	T3	105	1.92	37	T14	92	1.80
11	T24	83	1.86	38	T12	78	1.96
12	K23	70	1.84	39	T6	96	1.86
13	K28	92	1.91	40	K11	104	1.98
14	T26	112	1.91	41	T9	77	1.98
15	K16	87	2.01	42	K34	90	1.91
16	K8	110	1.90	43	T28	84	1.80
17	K4	91	1.80	44	K5	89	1.80
18	T30	114	1.81	45	T23	110	2.00
19	T11	91	1.87	46	T1	122	1.81
20	K13	86	2.01	47	T29	84	1.80
21	K2	80	1.81	48	T21	96	1.80
22	KL3	78	1.80	49	K32	98	1.87
23	K27	102	1.80	50	K36	102	2.03
24	T2	122	1.92	51	K7	98	1.88
25	AP4	97	1.80	52	*S. spicatum* 1	72	1.91
26	K14	66	1.81	53	*S. spicatum* 2	67	1.80
27	T8	120	1.83	54	*S. spicatum* 3	96	1.80

4.2.2. Reaction parameters

It is important to optimize the concentration of PCR mixture, in order to produce informative and reproducible RAPD fingerprints. Hence different concentrations of template DNA (10-15 ng, 25-30 ng and 40-50 ng), $MgCl_2$ (1.0 mM, 1.5 mM, 2.0 mM and 2.5 mM) and dNTPs (150 μM, 200 μM, 215 μM and 225 μM) were tried with the similar amplification conditions. A concentration of 25-30 ng of template DNA, 2.0 mM of $MgCl_2$ and 215 μM of dNTPs per reaction was found optimum for obtaining intense, clear and reproducible banding pattern in sandalwood (Table 5). In all the cases, 5 pico moles of primer and 1 unit of *Taq* polymerase per reaction were used. However, fluctuation in the concentration of template DNA did affect the amplification, with too little DNA (10-15 ng) causing either reduced or no amplification of small fragments and higher concentration of DNA (40-50 ng) producing a smear (Fig. 7a).

A dNTP concentration of 215 μM was found adequate for generating reproducible RAPDs. At lower concentrations (150 μM) the intensity of stained bands in the gel became progressively weaker, and at higher concentrations smearing of the bands was evident (Fig. 7b). Magnesium ion concentration did affect the relative intensity of amplified bands. As the magnesium ion concentration increases, some DNA segments are amplified more efficiently while others are amplified less efficiently (Fig. 7c). For reproducibility of RAPDs, 2.0 mM $MgCl_2$ over the total nucleotide concentration was found optimum.

4.2.3 Primer selection for RAPD analysis

After screening 220 random primers of arbitrary sequence (*viz.*, Operon primers A to K series), eleven primers (OPB12, OPE15, OPE20, OPF2, OPF5, OPF10, OPF12, OPH13, OPJ10, OPJ20 & OPK12) that yielded maximum number of bands that were consistent and clear were selected for fingerprinting and diversity analysis (Table 6).

Table 5 : Optimum concentration and conditions for RAPD analysis

Variable	Concentrations / Conditions	
	Evaluated	Optimum
PCR amplification		
Hot start (95^0 C)	2 min, 3 min, 4 min, 5 min	4 min
Denaturation (94^0 C)	30 sec, 1 min, 1.5 min	1 min
Annealing (35^0 C)	1 min, 1.5 min, 2.0 min	2.0 min
Extension (72^0 C)	1 min, 1.5 min, 2.0 min	2.0 min
Number of cycles	30, 40, 45 cycles	45 cycles
RAPD protocol		
Template DNA	10-15 ng, 25-30 ng, 40-50 ng	25-30 ng
$MgCl_2$	1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM	2.0 mM
dNTPs	150 μM, 200 μM, 215 μM, 22 5 μM	215 μM

Table 6 : The random primers with their nucleotide sequence selected for RAPD analysis

Sl. No.	Primer code	Nucleotide sequence (5′-3′)
1	OPB12	CCTTGACGCA
2	OPE15	ACGCACAACC
3	OPE20	AACGGTGACC
4	OPF2	GAGGATCCCT
5	OPF5	CCGAATTCCC
6	OPF10	GGAAGCTTGG
7	OPF12	ACGGTACCAG
8	OPH13	GACGCCACAC
9	OPJ10	AAGCCCGAGG
10	OPJ20	AAGCGGCCTC
11	OPK12	TGGCCCTCAC

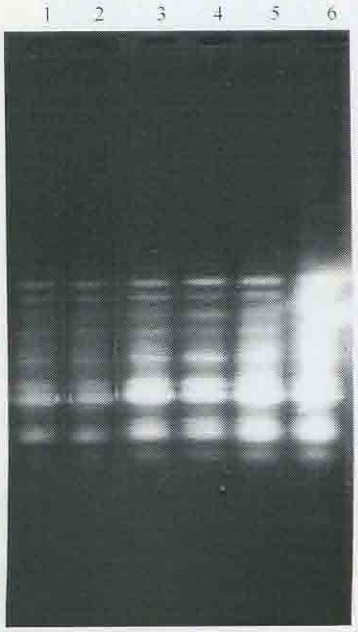

Fig. 7a : Effect of sandalwood genomic DNA concentration on PCR amplification (Different amounts of sandalwood genomic DNA were amplified under standard PCR conditions with the primer 5'- ACGGTACCAG. Each sample was amplified in duplicate to assess the reproducibility of the reaction. Lanes 1 & 2 : 10-15 ng DNA, Lanes 3 & 4 : 25-30 ng DNA, Lanes 5 & 6 : 40-50 ng DNA. Note: Too little DNA causing reduced amplification and higher amounts producing a smear.

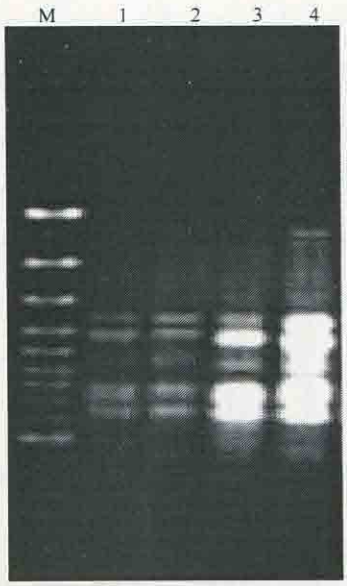

Fig. 7b : Effect of dNTPs concentration on PCR amplification
Sandalwood genomic DNA was amplified with the primer 5'-GGAAGCTTGG under standard PCR conditions. Lane M : 100 bp DNA ladder, Lanes 1, 2, 3 & 4 corresponds to dNTP concentrations 150 μM, 200 μM, 215 μM and 225 μM respectively. Note : As the dNTP concentration increases, smearing of the bands was observed.

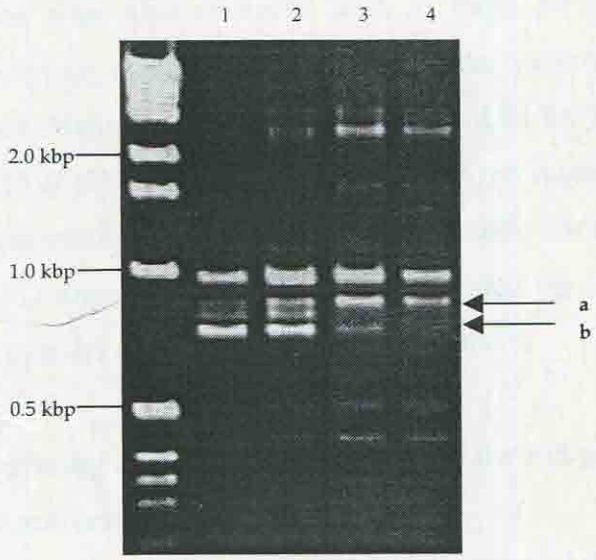

Fig. 7c : Effect of magnesium chloride concentration on PCR amplification
Sandalwood genomic DNA was amplified with the primer 5'-ACGGTACCAG under standard PCR conditions. Lanes 1, 2, 3 & 4 corresponds to$MgCl_2$ concentration 1.0 mM, 1.5 mM, 2.0 mM & 2.5 mM respectively. Note: As the $MgCl_2$ concentration increases, amplification of band '**a**' increases, while that of band '**b**' decreases.

4.3 DNA fingerprinting and genetic diversity analysis in sandalwood

4.3.1 RAPD profile analysis

A total of 156 RAPD markers ranging from 100 bp to 4 kbp that were consistent, unambiguous and repeatable produced from the selected eleven primers were used for fingerprinting and to estimate genetic diversity among 54 genotypes of sandalwood. The number of markers or bands scored for each primer varied from 12 to 17 with an average of 14.1 bands per primer (Table 7).

Out of 156 amplification products recorded, 8.3 per cent were monomorphic common to all the genotypes, 7.05 per cent were unique present in only one genotype and 84.6 per cent were polymorphic (shared by atleast two genotypes), which were informative in revealing the relationship between the genotypes. Among the selected primers, OPJ10 produced maximum number of polymorphic bands (16) followed by OPF5 and OPK12 (15 bands each), while OPJ20 produced maximum number of unique bands (6). In addition, bands that could clearly distinguished between the Indian and exotic genotypes were also observed such as band A^1 (monomorphic across Indian genotypes and absent in Australian genotypes) and S^1 (monomorphic across Australian genotypes and absent in Indian genotypes) (Table 7; Figures 9, 15 & 17). This pool of primers yielded reasonable number of polymorphic fragments for all the genotypes examined. For the purpose of illustration, the RAPD fingerprints/gel profiles generated for 54 sandalwood genotypes using 11 primers are presented in figures 8 to 18.

4.3.2 DNA fingerprinting and identification of sandalwood genotypes using RAPD markers

To determine the suitability of RAPDs to fingerprint 54 sandalwood genotypes, some of which were closely related, eleven primers of arbitrary nucleotide sequence (Table 6) were used to amplify genomic DNA. Before all eleven primers were used on each genotype, a reproducibility study was

Table 7 : Total number of amplified fragments and number of polymorphic bands generated by PCR using eleven selected random primers

Primer	Polymorphic bands		Monomorphic bands			Total	Band size
	Shared	Unique	A^1	S^1	Both		
OPB12	14	0	0	0	0	14	300bp-2.8kbp
OPE15	8	1(A)	1	1	1	12	400bp-2.8kbp
OPE20	11	2(S)	0	0	0	13	100bp-3.0kbp
OPF2	11	0	0	0	1	12	250bp-3.0kbp
OPF5	15	0	0	0	0	15	400bp-2.8kbp
OPF10	10	0	0	0	4	14	200bp-3.0kbp
OPF12	14	0	0	0	0	14	200bp-2.9kbp
OPH13	10	1(A)	1	0	1	13	500bp-2.6kbp
OPJ10	16	0	0	0	0	16	200bp-4.0kbp
OPJ20	8	6(A)	0	1	2	17	400bp-3.0kbp
OPK12	15	1	0	0	0	16	300bp-3.0kbp
Total	132	11	2	2	9	156	

A : *S. album* S : *S. spicatum*

A^1 : Bands found to be monomorphic across *S. album* and absent in *S. spicatum*

S^1 : Bands found to be monomorphic across *S. spicatum* and absent in *S. album*

carried out on one primer chosen at random to ensure fingerprints produced from all the 54 genotypes were accurate and reproducible. The primer OPE15 was, therefore, used in ten PCR reactions under the same conditions. Twelve markers were produced with the band size ranging from 400 bp to 2.8 kb (Table 7), eight of which were completely reproducible in whatever genotypes they occurred. The 965 bp band was only completely reliable in the genotype K37. The 1115 bp molecular mass band was reliable in all the Indian genotypes and absent in exotics (Fig. 9).

For fingerprinting, eleven random primers were used (Table 6). The PCR reactions were repeated five times per primer, and the bands that appeared consistently in 4 or 5 gels were used in the study. An average of 14.1 bands were produced per primer. The number of genotypes identified per primer is shown in Table 8.

Eleven primers used in the present study were able to identify 50 of the 54 genotypes, ranging from 7 to 26 genotypes per primer. The genotypes T2, T12, T13 and T27 could not be differentiated with the eleven primers examined. No primer was able to completely resolve all the 54 genotypes studied. For fingerprinting, each band was treated on an equal footing. The primer OPJ20 produced maximum number of bands (17) and could distinguish only seven genotypes from the others (Table 8). OPE20 on the other hand generated only 13 bands that could distinguish 26 genotypes (Table 8). A combination of three primers *viz.*, OPB12, OPE20 and OPF12 resolved majority of the genotypes (41) by atleast three markers/bands.

4.3.3 Genetic diversity analysis of sandalwood genotypes

4.3.3.1 Cluster analysis and genetic dissimilarity matrix of 54 sandalwood genotypes

RAPD bands were manually scored from the gel profile, '1' for the presence and '0' for the absence and the binary data generated from all the

Table 8 : Decamer primer and the sandalwood genotypes identified by the bands produced

Sl. No.	Primer code	Nucleotide sequence (5′-3′)	Number of sandalwood genotypes identified	
1	OPB12	CCTTGACGCA	23	T4, KL1, K35, K10, K37, K19, T20, K13, KL3, K27, T8, K31, T7, K6, K11, T28, K5, T23, K32, K7, *S. spicatum* 1, *S. spicatum* 2, *S. spicatum* 3
2	OPE15	ACGCACAACC	10	KL1, K29, K8, K31, T7, K6, T1, *S. spicatum* 1, *S. spicatum* 2, *S. spicatum* 3
3	OPE20	AACGGTGACC	26	T4, KL1, K35, K10, K29, K37, T20, T22, T24, K23, K28, K8, K4, K13, K27, T8, T5, T19, K9, T14, K11, T28, K32, *S. spicatum* 1, *S. spicatum* 2, *S. spicatum* 3
4	OPF2	GAGGATCCCT	12	KL1, T26, K4, K14, K6, T19, T6, K11, K5, K36, K7, K32
5	OPF5	CCGAATTCCC	7	T3, K23, K8, K4, K9, T21, *S. spicatum* 3
6	OPF10	GGAAGCTTGG	11	KL1, K35, K10, T20, K31, T7, K6, K5, T21, K36, *S. spicatum* 3
7	OPF12	ACGGTACCAG	24	T4, K35, K29, K37, T20, T24, K16, K4, T11, K13, K2, K27, AP4, K14, K30, K31, K9, T6, K11, T28, T21, K32, *S. spicatum* 2, *S. spicatum* 3
8	OPH13	GACGCCACAC	7	K37, T3, K6, K7, *S. spicatum* 1, *S. spicatum* 2, *S. spicatum* 3
9	OPJ10	AAGCCCGAGG	10	K35, K37, T26, T30, K2, K5, T23, T1, T21, K36
10	OPJ20	AAGCGGCCTC	7	K37, K4, K31, T7, K9, K5, T29
11	OPK12	TGGCCCTCAC	11	K37, T20, T22, T3, K23, K8, K13, K14, K6, T9, K34

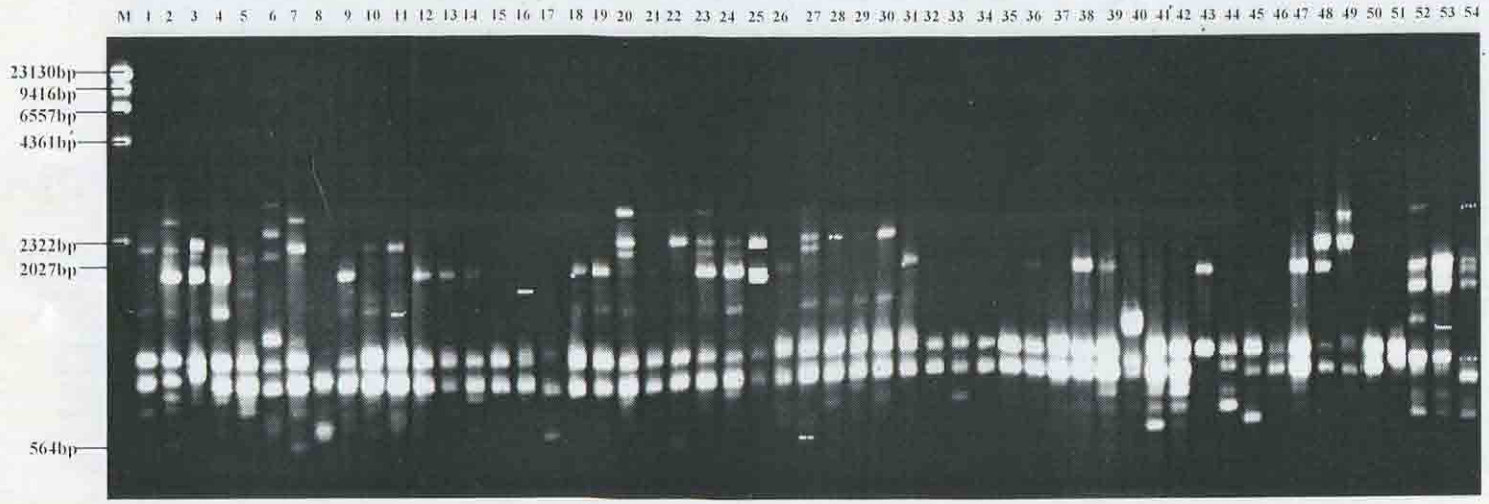

Fig. 8 : RAPD gel profile of sandalwood clones generated using 10-mer random primer OPB12

Lane M : λ DNA/*Hind*III Digest, Lanes 1 – 54 sandalwood clones (lanes 1 : T4, 2 : KL1, 3 : K35, 4 : K10, 5 : K29, 6 : K37, 7 : K19, 8 : T20, 9 : T22, 10 : T3, 11 : T24, 12 : K23, 13 : K28, 14 : T26, 15 : K16, 16 : K8, 17 : K4, 18 : T30, 19 : T11, 20 : K13, 21 : K2, 22 : KL3, 23 : K27, 24 : T2, 25 : AP4, 26 : K14, 27 : T8, 28 : T5, 29 : T13, 30 : K30, 31 : K31, 32 : T7, 33 : K6, 34 : T19, 35 : T27, 36 : K9, 37 : T14, 38 :T12, 39 : T6, 40 : K11, 41 : T9, 42 : K34, 43 : T28, 44 : K5, 45 : T23, 46 : T1, 47 : T29, 48 : T21, 49 : K32, 50 : K36, 51 : K7, 52 : *S.spicatum* 1, 53 : *S. spicatum* 2, 54 : *S.spicatum* 3)

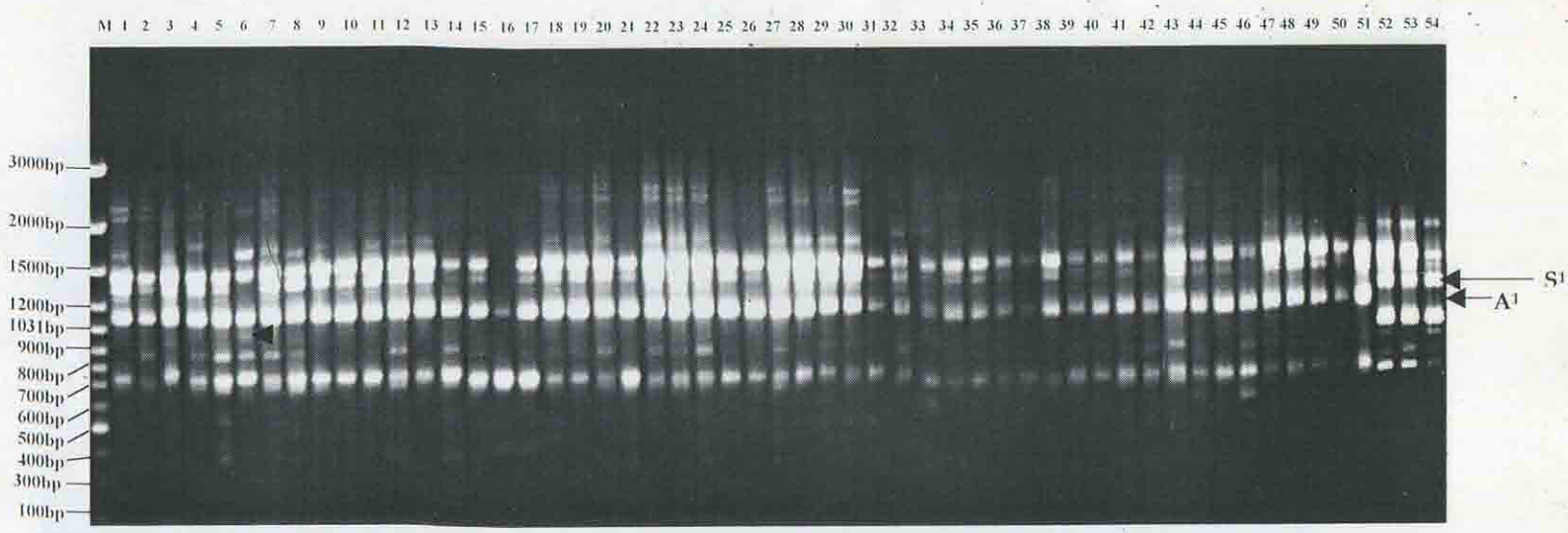

Fig. 9 : RAPD gel profile of sandalwood clones generated using 10-mer random primer OPE15

Lane M : 100bp DNA Ladder, Lanes 1 – 54 sandalwood clones (lanes 1 : T4, 2 : KL1, 3 : K35, 4 : K10, 5 : K29, 6 : K37, 7 : K19, 8 : T20, 9 : T22, 10 : T3, 11 : T24, 12 : K23, 13 : K28, 14 : T26, 15 : K16, 16 : K8, 17 : K4, 18 : T30, 19 : T11, 20 : K13, 21 : K2, 22 : KL3, 23 : K27, 24 : T2, 25 : AP4, 26 : K14, 27 : T8, 28 : T5, 29 : T13, 30 : K30, 31 : K31, 32 : T7, 33 : K6, 34 : T19, 35 : T27, 36 : K9, 37 : T14, 38 :T12, 39 : T6, 40 : K11, 41 : T9, 42 : K34, 43 : T28, 44 : K5, 45 : T23, 46 : T1, 47 : T29, 48 : T21, 49 : K32, 50 : K36, 51 : K7, 52 : *S.spicatum* 1, 53 : *S. spicatum* 2, 54 : *S.spicatum* 3) , **S^1** : 1400bp band monomorphic across *S. spicatum*, **A^1** : 1115bp band monomorphic across *S. album*, Arrowhead in lane 6 shows 965bp genotype specific band for clone K37

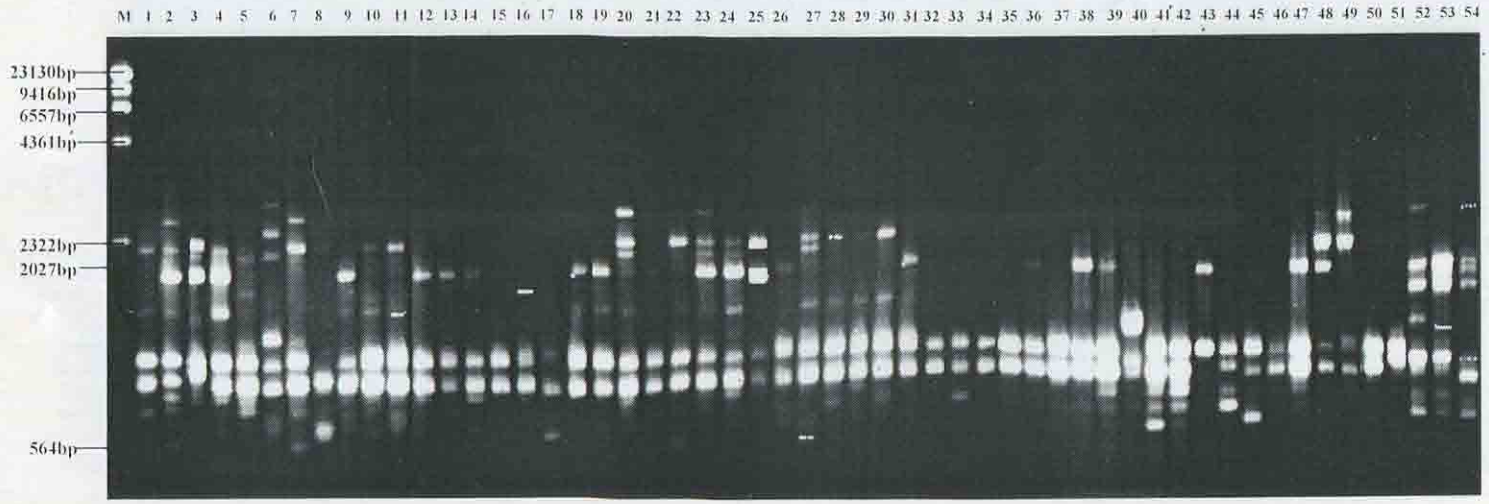

Fig. 8 : RAPD gel profile of sandalwood clones generated using 10-mer random primer OPB12

Lane M : λ DNA/*Hind*III Digest, Lanes 1 – 54 sandalwood clones (lanes 1 : T4, 2 : KL1, 3 : K35, 4 : K10, 5 : K29, 6 : K37, 7 : K19, 8 : T20, 9 : T22, 10 : T3, 11 : T24, 12 : K23, 13 : K28, 14 : T26, 15 : K16, 16 : K8, 17 : K4, 18 : T30, 19 : T11, 20 : K13, 21 : K2, 22 : KL3, 23 : K27, 24 : T2, 25 : AP4, 26 : K14, 27 : T8, 28 : T5, 29 : T13, 30 : K30, 31 : K31, 32 : T7, 33 : K6, 34 : T19, 35 : T27, 36 : K9, 37 : T14, 38 :T12, 39 : T6, 40 : K11, 41 : T9, 42 : K34, 43 : T28, 44 : K5, 45 : T23, 46 : T1, 47 : T29, 48 : T21, 49 : K32, 50 : K36, 51 : K7, 52 : *S.spicatum* 1, 53 : *S. spicatum* 2, 54 : *S.spicatum* 3)

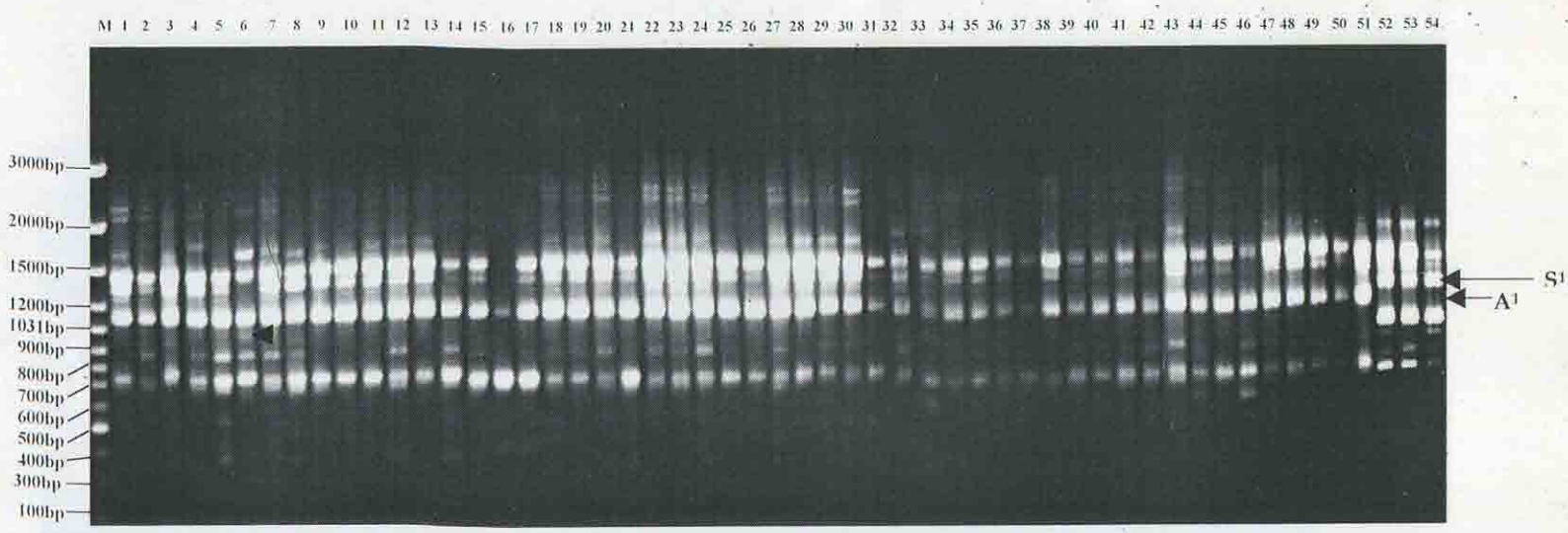

Fig. 9 : RAPD gel profile of sandalwood clones generated using 10-mer random primer OPE15

Lane M : 100bp DNA Ladder, Lanes 1 – 54 sandalwood clones (lanes 1 : T4, 2 : KL1, 3 : K35, 4 : K10, 5 : K29, 6 : K37, 7 : K19, 8 : T20, 9 : T22, 10 : T3, 11 : T24, 12 : K23, 13 : K28, 14 : T26, 15 : K16, 16 : K8, 17 : K4, 18 : T30, 19 : T11, 20 : K13, 21 : K2, 22 : KL3, 23 : K27, 24 : T2, 25 : AP4, 26 : K14, 27 : T8, 28 : T5, 29 : T13, 30 : K30, 31 : K31, 32 : T7, 33 : K6, 34 : T19, 35 : T27, 36 : K9, 37 : T14, 38 :T12, 39 : T6, 40 : K11, 41 : T9, 42 : K34, 43 : T28, 44 : K5, 45 : T23, 46 : T1, 47 : T29, 48 : T21, 49 : K32, 50 : K36, 51 : K7, 52 : *S.spicatum* 1, 53 : *S. spicatum* 2, 54 : *S.spicatum* 3) , **S^1** : 1400bp band monomorphic across *S. spicatum*, **A^1** : 1115bp band monomorphic across *S. album*, Arrowhead in lane 6 shows 965bp genotype specific band for clone K37

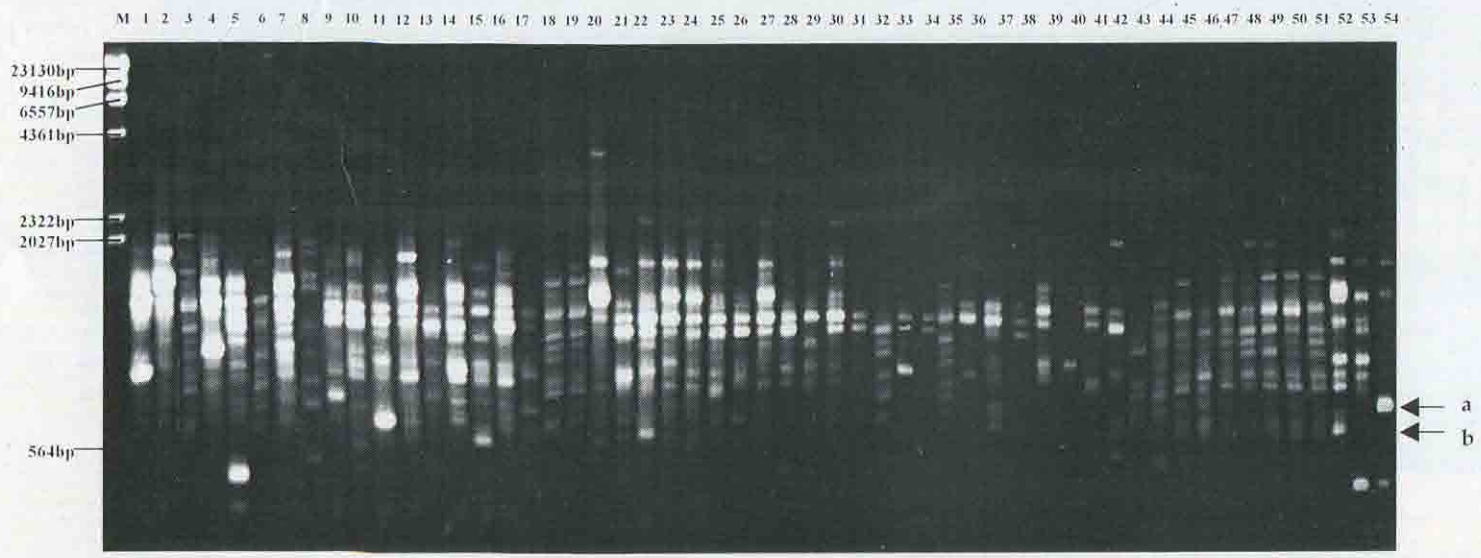

Fig. 10 : RAPD gel profile of sandalwood clones generated using 10-mer random primer OPE 20

Lane M : λ DNA/*Hind*III Digest, Lanes 1 – 54 sandalwood clones (lanes 1 : T4, 2 : KL1, 3 : K35, 4 : K10, 5 : K29, 6 : K37, 7 : K19, 8 : T20, 9 : T22, 10 : T3, 11 : T24, 12 : K23, 13 : K28, 14 : T26, 15 : K16, 16 : K8, 17 : K4, 18 : T30, 19 : T11, 20 : K13, 21 : K2, 22 : KL3, 23 : K27, 24 : T2, 25 : AP4, 26 : K14, 27 : T8, 28 : T5, 29 : T13, 30 : K30, 31 : K31, 32 : T7, 33 : K6, 34 : T19, 35 : T27, 36 : K9, 37 : T14, 38 :T12, 39 : T6, 40 : K11, 41 : T9, 42 : K34, 43 : T28, 44 : K5, 45 : T23, 46 : T1, 47 : T29, 48 : T21, 49 : K32, 50 : K36, 51 : K7, 52 : *S.spicatum* 1, 53 : *S. spicatum* 2, 54 : *S.spicatum* 3), Arrowhead shows the genotype specific bands '**a**' and '**b**' specific to *S. spicatum* 3 and *S. spicatum* 1 respectively.

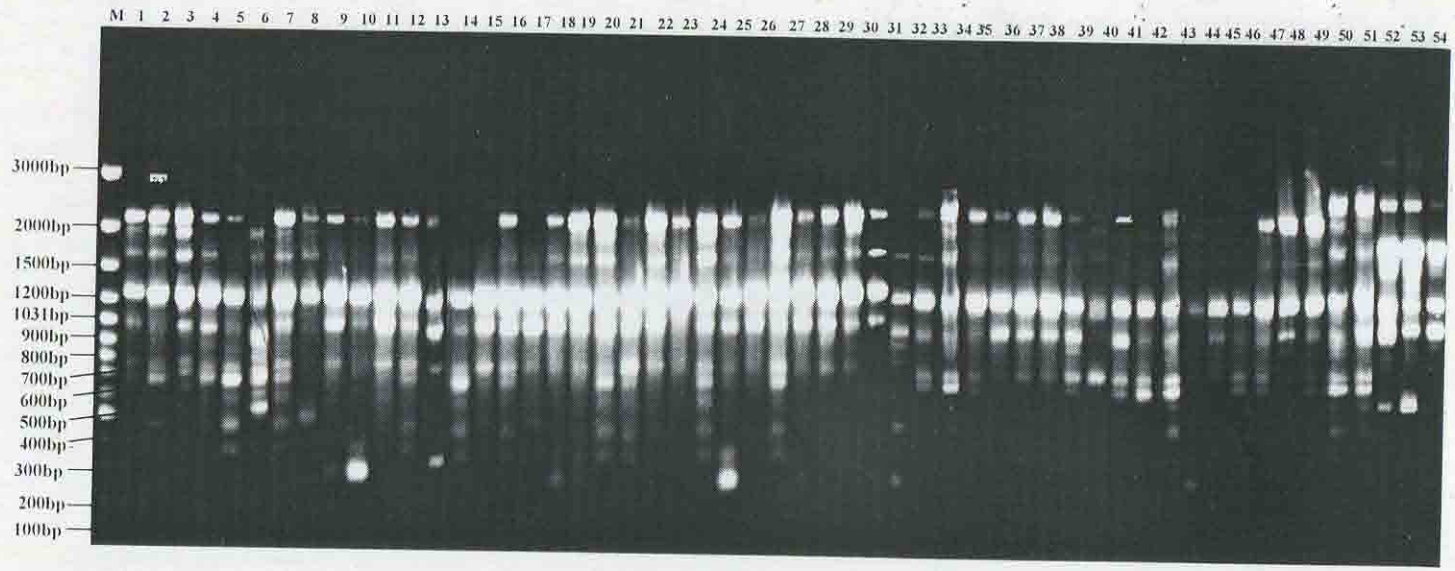

Fig. 11 : RAPD gel profile of sandalwood clones generated using 10-mer random primer OPF2

Lane M : 100bp DNA Ladder, Lanes 1 – 54 sandalwood clones (lanes 1 : T4, 2 : KL1, 3 : K35, 4 : K10, 5 : K29, 6 : K37, 7 : K19, 8 : T20, 9 : T22, 10 : T3, 11 : T24, 12 : K23, 13 : K28, 14 : T26, 15 : K16, 16 : K8, 17 : K4, 18 : T30, 19 : T11, 20 : K13, 21 : K2, 22 : KL3, 23 : K27, 24 : T2, 25 : AP4, 26 : K14, 27 : T8, 28 : T5, 29 : T13, 30 : K30, 31 : K31, 32 : T7, 33 : K6, 34 : T19, 35 : T27, 36 : K9, 37 : T14, 38 :T12, 39 : T6, 40 : K11, 41 : T9, 42 : K34, 43 : T28, 44 : K5, 45 : T23, 46 : T1, 47 : T29, 48 : T21, 49 : K32, 50 : K36, 51 : K7, 52 : *S.spicatum* 1, 53 : *S. spicatum* 2, 54 : *S.spicatum* 3)

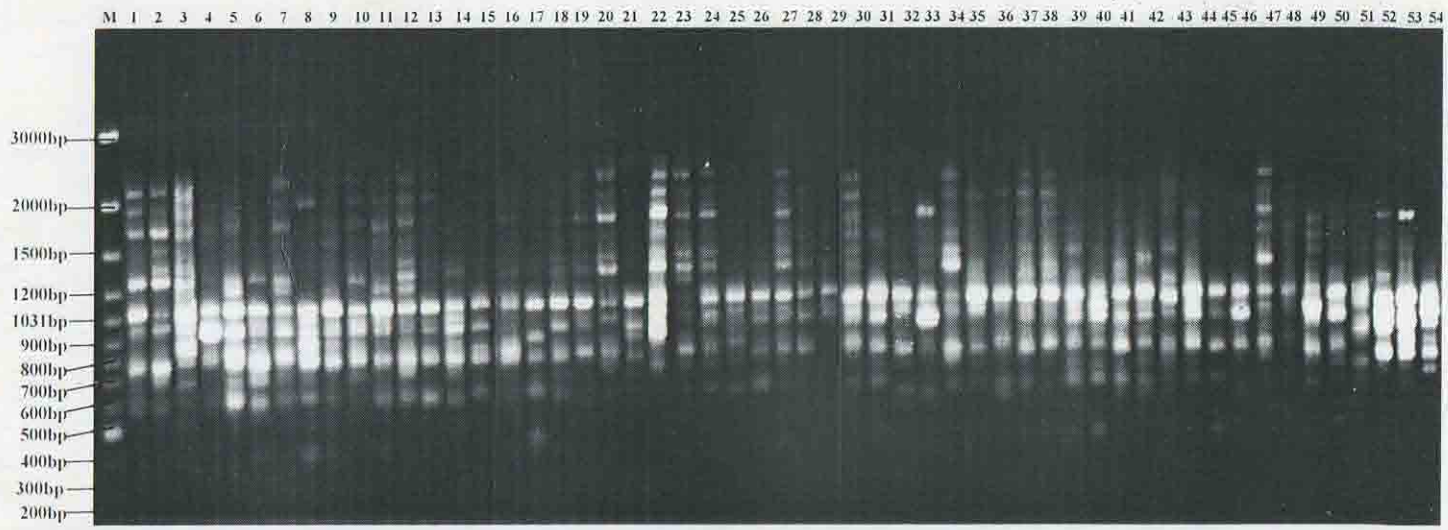

Fig. 12 : RAPD gel profile of sandalwood clones generated using 10-mer random primer OPF5

Lane M : 100bp DNA Ladder, Lanes 1 – 54 sandalwood clones (lanes 1 : T4, 2 : KL1, 3 : K35, 4 : K10, 5 : K29, 6 : K37, 7 : K19, 8 : T20, 9 : T22, 10 : T3, 11 : T24, 12 : K23, 13 : K28, 14 : T26, 15 : K16, 16 : K8, 17 : K4, 18 : T30, 19 : T11, 20 : K13, 21 : K2, 22 : KL3, 23 : K27, 24 : T2, 25 : AP4, 26 : K14, 27 : T8, 28 : T5, 29 : T13, 30 : K30, 31 : K31, 32 : T7, 33 : K6, 34 : T19, 35 : T27, 36 : K9, 37 : T14, 38 :T12, 39 : T6, 40 : K11, 41 : T9, 42 : K34, 43 : T28, 44 : K5, 45 : T23, 46 : T1, 47 : T29, 48 : T21, 49 : K32, 50 : K36, 51 : K7, 52 : *S.spicatum* 1, 53 : *S. spicatum* 2, 54 : *S.spicatum* 3)

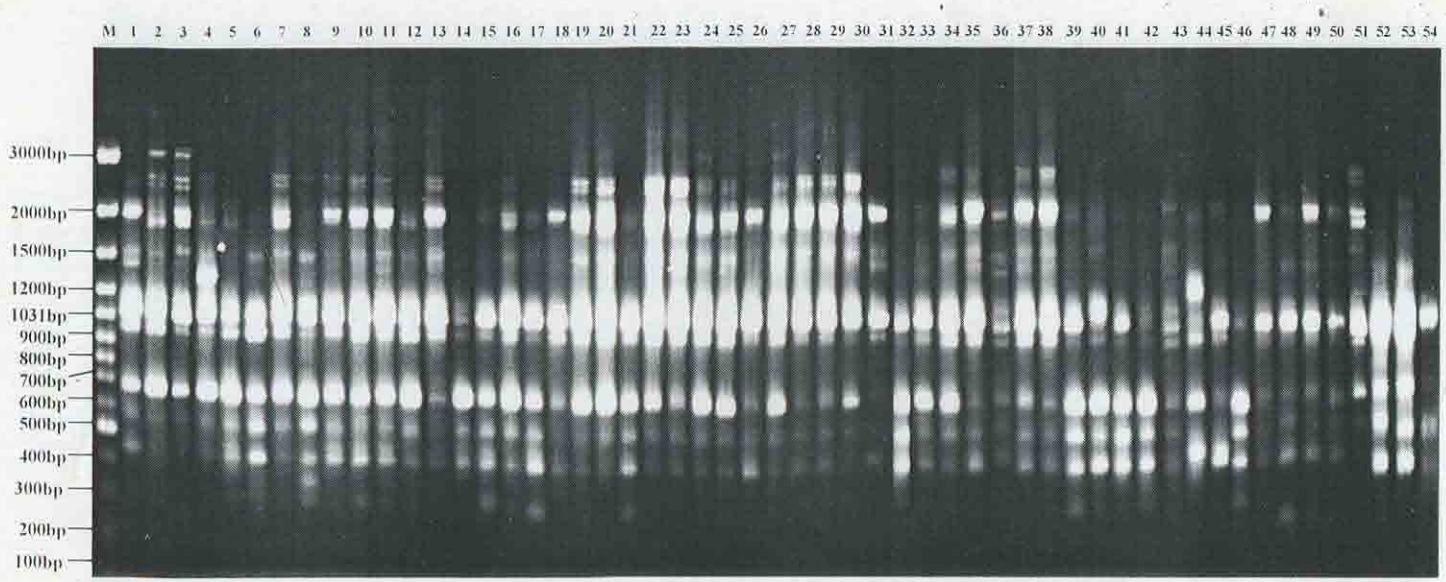

Fig. 13 : RAPD gel profile of sandalwood clones generated using 10-mer random primer OPF10

Lane M : 100bp DNA Ladder, Lanes 1 – 54 sandalwood clones (lanes 1 : T4, 2 : KL1, 3 : K35, 4 : K10, 5 : K29, 6 : K37, 7 : K19, 8 : T20, 9 : T22, 10 : T3, 11 : T24, 12 : K23, 13 : K28, 14 : T26, 15 : K16, 16 : K8, 17 : K4, 18 : T30, 19 : T11, 20 : K13, 21 : K2, 22 : KL3, 23 : K27, 24 : T2, 25 : AP4, 26 : K14, 27 : T8, 28 : T5, 29 : T13, 30 : K30, 31 : K31, 32 : T7, 33 : K6, 34 : T19, 35 : T27, 36 : K9, 37 : T14, 38 :T12, 39 : T6, 40 : K11, 41 : T9, 42 : K34, 43 : T28, 44 : K5, 45 : T23, 46 : T1, 47 : T29, 48 : T21, 49 : K32, 50 : K36, 51 : K7, 52 : *S.spicatum* 1, 53 : *S. spicatum* 2, 54 : *S.spicatum* 3)

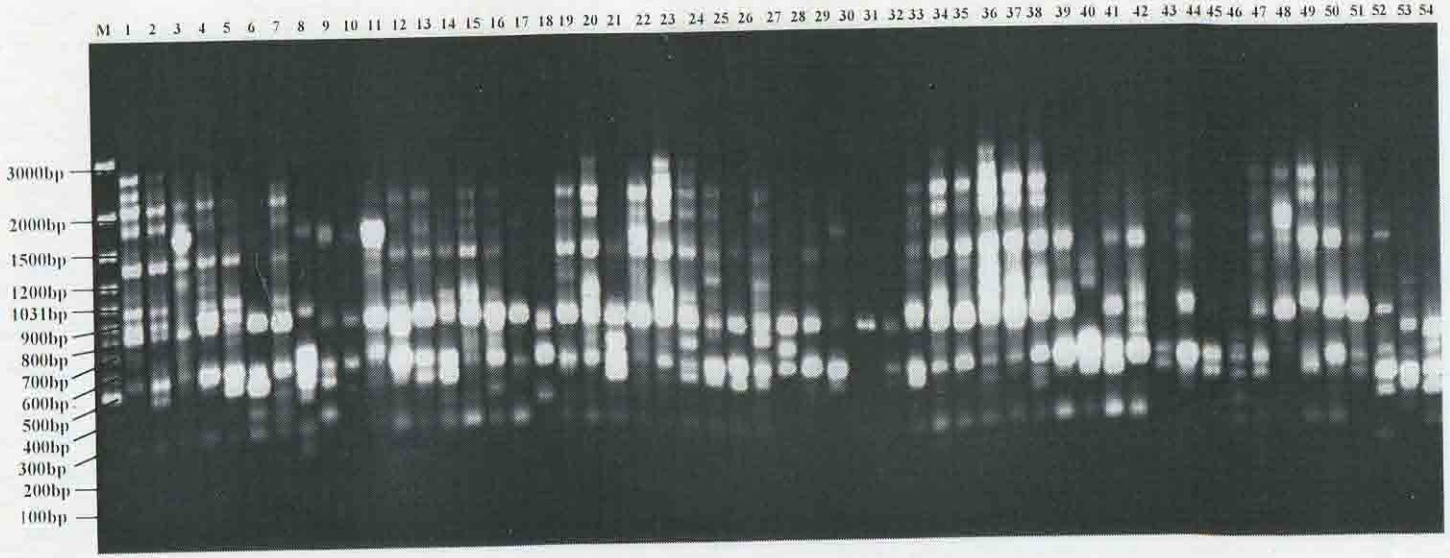

Fig. 14 : RAPD gel profile of sandalwood clones generated using 10-mer random primer OPF12

Lane M : 100bp DNA Ladder, Lanes 1 – 54 sandalwood clones (lanes 1 : T4, 2 : KL1, 3 : K35, 4 : K10, 5 : K29, 6 : K37, 7 : K19, 8 : T20, 9 : T22, 10 : T3, 11 : T24, 12 : K23, 13 : K28, 14 : T26, 15 : K16, 16 : K8, 17 : K4, 18 : T30, 19 : T11, 20 : K13, 21 : K2, 22 : KL3, 23 : K27, 24 : T2, 25 : AP4, 26 : K14, 27 : T8, 28 : T5, 29 : T13, 30 : K30, 31 : K31, 32 : T7, 33 : K6, 34 : T19, 35 : T27, 36 : K9, 37 : T14, 38 :T12, 39 : T6, 40 : K11, 41 : T9, 42 : K34, 43 : T28, 44 : K5, 45 : T23, 46 : T1, 47 : T29, 48 : T21, 49 : K32, 50 : K36, 51 : K7, 52 : *S.spicatum* 1, 53 : *S. spicatum* 2, 54 : *S.spicatum* 3)

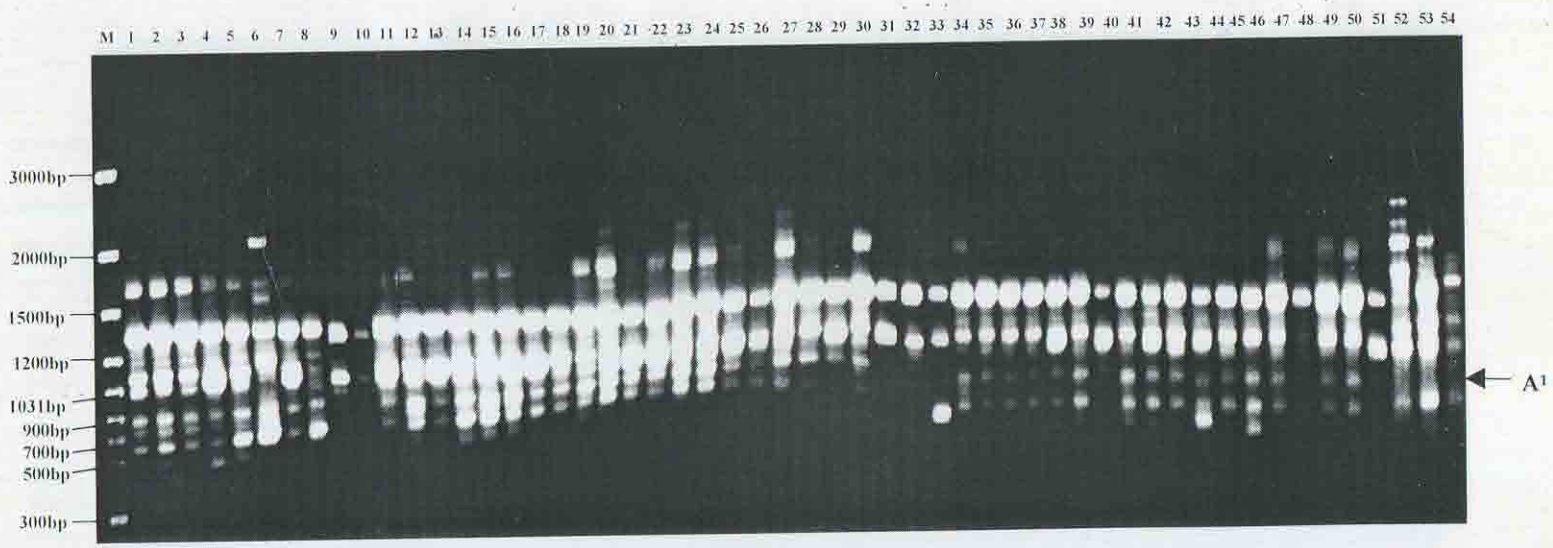

Fig. 15 : RAPD gel profile of sandalwood clones generated using 10-mer random primer OPH13

Lane M : 100bp DNA Ladder, Lanes 1 – 54 sandalwood clones (lanes 1 : T4, 2 : KL1, 3 : K35, 4 : K10, 5 : K29, 6 : K37, 7 : K19, 8 : T20, 9 : T22, 10 : T3, 11 : T24, 12 : K23, 13 : K28, 14 : T26, 15 : K16, 16 : K8, 17 : K4, 18 : T30, 19 : T11, 20 : K13, 21 : K2, 22 : KL3, 23 : K27, 24 : T2, 25 : AP4, 26 : K14, 27 : T8, 28 : T5, 29 : T13, 30 : K30, 31 : K31, 32 : T7, 33 : K6, 34 : T19, 35 : T27, 36 : K9, 37 : T14, 38 :T12, 39 : T6, 40 : K11, 41 : T9, 42 : K34, 43 : T28, 44 : K5, 45 : T23, 46 : T1, 47 : T29, 48 : T21, 49 : K32, 50 : K36, 51 : K7, 52 : *S.spicatum* 1, 53 : *S. spicatum* 2, 54 : *S.spicatum* 3), Arrowheads indicated in the lane 6 is the genotype specific bands for the clone K37, A^1 : 900bp band monomorphic across *S. album* that were absent in *S. spicatum*

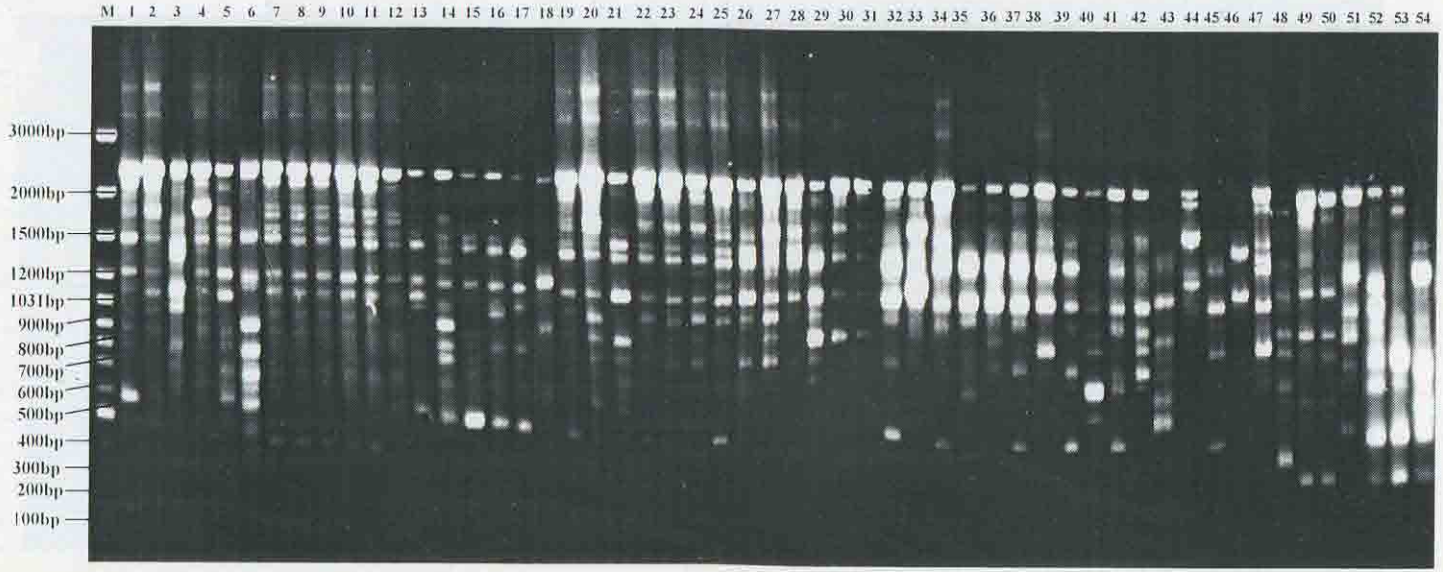

Fig. 16 : RAPD gel profile of sandalwood clones generated using 10-mer random primer OPJ10

Lane M : 100bp DNA Ladder, Lanes 1 – 54 sandalwood clones (lanes 1 : T4, 2 : KL1, 3 : K35, 4 : K10, 5 : K29, 6 : K37, 7 : K19, 8 : T20, 9 : T22, 10 : T3, 11 : T24, 12 : K23, 13 : K28, 14 : T26, 15 : K16, 16 : K8, 17 : K4, 18 : T30, 19 : T11, 20 : K13, 21 : K2, 22 : KL3, 23 : K27, 24 : T2, 25 : AP4, 26 : K14, 27 : T8, 28 : T5, 29 : T13, 30 : K30, 31 : K31, 32 : T7, 33 : K6, 34 : T19, 35 : T27, 36 : K9, 37 : T14, 38 :T12, 39 : T6, 40 : K11, 41 : T9, 42 : K34, 43 : T28, 44 : K5, 45 : T23, 46 : T1, 47 : T29, 48 : T21, 49 : K32, 50 : K36, 51 : K7, 52 : *S.spicatum* 1, 53 : *S. spicatum* 2, 54 : *S.spicatum* 3)

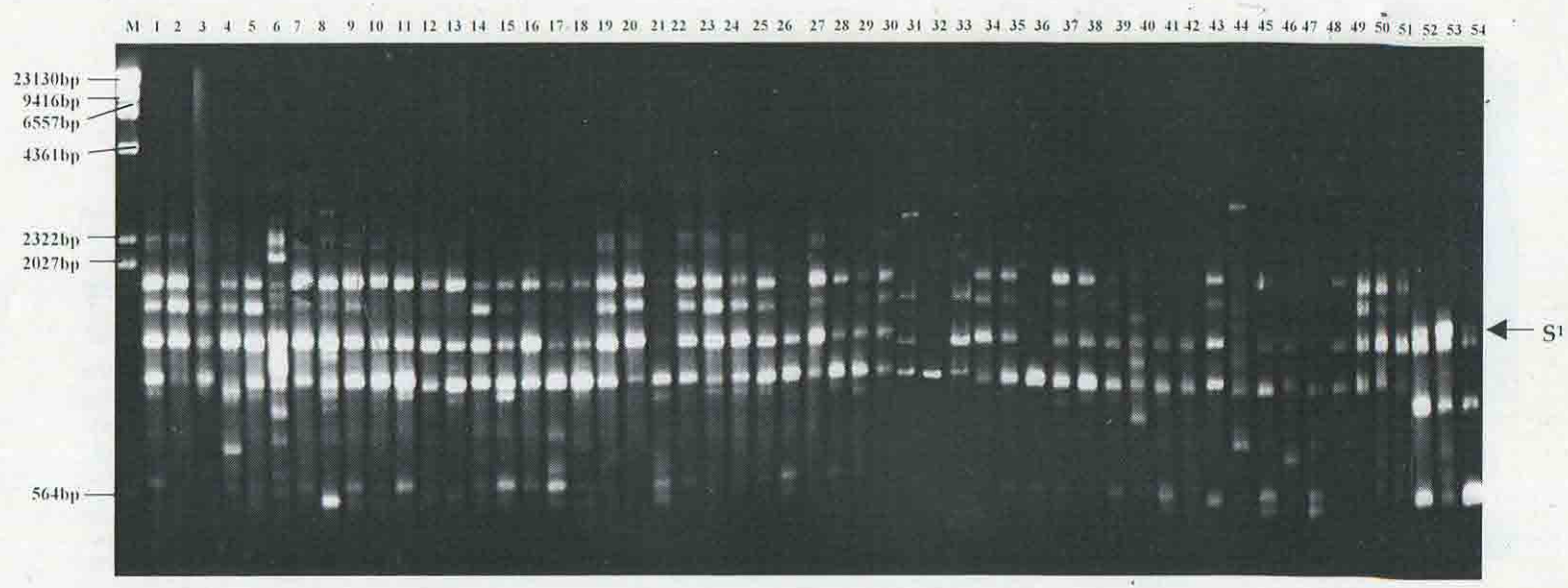

Fig. 17 : RAPD gel profile of sandalwood clones generated using 10-mer random primer OPJ20

Lane M : λ DNA/*Hind*III Digest, Lanes 1 – 54 sandalwood clones (lanes 1 : T4, 2 : KL1, 3 : K35, 4 : K10, 5 : K29, 6 : K37, 7 : K19, 8 : T20, 9 : T22, 10 : T3, 11 : T24, 12 : K23, 13 : K28, 14 : T26, 15 : K16, 16 : K8, 17 : K4, 18 : T30, 19 : T11, 20 : K13, 21 : K2, 22 : KL3, 23 : K27, 24 : T2, 25 : AP4, 26 : K14, 27 : T8, 28 : T5, 29 : T13, 30 : K30, 31 : K31, 32 : T7, 33 : K6, 34 : T19, 35 : T27, 36 : K9, 37 : T14, 38 :T12, 39 : T6, 40 : K11, 41 : T9, 42 : K34, 43 : T28, 44 : K5, 45 : T23, 46 : T1, 47 : T29, 48 : T21, 49 : K32, 50 : K36, 51 : K7, 52 : *S.spicatum* 1, 53 : *S. spicatum* 2, 54 : *S.spicatum* 3), **S¹** : indicates monomorphic band across *S. spicatum* that were absent in the Indian genotypes, The bands indicated by arrow heads in the lanes 6, 40 & 44 are genotype Specific bands for K37, K11 & K5 respectively.

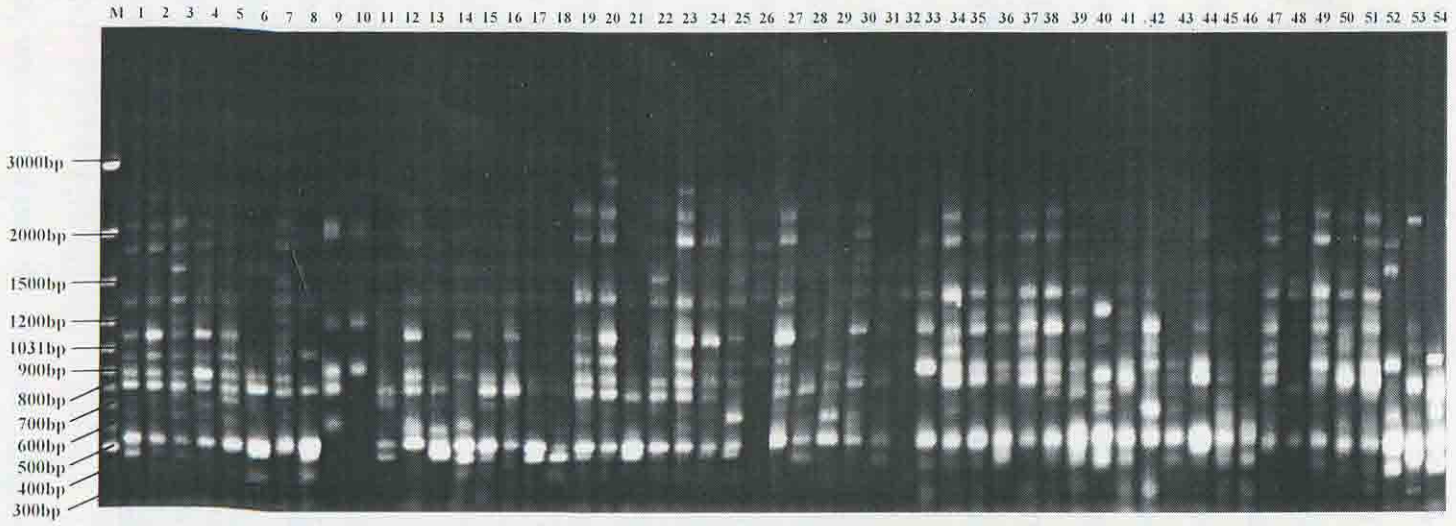

Fig. 18 : RAPD gel profile of sandalwood clones generated using 10-mer random primer OPK12

Lane M : 100bp DNA Ladder, Lanes 1 – 54 sandalwood clones (lanes 1 : T4, 2 : KL1, 3 : K35, 4 : K10, 5 : K29, 6 : K37, 7 : K19, 8 : T20, 9 : T22, 10 : T3, 11 : T24, 12 : K23, 13 : K28, 14 : T26, 15 : K16, 16 : K8, 17 : K4, 18 : T30, 19 : T11, 20 : K13, 21 : K2, 22 : KL3, 23 : K27, 24 : T2, 25 : AP4, 26 : K14, 27 : T8, 28 : T5, 29 : T13, 30 : K30, 31 : K31, 32 : T7, 33 : K6, 34 : T19, 35 : T27, 36 : K9, 37 : T14, 38 :T12, 39 : T6, 40 : K11, 41 : T9, 42 : K34, 43 : T28, 44 : K5, 45 : T23, 46 : T1, 47 : T29, 48 : T21, 49 : K32, 50 : K36, 51 : K7, 52 : *S.spicatum* 1, 53 : *S. spicatum* 2, 54 : *S.spicatum* 3)

profiles were used for statistical analysis. The dissimilarity matrix was computed using Squared Euclidean Distance (SED) that estimated all pair wise differences in the amplification product (Sokal and Sneath, 1973). The genetic dissimilarity value ranged from 15 per cent to 91 per cent suggesting a wide genetic base within the sandalwood genotypes used in the present investigation (Table 9). The highest dissimilarity (91%) was observed between genotypes collected from Chamarajnagar, Karnataka (K13) and Australia (*S. spicatum* 3) and the least (15%) between genotypes collected from Tamil Nadu (T3 and T22). When the dissimilarity was estimated among the Indian genotypes, it was found to range from 15 per cent (between T3 and T22) to 75 per cent (between T1 and K13) (Table 10).

The dendrogram was constructed by Ward's method of clustering using minimum variance algorithm (Ward, 1963). Cluster analysis based on 156 RAPD markers revealed that the 54 genotypes examined, clustered at a linkage distance of 280 units on the dendrogram with T4 (Vellore, Tamil Nadu) and *S. spicatum* 3 (Australia) spanning the extremes. All the accessions grouped into two major groups at about 160 units. The resultant dendrogram grouped all the exotic genotypes in a separate distinct sub cluster at about 35 units on the dendrogram (Fig. 19).

With in each major group, four distinct sub groups could be visualized. Major group I comprised of 28 genotypes, all belonging to India with T12 (Salem, Tamil Nadu) and T4 (Vellore, Tamil Nadu) spanning the extremes at a linkage distance of 130 units in the dendrogram. The genetic dissimilarity value for this group ranged from 15 to 57 per cent (Table 9).

The first sub cluster Ia was the largest sub cluster comprising of 9 genotypes with K30 from Karnataka and T4 from Tamil Nadu spanning the extremes (at 55 units in the dendrogram). This cluster included T2, T4, T8 (from Tamil Nadu), K13, K27, K30, K35 (from Karnataka) and all the

genotypes from Kerala (KL1 and KL3). The genetic dissimilarity value for this sub cluster ranged from 19 per cent (between K27 and K13) to 47 per cent (between K27 and K35).

The genetic dissimilarity value for sub cluster Ib ranged from 25 per cent (between K23 and K29) to 47 per cent (between K7 and K10). This group comprised of 7 genotypes belonging to Karnataka (K7, K10, K19, K23 and K29), Tamil Nadu (T11) and Andhra Pradesh (AP4). The only genotypes examined from Andhra Pradesh (AP4) was clustered in this sub cluster, which was placed next to T11 (Salem, Tamil Nadu) at a distance of 30 units showing a dissimilarity of 31 per cent.

Sub cluster Ic also consisted of 7 genotypes, out of which 6 were from Tamil Nadu (T3, T5, T13, T20, T22, T24) and K28 from Karnataka. K28 (Hassan, Karnataka) was placed next to T24 (Dharmpuri, Tamil Nadu) and exhibited a genetic dissimilarity of 28 per cent. This sub cluster has T13 and T20 from Tamil Nadu at the extremes of dendrogram with the dissimilarity value ranging from 15 (between T3 and T22) to 43 per cent (between T13 and T20).

A small sub cluster Id comprising of 5 genotypes, 4 from Tamil Nadu regions of Tirunelveli (T19), Dharmpuri (T27), Salem (T14 and T12) and one from Bangalore region of Karnataka (K9) exhibiting the maximum dissimilarity of 30 per cent (between T12 and K9). In this sub cluster, grouping was according to their geographical locations.

Four sub clusters can be visualized in the II major group, which comprised of 26 genotypes that are linked at a distance of 160 units on the dendrogram (Fig. 19). The dissimilarity values for this group ranged from 18 to 71 per cent (Table. 9). The sub cluster IIa composed of 4 genotypes from Karnataka *viz.*, K5 (Bangalore), K11 (Chamarajnagar), K31 (Kadur), K37

[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]
K10	32	30	35	0
K29	40	40	47	30	0
K37	61	67	66	55	51	0
K19	39	37	46	27	29	54	0
T20	44	50	57	38	46	55	37	0
T22	43	53	52	37	37	54	36	37	0
T3	46	54	55	40	40	57	35	38	15	0
T24	41	47	48	35	35	54	30	37	34	29	0
K23	41	33	42	31	25	62	28	35	34	29	26	0
K28	43	55	48	39	41	52	46	39	30	29	28	32	0
T26	53	51	56	45	27	52	44	49	44	45	42	34	40	0
K16	42	52	53	34	30	47	45	46	43	44	33	39	31	25	0
K8	41	47	52	35	29	50	40	47	36	41	38	34	32	32	21	0
K4	54	64	65	48	44	39	53	42	45	44	39	49	33	31	22	29	0
T30	54	58	57	48	50	47	53	48	41	38	49	49	37	39	32	41	26	0
T11	30	42	43	38	42	55	35	46	41	36	39	41	39	49	38	39	46	36	0
K13	33	33	44	39	39	62	32	51	52	45	42	36	50	50	55	50	61	61	31	0
K2	51	57	60	47	37	46	46	41	38	41	38	40	32	32	29	34	25	29	41	52	0
KL3	39	41	30	37	45	60	40	53	48	47	36	42	48	54	45	44	55	57	27	32	54	0
K27	32	38	47	38	46	67	45	56	57	48	45	47	51	59	48	53	58	62	32	19	59	29	0
T2	31	23	42	33	35	58	30	47	50	45	34	32	48	42	45	40	53	55	31	20	42	28	23	0
AP4	37	43	50	31	37	56	34	45	36	43	40	40	40	44	35	34	45	53	31	38	38	34	37	30	0
K14	45	57	64	41	35	52	40	37	28	31	38	40	30	32	25	28	25	37	35	52	24	50	49	48	32	0
T8	36	32	45	40	44	65	33	52	51	50	45	41	57	55	54	47	66	64	30	25	57	25	32	27	39	51	0
T5	43	51	54	39	45	56	50	43	32	33	34	44	32	48	39	36	37	39	31	44	38	36	37	36	36	32	43	0
T13	39	45	46	39	41	56	42	43	32	35	36	38	34	40	33	30	39	37	31	46	36	34	43	34	38	32	41	18	0
K30	39	35	40	43	45	62	40	45	42	39	40	30	42	48	49	44	55	53	35	34	50	26	37	28	44	42	29	38	28	0
K31	52	60	55	52	50	57	49	44	37	40	45	51	37	41	32	39	34	30	44	63	37	57	64	59	55	33	66	41	37	51	0
T7	55	61	62	53	43	50	52	51	34	37	48	46	40	34	31	34	27	35	43	60	32	60	65	56	46	28	61	38	36	54	29	0
K6	46	54	55	46	38	57	45	56	39	40	39	43	37	31	32	35	40	46	46	57	41	53	58	51	45	31	58	41	37	47	34	27	0
T19	39	45	46	47	39	66	42	49	44	41	40	36	34	44	43	38	49	61	39	38	46	42	43	40	46	36	35	40	40	38	47	42	37	0
T27	42	48	41	40	40	61	45	48	39	40	45	35	33	41	36	27	44	46	36	47	43	39	46	43	43	33	40	37	29	33	40	39	36	21	0
K9	42	54	49	44	46	63	49	50	45	42	45	39	35	39	34	33	36	44	42	45	39	49	44	47	43	37	52	41	39	47	38	39	36	27	22	0
T14	49	57	48	45	39	64	42	51	42	37	38	34	32	44	35	32	43	55	35	42	46	38	39	42	42	30	39	36	36	38	47	42	35	18	21	25	0
T12	46	50	45	44	50	69	45	52	45	40	37	37	37	51	42	45	52	46	32	39	47	35	36	37	47	41	40	33	35	35	48	51	42	27	30	30	17	0
T6	46	46	51	36	32	45	47	46	39	42	41	35	37	27	22	31	32	40	42	49	31	41	46	39	35	23	50	37	37	41	36	33	32	39	30	36	35	40	0
K11	57	67	64	61	55	50	56	49	50	49	54	56	42	48	39	48	39	35	47	66	38	62	63	60	54	38	61	48	46	52	37	48	49	50	41	37	46	47	37	0
T9	48	56	57	44	36	47	49	50	41	44	43	43	33	31	24	33	30	36	38	55	25	49	54	45	39	25	58	33	35	45	38	33	32	39	34	34	39	42	22	33	0
K34	53	53	58	43	31	48	40	47	44	41	42	32	48	30	29	34	33	41	43	48	30	56	57	44	40	30	49	48	46	50	47	32	39	40	41	37	36	43	25	42	23	0
T28	65	65	54	57	43	46	56	51	52	47	44	46	40	42	37	42	39	35	47	58	38	50	61	54	58	46	59	46	42	52	45	44	47	46	39	49	42	41	37	38	37	40	0
K5	59	61	54	51	45	52	56	51	50	53	52	52	54	42	41	46	41	43	41	62	46	52	63	58	50	40	59	46	48	54	43	36	41	50	39	43	48	49	33	44	33	36	48	0
T23	52	64	57	52	46	47	55	48	43	48	47	51	39	33	26	35	26	28	40	63	33	55	62	59	49	33	60	41	39	51	32	33	42	53	38	42	45	46	30	35	28	33	35	31	0
T1	60	72	65	52	48	47	61	50	49	54	49	55	37	39	30	41	24	28	54	75	35	67	72	67	53	35	72	47	43	57	38	39	44	61	48	52	55	58	40	41	32	41	39	37	20	0
T29	49	51	48	39	41	66	46	57	52	49	46	42	44	50	45	44	51	51	45	48	48	52	49	50	52	42	49	50	48	44	47	48	43	48	43	45	46	49	43	54	43	42	58	50	43	45	0
T21	58	62	59	56	56	63	61	58	55	54	53	53	43	53	36	41	44	42	48	63	43	59	58	59	49	41	64	49	47	57	44	43	44	55	42	44	49	52	44	51	40	45	51	47	40	42	43	0
K32	49	43	50	39	39	54	40	51	54	55	50	46	50	48	43	42	51	49	43	46	44	46	51	44	44	46	39	54	52	44	53	54	47	40	39	49	48	51	41	48	39	38	42	48	47	49	38	41	0
K36	39	41	44	39	33	48	38	49	44	47	46	40	42	38	37	36	41	37	35	46	32	46	55	44	42	40	45	54	46	42	43	48	43	40	37	41	46	49	39	44	37	36	46	44	39	41	34	43	18	0
K7	47	49	52	47	37	50	42	53	42	47	50	46	40	42	41	36	43	47	39	50	34	46	57	46	32	36	47	50	42	48	51	40	43	44	41	45	42	53	39	50	39	44	52	42	47	47	50	53	40	26	0
S. spicatum 1	77	75	66	67	61	56	68	63	72	71	68	70	64	48	61	70	57	55	73	76	60	68	75	72	72	60	77	74	68	72	61	60	59	74	67	71	72	73	57	62	61	64	58	60	55	57	70	65	62	58	62	0
S. spicatum 2	67	71	66	67	59	60	68	51	60	61	58	66	58	52	61	64	55	55	67	74	58	70	75	68	72	58	77	62	60	68	49	56	55	64	61	67	64	67	57	56	59	58	52	60	51	57	66	63	60	56	64	22	0
S. spicatum 3	82	80	79	76	70	63	81	56	71	70	73	73	63	53	58	67	50	52	78	91	57	81	88	81	77	61	88	71	63	77	56	55	62	77	70	68	79	80	60	55	58	63	57	65	52	50	71	62	69	67	69	33	31	0

Table 9 : Genetic dissimilarity matrix of 54 sandalwood genotypes developed by Squared Euclidean distance

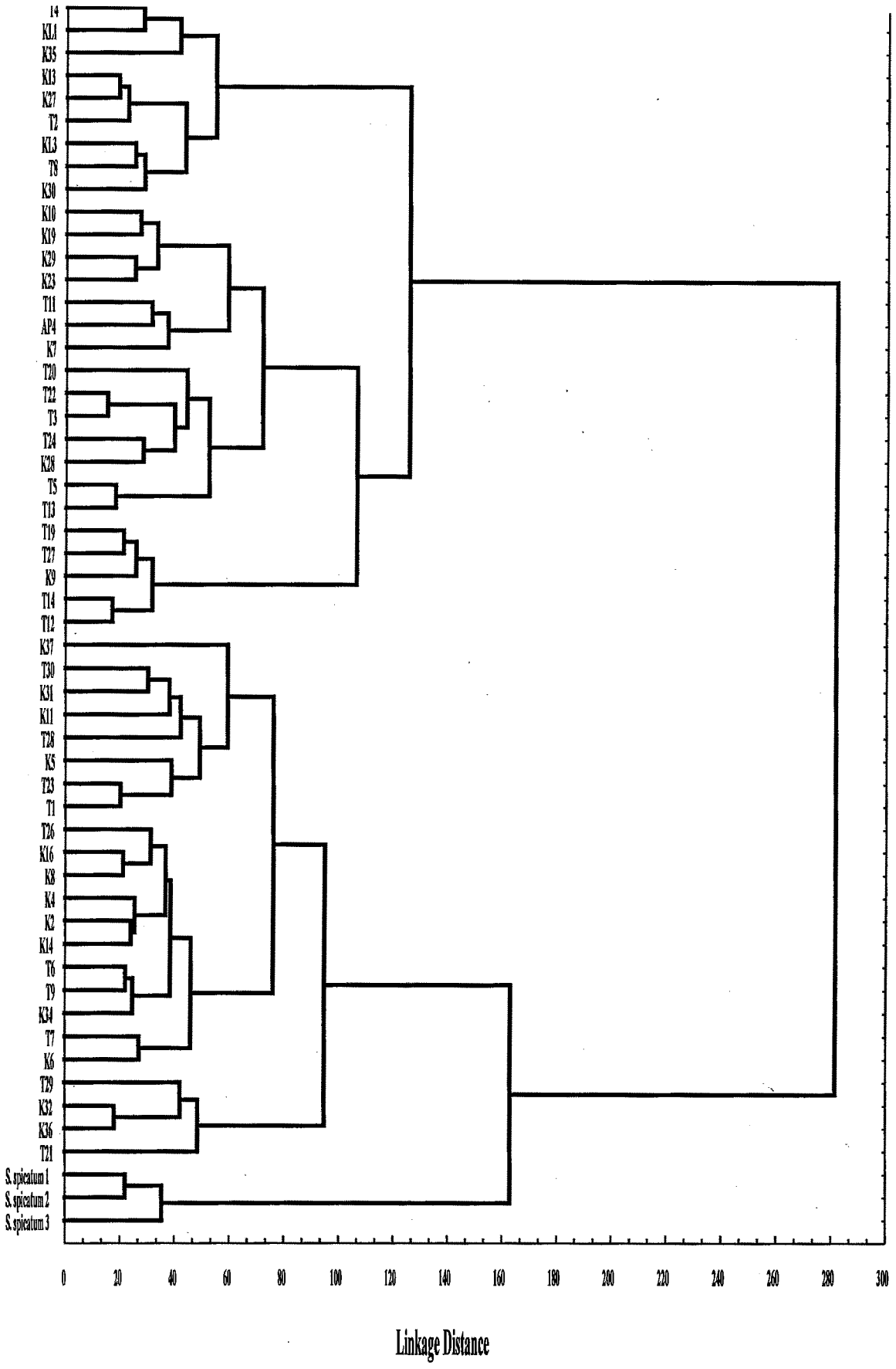

Figure 19 : Dendrogram showing the genetic relationships among 54 sandalwood genotypes based on RAPD markers according to Ward's method

(Mudigere) and 4 from Tamil Nadu *viz.*, T1 (Kumbakonam), T23 (Dharmpuri), T28 (Hanur) and T30 (Hosur) clustered at a distance of 65 units in the dendrogram. The genotypes T1 and K37 were at the extremes. Within this sub cluster, K37 from Karnataka was quite distinct and appeared as a separate entity. It was placed next to T30 exhibiting 47 per cent dissimilarity. The remaining genotypes formed two minor groups with the dissimilarity values ranging from 20 (between T1 and T23) to 57 per cent (between K31 and K37).

The sub cluster IIb was the largest group comprising of 11 genotypes *viz.*, T6, T7, T9, T26 (from Tamil Nadu), K2, K4, K6, K8, K14, K16 and K34 (from Karnataka) clustered at about 50 units, with T26 (Dharmpuri) and K6 (Bangalore) spanning the extremes. Several minor clusters were recognizable with in this sub cluster IIb exhibiting the dissimilarity of 21 (between K8 and K16) to 41 per cent (between K2 and K6). Based on their order of clustering, there were four small groups, which more or less grouped according to their geographical locations, with the exception of K6 (Bangalore) and K34 (Somwarpet). K6 was placed next to T7 (Vellore) showing 27 per cent dissimilarity, while K34 was clustered together with T6 (25% dissimilarity) and T9 (23% dissimilarity) from Tamil Nadu.

The sub cluster IIc is a small group consisting of only four genotypes from Tirunelveli (T21), Dharmpuri (T29), Kadur (K32) and Hunsur (K36). Within this sub cluster, a distinct minor cluster comprised of two genotypes from Karnataka (K32 and K36) could be visualized. They were clustered in between the genotypes from Tirunelveli (T21) and Dharmpuri (T29) regions of Tamil Nadu at about 50 units in the dendrogram. The dissimilarity values for this group ranged from 18 (between K32 and K36) to 43 per cent (between T21 and T29).

As expected, all the exotic genotypes collected from Australia formed a distinct group (sub cluster IId). They were clearly discernible from the rest of Indian genotypes. Without any exception, all the 51 genotypes procured from different geographical regions of India were linked to the exotics at a distance of 160 units in the dendrogram. Within the exotic genotypes, *S. spicatum* 3 was quite distinct forming a separate entity, whereas *S. spicatum* 1 and 2 were placed close to each other exhibiting 22 per cent dissimilarity.

Matching the clustering results of these 54 genotypes with their collection sites revealed that, the geographical distribution of most genotypes in each of the sub clusters were well defined. Majority of the genotypes in sub cluster Ia and IIb were collected from Karnataka. With the exception of T11 and AP4, all the individuals in sub cluster Ib were from Karnataka. Similarly 6 of the 7 genotypes in the sub cluster Ic and 4 of the 5 genotypes in the sub cluster Id was procured from Tamil Nadu. Without exception, all the individuals in sub cluster IId were collected from Australia. From the resultant dendrogram, it is observed that, most of the genotypes assessed clustered according to their geographical regions with few exceptions.

4.3.3.2 PCA of 54 sandalwood genotypes based on RAPD markers

To visualize the genetic relatedness among the sandalwood genotypes in detail, Principal Component Analysis (PCA) was made for 156 RAPD markers generated by eleven decamer random primers. The description of the data was done using three dimensions and the same is presented in figure 20. From the graph, it is evident that sandalwood genotypes were more dispersed on the PCA plot, which is a reflection of wider genetic base. The results of PCA show a clear-cut separation of 51 Indian genotypes from the 3 Australian accessions. However, as indicated in the figure 20, some of the Indian genotypes appear to be overlapping with each other depicting redundancy in these genotypes. Among the Indian genotypes, K37 collected from Mudigere region of Karnataka was quite distinct from the others

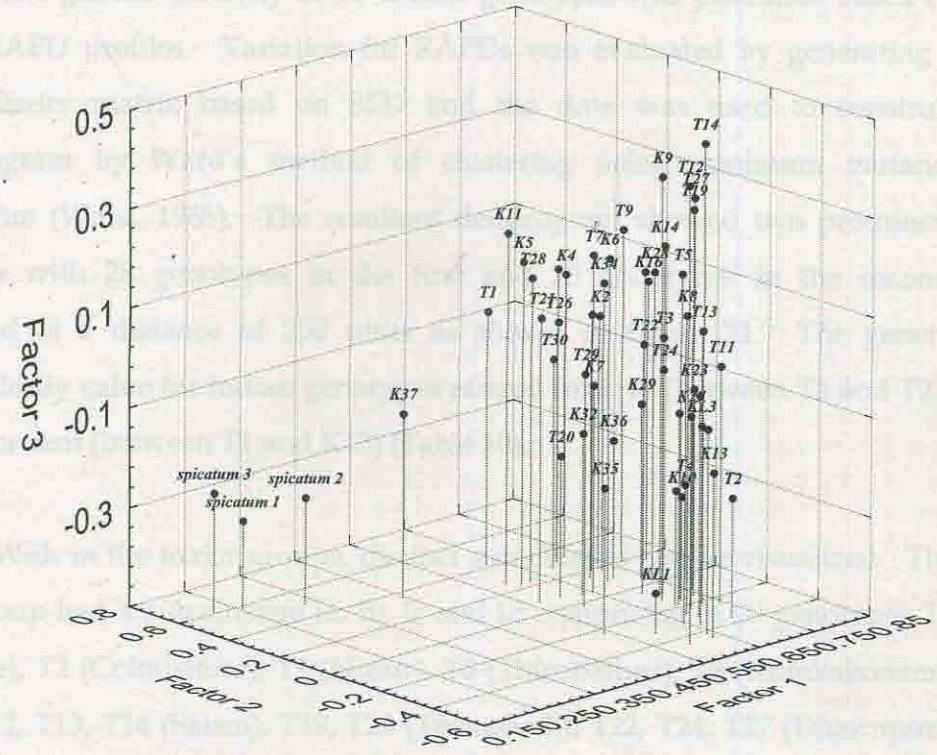

Figure 20 : Principal Component Analysis of 54 sandalwood genotypes generated by RAPD markers

forming a separate entity. T24, K9, K11, T1, T20, KL1, T2 and T11 are some of the genotypes found at the periphery of the PCA. In general, the results obtained from PCA is in agreement with the dendrogram generated by Wards method, is a further confirmation of the genetic relationships delineated by cluster analysis.

4.3.4 Genetic diversity and relatedness of the 51 Indian genotypes

The genetic diversity of 51 Indian genotypes was examined based on their RAPD profiles. Variation for RAPDs was evaluated by generating a dissimilarity matrix based on SED and the data was used to construct dendrogram by Ward's method of clustering using minimum variance algorithm (Ward, 1963). The resultant dendrogram showed two prominent clusters with 28 genotypes in the first and 23 genotypes in the second, grouped at a distance of 250 units as shown in figure 21. The genetic dissimilarity value for Indian genotypes ranged from 15 (between T3 and T22) to 75 per cent (between T1 and K13) (Table 10).

With in the major groups, distinct sub clusters can be visualized. The first group had 4 sub clusters Ia, Ib, Ic and Id comprising of 28 genotypes T4 (Vellore), T2 (Coimbatore), T3 (Hosur), T5 (Thirupathur), T8 (Kumbakonam), T11, T12, T13, T14 (Salem), T19, T20 (Tirunelveli), T22, T24, T27 (Dharmpuri) from Tamil Nadu, K7, K9, K10 (Bangalore), K13 (Chamarajnagar), K19 (Siddapura), K23 (Bhadravathi), K27 (Shikaripura), K28, K29 (Hassan), K30 (Chikkamangalur), K35 (Kushalnagar) from Karnataka, KL1, KL3 (Munnar) from Kerala and AP4 (Hyderabad) from Andhra Pradesh clustered at a distance of 130 units, with T4 and T12 spanning the extremes exhibiting the dissimilarity of 46 per cent.

Group II was divided into three sub clusters IIa, IIb and IIc comprising of 23 genotypes *viz.*, K4 (Chitradurga), K2, K5, K6, K8 (Bangalore), K11, K16 (Chamarajnagar), K14 (Kolar), K31, K32 (Chikkamangalur), K34 (Madikeri),

T4	0																																																		
KL1	28	0																																																	
K35	41	35	0																																																
K10	32	30	35	0																																															
K29	40	40	47	30	0																																														
K37	61	67	66	55	51	0																																													
K19	39	37	46	27	29	54	0																																												
T20	44	50	57	38	46	55	37	0																																											
T22	43	53	52	37	37	54	36	37	0																																										
T3	46	54	55	40	40	57	35	38	15	0																																									
T24	41	47	48	35	35	54	30	37	34	29	0																																								
K23	41	33	42	31	25	62	28	35	34	29	26	0																																							
K28	43	55	48	39	41	52	46	39	30	29	28	32	0																																						
T26	53	51	56	45	27	52	44	49	44	45	42	34	40	0																																					
K16	42	52	53	34	30	47	45	46	43	44	33	39	31	25	0																																				
K8	41	47	52	35	29	50	40	47	36	41	38	34	32	32	21	0																																			
K4	54	64	65	48	44	39	53	42	45	44	39	49	33	31	22	29	0																																		
T30	54	58	57	48	50	47	53	48	41	38	49	49	37	39	32	41	26	0																																	
T11	30	42	43	38	42	55	35	46	41	36	39	41	39	49	38	39	46	36	0																																
K13	33	33	44	39	39	62	32	51	52	45	42	36	50	50	55	50	61	61	31	0																															
K2	51	57	60	47	37	46	46	41	38	41	38	40	32	32	29	34	25	29	41	52	0																														
KL3	39	41	30	37	45	60	40	53	48	47	36	42	48	54	45	44	55	57	27	32	54	0																													
K27	32	38	47	38	46	67	45	56	57	48	45	47	51	59	48	53	58	62	32	19	59	29	0																												
T2	31	23	42	33	35	58	30	47	50	45	34	32	48	42	45	40	53	55	31	20	42	28	23	0																											
AP4	37	43	50	31	37	56	34	45	36	43	40	40	40	44	35	34	45	53	31	38	38	34	37	30	0																										
K14	45	57	64	41	35	52	40	37	28	31	38	40	30	32	25	28	25	37	35	52	24	50	49	48	32	0																									
T8	36	32	45	40	44	65	33	52	51	50	45	41	57	55	54	47	66	64	30	25	57	25	32	27	39	51	0																								
T5	43	51	54	39	45	56	50	43	32	33	34	44	32	48	39	36	37	39	31	44	38	36	37	36	36	32	43	0																							
T13	39	45	46	39	41	56	42	43	32	35	36	38	34	40	33	30	39	37	31	46	36	34	43	34	38	32	41	18	0																						
K30	39	35	40	43	45	62	40	45	42	39	40	30	42	48	49	44	55	53	35	34	50	26	37	28	44	42	29	38	28	0																					
K31	52	60	55	52	50	57	49	44	37	40	45	51	37	41	32	39	34	30	44	63	37	57	64	59	55	33	66	41	37	51	0																				
T7	55	61	62	53	43	50	52	51	34	37	48	46	40	34	31	34	27	35	43	60	32	60	65	56	46	28	61	38	36	54	29	0																			
K6	46	54	55	46	38	57	45	56	39	40	39	43	37	31	32	35	40	46	46	57	41	53	58	51	45	31	58	41	37	47	34	27	0																		
T19	39	45	46	47	39	66	42	49	44	41	40	36	34	44	43	38	49	61	39	38	46	42	43	40	46	36	35	40	40	38	47	42	37	0																	
T27	42	48	41	40	40	61	45	48	39	40	45	35	33	41	36	27	44	46	36	47	43	39	46	43	43	33	40	37	29	33	40	39	36	21	0																
K9	42	54	49	44	46	63	49	50	45	42	45	39	35	39	34	33	36	44	42	45	39	49	44	47	43	37	52	41	39	47	38	39	36	27	22	0															
T14	49	57	48	45	39	64	42	51	42	37	38	34	32	44	35	32	43	55	35	42	46	38	39	42	42	30	39	36	36	38	47	42	35	18	21	25	0														
T12	46	50	45	44	50	69	45	52	45	40	37	37	37	51	42	45	52	46	32	39	47	35	36	37	47	41	40	33	35	35	48	51	42	27	30	30	17	0													
T6	46	46	51	36	32	45	47	46	39	42	41	35	37	27	22	31	32	40	42	49	31	41	46	39	35	23	50	37	37	41	36	33	32	39	30	36	35	40	0												
K11	57	67	64	61	55	50	56	49	50	49	54	56	42	48	39	48	39	35	47	66	38	62	63	60	54	38	61	48	46	52	37	48	49	50	41	37	46	47	37	0											
T9	48	56	57	44	36	47	49	50	41	44	43	43	33	31	24	33	30	36	38	55	25	49	54	45	39	25	58	33	35	45	38	33	32	39	34	34	39	42	22	33	0										
K34	53	53	58	43	31	48	40	47	44	41	42	32	48	30	29	34	33	41	43	48	30	56	57	44	40	30	49	48	46	50	47	32	39	40	41	37	36	43	25	42	23	0									
T28	65	65	54	57	43	46	56	51	52	47	44	46	40	42	37	42	39	35	47	58	38	50	61	54	58	46	59	46	42	52	45	44	47	46	39	49	42	41	37	38	37	40	0								
K5	59	61	54	51	45	52	56	51	50	53	52	52	54	42	41	46	41	43	41	62	46	52	63	58	50	40	59	46	48	54	43	36	41	50	39	43	48	49	33	44	33	36	48	0							
T23	52	64	57	52	46	47	55	48	43	48	47	51	39	33	26	35	26	28	40	63	33	55	62	59	49	33	60	41	39	51	32	33	42	53	38	42	45	46	30	35	28	33	35	31	0						
T1	60	72	65	52	48	47	61	50	49	54	49	55	37	39	30	41	24	28	54	75	35	67	72	67	53	35	72	47	43	57	38	39	44	61	48	52	55	58	40	41	32	41	39	37	20	0					
T29	49	51	48	39	41	66	46	57	52	49	46	42	44	50	45	44	51	51	45	48	48	52	49	50	52	42	49	50	48	44	47	48	43	48	43	45	46	49	43	54	43	42	58	50	43	45	0				
T21	58	62	59	56	56	63	61	58	55	54	53	53	43	53	36	41	44	42	48	63	43	59	58	59	49	41	64	49	47	57	44	43	44	55	42	44	49	52	44	51	40	45	51	47	40	42	43	0			
K32	49	43	50	39	39	54	40	51	54	55	50	46	50	48	43	42	51	49	43	46	44	46	51	44	44	46	39	54	52	44	53	54	47	40	39	49	48	51	41	48	39	38	42	48	47	49	38	41	0		
K36	39	41	44	39	33	48	38	49	44	47	46	40	42	38	37	36	41	37	35	46	32	46	55	44	42	40	45	54	46	42	43	48	43	40	37	41	46	49	39	44	37	36	46	44	39	41	34	43	18	0	
K7	47	49	52	47	37	50	42	53	42	47	50	46	40	42	41	36	43	47	39	50	34	46	57	46	32	36	47	50	42	48	51	40	43	44	41	45	42	53	39	50	39	44	52	42	47	47	50	53	40	26	0

Table 10 : Genetic dissimilarity matrix of 51 In-dian sandalwood genotypes developed by Squared Euclidean distance

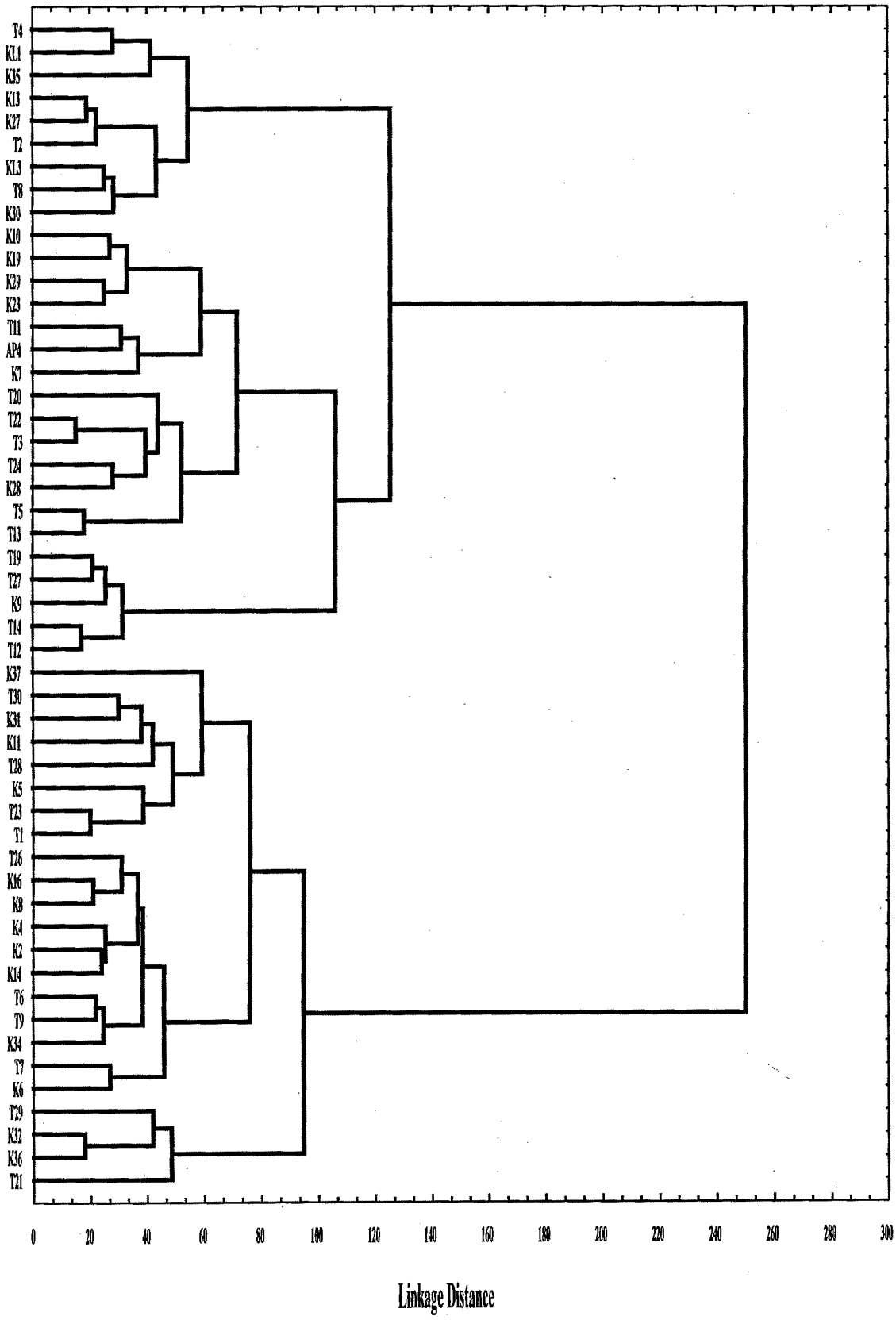

Figure 21 : Dendrogram showing the genetic relationships among 51 Indian sandalwood genotypes based on RAPD markers according to Ward's method

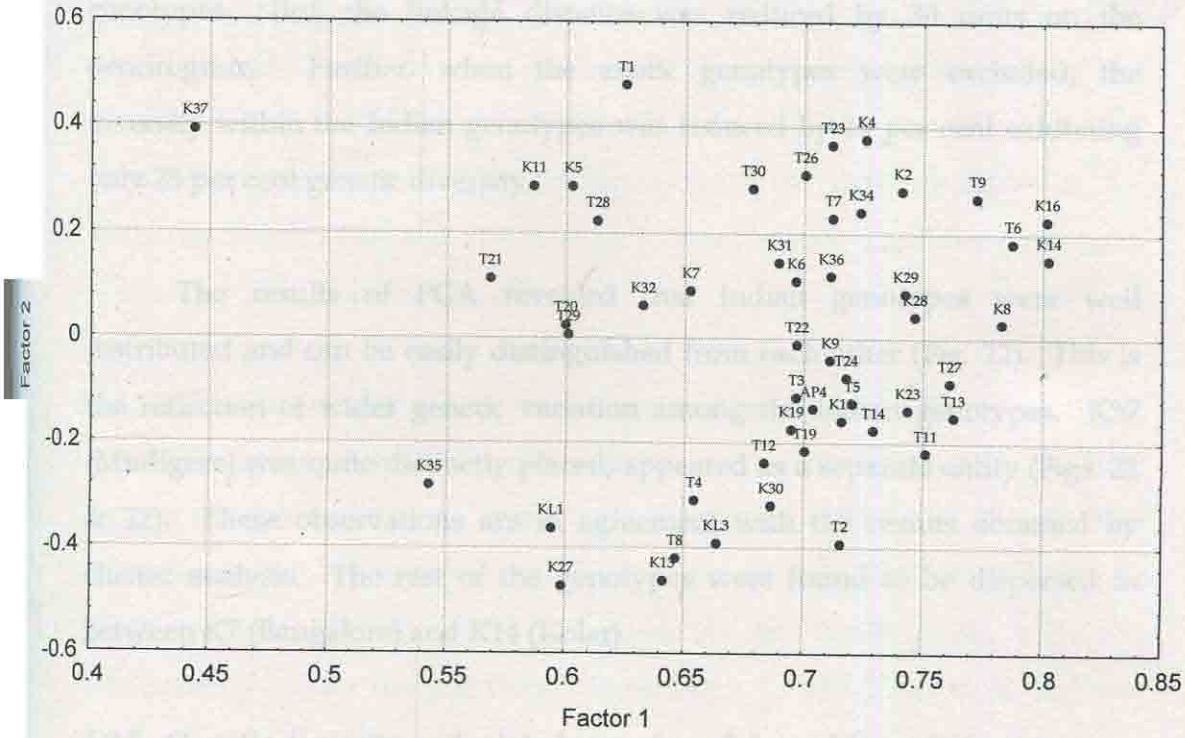

Figure 22 : Principal Component Analysis of 51 Indian sandalwood genotypes generated by RAPD markers

K36 (Hunsur), K37 (Mudigere), T1 (Kumbakonam), T6, T7 (Vellore), T9 (Salem), T21 (Tirunelveli) T23, T26, T29 (Dharmpuri), T28 (Hanur) and T30 (Hosur), clustered at a distance of 95 units in the dendrogram. K37 and T21 were found spanning the extremes of the dendrogram (63% dissimilarity).

Although, the Australian genotypes were not included, the sub groups assigned by cluster analysis was found to be consistent within the Indian genotypes. But, the linkage distance was reduced by 30 units on the dendrogram. Further, when the exotic genotypes were excluded, the diversity within the Indian genotypes was reduced by 16 per cent exhibiting only 75 per cent genetic diversity.

The results of PCA revealed that Indian genotypes were well distributed and can be easily distinguished from each other (Fig. 22). This is the reflection of wider genetic variation among the Indian genotypes. K37 (Mudigere) was quite distinctly placed, appeared as a separate entity (Figs. 21 & 22). These observations are in agreement with the results obtained by cluster analysis. The rest of the genotypes were found to be dispersed in between K7 (Bangalore) and K14 (Kolar).

4.3.5 Genetic diversity and relatedness of sandalwood from different geographical regions

4.3.5.1 Genetic relatedness of the genotypes from Karnataka

The 24 genotypes from Karnataka fall in to three groups, clustered at 145 units in the dendrogram with K35 (Kushalnagar, Madikeri) and K34 (Somwarpet, Madikeri) spanning the extremes (Fig. 23). The first cluster consisted of 8 genotypes K35, K30, K13, K27, K10, K19, K29 and K23 that are distinct from each other. The second cluster is a small group comprised of only 4 genotypes K37, K32, K36 and K7 that are well distributed on the PCA plot. K37 (Mudigere) is quite distinct appeared as a separate entity. The third cluster is a major group consisting of 12 genotypes *viz.*, K28, K9, K31, K6, K11,

K5, K16, K8, K4, K2, K14 and K34. Matching the dendrogram results of the third cluster with their collection sites indicated that 5 out of 7 genotypes from Bangalore, 2 out of 3 from Chamarajnagar were placed in this group.

All the 24 genotypes collected from different geographical regions of Karnataka were distinct from each other and none of them were found overlapping. This difference was clearly observed in the gels after electrophoresis (Figs. 8 to 18), as well as in the dendrogram (Fig. 23). K37, K11, K27, K32 and K36 were placed on the periphery, while rest of the genotypes scattered in between K5 and K23 on the PCA plot (Fig. 24). K27 (Shikaripura, Sagar) and K37 (Mudigere) were the most dissimilar (67%), where as K32 (Kadur) and K36 (Hunsur) were the least dissimilar genotypes, differed by only 18 per cent (Table 11).

4.3.5.2 Genetic relatedness of the genotypes from Tamil Nadu

Dendrogram and the dissimilarity matrix indicating the diversity among the genotypes from Tamil Nadu are presented in figure 25 and table 12 respectively. Twenty-four genotypes procured from Tamil Nadu fall in to three prominent clusters (at 145 units in the dendrogram) with the dissimilarity values ranging from 15 (between T3 and T22) to 72 per cent (between T1 and T8).

The genotype T4 (Vellore), T2 (Coimbatore), T8 (Kumbakonam), T19 (Tirunelveli), T27 (Dharmpuri), T11, T12 and T14 (Salem) were grouped in the first cluster with the total dissimilarity of 46 per cent between them. They are distinct from each other and were placed apart in the PCA. In the dendrogram, the only genotype, T2 collected from Coimbatore was placed next to T8 from Kumbakonam showing a dissimilarity of 27 per cent. Six of the 24 genotypes were grouped in the second cluster exhibiting the genetic dissimilarity of 43 per cent (between T20 and T13). T20 and T24, T3 and T22, T5 and T13 were placed next to each other, clustered at a distance of 55 units

K35	0																								
K10	35	0																							
K29	47	30	0																						
K37	66	55	51	0																					
K19	46	27	29	54	0																				
K23	42	31	25	62	28	0																			
K28	48	39	41	52	46	32	0																		
K16	53	34	30	47	45	39	31	0																	
K8	52	35	29	50	40	34	32	21	0																
K4	65	48	44	39	53	49	33	22	29	0															
K13	44	39	39	62	32	36	50	55	50	61	0														
K2	60	47	37	46	46	40	32	29	34	25	52	0													
K27	47	38	46	67	45	47	51	48	53	58	19	59	0												
K14	64	41	35	52	40	40	30	25	28	25	52	24	49	0											
K30	40	43	45	62	40	30	42	49	44	55	34	50	37	42	0										
K31	55	52	50	57	49	51	37	32	39	34	63	37	64	33	51	0									
K6	55	46	38	57	45	43	37	32	35	40	57	41	58	31	47	34	0								
K9	49	44	46	63	49	39	35	34	33	36	45	39	44	37	47	38	36	0							
K11	64	61	55	50	56	56	42	39	48	39	66	38	63	38	52	37	49	37	0						
K34	58	43	31	48	40	32	48	29	34	33	48	30	57	30	50	47	39	37	42	0					
K5	54	51	45	52	56	52	54	41	46	41	62	46	63	40	54	43	41	43	44	36	0				
K32	50	39	39	54	40	46	50	43	42	51	46	44	51	46	44	53	47	49	48	38	48	0			
K36	44	39	33	48	38	40	42	37	36	41	46	32	55	40	42	43	43	41	44	36	44	18	0		
K7	52	47	37	50	42	46	40	41	36	43	50	34	57	36	48	51	43	45	50	44	42	40	26	0	

Table 11 : Genetic dissimilarity matrix of sandalwood from Karnataka developed by Squared Euclidean distance

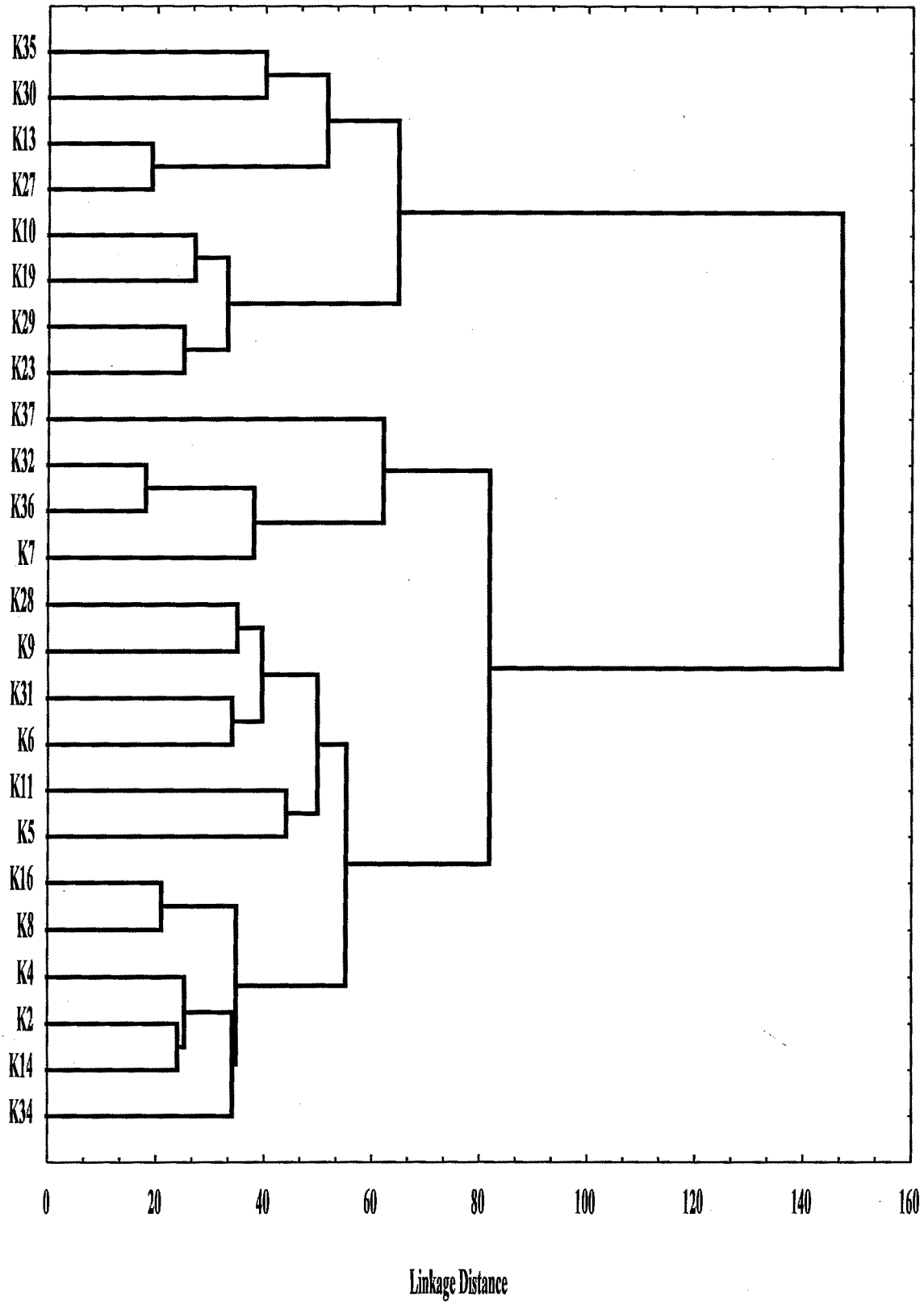

Figure 23 : Dendrogram of the 24 sandalwood genotypes from Karnataka generated by RAPD markers

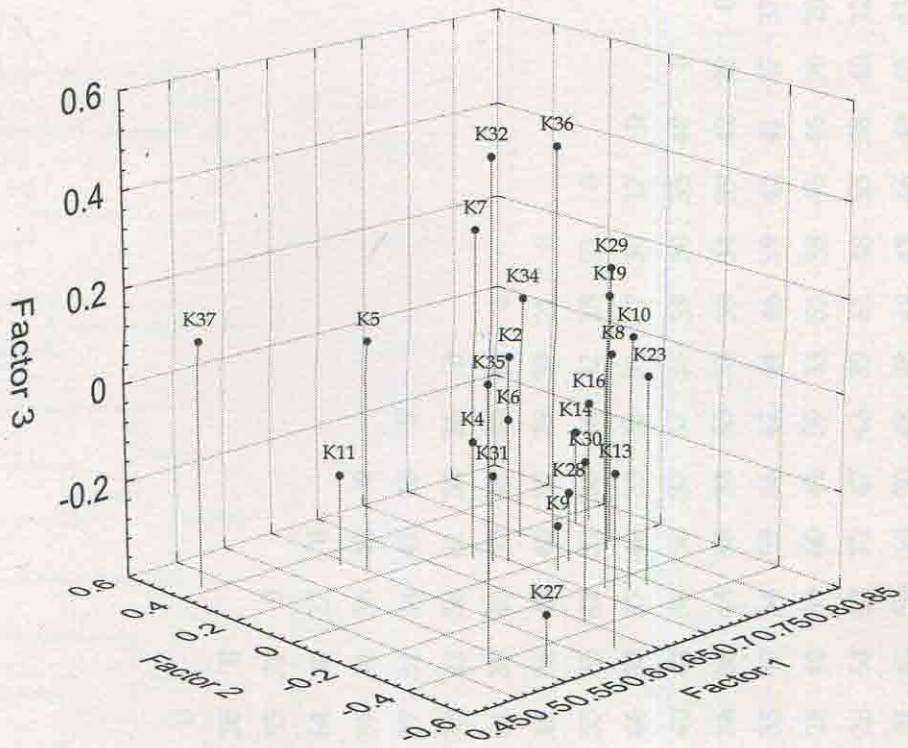

Figure 24 : Principal Component Analysis of 24 sandalwood genotypes from Karnataka generated by RAPD markers

T4	0																							
T20	44	0																						
T22	43	37	0																					
T3	46	38	15	0																				
T24	41	37	34	29	0																			
T26	53	49	44	45	42	0																		
T30	54	48	41	38	49	39	0																	
T11	30	46	41	36	39	49	36	0																
T2	31	47	50	45	34	42	55	31	0															
T8	36	52	51	50	45	55	64	30	27	0														
T5	43	43	32	33	34	48	39	31	36	43	0													
T13	39	43	32	35	36	40	37	31	34	41	18	0												
T7	55	51	34	37	48	34	35	43	56	61	38	36	0											
T19	39	49	44	41	40	44	61	39	40	35	40	40	42	0										
T27	42	48	39	40	45	41	46	36	43	40	37	29	39	21	0									
T14	49	51	42	37	38	44	55	35	42	39	36	36	42	18	21	0								
T12	46	52	45	40	37	51	46	32	37	40	33	35	51	27	30	17	0							
T6	46	46	39	42	41	27	40	42	39	50	37	37	33	39	30	35	40	0						
T9	48	50	41	44	43	31	36	38	45	58	33	35	33	39	34	39	42	22	0					
T28	65	51	52	47	44	42	35	47	54	59	46	42	44	46	39	42	41	37	37	0				
T23	52	48	43	48	47	33	28	40	59	60	41	39	33	53	38	45	46	30	28	35	0			
T1	60	50	49	54	49	39	28	54	67	72	47	43	39	61	48	55	58	40	32	39	20	0		
T29	49	57	52	49	46	50	51	45	50	49	50	48	48	48	43	46	49	43	43	58	43	45	0	
T21	58	58	55	54	53	53	42	48	59	64	49	47	43	55	42	49	52	44	40	51	40	42	43	0

Table 12 : Genetic dissimilarity matrix of sandalwood from Tamil Nadu developed by Squared Euclidean distance

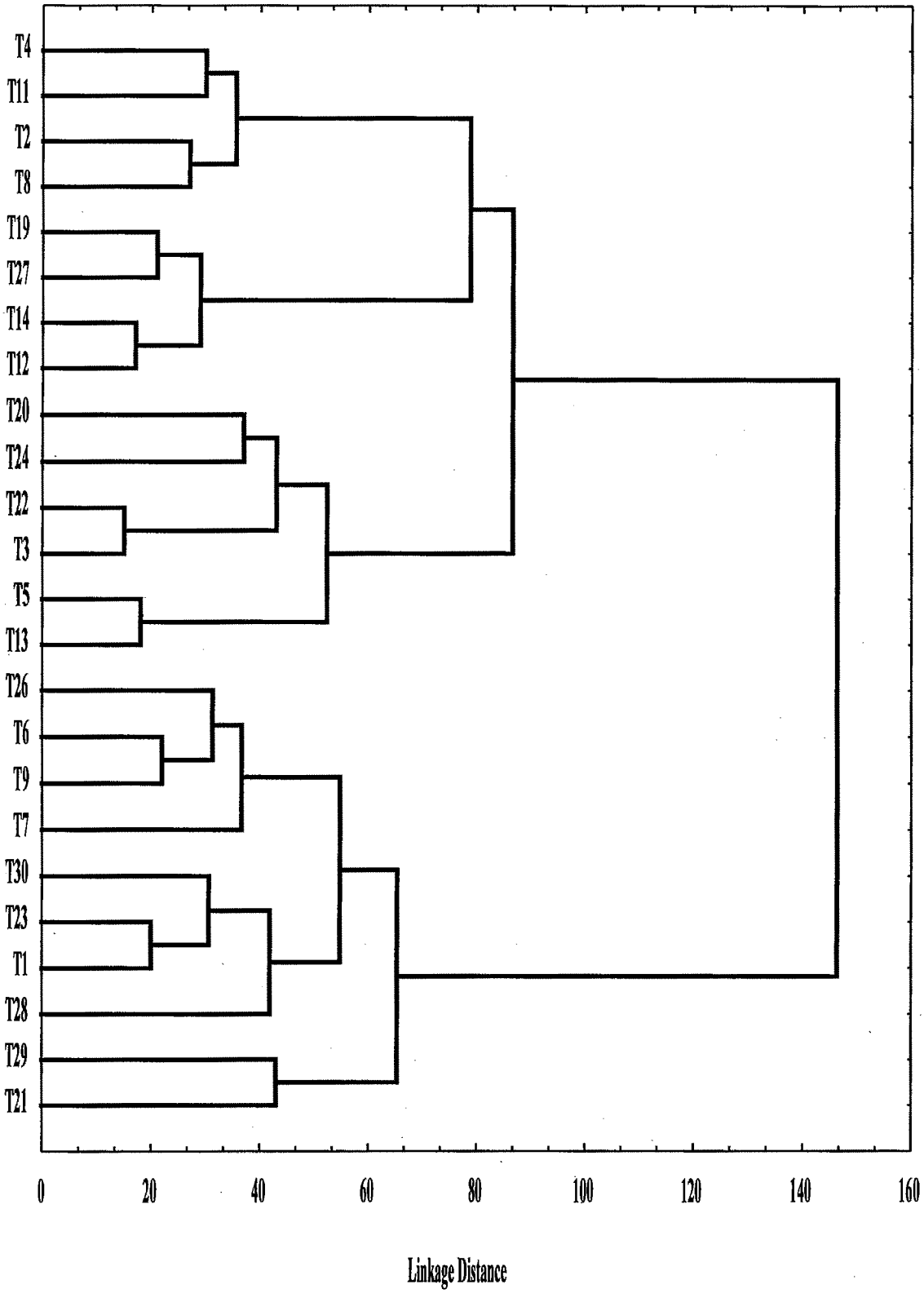

Figure 25 : Dendrogram of the 24 sandalwood genotypes from Tamil Nadu generated by RAPD markers

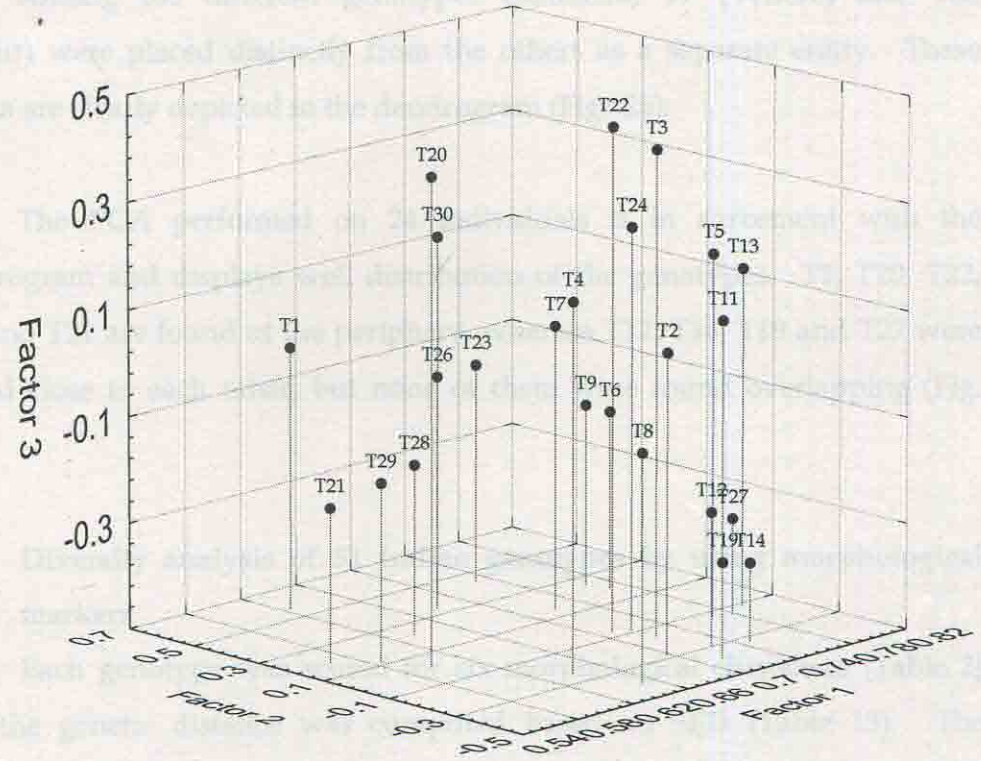

Figure 26 : Principal Component Analysis of 24 sandalwood genotypes from Tamil Nadu generated by RAPD markers

in the dendrogram. In the third cluster, 3 sub groups are recognizable, grouped at 65 units, spanning T26 and T21 as the extremes in the dendrogram exhibiting 53 per cent of dissimilarity. T6, T7, T9 and T26 formed the first sub group, while T1, T23, T28 and T30 formed the second sub group. The rest of the genotypes fall into third sub group exhibiting 43 per cent of genetic dissimilarity.

Among the different genotypes examined, T7 (Vellore) and T28 (Hanur) were placed distinctly from the others as a separate entity. These results are clearly depicted in the dendrogram (Fig. 25).

The PCA performed on 24 individuals is in agreement with the dendrogram and displays well distribution of the genotypes. T1, T20, T22, T13 and T21 are found at the periphery, whereas T12, T14, T19 and T27 were placed close to each other, but none of them were found overlapping (Fig. 26).

4.3.6 Diversity analysis of 51 Indian genotypes by using morphological markers

Each genotype was scored for six morphological characters (Table 2) and the genetic distance was computed based on SED (Table 13). The phenotypic diversity in the germplasm was estimated by cluster analysis based on Ward's method (Ward, 1963). The resultant dendrogram grouped the 51 genotypes tested in to 5 clusters (107 units in the dendrogram), with 13 genotypes in the first cluster, 9 in the second, 11 each in the third and fourth cluster and 7 in the fifth cluster (Fig. 27). All the clusters had individuals from different geographical regions with K4 (Chitradurga, Karnataka) and T4 (Vellore, Tamil Nadu) at the extremes. Genetic dissimilarity values are low ranging from 0.5 (between K8 and K35) to 33 per cent (between K7 and T23) that is a reflection of narrow variability (Table 13).

[illegible]	[illegible]																																																		
KL1	3.1	0																																																
K35	15	7.9	0																																														
K10	2.9	2.1	9.3	0																																													
K29	13	7.2	7.6	7.2	0																																												
K37	17	9.4	16	13	4.6	0																																											
K19	14	6.2	3.8	9.1	3.8	4.7	0																																										
T20	10	2.9	9.3	6.9	5	4.8	4.9	0																																									
T22	17	9.6	8	8.8	2.6	8.2	5.5	8.5	0																																								
T3	9.9	3.5	1.7	5.2	4.3	8.9	2	3.6	6.9	0																																							
T24	18	15	8.1	11	12	21	10	22	7.8	11	0																																						
K23	13	6.6	21	8	15	15	17	5.5	18	12	32	0																																					
K28	15	7.4	4.6	7.9	2.2	8.4	3.7	6.4	0.7	4.1	7.9	17	0																																				
T26	4	5.1	8.5	6.3	12	20	11	13	13	8.3	11	22	10	0																																			
K16	14	6.5	5.3	7.4	2.5	4	0.8	5.6	2.9	3	8	15	2.6	12	0																																		
K8	13	6.4	0.5	7.8	5.1	12	2.9	6.8	7.2	0.8	9.4	18	3.9	7.9	4.3	0																																	
K4	17	15	17	9	11	26	22	17	11	15	19	17	11	18	18	15	0																																
T30	0.6	2.5	15	3	14	17	14	10	17	9.9	17	11	16	4.9	13	13	20	0																															
T11	4.2	0.9	10	3.6	8.9	7.9	5.9	3.7	12	4.6	17	7.3	10	8.1	6.5	8.4	21	2.8	0																														
K13	9.5	7.6	22	11	14	9.1	12	12	15	16	19	18	16	12	11	20	33	7.4	5.9	0																													
K2	4	4.8	8.2	6.6	12	17	9.2	12	14	7.5	10	22	11	0.8	11	7.5	23	4.3	6.2	9.7	0																												
KL3	3.6	0.9	4.6	2.3	6.1	12	5.6	4.5	8	2.4	11	11	5.3	2.6	6.1	3.6	13	3.8	3	11	2.9	0																													
K27	13	8.3	15	11	9.9	6.3	7.4	6.6	18	7.3	26	12	16	20	8.2	12	29	13	5.1	15	16	11	0																												
T2	8.9	9.1	9.6	5.1	9.3	15	8.7	16	11	7.7	5.7	22	11	10	6.8	8.6	18	9.2	8.7	15	8.1	8	11	0																											
AP4	16	16	16	11	6.2	16	14	20	6.4	15	7.6	30	8	14	9.6	14	13	19	19	19	14	13	24	8.2	0																										
K14	11	6.2	4.8	5.4	5.4	8.8	2.9	7.5	8.4	2.1	9.4	15	7	12	2.6	3.7	17	11	5.4	15	10	5.8	5.1	3.2	13	0																									
T8	3.3	4.2	8.6	5.9	12	17	9.2	12	14	7.4	11	21	11	0.8	10	7.7	22	3.5	5.4	9	0	2.7	16	7.9	14	9.7	0																								
T5	2.6	1.3	5.7	1	7.3	14	7.3	6	9.9	3	11	10	7.5	3.6	7.3	4.5	11	2.9	3	12	3.8	0.6	10	5.8	13	4.8	3.4	0																							
T13	12	4.3	2.7	7.9	4.4	8.9	2.9	2.9	5.5	1.5	13	13	2.6	8.4	4.3	1.9	15	12	6.8	16	8.7	2.9	12	14	16	6.6	8.7	5.2	0																						
K30	22	15	7	15	7.7	14	7.3	12	14	5.1	20	23	11	21	8.5	4.9	20	24	15	32	19	13	8.9	12	19	4.9	19	12	9.1	0																					
K31	21	11	11	12	14	11	5.9	12	11	9.1	12	18	11	21	5	12	30	17	9	13	17	13	13	11	23	6.4	17	13	12	19	0																				
T7	16	13	12	8.8	20	28	16	23	16	13	5.8	24	15	15	13	14	21	14	14	21	14	12	25	6.9	19	10	14	10	18	26	9.1	0																			
K6	3.4	4.5	15	1.8	7.7	13	14	8	13	8.8	19	8.1	12	10	11	12	8.9	4.9	5.9	14	11	5.4	10	8.3	12	8.7	9.8	3.6	12	16	20	18	0																		
T19	7.2	8.5	13	2.9	15	25	16	17	16	10	12	15	15	11	14	12	11	7.4	10	21	12	7.8	17	4.8	15	7.9	11	4.3	16	18	17	5.6	5.9	0																	
T27	15	10	24	10	28	29	24	17	25	18	27	8.5	24	21	22	24	26	11	11	19	22	14	25	22	39	20	20	12	21	38	15	12	17	13	0																
K9	21	17	17	13	22	26	16	26	17	17	6.8	29	19	21	13	19	30	17	16	19	18	17	24	7.1	20	11	17	15	23	28	6.7	1.9	22	10	17	0															
T14	22	13	4	14	17	26	11	16	13	7.7	10	23	9.3	14	12	6.7	21	20	16	27	14	9.4	27	17	26	12	15	11	7.6	19	12	9.4	24	15	17	15	0														
T12	13	5.1	1.8	8.3	4.9	7.9	0.9	4.5	7.5	0.6	11	16	4.4	9.3	2.6	1	20	12	5.6	15	7.7	3.9	7.7	9.2	16	2.9	7.8	5.2	1.6	5.5	8.6	16	13	15	23	18	8.4	0													
T6	5.9	5.6	4.6	3.9	9.1	21	10	11	11	4.7	9.1	18	7.9	3.2	10	4	9.6	7.5	9.1	20	4.6	2.3	18	7.1	12	7.5	4.6	1.8	6.6	12	19	11	7.2	5.3	20	18	9.5	7.1	0												
K11	5.6	1.8	3.8	4	8.1	11	3.8	6	9.3	2.3	8.9	13	6.6	3.8	4.9	3.5	19	4.6	2.4	9.1	2.6	1.2	10	6.5	15	4.3	2.4	1.8	3.8	13	8	9.2	9.1	8.7	13	12	7.5	2.7	4.3	0											
T9	18	14	6.3	13	13	18	7.2	17	18	6	13	28	15	17	8.8	5.7	28	18	12	26	13	12	9.6	5.9	20	3.1	13	10	13	4.9	13	15	18	13	32	16	16	5.8	11	8.5	0										
K34	9.4	5.4	6.5	6	3.9	9.8	5.4	7.9	2.5	5.8	6.2	18	1.8	4.9	4.2	5.8	13	9.8	8.3	9.5	5.6	3.4	18	9.3	6.3	8.7	5.7	5.6	4.3	17	13	14	10	13	22	17	11	6.2	6.2	4.7	17	0									
T28	11	8.4	10	6.2	5.1	13	9.5	12	2.3	9.3	5.8	19	3	7.7	6.5	9.4	9.2	12	12	13	9.4	6.2	22	9.5	3.8	11	9.3	7.6	8.3	20	15	13	9.7	12	23	16	14	11	7.5	8.5	21	1.2	0								
K5	9.4	16	22	11	26	34	24	28	32	18	20	30	30	15	23	20	31	9.8	14	24	12	15	19	6.7	25	13	12	11	28	25	26	14	13	6.7	25	16	30	22	13	14	12	25	26	0							
T23	15	14	18	9.3	28	37	24	24	26	16	18	18	25	18	23	19	22	12	15	28	18	14	26	13	32	15	17	10	23	30	17	4.8	16	4.3	6.7	10	13	21	12	12	20	23	22	12	0						
T1	18	17	22	9.8	11	19	18	22	8	19	9.3	24	12	20	12	21	13	18	19	17	20	16	26	9.4	4.8	15	20	15	22	28	18	13	12	12	27	13	27	22	17	18	27	9.8	5.2	26	24	0					
T29	15	8.1	13	6.1	6.9	7.8	7.3	8.7	5.1	8.3	11	10	6.8	18	3.8	12	15	13	7.9	11	17	9.9	12	8.2	12	5.6	16	9.4	11	17	4.6	10	9.7	11	14	9.3	16	10	15	9.2	16	8.3	7.7	24	17	6.9	0				
T21	9.8	5	8.1	5.2	8.9	10	5.7	6.1	14	3.2	17	9.4	11	15	5.9	6.4	20	9	3.5	16	12	6.3	2.9	6.3	20	1.5	11	4.7	8.2	7	8	13	7.5	7.8	15	14	15	4.8	9.5	5	5.5	13	16	12	13	20	7.2	0			
K32	8.8	10	18	6.2	17	20	16	17	22	12	17	17	21	17	14	16	23	8.2	8	17	14	12	9	4	20	6.3	13	7.9	21	17	14	11	7.5	4.5	17	11	25	15	13	11	10	19	20	4.1	10	17	11	4.8	0		
K36	12	6.3	10	7.1	0.6	2.1	3.8	3.5	3.6	4.9	15	12	3.6	14	2.3	7.1	14	13	6.9	11	13	6.7	7.2	10	8.7	5.5	13	7.9	5.2	9.2	12	21	7.2	16	26	22	20	5.5	12	8.3	14	5.2	6.8	27	29	12	5.4	7.7	16	0	
K7	16	10	7.3	11	1.4	4.3	2.4	7.7	4.6	5.1	11	22	3.9	14	2	5.1	19	18	11	15	12	8.6	9.4	9.1	7.6	5.1	12	10	5.9	6.7	13	22	13	19	35	22	19	4	12	8.7	9.9	5.6	8.2	27	33	15	9.5	9.8	18	2.1	0

Table 13 : Genetic dissimilarity matrix of Indian sandalwood genotypes based on morphological characters

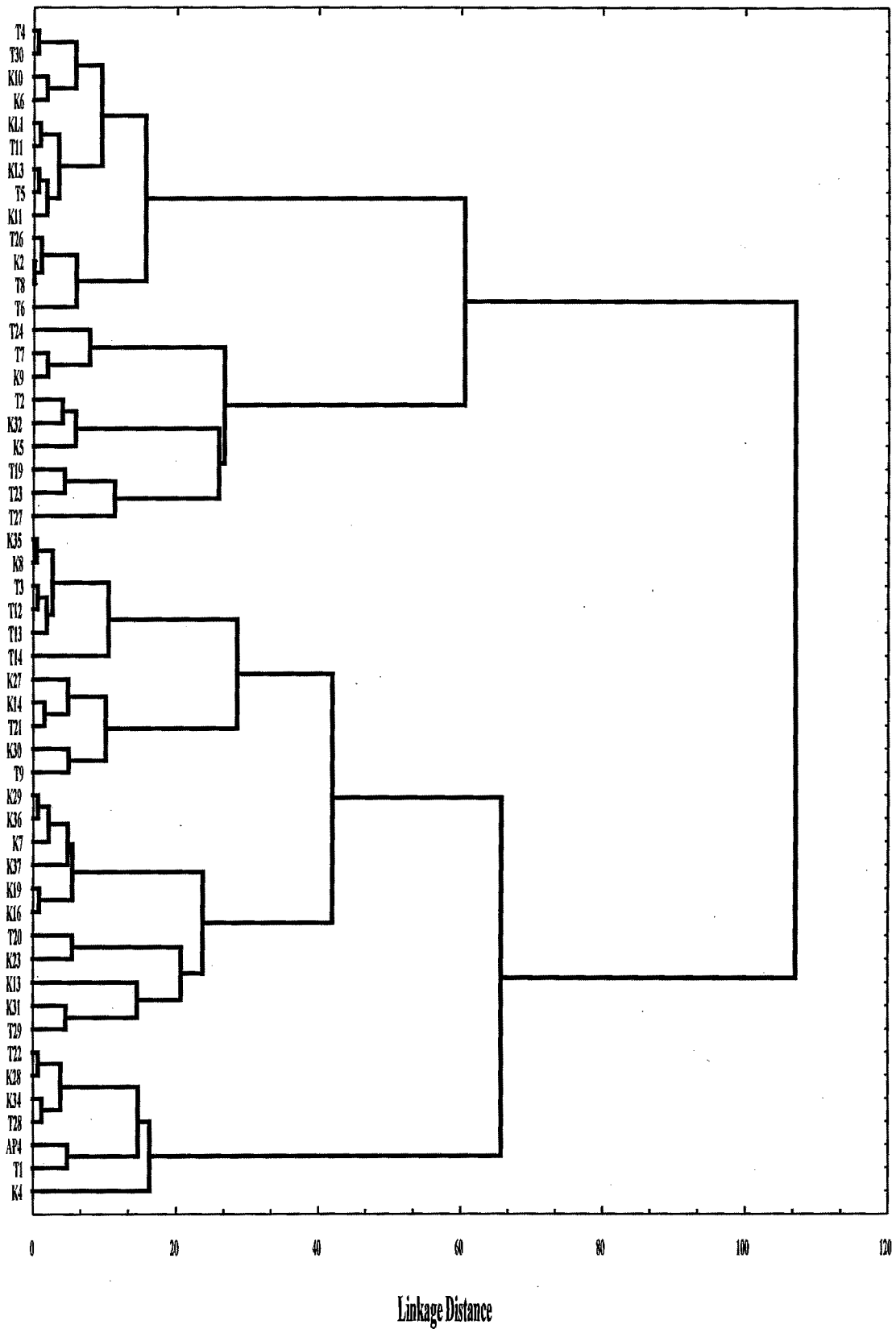

Figure 27 : Dendrogram showing the relationship among Indian sandalwood genotypes based on morphological markers according to Ward's method

Seven genotypes from Tamil Nadu (T4, T5, T6, T8, T11, T26, T30), four from Karnataka (K2, K6, K10, K11) and two from Kerala (KL1, KL3) were grouped in the first cluster. Elliptic or elliptic-lanceolate leaves, entire or wavy leaf margin and acute or acuminate leaf tip morphologically characterize sandalwood genotypes in this group. These accessions showed low dissimilarity of 5.9 per cent and in addition were divided into three small groups according to cluster analysis.

The second cluster is a small group comprised of six genotypes (T2, T7, T19, T23, T24, T27) from Tamil Nadu and three (K5, K9, K32) from Karnataka showing a dissimilarity of 27 per cent. All the accessions in this group have ovate or ovate-lanceolate leaves, wavy or entire leaf margin with acute or acuminate leaf tip, with the exception of T2 that has got obtuse-elliptic leaves. Morphologically, T24, T27 and K5 are clustered distinctly in the dendrogram and are quite diverse from each other (Fig. 27).

Six out of twenty four genotypes from Tamil Nadu (T3, T9, T12, T13, T14, T21) and five from Karnataka (K8, K14, K27, K30, K35) were placed in the third cluster at about 30 units of linkage distance in the dendrogram. Phenotypically, elliptic-lanceolate or ovate-lanceolate leaves with entire leaf margin and acute or mucronate leaf tip characterize this group, exhibiting a narrow variability. Morphologically, T14 (Salem) from Tamil Nadu appeared as a separate entity in the dendrogram (Fig. 27).

Maximum number of genotypes (9) from Karnataka *viz.*, K7, K13, K16, K19, K23, K29, K31, K36 and K37 were grouped in the fourth cluster along with two genotypes from Tamil Nadu *viz.*, T20 and T29, showing a dissimilarity of 6.9 per cent. This group had the genotype with maximum girth class (K23) and minimum leaf area (K37) (Table 2). The most distinct genotype appears to be K13 from Karnataka, which was placed next to K31 in the dendrogram. All the accessions in this group are morphologically

characterized by lanceolate or elliptic-lanceolate leaves with wavy or entire leaf margin, except K31. K31 is characterized by ovate leaves.

The last group contains seven genotypes collected from Tamil Nadu (T1, T22, T28), Karnataka (K4, K28, K34) and Andhra Pradesh (AP4). The only accession examined from Andhra Pradesh (AP4) was found to cluster with T1 in the dendrogram with the genetic dissimilarity of 4.8 per cent. Morphologically, this group is characterized by elliptic leaves with entire leaf margin. K4 appears to be distinct in the dendrogram, which has maximum leaf area. These results are illustrated by cluster analysis in figure 27.

PCA was performed in order to visualize the genetic relationships among sandalwood genotypes in detail. The results of PCA are presented in figure 28 and the description of relationship was done using three dimensions. From the graph, it is evident that most of the genotypes were overlapping with each other depicting similarity in genetic constitution. Hence, it was not easy to distinguish all the genotypes from each other. However, T26, T7, T5, T22, T11, K6, K13, K32, K31, K29, K25, K28 and KL1 were placed apart. The rest of the genotypes were dispersed close to each other on the PCA plot indicating that morphological traits are not as effective as RAPD markers in diversity studies and in discriminating the different genotypes.

4.3.7 Correlation of genetic distances generated from RAPDs and Morphological markers

The distance matrices of 51 Indian genotypes were developed from the RAPD and morphological data based on SED using the computer package "STATISTICA". The two matrices were correlated using the Excel programme from Windows. The pattern of variation of molecular and morphological distances is quite different and both the data were discordant.

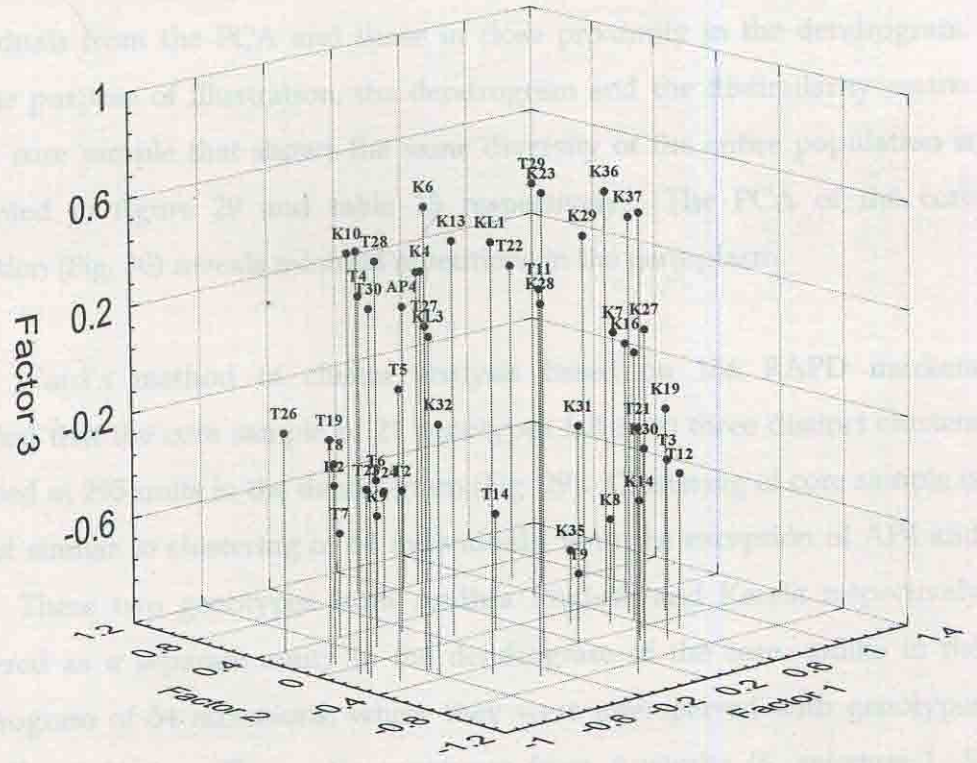

Figure 28 : Principal Component Analysis of Indian sandalwood genotypes generated by morphological markers

However, the results indicated a low correlation between the two distance matrices derived from the molecular and phenotypic data (r = 0.185).

4.4 Core collection

The genetic diversity among the 54 sandalwood genotypes was assessed using RAPD markers. Twenty-one genotypes were identified as a core collection, which revealed the same diversity of the 54 genotypes (Table 14). The "Core Sample" was identified by eliminating the overlapping individuals from the PCA and those in close proximity in the dendrogram. For the purpose of illustration, the dendrogram and the dissimilarity matrix of the core sample that shows the same diversity of the entire population is presented in figure 29 and table 15 respectively. The PCA of the core collection (Fig. 30) reveals minimal repetitions in the germplasm.

Ward's method of cluster analysis based on 156 RAPD markers revealed that the core sample of 21 genotypes fall in to three distinct clusters grouped at 295 units in the dendrogram (Fig. 29). Clustering of core sample is almost similar to clustering of 54 individuals, with the exception of AP4 and KL1. These two genotypes from Andhra Pradesh and Kerala respectively clustered as a separate entity in the dendrogram of the core, unlike in the dendrogram of 54 accessions, where they were interspersed with genotypes from Tamil Nadu. The exotic genotypes from Australia (*S. spicatum* 1, *S. spicatum* 2 and *S. spicatum* 3), the only genotype from Andhra Pradesh (AP4), both the genotypes from Kerala (KL1 and KL3), eight out of twenty four genotypes from Karnataka, seven out of twenty four genotypes from Tamil Nadu formed the core collection exhibiting the genetic dissimilarity of 91 per cent (Table 15).

The PCA of the core sample clearly demarcated the three exotics from the Indian genotypes (Fig. 30). The Indian genotypes were distributed in to two distinct groups similar to the results depicted in the dendrogram. The

Table 14 : Sandalwood core collection (21) identified from the 54 genotypes

Sl. No.	Genotype	Source of collection	Origin
1.	KL1	Munnar, Kerala	India
2.	K10	Bangalore, Karnataka	India
3.	K19	Siddapura, Sagar, Karnataka	India
4.	K4	Chitradurga, Karnataka	India
5.	T30	Hosur, Tamil Nadu	India
6.	K13	Chamarajnagar, Karnataka	India
7.	KL3	Munnar, Kerala	India
8.	K27	Shikaripura, Sagar, Karnataka	India
9.	T2	Coimbatore, Tamil Nadu	India
10.	AP4	Hyderabad, Andhra Pradesh	India
11.	T8	Kumbakonam, Tamil Nadu	India
12.	K30	Chikkamangalur, Karnataka	India
13.	K31	Kadur, Karnataka	India
14.	T7	Vellore, Tamil Nadu	India
15.	K11	Chamarajnagar, Karnataka	India
16.	T28	Hanur, Tamil Nadu	India
17.	T23	Dharmpuri, Tamil Nadu	India
18.	T1	Kumbakonam, Tamil Nadu	India
19.	*S. spicatum* 1	Western Australia	Australia
20.	*S. spicatum* 2	Western Australia	Australia
21.	*S. spicatum* 3	Western Australia	Australia

KL1	0																					
K10	30	0																				
K19	37	27	0																			
K4	64	48	53	0																		
T30	58	48	53	26	0																	
K13	33	39	32	61	61	0																
KL3	41	37	40	55	57	32	0															
K27	38	38	45	58	62	19	29	0														
T2	23	33	30	53	55	20	28	23	0													
AP4	43	31	34	45	53	38	34	37	30	0												
T8	32	40	33	66	64	25	25	32	27	39	0											
K30	35	43	40	55	53	34	26	37	28	44	29	0										
K31	60	52	49	34	30	63	57	64	59	55	66	51	0									
T7	61	53	52	27	35	60	60	65	56	46	61	54	29	0								
K11	67	61	56	39	35	66	62	63	60	54	61	52	37	48	0							
T28	65	57	56	39	35	58	50	61	54	58	59	52	45	44	38	0						
T23	64	52	55	26	28	63	55	62	59	49	60	51	32	33	35	35	0					
T1	72	52	61	24	28	75	67	72	67	53	72	57	38	39	41	39	20	0				
S. spicatum 1	75	67	68	57	55	76	68	75	72	72	77	72	61	60	62	58	55	57	0			
S. spicatum 2	71	67	68	55	55	74	70	75	68	72	77	68	49	56	56	52	51	57	22	0		
S. spicatum 3	80	76	81	50	52	91	81	88	81	77	88	77	56	55	55	57	52	50	33	31	0	

Table 15 : Genetic dissimilarity matrix of core collection developed by Squared Euclidean distance

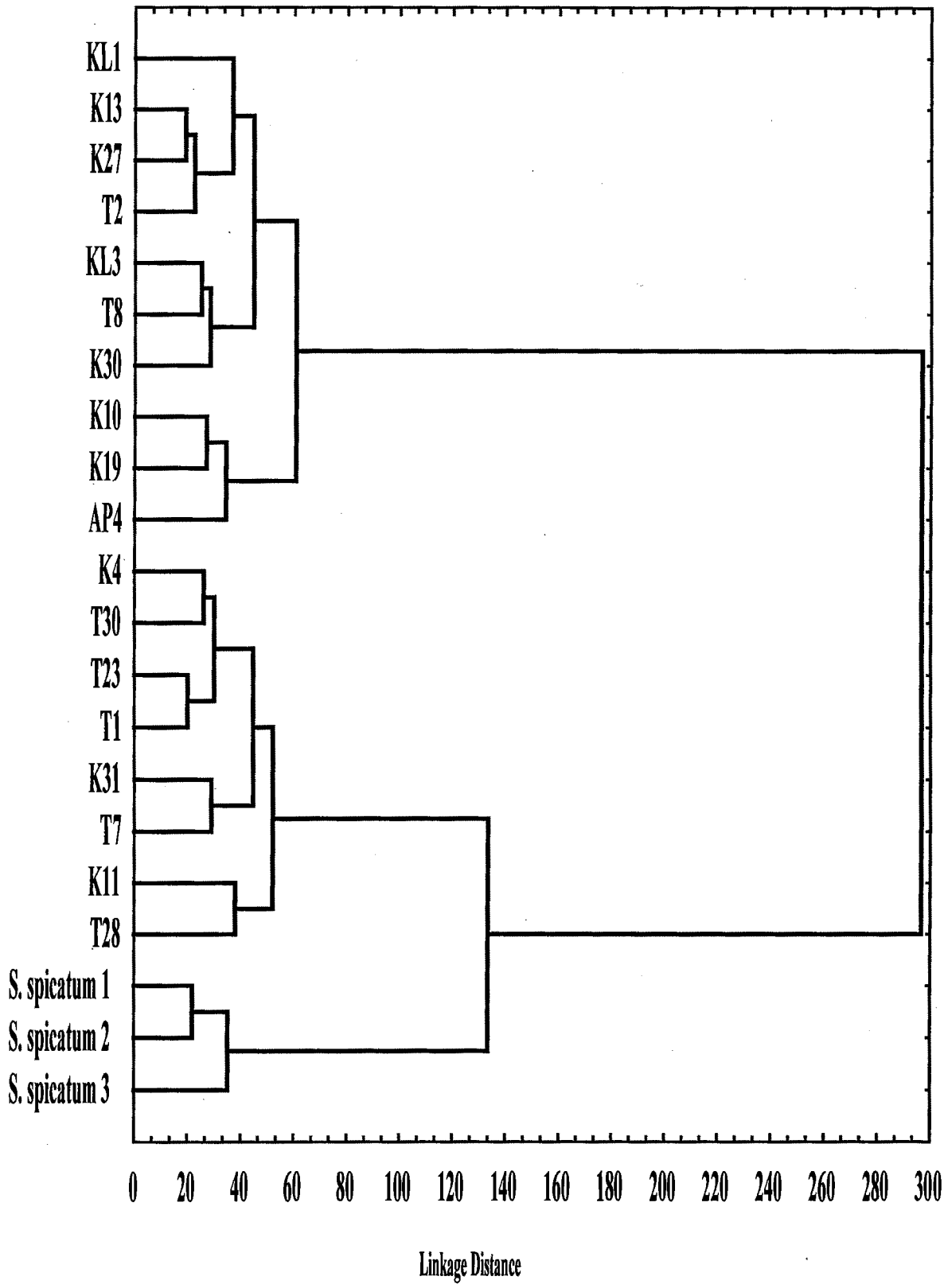

Figure 29 : Dendrogram of the sandalwood core collection identified by RAPD markers

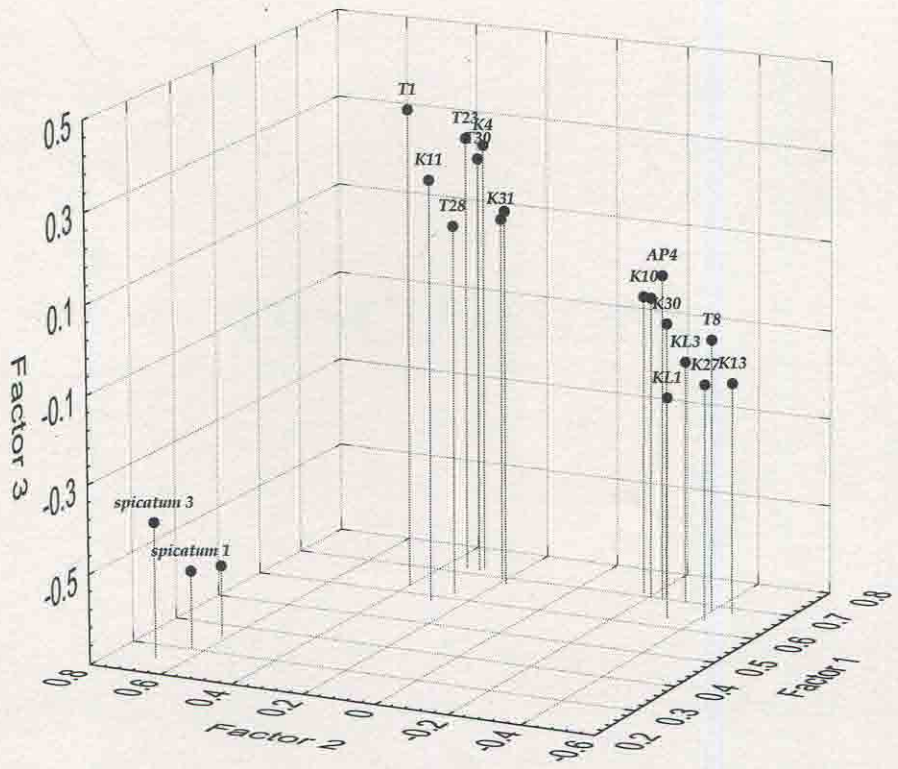

Figure 30 : Principal Component Analysis of the sandalwood core collection identified by RAPD markers

results indicated that, cluster analysis is in agreement with the Principal Component Analysis representing minimal duplication in the germplasm.

DISCUSSION

V. DISCUSSION

The results of the present investigation entitled "Genetic evaluation of elite sandalwood (*Santalum album* L.) clones using RAPD markers" have been discussed in this chapter under the various heads.

5.1 Standardization of protocol for DNA isolation

5.1.1 Preparation and processing of leaf samples

In the present study, recently matured leaves before flowering were chosen as source material to facilitate easy cell disruption for DNA extraction. Sandalwood leaves collected after the onset of reproductive phase posed difficulty in isolating pure DNA (Lodhi *et al.*, 1994), as it is associated with higher phenol content. Processing of too young leaves resulted in charring of tissue while drying. Similar results were also reported by Suneetha (2000). Leaves were dried uniformly in hot air oven at 40^0 C for 24 hrs. Prolonged drying of leaves resulted in poor DNA yield and quality. This can be attributed to the formation of irreversible complexes between polyphenols and DNA as reported by Wilfinger (1999).

Early protocols for isolation of plant DNA called for the use of liquid nitrogen to assist in the grinding of leaf material (Murray and Thompson, 1980; Porebski *et al.*, 1997). Although these protocols yielded good quantities of high quality DNA, the use of liquid nitrogen presented some problems. Once exposed to liquid nitrogen, it is imperative that the tissue should not be allowed to thaw before extraction. Again if large number of samples were to be extracted, then processing of tissue to extractable form and storage of tissue in freezer may lead to problems involving lack of space (Jennifer and Paul, 1990). In addition, plant material that are available from distant sources must be shipped frozen at high cost. Liquid nitrogen is also expensive. To circumvent these problems, it has become a standard practice to lyophilize tissue before extraction. In this freeze dried state, it is not necessary to keep

the tissue in a freezer. Shipping of such material is relatively easy and inexpensive but lyophilizers are quite expensive and have only a limited capacity. Besides, lyophilization requires several days to complete and errors in operation may result in degradation of DNA. Therefore, a new alternate method reported by Tai and Tanksley (1990) has been used in the present study, by which plant tissue can be inexpensively dehydrated, efficiently processed and extracted to consistently yield high molecular weight DNA comparable to lyophilized and fresh frozen material. The present attempt to standardize a simple, cheap and yet efficient method of sample preparation for isolation of genomic DNA from large number of samples yielded favorable results. The DNA extracted from dried sandalwood leaves were of high quality (OD 260/280= 1.70 to 2.01) and is adequate for PCR amplification and RAPD analysis.

Further, the dried leaves can be stored for over three months in a sealed polythene cover without degradation in the quality of DNA, while the leaf powder cannot be stored for longer time because of its hygroscopic property. This is the first attempt in sandalwood and offers enormous flexibility for scientists in large-scale sample preparation and storage.

5.1.2 DNA isolation and purification

The first step for the application of a molecular biology to any crop species is to develop protocol to extract high quality DNA. Extraction of high quality DNA from sandalwood leaves is a difficult task due to their rigid cell wall composed of complex carbohydrates. Sandalwood leaves are also rich in secondary metabolites, such as polyphenols, tannins and polysaccharides, which pose major problem in DNA purification, as they are difficult to separate from DNA. Many of the initial problems encountered in the extraction of high quality DNA have been attributed to these contaminants (Murray and Thompson, 1980).

In the present study, most recently matured leaves were sampled and therefore possibilities of high amount of polyphenols, tannins and polysaccharides were expected. Dealing with such components in mature leaves becomes necessary. Aware of these difficulties, four methods (1 and 2 using CTAB; 3 and 4 using SDS) were compared for isolation of DNA from sandalwood leaves. The protocols involving SDS were uniformly unsuccessful in attempts to extract high quality DNA. The main obstacle was difficulty in re-dissolving isopropanol precipitate DNA in TE buffer. Further, the resulting suspension was sticky and impossible to draw. A brownish yellow coloured substance, presumably one or more of the polyphenols present in sandalwood, often co-purified with the DNA. Similar observations were also reported in other crops such as cocoa (Jennifer and Paul, 1990), Bhendi (Kochko and Hamon, 1990) and cotton (Franklin and Arkesh, 1991) where polyphenols were shown to co-purify with DNA.

Based on the problems encountered with SDS method, the protocols using CTAB (i.e. Method 1 and Method 2) was employed. It was found that CTAB method resulted in good amounts of quality DNA (46.2 to 116.7 μg/g dry tissue) that was suitable for PCR amplification.

The original CTAB method (Method 1 using 1% CTAB) resulted in low DNA yield (46.2 μg/g dry wt) and the PCR amplification was poor, when compared to modified CTAB method (Method 2 using 3% CTAB). The modified CTAB method gave very high DNA yield (116.7 μg/g dry wt) and the PCR amplification was also good. According to Barnwell *et al.* (1998), the most widely used method to avoid co-precipitation of polysaccharides and DNA is the CTAB method.

CTAB is a cationic detergent that precipitates DNA leaving the neutral polysaccharides in solution at the DNA precipitation step. The DNA-CTAB pellets were treated with 1.4 M NaCl (Method 2), so that the sodium

exchanges with the CTAB resulting in clean DNA. The inclusion of PVP (2%) in the extraction buffer helped to avoid browning of isolated DNA. The browning occurred chiefly due to binding of tannins to DNA, which leads to degradation of DNA (John, 1992). Similar observations were also made by Borthakur *et al.* (1998), who used 200 mg of PVP in the extraction buffer for isolation of chloroplast DNA from tea. The PVP acts as an antioxidant, which binds to the phenolic compounds and co-precipitates in subsequent centrifugation resulting in DNA free of polyphenol contamination (Maliyakal, 1992). A concentration of 1% β-mercaptoethanol was found to be optimal in order to keep the nucleic acids in a non-oxidative environment and to denature endonuclease activities (Lefort and Douglas, 1999).

Further repeated washings with chloroform-isoamylalcohol (24:1) resulted in clean DNA, consistently amplifiable by PCR. Centrifugation for 5 min at 6000 rpm, as the original protocol stated (Method 1), produced a loose mass that impeded liquid phase separation. Increasing centrifugation time to 20 min at 9000 rpm improved adherence of the solid phase to the tube and allowed better separation of debris and supernatant after cell disruption. Further using higher concentration of PVP (2%), NaCl (1.4M) and repeated washings with chloroform-isoamylalcohol (4 times) removed the interfering contaminants resulting in high quality DNA, as compared to original CTAB method (method 1). The improvement in DNA yield may be explained by the electrostatic interactions between the different chemicals, nucleic acids and proteins. This combination of chemicals seems to prevent more efficiently formation of insoluble complexes of DNA than the classical combination of one detergent, one reductant, one salt offered by other protocols (Lefort and Douglas, 1999).

Removal of RNA and protein is an important step in DNA purification, since it may hinder PCR amplification (Porebski *et al.*, 1997; Dhanaraj *et al.*, 2002). However Tapan *et al.* (2000) reported the non-interference of RNA in

DNA amplification in tea. In sandalwood it was observed that RNA and Protein interfered with PCR amplification and its removal is essential. Though it has been reported that high concentration of RNAse (10 μg/ml) is essential for removing RNA, it was able to obtain equivalent results in sandalwood with lower concentration of RNAse (3 μg/ml at 37^0 C overnight). The proteins were removed by subjecting to phenol-chloroform extraction. Similar results were reported by Dhanaraj *et al.* (2002) in cashew.

The present protocol standardized for sandalwood is a modified CTAB method (method 2), which is relatively quick and inexpensive. The method involves use of high salt concentration (3% CTAB and 1.4% NaCl) to remove polysaccharides, use of PVP (2%) and β-mercaptoethanol (1%) to remove polyphenols and to keep DNA in a non-oxidative state and an extended RNAse treatment followed by phenol-chloroform extraction, which has consistently given high amounts of good quality DNA suitable for RAPD analysis. The purity of extracted DNA as indicated by A_{260}/A_{280} ratio (1.70 to 2.01) was generally of high purity, as the values are near to the requisite values of purified DNA.

DNA yields for sandalwood genotypes varied from 58 to 126 μg/g dry tissue. Such variation in DNA yields could be attributed to the nature of the leaf material used. Though only recently matured leaves were collected for DNA extraction, in some genotypes such leaves were not available and mature leaves were collected. Yields from such plant material were lower due to the interference of other secondary metabolites like complex carbohydrates and polyphenols.

5.2. Standardization of protocol for PCR based RAPD analysis

5.2.1 Amplification conditions

PCR is an enzymatic method of making multiple copies of a predicted segment of DNA. It commences with the denaturation step, which ensures

complete strand separation of the template DNA. An initial hot start for 4 minutes at 95^0 C followed by denaturation for 1 minute at 94^0 C was found optimum for sandalwood DNA. Denaturation step that are too long (>90 sec) leads to the unnecessary loss of enzyme activity. In contrast, incomplete denaturation (<30 sec) allows the DNA strand to snap back and thus, reduce the product yield (Innis and Gelfand, 1990).

Annealing refers to hybridization of the primer to single stranded DNA. An annealing temperature of 35^0 C was selected since this was the optimum temperature found to amplify DNA with decamer random primers. Below and above 35^0 C either failed to amplify or produced smearing on the gel electrophoresis (Tapan *et al.*, 2000). Annealing for a period of 2 minutes was found effective for good banding pattern than one minute. Similar observations were also made by Mukund (2000) in jasmine.

The temperature and length of time required for primer annealing depend upon the base composition, length and concentration of the primer (Innis and Gelfand, 1990). Primer extension was performed for 2 minutes at 72^0 C because this temperature was found optimum for extending the primer. Though the extension time of one minute at 72^0 C has been reported sufficient for products up to 2 kb in length, longer extension time will be helpful in early cycles if the substrate concentration is very low and at late cycles when product concentration exceeds enzyme concentration (Innis and Gelfand, 1990).

Forty-five cycles were found optimal for amplification of sandalwood DNA. Similar results were reported in cashew (Dhanaraj *et al.*, 2002), guava (Prakash, 2000), jasmine (Mukund, 2000) and china aster (Suneetha, 2000). Too many cycles (> 45) lead to increase in the amount and complexity of non-specific background products. In contrast, few cycles (< 30) resulted in low product yield (Innis and Gelfand, 1990).

5.2.2 Reaction parameters

It is important to optimize the concentration of the various components in the PCR mixture, in order to produce informative and reproducible fingerprints. The results revealed that, one of the most important variables is the concentration of template DNA. Different extraction methods produce DNA of widely different purity. Assays to optimize the template concentration were conducted over the range of 10-50 ng DNA in 25 µl reaction volume. For sandalwood, 25-30 ng DNA per 25 µl reaction volume gave reproducible banding pattern. Too much template (40-50ng) showed smears or lack of clearly defined bands in the gel. Too little DNA (10-15ng) gave irreproducible patterns. Similar findings were reported by William's *et al.* (1993) in soybean.

Both the magnesium ion and dNTPs concentration are known to affect the relative intensity and the number of amplified bands. It was found that a magnesium concentration of 2.0 mM was optimal, since the lower concentration resulted in less intense bands. Similar findings were reported by Murali (1999) in cashew. As the magnesium concentration increases, some DNA segments are amplified more efficiently while others are amplified less efficiently. Further *Taq* polymerase activity is inhibited at higher concentration of Mg ion. Similar results were observed in soybean (William's *et al.*, 1993). The magnesium concentration required for optimum activity of *Taq* polymerase depends on the dNTPs concentration, since dNTPs binds magnesium (Innis and Gelfand, 1990). The results showed a dNTPs concentration of 215 µM for each of the four bases is optimal for generating satisfactory RAPDs. At lower concentrations (< 150 µM) the intensity of stained bands in the gel becomes progressively weaker and at higher concentration (> 225 µM) smearing of the band was evident. Baumforth *et al.* (1999) reported that higher concentration of dNTPs encourage misincorporation by *Taq* polymerase. Similar findings were also reported by Prakash in Guava (2000). Therefore one should decide on the optimum

concentration of dNTPs appropriate for the length and composition of targeted sequences (Innis *et al.*, 1988).

5.3 DNA fingerprinting and genetic diversity analysis of sandalwood germplasm

5.3.1 DNA fingerprinting and identification of sandalwood genotypes using RAPD markers

Sandalwood, which is a very important aromatic tree, is native to India and found growing in many parts of peninsular India. The present investigation revealed that the genetic resources and diversity of sandalwood in India is large. Hence, there is an immediate need to conserve this genetic resource and utilize them for further commercial exploitation. The DNA fingerprints of the sandalwood genotypes developed during the present study would be of immense use in identifying these genotypes individually which would be useful in future patenting and Plant Varietal Rights to safeguard the country's genetic resources.

In sandalwood, identification of elite trees still relies on morphological characters of the leaf, flower, fruit etc. However, many of them cannot be readily distinguished by morphological indices, particularly if they are closely related. Furthermore, phenotype identification based on morphological traits is subject to environmental variation (Nielson, 1985). Isoenzymes have been used in distinguishing sandalwood genotypes, but the main limitation is the small number of loci that can be examined (Angadi *et al.*, 1997). This limitation is overcome by the RAPD method, based on amplification of multiple random segments of the genome using arbitrary primers (Welsh and McClelland, 1990; Williams *et al.*, 1990).

One of the objectives in the present study was to identify and distinguish the sandalwood genotypes, some of which are closely related, using eleven selected random primers. Initially the reproducibility of PCR

technique was examined using the primer OPE15, as we had received mixed reports on the ability of PCR and random primers to produce continually set patterns for use in such an application. The results revealed that the banding patterns produced were consistent and reproducible. Once the usefulness of the system giving a set banding pattern had been demonstrated, then the remaining ten primers were used for fingerprinting. The unique and polymorphic bands generated by eleven random primers were able to identify 50 of the 54 genotypes. While no single primer was able to completely resolve all the 54 genotypes examined. However, a combination of three primers (OPB12, OPE20 and OPF12) was found to identify most (41) of the genotypes. Similar findings were reported in red raspberry (Julie *et al.*, 1994) and strawberry (Degoni *et al.*, 1998). Four genotypes (T2, T12, T13 and T27) could not be distinguished with the eleven primers examined. This could be attributed to the similarity in genomic composition and the inability of the primers used to detect polymorphisms between them. Ye *et al.* (1998) also reported similar observations in grapes with regard to certain accessions.

The results obtained in this study demonstrated that RAPD markers could be used to distinguish different sandalwood genotypes. Consequently DNA fingerprinting using RAPD markers should be useful in maintaining genotype purity in sandalwood germplasm.

5.3.2 Genetic diversity and relatedness estimated by RAPD markers

Molecular diversity has great potential for quality control in tree breeding. Assessment of genetic diversity could be of great importance for identification and classification of genotypes, estimating the genetic relationship between them and selection of superior parental combinations useful in hybridization programmes. With the objective of selecting and maintaining elite trees to bring about an overall genetic improvement in sandalwood for enhanced output of oil and scented heartwood, genotypes have been collected from various regions of the Indian sub continent and are

at present maintained by Institute of Wood Science and Technology, Bangalore. However, the evaluation and characterization of these genotypes have not been conducted hitherto using molecular markers. The present investigation was, therefore, undertaken to document and measure genetic diversity and relatedness in sandalwood using RAPD markers.

An advantage of RAPD markers over morphological markers is that they are seldom influenced by the environment, are more polymorphic and can be assessed during any stage of plant development. Assessment of genetic diversity and relatedness in the present study is based on 156 RAPD markers generated by eleven (10 mer) random primers. This was comparable with the results obtained by Murali (1999) in cashew (157 bands from 10 primers), Dhanaraj *et al.* (2002) in cashew (123 bands from 7 primers), Galderisi *et al.* (1999) in fig (131 bands from 20 primers), Ko *et al.* (1998) in *Viola* (56 bands from 13 primers), and Shin *et al.* (1995) in watermelon (162 bands from 15 primers). The genome size of sandalwood was estimated by Flow Cytometry analysis by Dr. Arumuganathan, University of Nebraska, Lincoln, USA to be 0.45 pg $2C^{-1}$, and 156 RAPD markers saturates the genome to an extent of one markers for every 1.39 mbp. Meaningful inferences can be drawn about genetic variability from this as reported in other crops like mango (Schnell *et al.*, 1995), olive (Fabbri *et al.*, 1995), eucalyptus (Grattapaglia and Bradshaw, 1994), asparagus (Khendka *et al.*, 1996) and cashew (Dhanaraj *et al.*, 2002).

The present investigation revealed that there is a large genetic diversity in sandalwood. Eighty four (84.6%) per cent polymorphism was detected with 156 RAPD markers indicating a high marker index. RAPD analysis revealed that the genetic diversity of 54 genotypes was high with K13 and *S. spicatum* 3 being 91 per cent dissimilar, while T3 and T22 differed by only 15 per cent. Such a wide range in dissimilarity values suggests that the sandalwood germplasm examined represents a genetically diverse

population. One of the major contributory factors to the high degree of polymorphism observed may be on account of its evolutionary status as an out-crossing angiosperm. Similar observations have been made in coconut by Perera *et al.* (1998) where the level of genetic diversity was shown to correlate with the breeding nature of the palms.

Cluster analysis of the sandalwood genotypes employing Ward's method as well as PCA led to the grouping of the accessions into two distinct groups. The 51 Indian genotypes grouped in to one cluster, while the three Australian genotypes grouped in to a separate cluster and they were clearly discernible from the rest of Indian genotypes. The results indicated clear separation of the two *Santalum* species. Similar conclusions were reached by Changtragoon *et al.* (1994) and Singh *et al.* (1999) when assessing genetic diversity in neem using isoenzymes and AFLP markers respectively.

A study of the dendrogram showing the genetic diversity/relatedness among the 54 genotypes, indicated that clustering was according to their geographical regions with a very few exceptions. Matching the dendrogram results with their collection sites revealed that the geographical distribution of most genotypes in each of the sub clusters were well defined. The sub cluster Ia and IIb had majority of the genotypes from Karnataka. Likewise six of the seven genotypes in the sub cluster Ic and four of the five genotypes in the sub cluster Id were procured from Tamil Nadu. With the exception of T11 and AP4, all the genotypes in sub cluster Ib were from Karnataka shared a dissimilarity values ranging from 25-47 per cent. AP4 is the only genotypes examined from Andhra Pradesh and inclusion of AP4 (Hyderabad) in this sub cluster is quite interesting, since it is geographically distant from the rest. It is grouped with T11 from Tamil Nadu (Salem) exhibiting 31 per cent genetic dissimilarity. It is possible that genotypes from different geographical regions can be genetically similar to genotypes with immediate spatial relationships. Similar results were also reported by Padmesh *et al.* (1999) in *Andrographis*

paniculata, where AP48 from Thailand was shown to cluster with genotypes from different parts of Tamil Nadu.

Major cluster II having genotypes from local and distant localities of Karnataka and Tamil Nadu respectively, showed genetic dissimilarity of 66 per cent (excluding exotic genotypes) and this association between genotypes from different regions may be attributed to their broad genetic base of the species, which enable it to maintain and exist in different gene combination. Genotypes from Karnataka (K32 and K36) were grouped in between genotypes from Tirunelveli (T21) and Dharmpuri (T29) regions of Tamil Nadu, constituted sub cluster IIc sharing genetic dissimilarity of 18-43 per cent. This association between genotypes from neighboring states could be attributed to seed movement and gene flow. It was also observed that K37 from Karnataka was quite distinct and appeared as a separate entity both in the dendrogram and PCA. Although K37 is a native genotype, it is found to be significantly diverse from the rest exhibiting 47 per cent dissimilarity with T30 from Tamil Nadu. This may be attributed to the unique genetic composition of K37 as compared to other genotypes. Similar observations have been made by Padmesh *et al.* (1999) in *Andrographis paniculata,* where isolation of AP29, a native genotype as a separate cluster has been reported.

On comparing the genetic diversity from the different states, as revealed by the PCA and dendrogram, it was evident that genotypes from Karnataka (67%) were less diverse compared to Tamil Nadu (72%). This could be partly attributed to the duplication of genotypes from Karnataka.

There is some discrepancy in the origin of sandalwood. Gode (1961) believed that sandal is indigenous to India since it has been often referred to in ancient literature. However, Thirawat (1955) reported that sandal is exotic to India since there are many species present in the Malayan archipelago region. Majumdar (1941) had a different theory for the origin of sandalwood.

He proposed its diphyletic origin from India and Timor from the Malayan archipelago. This hypothesis states that sandal could have spontaneously originated in different places about the same time.

In order to confirm any of these hypotheses, the Indian genotypes used in the present investigation were grouped into three classes based on their distance from Cape Comorin (Kanyakumari) and the diversity was estimated from each of these groups. The first group consisted of 16 genotypes from 8.04 ^{0}N to 11.39 ^{0}N, second had 27 genotypes from 11.40 ^{0}N to 13.56 ^{0}N and the third group consisted of 8 genotypes from 13.57 ^{0}N to 17.2 ^{0}N. The diversity of the genotypes from the first group (southern most tip of the Indian peninsular) was the maximum with T1 and T4 being most dissimilar (71 per cent). The diversity of the other two regions were relatively lesser than that of the first region. In the second group, K11 and K13 were dissimilar by 66 per cent and K23 and K19 were dissimilar by 67 per cent in the third group. Since the diversity of the genotypes from the southern tip of the peninsular region was maximum when compared to the interior regions, it could possibly indicate that sandal came to India *via* Cape Comorin (Kanyakumari) from the Malayan archipelago. In cashew it has been reported that it was first introduced to the Malabar Coast of India from where it spread to other interior parts of the country over the years. Hence, the diversity of the accession from the Malabar Coast was more when compared to other regions of the country (Dhanaraj, 2002).

In sandalwood the diversity of genotypes from other regions of south east Asia should be estimated and compared with that of the Indian genotypes in order to strengthen the earlier reports regarding its origin. If the diversity of the genotypes from other regions is lesser than that of the Indian ones, then it could be confirmed that India is the center of origin of sandalwood, and as revealed by this study the peninsular tip being a hot spot for sandalwood with the most genetically diverse genotypes.

When the exotic genotypes (Australia) were excluded, only 75 per cent genetic dissimilarity was observed within the Indian genotypes. However, the sub groups assigned by cluster analysis were not altered and were found to be consistent within the Indian genotypes. But the diversity was reduced by 16 per cent indicating the need for introduction of exotic genotypes in order to strengthen the genetic base of sandalwood in India. Similar observations were reported by Dhanaraj in cashew (2002).

Eleven unique bands were identified using eleven random primers. Such unique bands can be converted into genotype specific RAPD markers, which may be used for the identification of genotypes. In addition, four species specific bands (A^1 and S^1) were identified that are specific to *S. album* and *S. spicatum,* respectively. Such species-specific bands that could distinguish clearly between the Indian and exotic genotypes were also reported in case of neem (Singh *et al.*, 1999).

5.4 Correlation of the RAPD and morphological data

Six morphological characters and 149 RAPD markers were used to discriminate the 51 Indian sandal genotypes. Cluster analysis revealed that morphological and RAPD data were discordant. Harrison *et al.* (1997) made similar observations in strawberry.

When compared to RAPD markers, the morphological markers could not discriminate the genotypes studied even though there was a positive correlation between the distance matrices generated by RAPD and morphological data ($r = 0.185$). The inability of the morphological markers to estimate diversity and to discriminate the genotypes could be due to the lesser number of markers used, and the lesser amount of polymorphism generated by these markers. In sandalwood it was thus evident that molecular markers were superior to morphological markers in discriminating

the genotypes. Similar findings have also been reported in perennial crops like cocoa (Lerceteau *et al.*, 1997), rhubarb (Persson *et al.*, 2000) and cotton (Tatineni *et al.*, 1996).

5.5 Core collection

A core collection is a set of accessions selected to represent the genetic diversity of a base collection with minimum redundancy (Frankel and Brown, 1984; Hamon *et al.*, 1995). This strategy was introduced with the intention of minimizing the cost of genetic conservation, while ensuring representation of maximum genetic variation. It also allows a rapid evaluation of germplasm and a better access to the base collection. A gene bank is meant to conserve the diversity of the genetic resources for the benefit of plant breeders and researchers. Often because of the large number of accessions, problems are encountered in documentation, conservation, multiplication and evaluation.

From the PCA of the 54 genotypes generated by 156 RAPD markers, it is evident that some of the genotypes were found overlapping each other depicting redundancy. A core collection of 21 genotypes was identified for the 54 by eliminating the overlapping individuals and those in close proximity in each quadrant in the PCA. It includes eight of the twenty four genotypes from Karnataka, seven of the twenty four genotypes from Tamil Nadu, both the genotypes from Kerala, the only genotype from Andhra Pradesh and the three exotics from Australia. They were distributed into three distinct groups on the PCA exhibiting 19-91 per cent dissimilarity.

The two main problems to solve are, the sampling strategy and the size of the sample to be used. There are different strategies for developing a core collection where morphological or molecular data can be used. Irrespective of the strategy used, it is recommended that atleast one accession from each group should be included in the core collection (Brown, 1989a). The size of the core is also an important factor and it is usually 5-10 per cent of the

original population, which represents 75-90 per cent diversity of the base collection (Bisht *et al.*, 1998). However, Noirot *et al.* (1996) believe that larger core samples (20-30%) are needed, particularly when the objective is to capture the diversity of quantitatively inherited traits. Sandalwood is a perennial tree and many of the important agronomic traits (e.g. Heartwood and oil contents) are governed by polygenes. In the present study, a core collection of 21 individuals was considered an adequate sample size, which represents the same diversity of the original population. When the core sample was identified, it was ensured that the genotypes from different regions/states were included so as to represent the diversity to the maximum extent. Similar work was reported by Dhanaraj *et al.* (2002) in cashew, where a core collection of 54 accessions was identified from the 90 that show the same diversity of the entire population.

The grouping for developing the core collection in sandalwood was based on only RAPD data. Similar strategy was used in case of *Poa pratensis* (Johnson *et al.*, 1999) and cashew (Dhanaraj *et al.*, 2002). Grouping can also be based on morphological data or taxonomy as reported in snow barley (Knupffer and Hintum, 1995) and sesame (Zhang *et al.*, 2000). However, molecular markers are more stable and efficient in estimating the genetic relatedness among the individuals. Hence, in the present investigation, grouping of the genotypes for developing a core sample was confined to the data generated by RAPD markers. Similar conclusions were drawn by Dhanaraj (2002), where core collection was identified based on RAPD data in cashew.

The results effectively demonstrated the use of RAPD markers in estimating genetic diversity and identifying a core collection in sandalwood. Such core collection can serve as efficient starting point for screening of base population in the search of desirable traits. Further, it can be the basis for future studies about the phylogenetic origin of sandalwood in the region.

This could be the first step towards efficient germplasm management of an important aromatic tree species like sandalwood where land, time and money can be saved.

Future line of work

The present study clearly reflects the utility of RAPDs in fingerprinting and estimation of genetic diversity within this important aromatic tree. Other applications of RAPDs are now possible; the most appealing is the search for linkages between specific amplified DNA sequences and genetic traits of economic relevance such as resistance to 'spike disease'. Apart from RAPDs, there are many other marker techniques (e.g. SSRs, SCAR, AFLP, STS) which find various applications in sandalwood improvement like mapping of genetic traits, synteny studies, phylogenetic studies, identifying clones having potential for oil content etc.

Although assessment of genetic variability was attempted by evaluating morphological traits, the number of markers studied was not conclusive to infer the phenetic relationship between the genotypes. Hence morphological characterization of the genotypes examined needs to be under taken.

Though the level of genetic variability detected is moderate to high, exclusion of exotics showed decrease in diversity within the Indian genotypes. This clearly indicates that introducing exotic germplasm can broaden the genetic base of the Indian sandalwood. A core collection developed in the present study can be strengthened through additional characterization and evaluation of other genotypes that are not part of this study. This will allow for refinements in the core sample, thereby increasing the knowledge about the genetic variation contained in it.

Future attempts in sandalwood improvement programme should therefore, take into consideration the findings of the present investigation, as these have direct implications in tree improvement programmes. The DNA profiles of the 54 sandalwood genotypes have been characterized using RAPD markers. Molecular breeding amongst the elite trees, as identified by their respective fingerprints, may be carried out to produce superior clones. Also, if conservation strategies have to be devised to protect sandalwood trees, then genotypes representing maximum genetic diversity needs to be conserved, followed by the genotypes which complements the previous one. Prior to this, an exhaustive study with regard to the existing genetic diversity of sandalwood in India needs to be undertaken.

SUMMARY

VI. SUMMARY

Investigations on "Genetic evaluation of elite sandalwood (*Santalum album* L.) clones using RAPD markers" were conducted in the Plant Molecular Biology Laboratory, at the Division of Horticulture, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bangalore, during 1998 to 2002. The salient findings of the study are summarized hereunder.

6.1 Standardization of protocol for DNA isolation and RAPD analysis

Selection of recently matured leaves and drying them at 40^0 C for 24 hours was found optimum for powdering and further processing for DNA extraction. Prolonged drying beyond 24 hours was found detrimental for the quality of DNA. Use of high salt concentrations (3% CTAB, 1.4M NaCl), 2 per cent PVP, 1 per cent β-mercaptoethanol in 20 ml extraction buffer for 400 mg of dried leaf powder and an extended RNAse treatment followed by phenol-chloroform (1:1) extraction resulted in high amounts of good quality DNA suitable for RAPD analysis.

Amplification conditions and reaction parameters for PCR/RAPD analysis was standardized for sandalwood DNA using Operon random primers. A reaction volume of 25 µl containing 25-30 ng of template DNA, 2.0 mM $MgCl_2$, 215 µM each of dNTPs, 1 unit of *Taq* polymerase and 5 pico moles of primer was found optimum to obtain intense, clear and reproducible fingerprints. Consistent banding pattern was obtained by initial hot start for 4 min (95^0 C), followed by 45 cycles with each cycle consisting of 1 min denaturation (94^0 C), 2 min annealing (35^0 C) and 2 min primer extension (72^0 C) with a final extension period of 10 min (72^0 C).

6.2 Fingerprinting and genetic diversity analysis of sandalwood germplasm

One hundred and fifty six RAPD markers generated by eleven selected random primers were used for fingerprinting and to estimate the genetic

diversity among 54 sandalwood genotypes. Each primer could identify between 7 to 26 genotypes. The unique and polymorphic bands produced by eleven random primers were able to identify 50 of the 54 genotypes. A combination of three primers (OPB12, OPE20 and OPF12) was found to distinguish most of the genotypes. However, four genotypes (T2, T12, T13 and T27) could not be differentiated with the eleven primers examined.

Ward's method of cluster analysis based on 156 RAPD markers grouped the 54 genotypes into two major clusters with four sub clusters within each major cluster. Clustering was according to their geographical regions with few exceptions. The three exotic genotypes from Australia formed a separate sub cluster and they were clearly discernible from the Indian genotypes. The genetic relationships within the genotypes were evaluated by generating dissimilarity matrix based on SED. Distance matrix indicated that sandalwood germplasm examined constitutes a broad genetic base with the values of genetic dissimilarity ranging from 15 per cent (between T3 and T22) to 91 per cent (between K13 and *S. spicatum* 3). Also, K37 from Karnataka was found to be significantly diverse from the rest of the native genotypes and appeared as a separate entity. It was also observed that genotypes from Karnataka are less diverse (67%) compared to genotypes from Tamil Nadu (72%). Several unique and four species-specific bands were identified. Such bands can be converted into genotype/species specific DNA markers, which may be used for the identification of genotypes.

6.3 Correlation of genetic distances generated from RAPD and morphological markers

Six morphological markers were used to estimate diversity among the 51 Indian genotypes. They were not efficient in discriminating all the genotypes examined and the pattern of variation between molecular and morphological markers is quite different. Though there was a low level of correlation between the morphological and RAPD distance matrices, the

results are not concordant indicating that phenotypic traits are not as effective as RAPDs in diversity studies.

6.4 Core collection

A core collection of 21 genotypes was identified with RAPD markers, which represents the same diversity of the 54 genotypes studied. The core collection included eight of the twenty four genotypes from Karnataka, seven of the twenty four genotypes from Tamil Nadu, both the genotypes from Kerala, the only genotype from Andhra Pradesh and the three exotics from Australia exhibiting 91 per cent dissimilarity. This core collection could be effectively used to identify duplicates in the germplasm and can serve as the basis for screening of desirable traits in the base population.

In the present study, RAPD markers were successfully used to assess the genetic diversity among the sandalwood genotypes. The results suggests that it is an efficient marker technology for delineating genetic relationships amongst genotypes and estimating genetic variability, thereby enabling the formulation of appropriate strategies for conservation and sandalwood improvement programmes.

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APPENDIX

APPENDIX

Extraction buffer 20 mM EDTA pH 8.0
100 mM Tris-Base pH 8.0
1.4 M NaCl
3 % CTAB
2% PVP
1 % β-mercaptoethanol

Assay solution (0.1 μg/ml H33258 in 1X TNE, 0.2 M NaCl, 10 mM Tris-Hcl, 1 mM EDTA pH 7.4)
H33258 dye (10 μl)
10X TNE buffer (10 ml)
DD filtered water (90 ml)

Loading buffer 0.25% Bromophenol blue
0.25% Xylene cyanol
40% sucrose (w/v)

Running buffer 1x TBE buffer (54 g Tris, 27.5 g Boric acid, 20 ml EDTA 0.5 M pH 8.0 in 1 liter double distilled water)

PCR buffer 10x stock (50 mM KCl, 10 mM Tris-Hcl pH 9.0, 1.5 mM $MgCl_2$, 0.1% Gelatin, 0.05% Triton-X 100 and 0.05% NP40)

EDTA : Ethylene Diamine Tetra Acetic acid
TE : Tris EDTA buffer (10 mM Tris-Hcl and 1 mM EDTA, pH 8.0)
PVP : Poly Vinyl Pyrolidone
CTAB : Cetyl Trimethyl Ammonium Bromide
dNTPs : dinitro-triphosphates

M	: Molar
μM	: micromolar
mM	: millimolar
g	: gram
mg	: milligram
μg	: micro gram
ng	: nanogram
pg	: picogram
ml	: milliliter
μl	: microliter
pmol	: picomoles
rpm	: rotation per minute
bp	: base pairs
kb	: kilo base pairs
MSL	: Mean Sea Level
SED	: Squared Euclidean Distance

Enzymes –

Taq	**Thermus aquaticus**
*Eco*RI	*Escherchia coli* RY13
*Bam*HI	*Bacillus amyloliquefaciens*
*Hind*III	*Haemophilus influenzae* Rd.
*Hae*III	*Haemophilus aegyptius*
*Pst*I	*Providencia stuartii*