

Sandalwood Research Newsletter

November 2009

Issue 24

ISSN 1321-002X

EDITOR'S NOTE

The heavy exploitation of many sandalwood species has raised concerns about sustainability across several countries. Efforts to conserve natural sandalwood stands can benefit from knowledge of their genetic structure and relationships with other populations, with the view to allocating resources to particularly diverse or distinct stands. In this issue Srikanta Dani examines the molecular diversity among three populations of *S. album* in southern India. These authors provide an insight of why the population genetic structure of these continental occurrences resemble that of island populations, and the implications it has for their conservation.

In Western Australia conservation efforts for *S. spicatum* are assisted by a robust plantation sector, and with recent increases in the rate of plantation establishment a high demand exists for its seed. Graham et al. explores some of the possible physiological factors that influence seed set in this species with the view to understanding the mechanisms affecting seed yield.

Tony Page



Figure 1. *S. album* growing in Kengeri, Bangalore University Campus, India

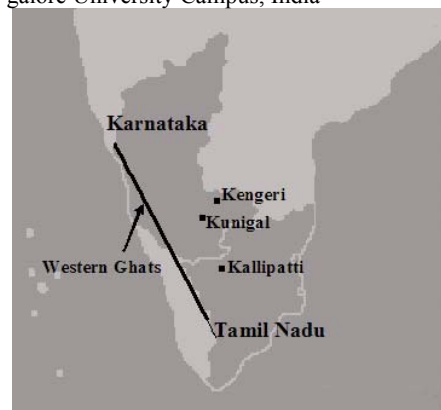


Figure 2. Three *S. album* populations Kengeri, Kunigal & Kallipatti in southern India. Western Ghats depicted as a line across the states of Karnataka and Tamil Nadu.

Genetic Diversity Estimates in Three Geographically Isolated Populations of *Santalum album* in India

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Abstract

Santalum album is an economically important aromatic tree that belongs to the family Santalaceae. The tropical and temperate deciduous forests of the Deccan plateau harbour an extensive sandalwood tree population. Illicit felling for its prized heartwood and cyclic spike epidemics together have drastically reduced the tree population placing it in the vulnerable category of IUCN Red list of threatened species. Conservation of the wild germplasm can be improved with more detailed information on the species genetic diversity. Systematic evaluation of genetic diversity among three geographically distinct natural populations from southern India was undertaken using 4 DAMD (Directed amplification of minisatellite DNA) and 7 RAPD (Random amplified polymorphic DNA) primers. The eleven primers used in this study amplified 65.99 % polymorphic bands and Analysis of Molecular Variance (AMOVA) revealed high genetic variation among and within the populations. Evidence of clonality within the populations was found, which may be attributed to habitat fragmentation and vegetative reproduction. The genetic diversity in this important tree species needs to be maintained through conservation activities to avoid further genetic erosion.

Introduction

In India, *Santalum album* is distributed in the states of Karnataka and Tamil Nadu (Rai 1990) and harvested primarily for their fragrant heartwood and oil. The global demand of Indian sandalwood is ever increasing but the legal harvest is highly restricted. Karnataka's annual sandalwood production has dropped from 3,000 tonnes to 50 tonnes in the last three decades.

The average price of wild sourced *S. album* has increased at a compound rate of 21% for 17 years to 2009 (TFS 2009). Given the high international

prices for sandalwood, smuggling is widespread in India. Government's effort to liberalize sandalwood trade has had no impact and there has been little gain from sandalwood farming (Hindu 2006b). Sandalwood farming initiatives in Western Australia may satisfy the market demands for sandalwood but the long-term economic feasibility of developing plantations relies on the preservation and use of genetic diversity within wild populations. *Santalum album* is currently recorded as 'vulnerable' in the International Union of Conservation of Nature and Natural Resources Red List of threatened spe-

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cies (IUCN 2009). There has been at least 20% loss over the last 10 years or three generations based on actual or potential levels of exploitation. The existing populations are devoid of trees of a commercial girth, due not only to illicit felling, but also grazing, recurrent fires and the lethal phytoplasmic spike epidemics. Conservation of genetic resources entails assessment and maintenance of important genetic variability among the germplasm of interest. Diversity estimates of a core collection of 51 elite sandalwood genotypes from different geographical locales in India were shown to originate from a broad genetic base (Shashidhara *et al.* 2003). Geographic information system studies confirm that sandalwood forests are concentrated in the Deccan plateau and the allozyme studies reveal such populations as having high genetic diversity (Rao *et al.* 2007).

In this study we have used RAPD (Random amplified polymorphic DNA) and DAMD (directed amplification of minisatellite DNA) markers for diversity estimates as a preliminary investigation of three fragmented populations in the Deccan plateau. Within we focus

on population fragmentation and local diversity levels, which influence its phylogeography and evolution. Despite the tendency for RAPDs to underestimate intra-population diversity because of dominance and biallelism, they are effective in detecting variations due to sequence duplications and accumulated errors of replication (Aagaard *et al.* 1998). DAMD PCR has been employed to determine both inter- and intraspecific variations in many eukaryotes (Somers *et al.* 1996; Bebeli *et al.* 1997; Bhattacharya and Ranade, 2001). While the distribution of mini-satellites (as revealed by DAMD) is not as prolific in plant genomes as other genetic primers, they are shown to be evenly distributed (and not clustered) (Tourment *et al.* 1998). The quickly evolving mini-satellites results in high levels of polymorphism levels and combined with the high abundance of RAPD loci can be used for quantifying intraspecific variation. This is one of the first reports on the genetic diversity analysis of *Santalum album* at the level of isolated populations, based on DNA markers with an emphasis on its germplasm conservation.

Materials and Methods

Collection of plant material

Sandalwood trees from three distinct geographical locations of Southern India (Kunigal (KN) and Kengeri (BU) in Karnataka and Kallipatti (KP) in Tamil Nadu) (Figure 2) were used for RAPD and DAMD analyses. The Kunigal and Kengeri populations were separated by a distance of 100km and each were approximately 400km from the Kallipatti population. The criteria for sampling were diameter (at breast height –DBH), distance from the previous individual sampled and visual phenotypic differences. The mean diameter (DBH) for the sampled individuals was 18 ± 5 cm (with two modal classes). Individual plant samples within the Kunigal and Kallipatti populations were each not less than 150 meters apart to avoid collecting clones. The samples from Kengeri population were each approximately 20 meters apart as it is an assorted collection of plants from Western Ghats. Two to three grams of leaf samples were collected from each tree and frozen in liquid nitrogen and stored at -80°C .

DNA extraction and Polymerase chain reaction

DNA from the collected leaf samples was extracted using CTAB extraction buffer by the method described by Doyle and Doyle (1990). The DNA was dissolved in TE (pH 8), purified by RNase treatment followed by chloroform and isoamyl alcohol (24: 1) extraction. The DNA was quantified spectrophotometrically and diluted to $25\text{ng}/\mu\text{L}$ for PCR amplification. PCR amplification was performed with custom synthesized random decamer primers (Appendix 1). Amplification was performed in a $25\mu\text{l}$ reaction volume that contained 35 ng of DNA template; $2.5\mu\text{l}$ 10X buffer (3Trishydroxy methyl methylamine propane sulphonic acid, pH 8.8; 2.5 mM MgCl_2 ; 50 mM KCl, 0.01% gelatin); 2.5 mM each of dATP, dCTP, dTTP, dGTP; 0.02 mM of primer; 0.3 U *Taq* polymerase. Amplification conditions were maintained at 94°C for 4 min and 45 cycles of 94°C for 1 min (denaturation), 40°C for 1 min (annealing) and 72°C for 2 min (elongation) followed by 10 min at 72°C . The random amplification was performed twice on all the samples and the primers with reproducibility of bands were selected for data analysis. Amplified products were examined by electrophoresis on 1.5% agarose gel run in 1X TAE buffer (100 mM Tris-HCl, pH 8.3; 83 mM acetic acid; 1 mM EDTA) at 60 V. The gels were stained with 0.5mgml^{-1} ethidium bromide solution and visualized under UV light. The size of the amplified products was compared to pBluescript *Hinf* I and lambda DNA *Hind* III digested markers.

DAMD PCR was carried out according to Zhou *et al.* (1997) using *Oryza* minisatellite core sequences (Winberg *et al.* 1993) and 35 ng of DNA at a stringent annealing temperature of 50°C . This technique involves the use

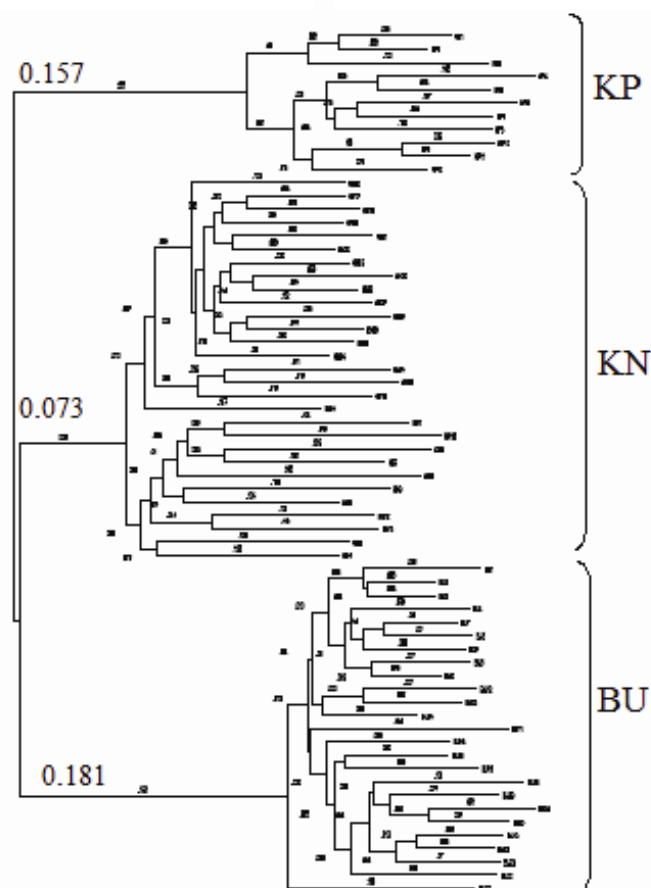
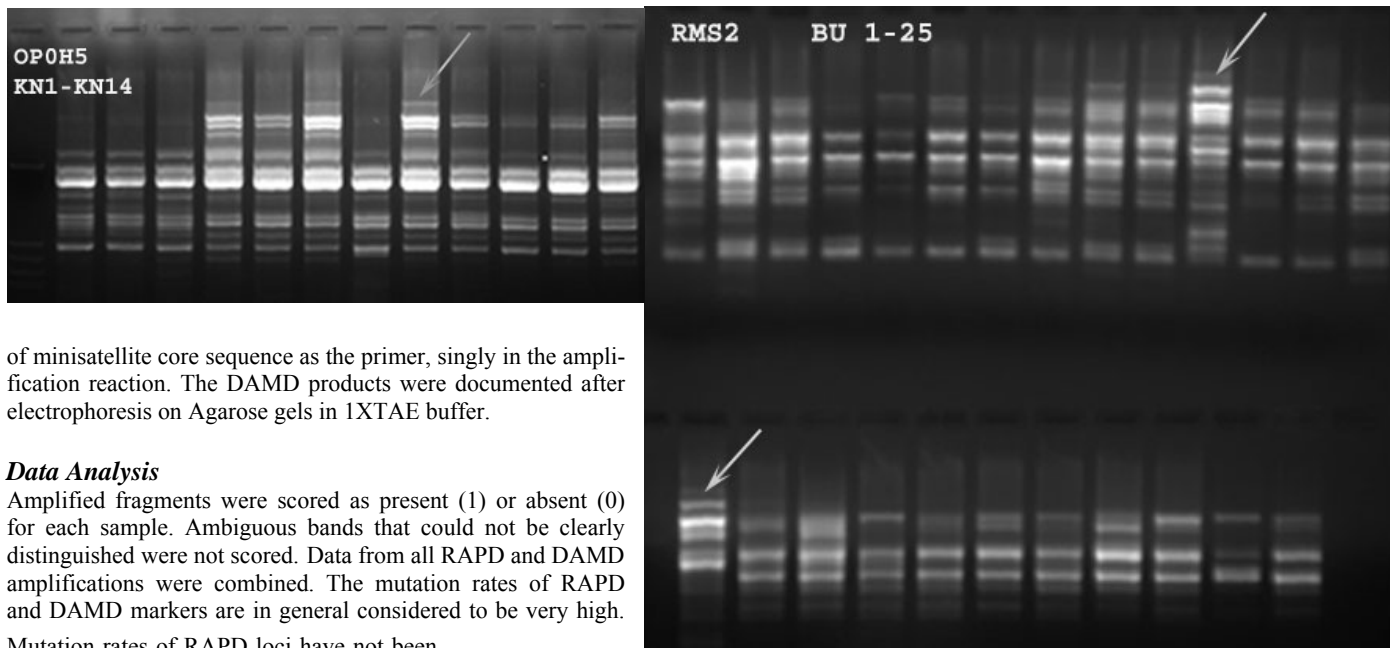


Figure 3. Dendrogram generated for the 65 samples from three populations of *S. album* in southern India (KP-Kallipatti, KN-Kunigal, BU- Kengeri) using neighbour joining method based on Dice distance



of minisatellite core sequence as the primer, singly in the amplification reaction. The DAMD products were documented after electrophoresis on Agarose gels in 1XTAE buffer.

Data Analysis

Amplified fragments were scored as present (1) or absent (0) for each sample. Ambiguous bands that could not be clearly distinguished were not scored. Data from all RAPD and DAMD amplifications were combined. The mutation rates of RAPD and DAMD markers are in general considered to be very high.

Mutation rates of RAPD loci have not been reliably quantified for plants. The mutation rate of mini-satellites (around 10^{-3} to 10^{-2}) could be faster than that of RAPDs (Spooner *et al.*, 2005), but it is proposed they are comparable. Homology and distinctness of the bands was ascertained by the co migration of the bands on the gel.

Pair wise distance and Bootstrap analysis

The Dice (Nei and Li) pair wise distances were calculated using RAPDALG program from RAPDistance software (Dice, 1945; Nei and Li, 1979). The distance values were used by NJTREE.EXE to produce Neighbor Joining tree and a dendrogram was generated by TDRAW.EXE. The strength of the statistical analysis was evaluated by bootstrapping data over loci for 1000 replications using the SEQBOOT program of PHYLIP. The phylogeny estimate was performed by PARS (Wagner parsimony method with multiple states) which allows freedom among all states and generates a consensus tree. PARS gives a list of equally parsimonious trees with branch lengths. These are computed using an algorithm by Hochbaum and Pathria (1997), a modified Wayne Maddison algorithm. The log likelihood differences were statistically estimated by using the mean and variance of step differences between trees, across populations by a test proposed by Templeton (1983) and Felsenstein (1985). The consensus tree obtained by the CONSENSE program of PHYLIP was displayed with TREEVIEW software (Page, 1996)

Analyses of population differentiation

The extent of population subdivision was examined using analysis of molecular variance (Excoffier *et al.* 1992). The AMOVA analyses were performed using the program WINAMOVA 1.04, which calculates Φ -statistics. The null distribution of pairwise Φ_{ST} values was obtained by 1000 permutations of the original data set.

Figure 4. Representative agarose gel electrophorogram displaying differentially amplified dominant loci using a RAPD primer .

Results and Discussion

RAPD and DAMD profile analysis

Eleven selected primers consistently amplified scorable bands ranging from 200 – 2000 bp (base pairs). Some bands were unique to a single population or differentially present in three populations. No genotype specific bands were observed. The high degree of polymorphism detected with 145 of total 220 RAPD and DAMD bands (Appendix 1) indicates a high marker index . The genome size of sandalwood estimated by flow cytometry is $0.45 \text{ pg}2\text{C}^{-1}$ (Arumuganathan *et al.* 2003; Shashidhara *et al.* 2003) and the 220 bands generated in this study saturate the genome, representing one marker for every 1.02 mbp. In the current study a high level (65.9%) of RAPD and DAMD primer polymorphism was found for *Santalum album*. *Plathymenia reticulata* (Fabaceae), a tree from Brazil and *Acacia raddiana* (Mimosaceae), a tree species from Israel exhibited high polymorphism (70.8 % and 90.69% respectively) when using RAPD markers (Lacerda *et al.* 2001; Shrestha *et al.* 2002).

Genetic diversity analysis

The AMOVA of the genetic distances of 65 individuals revealed variation among (54.9%) and within (45.1%) populations (Table 1). A high variance percentage of 54.9 among populations indicate genetically diverse collection of trees in three different populations. A

marginally lower variance of 45.1% within the populations is obtained and significantly high f_{ST} value of 0.549 for variance among populations was observed. Long-lived woody plants typically harbour a greater percentage of variation within populations (Hamrick and Loveless, 1989; Hamrick *et al.* 1992; Nanda *et al.* 2004; Li *et al.* 2008). But in the Indian context the typical population structure and fragmentation has given rise to isolated population systems. In our study we have made deliberate attempts to avoid incorporation of clones by stringent inter sample distance parameters. The mechanism of reproduction is the most important factor in determining the genetic structure of plant populations. Out crossing plant species tend to have 10-20% of the genetic variation among populations, whereas 'selfing' species have on average, 50% variation among populations (Hamrick and Godt 1989). Sandalwood is a predominantly out-breeding species with pollination carried out by bees, butterflies and beetles (Veerendra *et al.* 1996) contributing to significant genetic heterogeneity. The startling observation from the statistical analysis is high Φ_{ST} value of 0.549, which is typical of insular systems with physical barriers to contain the gene flow. Incidentally, such high values are reported for different endemic species of *Santalum* from the pacific islands (Bottin *et al.*, 2005; Lhuillier *et al.* 2006). Although, the molecular markers used in those studies

are different from this study, there are several points that make this comparison valid. It is supposed that sandalwood in India has its origin in Indonesian island of Timor (Rajagopal Shetty, 1977). There is molecular evidence to show that generally island populations exhibit lower genetic variations than their counterparts on the mainland (Frankham, 1997). Previously reported allozyme marker based heterozygosity estimates of the Timor [$H_0=14\%$] and Indian [$H_0=31\%$] sandalwood populations may apparently be different (Brand 1994; Rao *et al.* 2007). However, the higher genetic diversity of the species in India may be the manifestation of a larger population size and distribution range compared with Indonesia.

The Dice pairwise distances used to develop an NJ tree dendrogram clearly depict genetically diverse tree populations with clonality within the populations. The maximum distance in the cluster is 0.181 of Kengeri and closest is Kunigal (0.073) population from origin of the tree. The samples from Tamil Nadu cluster uniquely with a distance value of 0.157 in the dendrogram. The phylogenetic analysis reveals genetic clustering of the trees of a single population with some genotypes spanning the extremes of sub cluster. The popula-

tions from Kallipatti, Kunigal and Kengeri cluster in separate clades. The phenogram (Figure 3) depicts that the Kunigal cluster is in proximity to the oldest hypothetical common ancestor and prominently diverged than others. The BOOTSTRAP values were less than 50% within all the populations and 100 % for the populations from Tamil Nadu and Karnataka, which is in accordance with the geographical topology. The radial NJ tree (Figure 5) indicates clonality within the populations. In the Kallipatti cluster samples KP4-5 and KP10-11 are on the same branch with BOOTSTRAP values higher than 50%. These high BOOTSTRAP values further endorse the sampling strategy wherein all the three populations exhibit affiliations to their closest geographical allies. Keeping these points in view, we can say that the Indian sandalwood populations would tend to show considerably lesser genetic variation than an average indigenous mainland plant species. The populations used in the present investigation are partly mimicking the insular systems because they are subjected to isolation by natural and human interference.

The variance among populations under this study is high (54.9%), suggesting limited gene flow between them. Under the current circumstance of quick popu-

lation depletion, restricted gene flow could be an advantage for management as it can help in clear identification of unique gene pools over geographic scales. The extent of outbreeding within the existing genotypes can be influenced by a number of factors including pollination dynamics and preponderance of pollinators. Existence of continuous populations of a hemiparasitic tree species also largely depends on the presence of host trees. These physical and biological barriers encountered by the populations in the present study are probably contributing to high f_{ST} values. These values appear to be particularly high as compared to values of other tropical tree species distributed in the mainland viz., 0.047 in *Vitellaria pandoxa* (Sanou *et al.* 2005), 0.080 in *Vauca poua americana* (Dutech *et al.* 2004), and 0.22 in *Grevillea macleayana* (England *et al.* 2002).

Habitat fragmentation of *S. album* in India can lead to a loss of population genetic variation through reduction in population size. It also creates genetic bottlenecks as the remaining trees contain only a small sample of the original gene pool. Issues of habitat fragmentation and over exploitation of this species are important factors in short-term evolutionary changes, which accumulate in a big way on longer time scales.

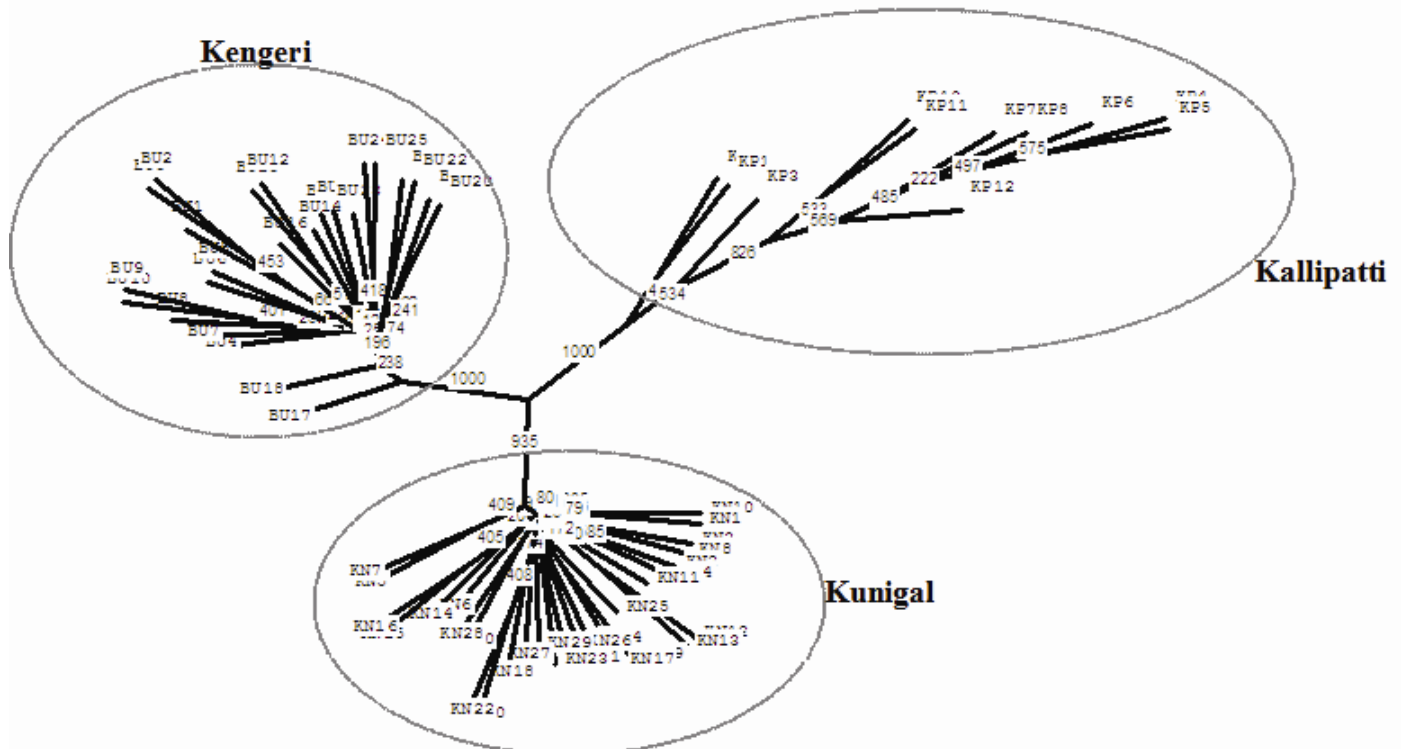


Figure 5. A Radial Neighbour Joining phylogram based on Excoffier distance matrix. three populations of *S. album* in southern India (KP- Kallipatti, KN- Kengeri, BU- Kunigal) BOOTSTRAP values (after 1000 permutations) are shown at the base of each branch.

	Sum of Squares	Degrees of freedom	Mean Squares	Variance components	%Total	Φ Stats*
Among Populations	1176.66	2	588.33	27.86	54.9	0.549
Within Populations	1419.16	62	22.89	22.88	45.1	

* $P < 0.001$ [significance test after 1000 permutations]

Table 1. Analysis of molecular variance (AMOVA) for 65 individuals in three populations of *Santalum album*.

The predicament of small and discontinuous populations in *S. album* is further aggravated by deadly spike epidemic (Hindu 2006a), which has led to loss of some important germplasm.

In recent years, natural regeneration of sandalwood has been poor. Although micropropagation (Rao and Akins 2005; Sanjaya *et al.* 2006) is very successful in and important for *Santalum* to sustain numbers, it should not become the only method of reproduction. It is plausible to believe that higher individual density in an area may not be positively correlated to the inherent diversity of the population, especially for a species that undergoes vegetative propagation such as root suckers. The parasitic nature of sandalwood, its palatability to grazing and poor seed dispersal contributes to poor rates of natural recruitment. Animal assisted seed dispersal through frugivory is a significant contributor to expanding the distribution of sandalwood. The restricted genetic exchange in the Indian *Santalum* species may be indicative of depletion in local frugivorous bat and avian populations.

Conclusion

From this study it may be concluded that small isolated natural populations of sandalwood in India are unique and appear to exhibit clonality. Close to exhaustive deforestation of India's sandalwood resources has resulted in small fragmented populations with limited genetic contact between them. This highlights the need for protecting populations harbouring small unique germplasm to sustain genetic diversity for the benefit of natural regeneration and commercial plantation development.

Acknowledgements

Dr SR Bhat, for valuable comments on the manuscript. Mr KS Sanjith and Mr Krishnappa for their help in field work. Financial assistance by Department of Biotechnology, Government of India is duly acknowledged.

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Primer	Sequence (5'-3')	Number of Bands		Percentage
		Total	Polymorphic	Polymorphic Bands
OP A01	CAGGCCCTTC	24	17	70.84
OPB 08	GTCCACACGG	16	11	68.75
OP D 12	CACCGTATCC	19	14	73.68
OP E02	GGTGCGGGAA	15	11	73.34
OP F05	CCGAATTCCC	24	14	58.34
OP G01	CTACGGAGGA	24	16	66.67
OP H05	AGTCGTCCCC	17	13	76.47
RMS1	GGCGGAGGTGGCCC	17	8	47.05
RMS2	GGAGGTTTTCA	23	14	60.86
RMS 3	GGTGTAGAGAGAGGGGT	21	15	71.42
RMS 4	CCTCCTCCCTCCT	20	12	60.00

Appendix 1. Details of the primer and the number of bands amplified using seven RAPD (OPA01-OPH05) and four DAMD (RMS1-RMS4) primers

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