Indian J. Genet., **64**(1): 13-16 (2004)

Isozyme variation in seven populations of *Santalum album* L. in Kerala

T. B. Suma and M. Balasundaran

Kerala Forest Research Institute, Peechi 680 653

(Received: September 2003; Revised: January 2004; Accepted: January 2004)

Abstract

Genetic diversity within and between seven sandal populations *viz.*, Marayoor, Wayanad, Thenmalai, Wadakkanchery, Ottappalam, Kasargod and Kannavam were investigated using metabolic enzymes *viz.*, peroxidase (PRX), shikimate dehydrogenase (SKDH), gluco phosphate isomerase (GPI), malate dehydrogenase (MDH) and esterase (EST). Eight out of the eleven resolved loci (72.73%) were found to be polymorphic at least in one of the individuals analysed. The average rate of gene flow between the populations was found to be very low (0.11). An examination of the partitioning of genetic diversity within and between populations indicated that 69% of the observed variation occurred between populations and the rest of the variation within populations. The genetic relatedness of the seven populations revealed by the UPGMA dendrogram, showed genetic grouping of populations in three clusters. Kasargod and Kannavam were the most genetically similar and Marayoor and Wayanad were the most diverse populations.

Key words: Sandal, genetic diversity, isozyme, UPGMA dendrogram

Introduction

The genus *Santalum* comprises 29 species distributed in India, Indonesia, Australia, New Guinea, New Caledonia and throughout the South Pacific [1]. The commercially important sandalwood, *Santalum album* L. occurs naturally in Southern India, in islands of Indonesia, notably Timor and in Sri Lanka [2]. It is highly valued for its fragrant heartwood and oil. India exports approximately 2000 tonnes of sandalwood and 100 tonnes of oil annually to various countries [3]. Both, wood and oil are used in incense, perfumes and in medicines. In India, sandal is distributed naturally in about 9600 km^2 mainly in Karnataka, Tamil Nadu and Kerala [4]. In Kerala, it is distributed in the forest ranges of Marayoor, Meppadi (Wayanad)), Arienkavu (Thenmalai), Wadakkanchery, Ottappalam, Kasargod and Kannavam. Marayoor provenance is considered as the best in India with respect to oil content, density of population [4] and genetic superiority [5]. Sandal trees in natural forests have been depleted considerably due to illicit felling. Extraction of heartwood and oil from dead and wind fallen trees only is legal. Marayoor population is further devastated due to a serious disease known as spike disease caused by phytoplasma. The large-scale destruction of sandal trees has necessitated a programme for conservation of these populations and to initiate research in tree improvement programme.

Genetic improvement programme depends on the existence, nature and extent of genetic variation available for manipulation within the species. Isozymes have proven to be an efficient and inexpensive tool for the study of genetic variation in tree species as compared to other molecular markers [6]. Isozyme techniques had been used to estimate variation between sandal plants within and among populations in Timor [7], to identify sandalwood provenances in Western Australia [8] and to estimate genetic variation in Indian sandal provenances [5, 9]. No information was available so far on the genetic variation between and within geographically isolated populations of *S. album* in Kerala.

This paper reports the level of genetic variation within and between seven sandal populations in Kerala using the five metabolic enzyme systems *viz.*, peroxidase (PRX), shikimate dehydrogenase (SKDH), gluco phosphate isomerase (GPI), malate dehydrogenase (MDH) and esterase (EST) detected through Polyacrylamide Gel Electrophoresis (PAGE).

Materials and methods

Seven sandal populations from different forest ranges of Kerala *viz.*, Marayoor, Meppadi (Wayanad), Arienkavu (Thenmalai), Wadakkanchery, Ottappalam, Kasargod and Kannavam were selected for isozyme characterization. Seed samples were collected from 15-20 parent trees located in different regions of these natural sandal reserve forests and the seeds were pooled together. From this bulk, samples were drawn randomly for raising seedlings. Seedlings at the four-leaf-stage (3-week-old) were used for the present study. Twenty samples from each of the seven populations were used for the analysis according to

modified Fox *et al.* [10] procedure. Samples were electrophoretically analyzed using the polyacrylamide gel electrophoresis procedure [11].

Enzyme visualization: Staining procedures for the selected five enzymes were adapted from Wendel and Weeden [12]. The stained gels were visualised under a bright light illuminator and photographed using Kodak DC120 gel electrophoresis documentation and analysis system (Kodak, USA). The isozymes were numbered in the order of decreasing mobility from the anode. The locus that specifies the isozyme with the least anodic migration was labeled as 1, the next as 2 and so on. At each locus, alleles of different isozymes were also designated in the order of decreasing mobility from the anode.

Statistical analysis: Genetic variation within populations: Within each population, the observed number of alleles per locus (Na), the effective number of alleles per locus (Ne), observed heterozygosity (Obs.Het.), expected heterozygosity (Exp.Het.) [13] and the number and per cent of polymorphic loci were determined. In addition, depending on the locus resolved, the observed average heterozygosity of an individual (Hi) and the expected average heterozygosity of an individual over the total analysed populations (Hs) were estimated [13]. The Wright's F-statistics [14] *viz.*, Fis - inbreeding coefficient within population; Fit - inbreeding coefficient over the total analysed populations; Fst - reduction in inbreeding coefficients due to differences among populations or genetic differentiation of populations at the level of all the loci, were also analysed. All these statistical measures were determined using the computer package Popgene version 1.31 [15].

Genetic diversity between populations: Estimates of Nei's genetic distance unbiased for sample size [16] for each pair-wise combination of populations were calculated using Popgene version 1.31 and an unweighted pair group method analysis (UPGMA) dendrogram was constructed [15]. The populations with the highest similarity (least genetic distance) were merged, forming nested classes (or clusters). This process was repeated until all the populations belonged to a single cluster.

Results and discussion

Genetic variation within populations: Five enzyme systems representing 11 loci were resolved with sufficient consistency and clarity (Fig. 1) Eight out of the eleven loci were found to be polymorphic for at least one of the analysed individuals (72.7%). The per cent of polymorphic loci was found to be 9.1 in Thenmalai, Wadakkanchery and Ottappalam, 18.2 per cent in Marayoor, Kasargod and Kannavam populations and 27.3 per cent in Wayanad population (Table 1). The

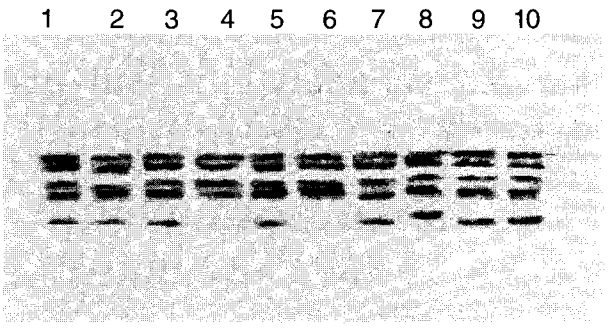

Fig. 1. Malate dehydrogenase isozyme polymorphism between the sandal plants in Marayoor population (Lanes 1-10 represents individual plants)

genetic "richness", as measured by the observed number of alleles, indicated that all loci were equally rich with two alleles except *Skdh-2, Mdh-2* and *Mdh-3* (single allele). The observed (Na) and effective number of alleles at the population level (averaged over all loci) and at the locus level (averaged over all populations) were almost identical (Table 2). Thus, there was not much loss of genetic richness (null allele formation) by the natural mutations or recombinations at the genic loci.

Table 1. Population genetic diversity parameters

Provenances	Na	Ne	Obs. Het.	Exp. Het.	No. P. loci	%P. loci
Marayoor	1.18	1.17	0.09	0.09	2	18.2
Wayanad	1.27	1.10	0.08	0.06	3	27.3
Thenmalai	1.09	1.04	0.04	0.03	1	9.1
Wadakkanchery	1.09	1.06	0.06	0.04	1	9.1
Ottappalam	1.09	1.07	0.05	0.04	1	9.1
Kasargod	1.18	1.18	0.18	0.09	2	18.2
Kannavam	1.18	1.18	0.18	0.09	2	18.2

Na: Observed number of alleles, Ne: Effective number of alleles, Obs. Het.: Observed heterozygosity, Exp. Het.: Expected heterozygosity, No. P. loci: Number of polymorphic loci, %P. loci: Percentage of polymorphic loci.

Observed average heterozygosity (Hi), expected average heterozygosity (Hs) and F-statistics (Fis, Fit and Fst) at the locus level are listed (Table 2). Six of the resolved loci *viz.*, *Prx-1, Skdh-2, Gpi-1, Mdh-1, Mdh-2* and *Mdh-3* (Hi = zero) were homozygous in all analysed individuals, four loci *viz.*, *Skdh-1, Gpi-2, Est-2* and *Est-3* showed heterozygosity values below 0.30 and the *Est-1* locus had an observed heterozygosity of 0.79. At the population level, the observed heterozygosities calculated directly from the genotypes (Hi) varied from 0.04 in Thenmalai to 0.18 in Kasargod and Kannavam. When observed and expected heterozygosities are compared, the observed heterozygosities are slightly higher than the expected heterozygosity values for all populations except Marayoor.

Table 2. Genetic diversity measures depending on the locus

Locus	Na	Ne	Hi	Hs	Fis	Fit	Fst
Prx-1	2	1.69	0.00	0.41	****	1.00	1.00
Skdh-1	2	1.69	0.29	0.41	–1.00	0.30	0.65
Skdh-2	1	1.00	0.00	0.00	****	****	0.00
Gpi-1	2	1.96	0.00	0.50	****	1.00	1.00
Gpi-2	2	1.85	0.14	0.46	–1.00	0.69	0.84
Mdh-1	2	1.64	0.00	0.39	1.00	1.00	0.64
Mdh-2	1	1.00	0.00	0.00	****	****	0.00
Mdh-3	1	1.00	0.00	0.00	****	****	0.00
Est-1	2	1.99	0.79	0.50	–0.87	–0.58	0.15
Est-2	2	1.08	0.06	0.07	0.04	0.17	0.14
Est-3	2	1.01	0.01	0.01	–0.03	–0.01	0.02
Mean	1.73	1.45	0.12	0.25	–0.52	0.53	0.69

Na: Observed number of alleles, Ne: Expected number of alleles, Hi: Observed heterozygosity, Hs: Expected average heterozygosity, and Wright's F-inbreeding coefficients [Fis (within population), Fit (over the total population) and Fst (genetic differentiation)].

F-statistics provided measures of deviations in gene frequencies from Hardy-Weinberg equilibrium (HWE) (Table 2). If all the loci are in HWE, Fis will be zero and Fit will be equal Fst. In the present study, except for *Mdh-1* (Fis = 1.00) which showed a positive value, all the heterozygous loci gave negative values for Fis (the inbreeding coefficient within population) and the average Fis was –0.52. These results indicated that all the loci showed deviations from HWE. Two of the resolved loci *viz.*, *Prx-1* and *Gpi-1* were found to be in HWE where Fit = Fst. When the species is considered as a single unit (over the total populations), the inbreeding coefficient (Fit) is 0.53, indicating that 53 % of the heterozygotes were lower than expected under Hardy-Weinberg equilibrium. This might be due to mating (gene flow) between similar genotypes caused by some natural evolutionary selection processes or due to least amount of gene flow.

The breeding system studies showed that *S. album*, like many other tree species is generally cross-pollinated [17]. Natural selfing at 5 % level and inbreeding coefficient of 0.025 are also reported in *S. album*. The sandal populations under study are separated by considerable distances and hence the chance for gene flow is minimal. It is reported that the possibilities of gene flow between natural populations of most species diminish rapidly with distance [18]. Hence, the populations might have evolved separately as discrete independent units.

Genetic differentiation among populations was high with mean Fst = 0.69, implying that 69 % of total variation was between populations and 31 % of total genetic variation was within populations. The low rate of gene flow (Nm = 0.11) might be the reason for the high level of genetic differentiation among the populations. Gene flow seemed to be an important factor in preventing the differentiation of natural populations. The sandal in Kerala exhibited a pattern of genetic diversity characterized by a moderate degree of intrapopulation variation and a rather high inter-population genetic diversity.

Genetic variation between populations: Genetic distance coefficients are provided in Table 3. Of the seven populations, Marayoor and Wayanad were the

Table 3. Estimates of Nei's (1978) genetic distance between populations based on data from 11 loci

Populations	Marayoor	Wayanad	Thenmalai	Wadakkanchery	Ottappalam	Kasargod	Kannavam
Marayoor	****						
Wayanad	0.56	****					
Thenmalai	0.15	0.52	****				
Wadakkanchery	0.26	0.17	0.12	****			
Ottappalam	0.37	0.21	0.27	0.28	****		
Kasargod	0.27	0.25	0.28	0.32	0.03	****	
Kannavam	0.27	0.25	0.28	0.32	0.03	0.01	****

most genetically distant populations (0.56) and Kasargod and Kannavam were the most genetically similar populations (0.01). Nei's [16] unbiased measures of genetic distance were used to construct a UPGMA dendrogram (Fig. 2). The genetic relatedness of the seven populations revealed by the UPGMA dendrogram, showed genetic grouping of some of the neighbouring populations but not fully in agreement with geographic separation. This pattern has also been observed in most of the studied populations of forest trees [19].

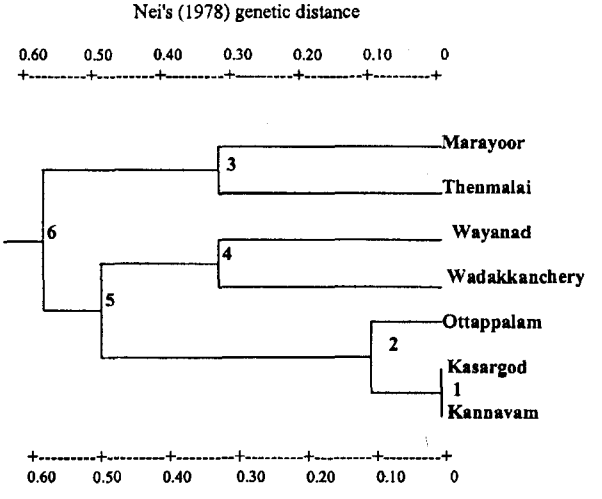

Fig. 2. Dendrogram showing clustering of seven sandal populations in Kerala based on Nei's [16] genetic distance: method = UPGMA

The branches in the dendrogram reflect the genetic differentiation among populations. The seven populations covered a broad geographic range and thus enzymatic differentiation is expected to exist among them. Differences in genetic variability within geographically separated populations of a species have been associated with climatic factors. *S. album* is found growing over a range of altitudes which can greatly affect climatic factors such as atmospheric temperature, moisture availability and rainfall. Among the seven selected sandal populations in Kerala, except Marayoor (>1000m) and Wayanad (>1000m), the rest five populations are lying in low altitude regions (<200m). Variable selection pressures may cause such populations to be more diverse [10]. This might have resulted in the high genetic differentiation of the sandal populations. Marayoor and Wayanad populations, the two most genetically distant ones are separated geographically by about 300 km whereas, Kasargod and Kannavam, the two genetically similar populations are only 90 km apart.

Acknowledgements

The authors wish to acknowledge the financial support provided by the Dept. of Biotechnology, Government of India (GOI). T. B. Suma would like to thank the Council of Scientific and Industrial Research, GOI for the award of a Senior Research Fellowship.

References

1. **Hewson H. J. and George A. S.** 1984. Santalaceae. *In*: Flora of Australia. Vol. 22 (ed. A.S. George). Australian Government Publishing Service, Canberra: 29-67.
2. **Tennakoon K. U., Ekanayake S. P. and Etampawala E. R. L. B.** 2000. An overview of *Santalum album* research in Sri Lanka. Sandalwood Research Newsletter, **11**: 1-4.
3. **Jain S. H., Angadi V. G. and Shankaranarayana K. H.** 2003. Edaphic, environmental and genetic factors associated with growth and adaptability of sandal (*Santalum album* L.) in provenances. Sandalwood Research Newsletter, **17**: 6-7.
4. **Srimathi R. A., Kulkarni H. D. and Venkatesan K. R.** 1995. Recent advances in research and management of sandal (*Santalum album* L.) in India. Associated Publishing Co., New Delhi: 416.
5. **Suma T. B. and Balasundaran M.** 2003. Isozyme variation in five sandal provenances of India. Aus. J. Bot., **51**: 271-278.
6. **Soltis D. C. and Soltis P. S.** 1989. Isozymes in plant biology. *In*: Advances in Plant Sciences Series (ed. T.R. Dudley). Discorides Press, Port land, Oregon, 268.
7. **Brand J. E.** 1994. Genotypic variation in *Santalum album* L. Sandalwood Research Newsletter, **2**: 2-4.
8. **Egerton-Warburton L. M.** 1990. Techniques for assessing isoenzyme activity in *Santalum album* L. and preliminary results. Mulga Research Centre Journal, **10**: 56-60.
9. **Angadi V. G., Jain S. H. and Shankaranarayana K. H.** 2003. Genetic diversity between sandal populations of different provenances in India. Sandalwood Research Newsletter, **17**: 4-5.
10. **Fox J. E. D., Brand J. E., Barrett D. R. and Effendi M.** 1995. Genetic variation in *Santalum album* in Timor. *In*: Sandalwood Nursery and Plantation Technology (eds. L. Gjerum, J.E.D. Fox and Y. Ehrhart). FAO, Suva, Fiji. Field Document No.8. RAS/ 92/ **361**: 93-110.
11. **Laemmli U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**: 680-685.
12. **Wendel J. F. and Weeden N. F.** 1989. Visualization and interpretation of plant isozymes. *In*: Isozymes in Plant Biology (eds. D.E. Soltis and P.G. Soltis). Dioscorides Press, Oregon, 5-45.
13. **Nei M.** 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci., **70**: 3321-3323.
14. **Wright S.** 1951. The genetical structure of populations. Annals of Eugenics, **15**: 323-354.
15. **Yeh F. C., Yang R. and Boyle T.** 1999. Population genetic analysis POPGENE version 1.31: Microsoft window-based freeware for population genetic analysis. A quick user's guide. A joint project developed by Centre for International Forestry Research and University of Alberta, Canada.
16. **Nei M.** 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, **89**: 583-590.
17. **Kulkarni H. D. and Muniyamma M.** 1998. Floral biology and breeding systems of sandal, *Santalum album* L. *In*: Sandal and Its Products (eds. A.M. Radomiljac, H.S. Ananthapadmanabha, R.M. Welbourn and K. Satyanarayana Rao). ACIAR Proceedings No. 84, Melbourne, Australia, 135-147.
18. **Varela M. C. and Eriksson G.** 1995. Multipurpose gene conservation in *Quercus suber* - a Portuguese example. Silvae Genetica, **44**: 28-37.
19. **Yang C., Huang Q., Li Y. and Liu W.** 1997. Study on the genetic structure of the best provenance of *Larix gmilinii* 11. Genetic variation and population differentiation. Journal of Northeast Forestry University, **25**: 1-5.