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Allozyme Variations to Measure Genetic Diversity in Clonal Accessions of Indian Sandalwood (*Santalum album*)

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Abstract: *Leaf samples of 40 Sandalwood (Santalum album L.) clones assembled at IWST, Bengaluru from natural growing areas and plantations in Tamil Nadu and Karnataka were analysed using horizontal starch gel electrophoresis to study isozyme variability for four enzyme systems: Peroxidase (POD), Aspartate amino transferase (AAT), Glutamate dehydrogenase (GDH) and Malate dehydrogenase (MDH). These systems are reported to show polymorphism in populations of Sandalwood in earlier studies. All the enzymes showed polymorphism in the present study. POD and MDH showing five and four different patterns differentiated the clones. Fourteen bands were scored with an average of 1.2 bands per clone. Presence of isozymes reflects the genetic complexity existing in the clones. No pattern of geographic distribution of isozyme variation was observed. The diversity observed among these clones would be useful in identifying diverse cross-combinations for deriving hybrids and establishing trials for production of seeds.*

Keywords: *Sandalwood clones, diversity, isozymes, variability*

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1. INTRODUCTION

Genetic diversity is a prerequisite for long-term survival and adaptability (Young et al., 2000; Bahuguna, 2007).The most serious consequence of its depletion is genetic erosion followed by extinction of species (Kemp et al., 1993). Genetic diversity in Sandalwood is under threat owing to the illegal felling and heavy infestation by spike disease (Arunkumar et al., 2016) and therefore International Union for Conservation of Nature has categorized it as 'Vulnerable'.

Allozymes are a good tool to estimate diversity (Gepts, 1990). They are polymorphic, co-dominant, follow simple inheritance patterns, and occur in plant tissues (Smith, 1989). Progenies from self and cross-pollinations may be differentiated using appropriate isozymes as the electrophoretic pattern obtained is a reflection of the genotype. The biggest advantage of allozyme analysis is that it requires no prior information about the species. The technique could be used directly without modification with any fresh tissue sample of animal, plant, or microbe (Allendorf, 2017).

Preliminary studies on isozyme analysis in Sandalwood were first reported by Parthasarathi et al. (1985) correlating isozyme activity and leaf variations. The authors also reported peroxidase activity in bark tissue as a marker for the oil-bearing capacity (Parthasarathi et al., 1986). Using isozymes, Brand, (1994) estimated genetic variation in Sandalwood plants within and between populations in Timor while Warburton, and (1990) used them to identify provenances in Western Australia. Angadi et al. (2003) reported that provenances of Sandalwood could be delineated using isozymes. Suma and Balasundaran (2003; 2004) assessed the level of genetic variation within and between southern Indian *S. album* populations using isoenzymes. Rao et al. (2007) analyzed the genetic diversity of 19 Sandalwood populations distributed over different parts of Peninsular India.

Differentiating clones using isozymes has not been reported in Sandalwood. This study, therefore, is an attempt to study diversity in clones of *Santalum album* to provide preliminary information on their genetic distances.

2. MATERIALS AND METHODS

Forty accessions of *Santalum album* (Table 1) from the clonal germplasm bank maintained by the Institute of Wood Science and Technology (IWST), Bangalore were used. These accessions were collected from natural populations/plantations in Karnataka and Tamil Nadu based on their growth and heartwood content. Based on information from literature, only those isozymes reported to reveal polymorphism in Sandalwood were used for the present study.

Table1. *Details of 40 clonal accessions used for the study*

Clonal Accession	Origin
K1	Anekad, Kushal Nagar, Madikeri, Karnataka
K2	Thindlu, Hoskote, Bangalore, Karnataka
K3	Hulihatti, Ranibennur, Gadag, Karnataka
K4	PWD guest house, Vanivilas Sagar, Chitradurga, Karnataka
K5	IWST, Bangalore, Karnataka
K6	IWST, Bangalore, Karnataka
K8	IWST, Bangalore, Karnataka
K9	IWST, Bangalore, Karnataka
K10	IWST, Bangalore, Karnataka
K11	Hardanhalli, Chamarajnagar, Karnataka
K13	Hardanhalli, Chamarajnagar, Karnataka
K14	Rayalpad (SF, Srinivaspura,) Kolar, Karnataka
K16	Hardanhalli, Chamarajnagar, Karnataka
K23	Honehatti MF, Bhadravathi, Karnataka
K27	Chandrakala, SF, Shikaripura, Sagar, Karnataka
K28	Ammankatte, Horticultural Farm, Hassan, Karnataka
K29	Muduganur Kaval, Arkalgud, Hassan, Karnataka
K31	Tangali sandal reserve, Kadur, Chickmagalur, Karnataka
K35	Sandal Koti, Kushalangar, Madikeri, Karnataka
K37	Silva Experiment Station, Mudigere, Bangalore, Karnataka
T1	Sholavaram Research Garden, RR Pudukottai, Tanjavur, Tamil Nadu
T3	Forest Guest House, Anchety, Hosur, Tamil Nadu
T4	Komateri, Polur, Vellore, Tamil Nadu
T5	Inner Javadhis RF, Alangayam, Thirupathur, Tamil Nadu
T6	Veerapannur RF, Polur, Vellore (1968 plantation), Tamil Nadu
T7	Veerapannur RF, Polur, Vellore (1970 plantation), Tamil Nadu
T8	Pavanamials Farm, Patta land, Shirkali, Tanjavur, Tamil Nadu
T9	FRC, Kurumbapatty, Shevroys, South Salem, Tamil Nadu
T11	FRC, Kurumbapatty, Shevroys, South Salem, Tamil Nadu
T13	FRC, Kurumbapatty, Shevroys, South Salem, Tamil Nadu
T14	FRC, Kurumbapatty, Shevroys, South Salem, Tamil Nadu
T19	Mundanthorai, Tirunelveli (1966 plantation), Tamil Nadu
T21	Mundanthorai, Tirunelveli (1966 plantation), Tamil Nadu
T22	Nachikotai, Harur, Chitteri Dharmpuri, Tamil Nadu
T23	Perieri Village, Pudur East, Chitteri, Harur, Dharmpuri, Tamil Nadu
T24	Thombakal, RF, Shanimadu, Harur, Chitteri, Dharmpuri, Tamil Nadu
T26	Thombakal, RF, Tholthuki Tending Plot, Harur, Chitteri, Dharmpuri, Tamil Nadu
T27	Parigam, Dharmpuri (1974 plantation), Tamil Nadu
T28	Jirgehalli, Hanur, Tamil Nadu
T29	Cattle Farm, Padak – 3 Hosur, Tamil Nadu

2.1. Isozyme Analysis

Four enzyme systems Peroxidase (POD), Aspartate amino transferase (AAT), Glutamate dehydrogenase (GDH) and Malate dehydrogenase (MDH) were studied (Suma and Balasundaram, 2007). Samples were collected from actively expanding leaves from each of the 40 accessions. About 0.02 g of these leaves were crushed in 100 µl of extraction buffer of 50 mM Tris buffer (pH 8.3) containing 1% vitamin C (w/v) and 1% Poly Vinyl Polypyrrolidone (w/v). After centrifugation at 12,500 *g* for 30 min, the supernatants were saved as crude extracts. Electrophoresis and staining procedures were adopted from Rovira *et al.* (1993). After electrophoresis, the gels were incubated in

staining solutions for a few minutes until clear bands appeared. The gels were washed with distilled water and photographed. The zones were numbered from the slowest to the fastest migration. The relative position of each band was drawn schematically and scored. Scoring was made for those bands which were clearly visible. Genetic interpretation of enzyme phenotypes was based on observed differences in zymogram profile after activity staining of the gel. Variation in banding patterns was determined by the migration from the origin towards the anode.

2.2. Data Analysis

POPGENE (Version 1.31) computer program for genetic data analysis was used to analyze allelic data. Standard genetic variability measures were computed for all accessions including the percentage of polymorphic loci (*P*), the mean observed number of alleles per locus (*na*), mean effective number of alleles per locus (*ne*), the average (*Avg_Hets*), observed (*Ho*) and expected (*He*) heterozygosities. The genetic distances obtained were mapped using Treeview 1.6.6 and Darwin 6.0.

3. RESULTS AND DISCUSSION

3.1. Isozyme analysis

The four enzyme systems provided a total of 14 loci for the 40 Sandalwood accessions. About forty percent (6/14) of the resolved isozyme loci were polymorphic (0.99 criterion). Out of these 14 loci, 8 were invariant in all the accessions and were monomorphic. The six alleles observed in MDH-1 MDH-2 AAT-1 GDH-1 POD-1 POD-2 were not equally distributed (Table 2). There were maximum variations in GDH-1 followed by POD-1 locus. Studies have been reported on isozyme variation in *S. album* (Manojkumar et al., 1998), *Acacia auriculiformis* (Wickneswari and Norwati, 1989), *Tectona grandis* (Kumaravelu, 1979), Eucalyptus (Aradhya and Phillips, 1993; Martin Corder and Lopes, 1997; Balaji, 1997) and *Azadirachta indica* (Philomina, 2000).

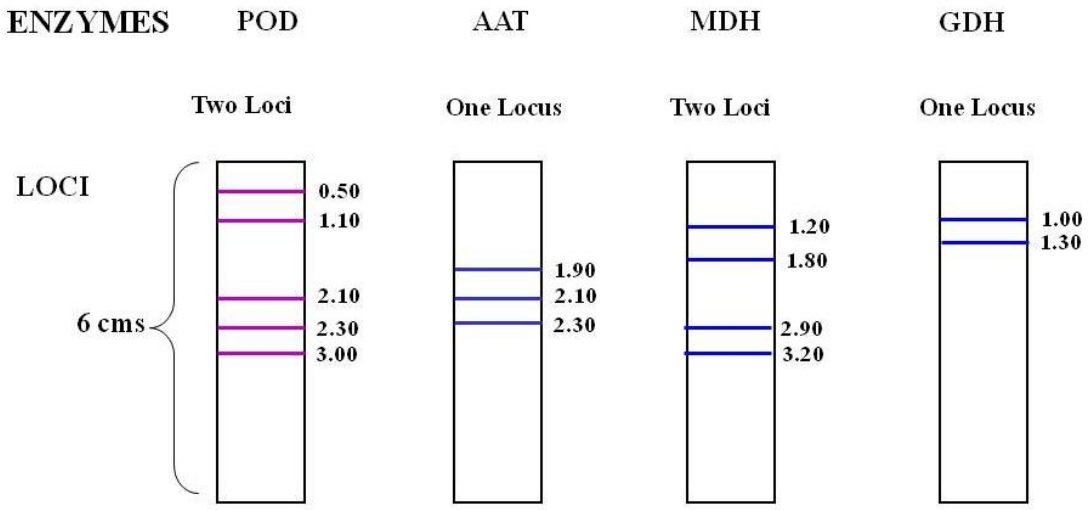

Fig1. *Relative mobility (Rf) and designation of electrophoretic variants at different loci found in sandalwood accessions*

Table2. *Summary of heterozygosity statistics for all loci*

Marker	Major Allele Frequency	Allele No.	Gene Diversity	Hetero-zygosity	Polymorphism information content
MDH-1	0.80	2	0.32	0.10	0.27
MDH-2	0.76	3	0.38	0.35	0.34
AAT-1	1.00	1	0.09	0.07	0.09
GDH-1	0.41	2	0.65	0.93	0.58
POD-1	0.61	2	0.48	0.78	0.36
POD-2	0.81	3	0.32	0.38	0.29

The genetic variability measures of the 40 accessions are presented in Table 3. The mean observed number of alleles per locus was the same as the mean effective number of alleles per locus. The percentage of polymorphic loci (*P*) (0.99 criterion) varied from 0.00 to 46.00 (mean 29.03±0.02), the

mean observed heterozygosity (*Ho*) was 0.21 ±0.02, the mean expected heterozygosity (*He*) from 0.00 to 0.67 (mean 0.42±0.02) and gene diversity (*I*) from 0.00 to 0.33 (mean 0.21±0.01).

The percentage of polymorphic loci was highest for K5, T7, T26, T27 and T28. The least was observed in K29 followed by K8, T3, T11 and T29. As the accessions were clones, the maximum average number of alleles per locus was also recorded in the clones with highest polymorphism while the minimum numbers of alleles were recorded in accessions exhibiting high homozygosity. Heterozygosity is directly applied as a measure of genetic variation (Crow, 1986). High levels of heterozygosity are important for survival over long life span of trees (Gregorius and Ziehe, 1986), which is related to the low mortality of seedlings and plants (Mejnartowicz and Lewandowski, 1994). Tree species in general are highly out crossing and highly heterozygous as compared to herbaceous perennials and annuals, the average level of heterozygosity being twice that of the herbaceous plants (Hamrick *et al.*, 1979). It was observed that the polymorphic loci ranged between 0 and 46 suggesting that there is a good chance for incorporation of new genes into the next generation. It may be noted that Sandalwood is an entomophillous species and hence a high level of out crossing is normally expected. However, the average heterozygosity was only 21 per cent indicating a lull in gene exchange despite having high polymorphism. The heterozygosity values suggests that if these clones have to be deployed in an orchard for improved seeds, more number of clones need to be included which would probably increase the polymorphic loci, as a result of which the expected heterozygosity and the average heterozygosity would simultaneously increase which is reflected in variation in the heterozygote levels doubling from 0.21 to 0.42. Reduced number of fertile unrelated trees and a low population size would also result in decreased diversity (Kang and Lindgren, 1998). In the present study, isozyme analyses have provided information about the relative amounts of genetic variation present within Sandalwood accessions distributed in the states of Tamilnadu and Karnataka in India. The genetic variability observed was lower than other plant species summarized by Hamrick *et al.* (1992). the mean number of alleles per locus per population was 1.42, which was on par with the average of 1.44 in dicots. The mean expected heterozygosity per population (0.21) in this study was higher than the averages of angiosperm (0.143), out crossing wind-pollinated woody plants (0.154) but equaled tropical tree species (0.217) reported by Martins Corder and Lopes (1997).

Although many woody species maintain relatively high levels of genetic variability (Hamrick and Godt, 1996), some examples exist of widespread conifers with limited genetic variation, such as *Pinus resinosa* (Fowler and Morris, 1977) and *Tsuga canadensis* (Zabinski, 1992), with 0 and 10% polymorphism, respectively. Though geographical range has been considered a good predictor of the levels of genetic variation in plant populations (Hamrick and Godt, 1989), the present study revealed otherwise. Other studies using isozymes have yielded similar results in trees, the rare Pacific yew (*Taxus brevifolia*) which occurs over a wide range, indicated low polymorphism (<50%) (Scher, 1996).

Table3. *Percentage of polymorphic loci (P), the mean observed number of alleles per locus (na), mean effective number of alleles per locus (ne), the average (Avg_Het), observed (Ho) and expected (He) heterozygosities, (I) Shannon Weiner index for the forty sandalwood accessions.*

POP	Obs_Hom	Exp_Het*	I*	na*	ne*	%P
K1	0.67	0.33	0.17	1.33	1.33	0.23
K2	0.67	0.33	0.17	1.33	1.33	0.23
K3	0.50	0.50	0.25	1.50	1.50	0.35
K4	0.50	0.50	0.25	1.50	1.50	0.35
K5	0.33	0.67	0.33	1.67	1.67	0.46
K6	0.50	0.50	0.25	1.50	1.50	0.35
K8	0.83	0.17	0.08	1.17	1.17	0.12
K9	0.67	0.33	0.17	1.33	1.33	0.23
K10	0.50	0.50	0.25	1.50	1.50	0.35
K11	0.50	0.50	0.25	1.50	1.50	0.35
K13	0.67	0.33	0.17	1.33	1.33	0.23
K14	0.67	0.33	0.17	1.33	1.33	0.23
K16	0.50	0.50	0.25	1.50	1.50	0.35
K23	0.50	0.50	0.25	1.50	1.50	0.35
K27	0.50	0.50	0.25	1.50	1.50	0.35

K28	0.67	0.33	0.17	1.33	1.33	0.23
K29	1.00	0.00	0.00	1.00	1.00	0.00
K31	0.50	0.50	0.25	1.50	1.50	0.35
K35	0.50	0.50	0.25	1.50	1.50	0.35
K37	0.67	0.33	0.17	1.33	1.33	0.23
T1	0.67	0.33	0.17	1.33	1.33	0.23
T3	0.83	0.17	0.08	1.17	1.17	0.12
T4	0.67	0.33	0.17	1.33	1.33	0.23
T5	0.50	0.50	0.25	1.50	1.50	0.35
T6	0.50	0.50	0.25	1.50	1.50	0.35
T7	0.33	0.67	0.33	1.67	1.67	0.46
T8	0.67	0.33	0.17	1.33	1.33	0.23
T9	0.50	0.50	0.25	1.50	1.50	0.35
T11	0.83	0.17	0.08	1.17	1.17	0.12
T13	0.50	0.50	0.25	1.50	1.50	0.35
T14	0.50	0.50	0.25	1.50	1.50	0.35
T19	0.50	0.50	0.25	1.50	1.50	0.35
T21	0.50	0.50	0.25	1.50	1.50	0.35
T22	0.67	0.33	0.17	1.33	1.33	0.23
T23	0.67	0.33	0.17	1.33	1.33	0.23
T24	0.67	0.33	0.17	1.33	1.33	0.23
T26	0.33	0.67	0.33	1.67	1.67	0.46
T27	0.33	0.67	0.33	1.67	1.67	0.46
T28	0.33	0.67	0.33	1.67	1.67	0.46
T29	0.83	0.17	0.08	1.17	1.17	0.12

na = Observed number of alleles

ne = Effective number of alleles [Kimura and Crow (1964)]

I = Shannon's Information index [Lewontin (1972)]

Expected homozygosty and heterozygosity were computed using Levene (1949)

3.2. Genetic Diversity

An enormous amount of protein diversity is characteristic of natural populations (Mallet, 1996). Their genetic structure is mainly influenced by the combined effects of random genetic drift, restricted gene flow and differential selection pressures. These effects lead to low within and comparatively high among population genetic variation in species consisting of small and isolated populations (Holderegger and Schneller, 1994). Generally, when populations are close together, having possibility of enough gene exchange, there should be few differences in gene frequency, but if they are far apart, there should be strong differences (Mallet, 1996). This was partly true in the case of Sandalwood, where accessions were selected from different latitudes. In our study, the genetic identity and distance values calculated between the 40 accessions represented as an UPGMA dendrogram was a fair representation of the Nei's (1972) genetic distances between the accessions, which showed many clusters (Fig. 2).

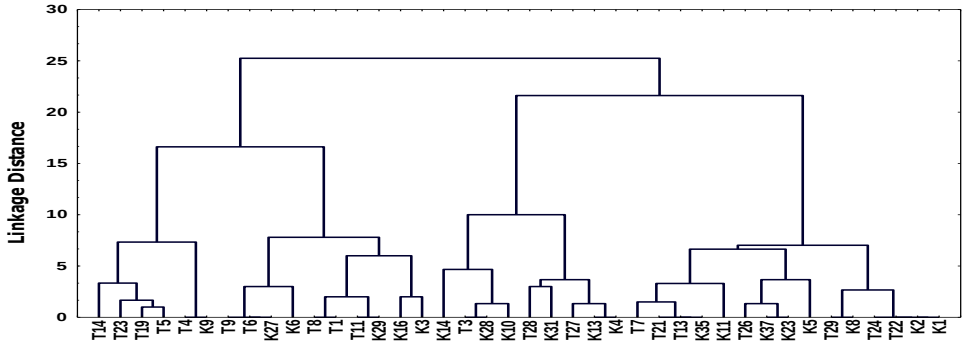

Fig2. *Isoenzyme dendrogram (UPGMA) showing clustering of forty sandalwood clones based on Nei's (1972) genetic distance.*

Analysis of genetic relationships revealed heterogeneous values for Nei's genetic identity (*i*). It ranged from 0.08 (minimum value) to 0.33 (maximum value). All the forty accessions clustered at various levels irrespective of the geographical similarity or distance. Most of the accessions showed similarity. The level of gene flow (*Nm*) was estimated to be 0.59 (Table 4). F-statistics (Table 4) provided the measure of deviations in gene frequencies from Hardy-Weinberg expectations.

Table4. *Summary of F-Statistics and gene flow for all loci*

	Fst	**Nm***	**na***	**ne***	**I***
Mean	0.41	0.36	2.33	1.72	0.59

Nm = Gene flow estimated from Fst = 0.25(1 - Fst)/Fst.

na = Observed number of alleles

ne = Effective number of alleles [Kimura and Crow (1964)]

I = Shannon's Information index [Lewontin (1972)]

A lack of alliance was observed between genetic data and geographical locations of the populations. Accessions studied from the same localities were grouped into different clusters separated by a fair distance suggesting that they were genetically distinct. Despite this, the genetic variability estimates were low. The accessions would have been brought from different places where they have grown in varied climatic and edaphic conditions, but the base material could have been genetically closer. Rao et al. (2007) reported that total variability in Sandalwood was due to differences among the populations rather than within geographical regions. Sandalwood trees are primarily out crossed and to avoid inbreeding depression they require sufficiently large populations for gene flow to occur. In this study, the clones though showed a *He* of 0.21, could be increased to 0.42 provided the genetic base is further widened which would then improve the gene flow and mating opportunities resulting in improved progenies.

Sandalwood trees in their natural stands have been depleted considerably due to illicit felling, (Rao, 2004), lack of regeneration (Sreenivasan et al. 1992) and spike disease (Radomiljac et al. 1998). This has led to an awareness on the need for conservation of the species both through *ex-situ* (Sreenivasan et al. 1992) and *in-situ* measures (Rao, 2004). Priority for genetic conservation has often been set based on the level of genetic diversity and allelic uniqueness of populations as well as on the degree of gene differentiation between populations (Coates and Sokolowski, 1992). A major bottleneck described by various researchers working on Sandalwood, is restricted gene flow and poor mating opportunities (Suma and Balasundaran, 2003; 2004; Rao et al., 2007). The present study, which comprised accessions from predominantly natural Sandalwood growing areas of Karnataka and Tamilanadu, showed that most alleles were shared. Therefore, conserving a single large and genetically diverse population may help capture most of the variation. Thus, in addition to identification of 'hot-spots' of genetic variability, the concept of a National Germplasm Bank for Sandalwood would enable not only conservation planning but also for collecting and maintaining diverse genetic material for Sandalwood tree improvement. With the demand for Sandalwood constantly on the rise, a consolidated approach as suggested here will help in its conservation and efficient utilisation.

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