Pak. J. Bot., 42(1): 79-88, 2010.

A MOLECULAR STUDY OF GENETIC DIVERSITY IN SHISHAM (*DALBERGIA SISSOO*) PLANTATION OF NWFP, PAKISTAN

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Abstract

Genetic diversity of 22 accessions of *Dalbergia sissoo* that were collected from the canal, road and farmer's field and forest sites of N.W.F.P, Pakistan has been studied, by using a finger printing technique "RAPD" (Random Amplified Polymorphism DNA). Out of 20 primers OPA-2 was the primer that allows distinguishing the diseased and healthy accessions. The selected primer was used for identification and for establishing a profiling system to estimate genetic relationships and to evaluate the genetic variability among the accessions. A total of 126 DNA bands or fragments were amplified by using the primers. The UPGMA cluster analysis revealed 2 main clusters among 22 accessions of *Dalbergia sissoo* based on coefficient of similarity and dissimilarity. Overall 72% similarity and 98% dissimilarity were observed. Low level of genetic variation and high level of genetic relatedness occurred among the canal, road, farmer's field and forest sites. The accessions were closely related with each other and showed mix pattern of genetic diversity. Thus RAPD markers have the potential to characterize and establish genetic relationships among the accessions of *Dalbergia sissoo.*

Introduction

The area of Shisham's natural distribution is the foothill of Himalayas from eastern Afghanistan through Pakistan to India and Nepal. The species is found in India, Nepal, Bhutan, Bangladesh, Malaysia, Pakistan, and Afghanistan. Pakistan has more than 10,000 ha of *Dalbergia sissoo* plantation. The area under such plantation in Punjab is 154,886 ha with annual production of 28,000-meter cube, introduced in Pakistan in mid 1800s (Khan & Khan, 2000). *Dalbergia sissoo* is an important species for rural as well as industrial plantations. It is popular for plantations due to its fast growth and multiple use properties (Dhakal, 2000), prefers well-drained, alluvial soils near rivers and streams (Acharya, 2000). Shisham trees in Pakistan are found to be attack and by two types of diseases, wilting and die back, the latter being more prevalent than the former (Bajwa *et al*., 2004). In the case of die back, during 1990-1991 disease incidence recorded was 5% which has raised to 25% in 19 districts of the Punjab during 1999-2000, especially in the linear plantations along the canal bank, road side and water channels. Since then no control measure has been adopted therefore the disease is on the rise (Gill *et al*., 2001). It is believed that primary cause of die back are a variety of physical and soil based physiological stresses resulting from intensive cultivation of agricultural crops, excessive use of chemicals and changed pattern of agricultural practices on the farm land (Anon, 2001).

Solberig (1992) stated that genetic diversity covers range of variability (for specific character) among representative trees or in their seeds. It describes homogeneity or heterogeneity of seeds which ultimately composes the trees into single variety. Diversity of

varieties growing in a given area includes variations within genes of an individual tree. These variations may be visible such as color, height or shape or it may be in the form of any disease. It makes possible to produce new varieties of trees and allows them to adapt in changing climate. Insights into the relative genetic diversity within and between populations of *Dalbergia* species would be useful in plant breeding and also for the development of strategies for *ex situ* conservation of plant genetic resources. DNA fingerprinting of all the genetic resources of the timber-yielding tree plants is a necessity for generating a molecular database, to catalogue as well as to utilize the information in a systematic manner. Moreover, the commercial value associated with identifying useful traits creates a direct value on gene banks, ensuring long-term preservation of a collection. The genetic relation-ship and markers can be useful for designing strategies for gene introgression and breeding programs to produce desired recombinant hybrid genotypes with both timber quality and biomass yields. Management decisions for the conservation of taxa ideally necessitate an understanding of their biology and other factors, including genetic variability, that influence their survival. Conservation management decisions for rare taxa, however, often have to be made quickly without adequate ecological data or information on their genetic variability (Gaston & Kunin, 1997).

The purpose of present study was to evaluate the genetic diversity in different accessions of *Dalbergia sissoo* by using RAPD analysis in relation to dieback disease. This will help to control disease and will be helpful in tree breeding programs and also provides important input into conservation biology.

Material and Methods

Leaves samples of 22 accessions of *Dalbergia sissoo* were collected from 4 different sites of N.W.F.P. The sites were Canal, forest, farmers' field and road. Each road site ranges approximately 1 km.

Extraction and purification of plant genomic DNA: Extraction and purification of plant genomic DNA was done by three methods.

1. Yang & Kang (2004) DNA extraction with modification: A modified extraction protocol by Yang & Knag (2004) has been reported to be utilized in many plant species. This DNA extraction protocol was employed to 8 random accessions from preserved material.

2. Modified extraction method by Dellaporta *et al.*, (1983): A modified extraction protocol by Dellaporta *et al*., (1983) was then utilized on previously 8 and 6 new accessions from preserved material.

3. Modified method by Doyle & Doyle (1990): A modified Cetyl trimethyle ammonium bromide (CTAB) extraction protocol (Doyle & Doyle, 1990) was also utilized. The DNA extraction protocols were analyses in two groups of data. The samples included fresh leaves from 5 accessions and 19 accessions from preserved material. Total genomic DNA was extracted as follows:

To ground each leaf sample, added 1mM extraction buffer (2% CTAB, 100Mm Tris/HCl, pH 7.5, 1.4 M NaCl, 2% Polyvinylpyrroliodine (PVP)-40, 20mM Ethylene diamiane tetra acetic acid (EDTA) pH 8.0), 20 µl/ mL β-mercaptoethanol was added immediately prior to use). Samples were incubated for 1 hr at 60°C with occasional swirling then samples were cooled at room temperature. 600 µl Chloroform:

Isoamylalcohol (24: 1) was added and mixed gently for 5 minutes. Samples were centrifuged for 15 minutes. Supernatant was transferred to new tube and equal volume of isopropanol was added, mixed gently and incubated at -20 °C overnight. Samples were centrifuged for 15 minutes then washed the DNA pellets with 70% Ethanol and centrifuged for 10 minutes. Washing was repeated twice, DNA pellet was dried and dissolve in 20-50 µl TE- RNase solution, samples were incubated for 1 hr at 37°C. The result was satisfactory after DNA quantification of extracted samples. DNA extracted by this method was free of impurities and used in PCR amplification.

PCR amplifications: A set of 20 random dacamer oligonucleotides purchased from operon technologies was used as single primers for the amplification of RAPD fragments. PCR was carried out in a final volume of 25 µl containing 20ng template DNA. 100µM of each deoxynucleotide triphosphate, 20 ng of decanucleotide primers, 1.5mM MgCl2, 1X Taq buffer (10mM Tris-HCl pH 9.0, 50mM KCl, 0.01% gelatin) and 0.5 U Taq DNA polymerase. Amplification was achieved in a thermal cycler (BioRad 48 well) programmed for preliminary 4 minutes denaturation steps at 94°C, followed by 45 cycles of denaturation at 94°C for 1 minute. Annealing at 37°C for 1 minute and extension at 72°C for 2 minute, finally at 72°C for 10 minutes. Amplification products were separated alongside a molecular weight marker (1 kb ladder) by electrophoresis on 1.2 % agrose gel run in 0.5 X TAE (Tris-acetate-EDTA) buffer, stained with Ethidium bromide and visualized under UV light. Gel photographs were scanned through a Gel Doc system (BioRad).

Data analysis: Data were recorded as presence (1) or absence (0) of band products from the examination of gel photographs. Bands with similar mobility to those detected in the negative control, if any were not scored. Similarity indexes were estimated using the Dice coefficient of similarity (Nei & Li, 1979). Cluster analysis was carried out on similarity estimates using the unweighted pair-group method arithmetic average (UPGMA) using NTSYS-PC.

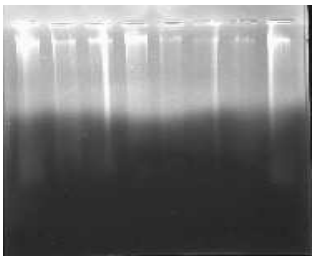
Results

Different modified methods were employed to extract the satisfactory DNA from *Dalbergia sissoo.* For example; Ang & Kang (2004) (Fig. 1), Dellaporta *et al*., 1983 (Fig. 2) and Doyle & Doyle (1990) (Fig. 3).

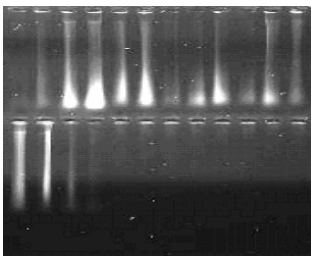
The method described by Yang & Kang (2004) with few modifications such as extra washings were done to further remove phenolic compounds (that made the appearance of solution yellow and dark brown in some cases). As the DNA was resuspended in TE in the final step. It was too viscous to be pipetted.

The presence of bulky phenolic compounds, however, posed the same problem as with the method of Yang & Kang (2004) and did not reveal any result in the further amplification results as by applying the modified method of Dellaporta *et al*., (1983).

The method described by Doyle & Doyle (1990) was therefore employed. This method utilizes Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method which has been reported to be successfully employed in plant species. This protocol is based on lysis and purification with CTAB that selectively precipitates DNA while maintaining the solubility of many polysaccharides. By this method the leaves of *Dalbergia sissoo* showed better DNA quality (Fig. 3). The spectrophotometric results in most cases showed desirable results.



(Fig. 1)



(Fig. 2)

(Fig. 3)

(Agarose gels showing DNA samples)

Table 1. Total number of amplified fragments and number of polymorphic fragments Generated by PCR using A-02.

Name of primer	sequence of primers	Total no. of amplified products	Total no. of polymorphic products	Size range (kb)	P (%)
A-02	-TGCCGAGCTG-	126	124	0.4-2.6	100

Table 2. Site data showing total number of healthy, diseased and dead trees per site.

Sample #	Date of collection	Location site	No. of trees	Diseased trees	Dead trees
1	5.7.07	Forest site	20	0	0
2	5.7.07	Forest site	20	10d/6h	4
3	5.7.07	Forest site	20	10d/6h	4
4	5.7.07	Forest site	20	10d/6h	4
5	5.7.07	Forest site	20	10d/6h	4
6	6.7.07	Canal site	60	28d/24h	8
7	6.7.07	Canal site	60	28d/24h	8
8	6.7.07	Canal site	60	28d/24h	8
9	6.7.07	Canal site	60	28d/24h	8
10	6.7.07	Canal site	60	28d/24h	8
11	7.7.07	Farmer's field site	50	35d/12h	3
12	7.7.07	Farmer's field site	50	35d/12h	3
13	7.7.07	Farmer's field site	50	35d/12h	3
14	7.7.07	Farmer's field site	50	35d/12h	3
15	7.7.07	Farmer's field site	50	35d/12h	3
16	7.7.07	Farmer's field site	50	35d/12h	3
17	7.7.07	Farmer's field site	50	35d/12h	3
18	7.7.07	Farmer's field site	50	35d/12h	3
19	7.7.07	Farmer's field site	50	35d/12h	3
20	7.7.07	Road site	30	24d/6h	0
21	7.7.07	Road site	30	24d/6h	0
22	7.7.07	Road site	30	24d/6h	0

Table 3. DNA Quantification results of 22 accessions of *Dalbergia sissoo*.

Sample No.	Concentration(µg/ml)	A 260:280
1	6.469	1.63
2	7.4	1.8
3	42.0	1.5
4	6.464	1.3
5	10.297	1.6
6	1.50	2.3
7	5.042	2.43
8	8.13	2.39
9	9.2	1.6
10	6.47	2.1
11	5.659	2.1
12	6.38	1.75
13	3.559	1.54
14	4.461	1.76
15	1.95	1.74
16	5.813	1.6
17	6.735	1.8
18	10.858	1.73
19	3.682	1.73
20	19.90	1.99
21	8.587	1.7
22	3.581	2.6
23	7.50	1.98
24	14.116	2.4

On the whole group 1 defines 44.7%, group 2 defines 55.55%, group 3 defines 25.39% and group 4 defines 60.31% of total genetic diversity.

The sharing of bands between the four groups was also observed in 22 accessions of *Dalbergia sissoo*. Group 1 shared 12 and 10 bands with group 2, 3 and 4 respectively. Similarly group 2 shared 10 and 2 bands with group 2 and 4 respectively and no any sharing of bands with group 1 and 3. Group 3 shared 2 bands with group 4 and no bands with groups 1, 2 and 3. Group 4 shared no bands with group 1, 2 and 3.

Cluster analysis based on coefficient of similarity: Two different coefficients were employed to contrast pair wise similarity and dissimilarity among accessions. The UPGMA cluster analysis revealed following results. The coefficient of similarity and UPGMA revealed two main clusters among 22 accessions of *Dalbergia sissoo* except accession number 22, i.e., cluster A and cluster B at 72% overall similarity the accessions have been grouped into:Cluster A at 75% similarity level was divided into two sub cluster A1 and A2 (Fig. 4). There were 5 accessions (1, 2, 3, 4, and 5) in sub cluster A1 and 4 accessions in A2 that was further divided into two groups. Accession number 18 and 20 in one group and 19 and 21 in another group.

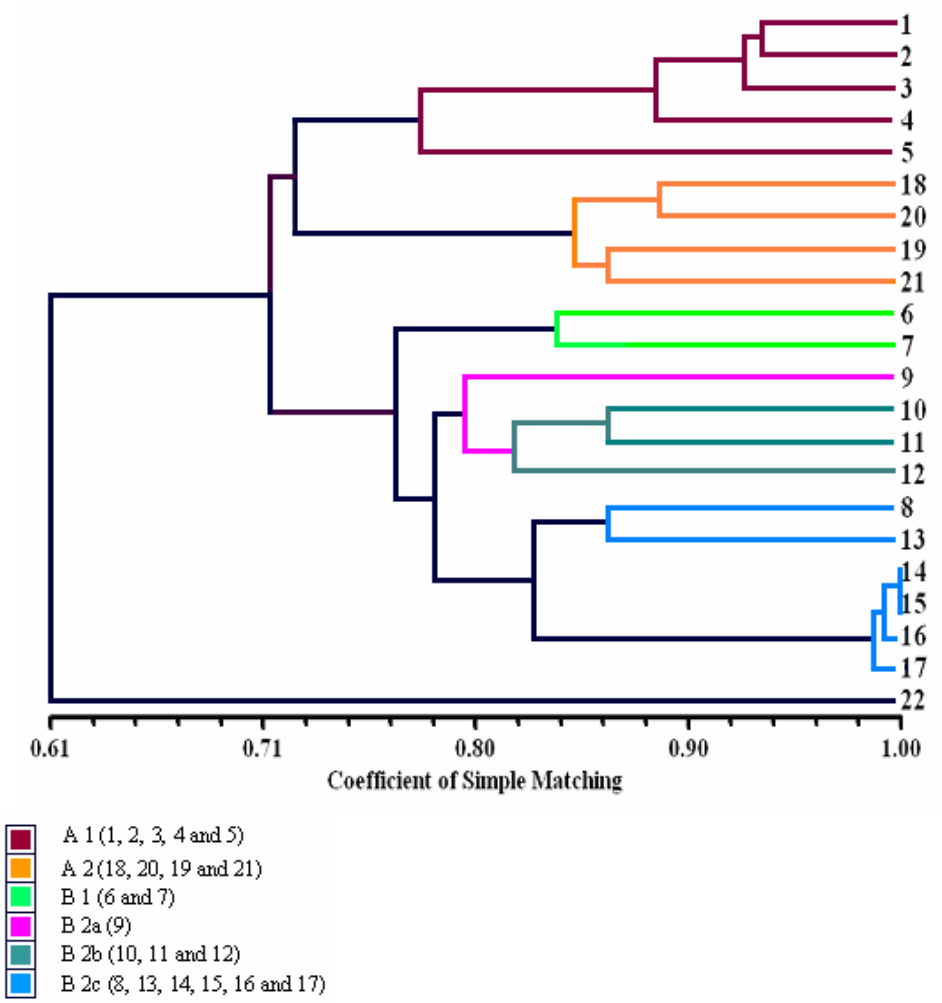


Fig. 4. UPGMA clustering of 22 accessions of *Dalbergia sissoo* based on coefficient of similarity.

Cluster B at 72% similarity divided into two sub clusters B1 and B2. Accession number 6 and 7 were included in B1 and showed 79% similarity. Cluster B2 was further divided into three groups that were B2a, B2b and B2c (figure) at 79% similarity level. There was one accession (9) in B2a, showed 80% similarity, three accessions (10, 11, and 12) in group B2b showed 82% similarity. Group B2c showed 86% similarity and further divided into two sub groups including accession number 8 and 13 in one sub group and 14, 15, 16, 17 in another subgroup of B2c.

Cluster analysis based on coefficient of dissimilarity: On the basis of coefficient of dissimilarity and UPGMA clustering method two main types of clusters were revealed (Figure 5 quite comparable to Figure 6) among 22 accessions of *Dalbergia sissoo* i.e., cluster A and cluster B. the overall dissimilarity depicted was 98% while the minimum dissimilarity was 32% between accessions 19 and 21 in cluster A and 55% in accession number 9, 10 and 11 in cluster B.

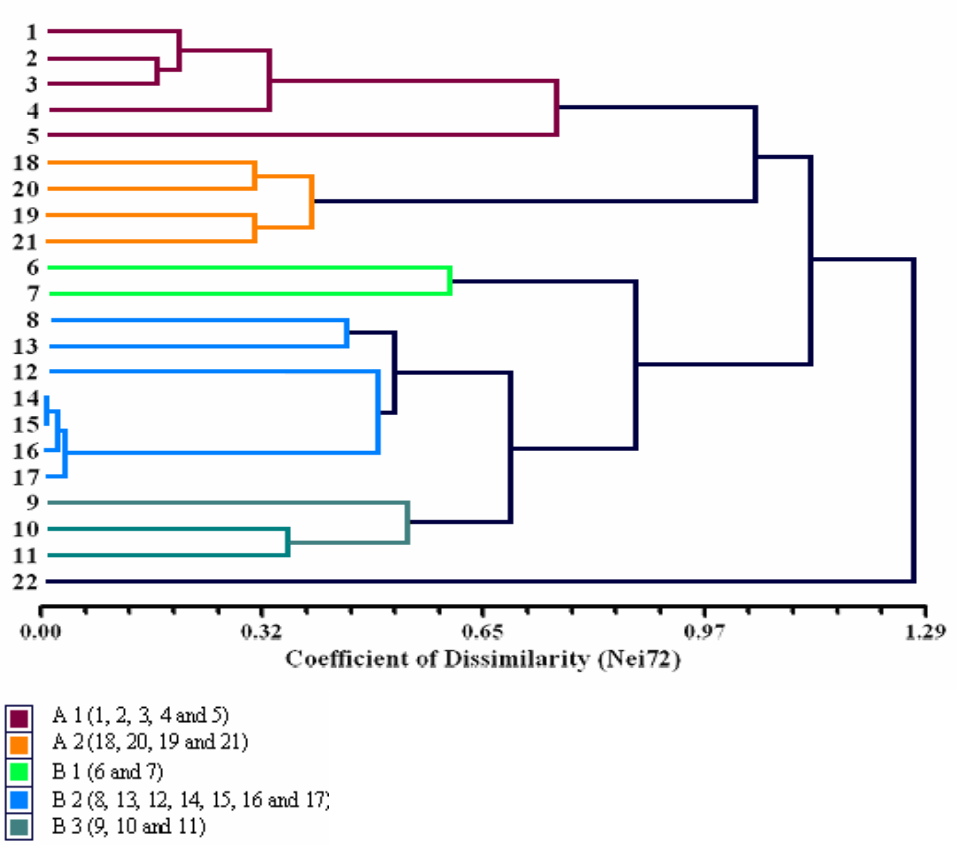


Fig. 5. UPGMA clustering of 22 accessions of *Dalbergia sissoo* based on coefficient of dissimilarity. The number in brackets corresponds to accessions as follows:

By the comparison of two coefficient it was concluded that the grouping in cluster A of both the coefficients are almost same among all the accessions except accession number 22, the accession number 5 behave like a separate group, while in the groups of cluster B of both the coefficients, accession number 9 and 12 behave like separate groups. Accession number 12 showed similarity with 10 and 11 in similarity coefficient and with 14, 15, 16 and 17 in dissimilarity coefficient.

Discussion

Success in the extraction of DNA depends on method of sampling in the field and preservation of the samples (Drabkova *et al*., 2002). *Dalbergia* leaves exhibit oxidation when left under humid conditions for some time after collection, which means that it is necessary to dry or freeze them as soon as possible to cease the metabolic activities in cytoplasm, thus saving DNA from getting digested. To yield a good quality and quantity DNA, methods for collection and long term preservation of herbarium specimens play an important role in success for molecular phylogenetic studies.

DNA extraction from leaves of *Dalbergia sissoo* was complicated due to the abundance of secondary metabolites. In a comparison of three protocols analyzed with preserved leaves the protocols described by Doyle & Doyle (1990), produced the best DNA quality that was further tested by successful amplification of PCR for these accessions. This method is applicable to both, fresh leaves and herbarium specimens. Generally fresh leaves produced better quality DNA while some level of degradation has been observed in the preserved samples during DNA extraction. The DNA obtained by Yang & Kang (2004) and Dellaporta (1983), the extracted DNA was yellow to dark brown in color. The DNA yield was not of required quality and quantity due to abundance of secondary metabolites, showed unsatisfactory results after quantification and consequently there was no PCR amplification, probably due to high degradation of samples.

According to Jobes *et al*., (1995), in the presence of PVP, phenolics adhere to DNA in solution forming a colored extract around the DNA that can be removed after several washings only. The addition of high molar concentration of NaCl increases the solubility of polysaccharides in ethanol effectively decreasing co precipitation of the polysaccharides and DNA (Fang *et al*., 1992). However protocol by Doyle & Doyle (1990) demonstrated desirable results in *Dalbergia sissoo*. Spectrophotometric results of the extracted DNA by Doyle & Doyle (1990) showed values for 260:280 ratio between1.5-2.0 and eventually resulted in successful PCR amplification.

A-02 primer showed polymorphism within 22 accessions of *Dalbergia sissoo*. A total of 126 amplified fragments were scored. Cluster analysis based on binary data revealed similarity and dissimilarity among accessions. The similarity and dissimilarity matrix obtained by Nei & Li ((1972) coefficients showed quite comparable results.

The DNA profiling in the accessions of *Dalbergia sissoo* clearly showed that it was possible to analyzed genetic diversity pattern and probably to correlate them with respect to shisham die back. By the comparison of the coefficient it was concluded that grouping pattern was almost same. Group 1 of both the coefficients was closely related and all the other groups showed mix pattern of genetic diversity among each other. Mix pattern of genetic diversity occurred for the canal, farmer's field and road sites while the members of forest site were less genetically diverse and more related to each other except accession number 22, which was genetically more diverse, healthy and resistant.

The results indicating that the mean level of genetic variation was low among the individual of *Dalbergia sissoo* as mentioned by the results obtained by Rout *et al*., (2002) by evaluating the genetic relationships in *Dalbergia* species using RAPD markers.

Conclusion

RAPD markers provide information on genetic relatedness among the 22 accessions of *Dalbergia sissoo*. It was concluded that among four sampling sites, the diseases incidence was more in canal, farmer's field and road site as compared to forest site either due to the same ecological conditions as these sites showed mix pattern of genetic diversity and lowest level genetic diversity occurred among the individual of *Dalbergia sissoo*. Level of genetic variation within accessions would prove significant in finding causes of Shisham dieback and also managing the disease incidence provide an important input into determining the appropriate management studies and causes of shisham die back.

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(Received for publication 4 December 2009)