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*Full Length Research Paper*

# Genetic divergence of *Dalbergia sissoo* through random amplified polymorphic DNA analysis at different districts of Punjab Pakistan

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**Genetic diversity of eight trees of *Dalbergia* germplasm as collected from three different ecological zones of the Punjab (Lahore, Bahawalpur and Faisalabad). All samples were characterized through random amplified polymorphic DNA (RAPD) with agarose gel electrophoreses techniques. The 25 primers screened out from 50 primers based on their polymorphic nature. Total numbers of 182 polymorphic fragments obtained and 35 and 14 polymorphic fragments selected in the Dalbergia healthy dark green and diseased yellow plants respectively. The healthy samples differentiated from diseased samples on the bases of their DNA base pairs and bands size. The healthy samples showed strong and bright bands 4200 bp with 25 ng and 500 bp with 50 ng DNA quantities. While diseased samples showed weak/faint bands at 4200 bp with 3 ng and 500 bp is only 8 ng quantities. The range of similarity matrix was 68 to 88% according to the DNA polymorphic loci estimated by Nei's similarity index. Trees of Bahawalpur and Lahore zone have least 68% genetic similarity. The trees from Faisalabad and Bahawalpur have 88% genetic similarity. Diseased trees from Lahore and Bahawalpur zones have 68% genetic similarity.**

**Key words:** Agarose gel electrophoresis, *Delbergia sissoo* L., Genetics analyisis, RAPD.

## INTRODUCTION

Forest trees are important as they contribute to economic development and play a great role in the protection of watersheds, maintenance of biodiversity and environment quality. Pakistan is forest deficient country, with only 0.3 ha per capita share as compared to world average of 1 ha per capita and is further declining. The study of adaptation is fundamental factor to forestry and forest genetic conservation, but the extent of biotechnology and use of molecular markers to study pattern of adaptation and genetic conservation is limited. Now, agro-forestry science spans the disciplinary spectrum from biological, physical and social sciences and molecular studies have become an integral part of farm-forestry (Shahid, 2002). Analysis of molecular diversity in forest trees, using RAPD markers is a subject of great interest and application (Cavers et al., 2003; Bouvet, 2004). Ahuja (2001) studied DNA-RFLP in trees of *Dalbergia latifolia*, *Cedrus deoda*ra and *Pinus sp* found polymorphism in all species except that the test species had small number of rare variants. Salam et al. (2000) described RAPD and the techniques involved regarding the use of short oligonucleotides of arbitrary sequences to prime amplification of DNA fragments through polymerase chain reaction. Due to advances in molecular biology techniques, large numbers of visible DNA markers developed for the identification of genetic polymorphism.

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**Table 1**. Locations and size of *Dalbergia sissoo* germplasm.

| Serial number | Leaves samples | Plant height | Diameter at breast height | Zone |
|---|---|---|---|---|
| 1 | Dark Green (Healthy) | 40 feet 13.1 m | 61 inch155 cm | Lahore |
| 2 | Yellowish Green (Diseased) | 30 feet 9.1m | 29 inch73.6 cm | Lahore |
| 3 | Yellowish Green (Diseased) | 32 feet 9.76 m | 36 inch91.4 cm | Faisalabad |
| 4 | Dark Green (Healthy) | 45 feet 14.8 m | 69 inch175 cm | Faisalabad |
| 5 | Dark Green (Healthy) | 39 feet 12.8 m | 57 inch144.8 cm | Faisalabad |
| 6 | Dark Green (Healthy) | 37 feet 12.13 m | 61 inch155 cm | Bahawalpur |
| 7 | Yellowish Green (Diseased) | 29 feet 9.5 m | 25 inch63.5 cm | Bahawalpur |
| 8 | Dark Green (Healthy) | 40 feet 13.11 m | 49 inch124.5 cm | Lahore |

In the last decade, the randomly amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers. RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotides primers, and thus do not require prior knowledge of a DNA sequence. Cluster analysis revealed a broad genetic base with coefficient ranging between 0.74 and 0.93 controls that neem (*Malia azadirech*) germplasm from India. Similarly Brunner et al. (2000) studied distribution of genetic diversity, based on RAPD markers, among the populations of several tree species, and made important observations on various aspects on farmland habitat for conservation of species, quantitative genetics in ecosystem, functioning models and phylogenic of forest trees.

*Dalbergia sissoo* is the most important cultivated timber tree in Pakistan as well in the other countries. *D. sissoo* makes first class cabinetry and furniture. It is being used for plywood, agricultural, and musical instruments, skis, carvings, boats, floorings, etc. Nelson and LIZ (2000) reported that *D. sissoo* is a folk remedy for excoriations, gonorrhea, and skin ailments Ayurvedics prescribe the leaf juice for eye ailments, considering the wood and bark abortifacient, anthelmintic, antipyretic, aperitif, aphrodisiac, expectorant, and refrigerant. *D. sissoo* germplasm in Pakistan is found to be attacked by wilting and die back diseases. Die back is more prevalent than wilting. During 1990 and 1991 disease incidence recorded was 5%, which has risen to 25% until 2000. Since no controlling measures were adopted. The cause of disease is still not clear but it is believed that primary cause of die back are a variety of physical and soil based physiological stresses. There is great need to cure *D. sissoo* by environmental stresses to increase its production on private and farmlands. The purpose of present study was to evaluate/identify the genetic diversity with in the species of *D. sissoo* at different zones of the Punjab province on which it is grown (Government and private level) by using RAPD analysis in relation to dieback disease.

## MATERIALS AND METHODS

Leave samples of eight *D. sissoo* germplasm collected from different zones of Punjab. The molecular characterization of forest tree species carried out at the Centre for Agricultural Biochemistry and Biotechnology (CABB) University of Agriculture, Faisalabad during 2006 and 2007.The plant material collected in three selected zones of the Punjab but at randomly selected places. Lahore zone consisting of vast plain bisected by rivers canals and water channels, 213 m above sea level with temperature range of 9 to 37°C and 160 to 440 mm annual precipitation. Faisalabad zone characterized by fertile, saline and waterlogged areas, hot and cold climate, 4 to 48°C, 400 mm rainfall in monsoon (July to September) and winter rains (January to March). Bahawalpur has vast desert area, dry summer and dry and cold winter: 7 to 48°C and 200 mm annual precipitation. *D. sissoo* (Roxb), family (papilionaceae) locally known as Shisham or Tali is selected for its identification and molecular characterization. Two types of trees were examined, one was dark green (healthy) and other was yellowish green (declining or deceased) (Table 1).Young leaves as harvested from selected trees placed in the moist muslin cloth, preserved in an auto-refrigerator and finally stored at -80°C until processed. For DNA extraction, tissues processed without any delay. Eight collected samples were marked specifically. DNA was extracted from young leaves using N-cetyle-N, N, N-dimethylammonium bromide method (Doyle and Doyle, 1990).

In the present work, both CTAB and Qiagen DNeasy® Kits were used for genomic DNA extraction according to the protocol suggested by (David et al., 1996). To ground each leaf sample, added 1mM extraction buffer (2% CTAB, 100 mM tris/HCl pH 7.5, 1.4 M NaCl, 2% polyvinylpyrroliodione (PVP)-40, 20 mM EDTA pH 8.0, 20 µl/ml β-mercaptoethanol were added immediately prior to use. Samples incubated for 1 h at 60°C with occasional swirling then samples cooled at room temperature. The solution of Chloroform and Isoamylalcohol (600 µl, 24:1) was added mixed gently in 5 min. Samples were centrifuged for 15 min and in supernatant portion added equal volume of isopropanol and incubated at -20°C overnight. Samples were centrifuged for 15 min then washed the DNA pallets with 70% ethanol and centrifuged for 10 min. DNA pellet was dried and dissolve in 20 to 50 µl TE-R Nase solution. Samples incubated for 1 h at 37°C. Extracted DNA as estimated by comparing two-fold dilution rDNA (from 6.2 mg/µl to 100 µg/µl). Results were evaluated by Popgen software (ver; 1.44), and 1% agarose gel was used for quantification genomic DNA. PCR conditions standardized using 10X PCR buffer, $MgCl_2$, dNTPs, primer and Taq DNA polymerase (Scherwater and Ender 1993). Template DNA at different concentrations (7, 10, 15, 20 and 25 ng/µl), Taq DNA (one unit concentration) polymerase and $MgCl_2$, with different concentration used to standardize PCR conditions in order to obtain bright and reproducible RAPD bands.

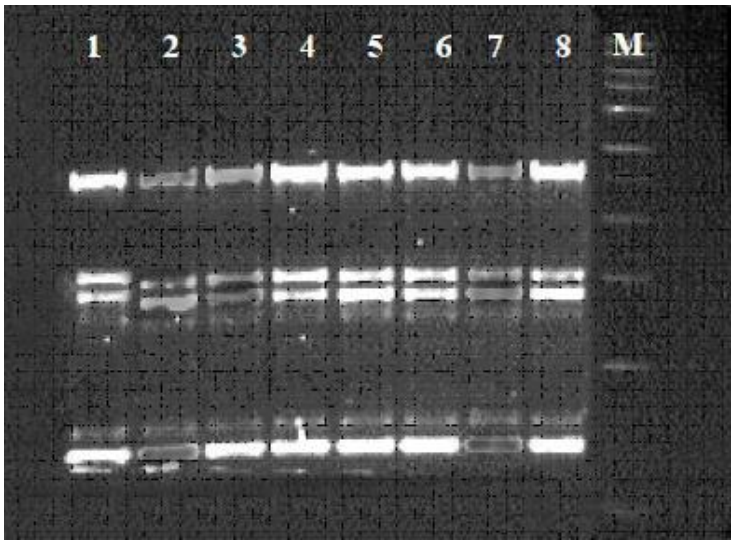


**Figure 1.** RAPD (PCR) of 8 samples of 1-8 *Dalbergia sissoo* germplasm, Primer GLA-19 on agarose gel Electrophoreses images, M 1Kb DNA ladder.

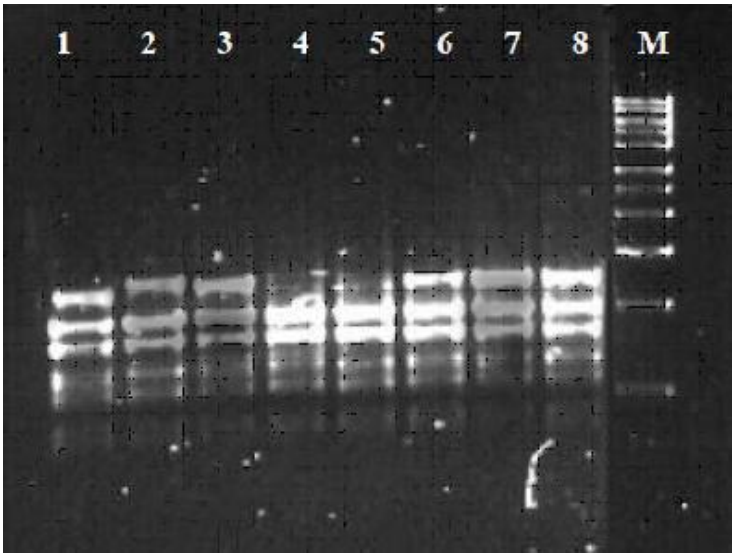


**Figure 2.** RAPD (PCR) of 8 samples of trees of, 1-8 *Dalbergia,* Primer GLC-17.

RAPD primers with their sequence and number of polymorphic bands were determined and cluster analysis was done based on Nei's similarity matrices (Figures 1 to 5).A set of 25 random dacamer oligonucleotides (Table 2) purchased from Gene Link Company as used for single primers amplification of RAPD fragments. PCR was carried out in a final volume of 25 µl containing 20 ng template DNA. Deoxynucleotide triphosphate (100 µg) was added decannucleotide primers (20 ng) with 1.5 mM $MgCl_2$, 1X Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, and 0.01% gelatin) and 0.5 U Taq DNA polymerase. Amplification was achieved in thermal cycler (BioRad 48 well) programmed for preliminary 4 min denaturation steps followed by 45 cycles of denaturation for 1 min at 94°C. Annealing at 37°C for 1 min and extension at 72°C for 2 min and finally at 72°C for 10 min.

Amplifications of products separated alongside a molecular weight marker (1 Kb ladder) by electrophoresis on 1.2% agarose gel run in 0.5 X TAE (Tris-acetate-EDTA) buffer, stained with Ethidium bromide and visualized under UV light. Gel photographs scanned through a Gel Doc system (BioRad).

## RESULTS AND DISCUSSION

Two sets of *D. sissoo* plants analyzed through PCR and RAPD for identifying genetic divergence in germplasm. First one set consisted of 1, 4, 5, 6, and 8 healthy and the second set consisted of 2, 3, and 7 diseased leaves

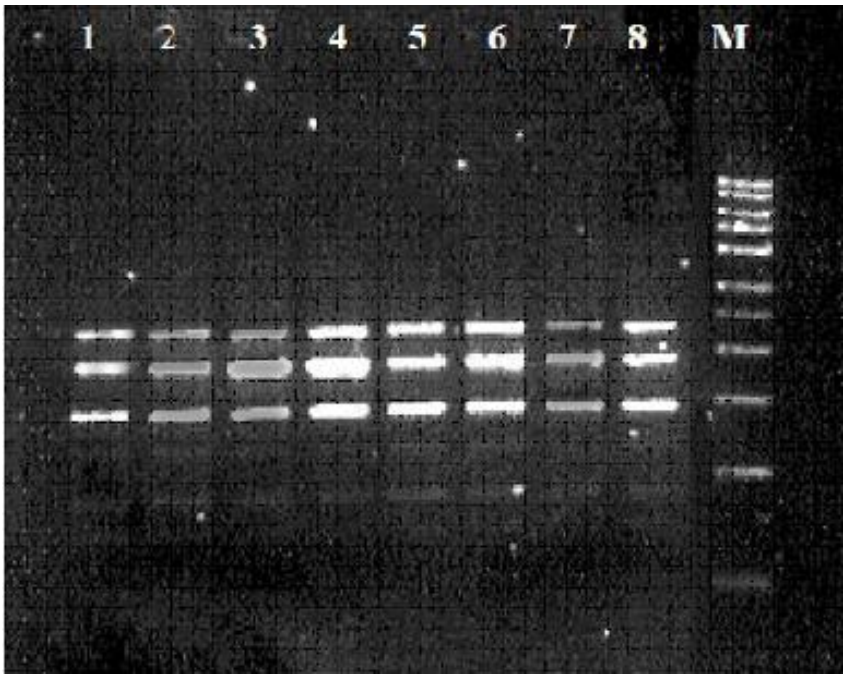


**Figure 3.** RAPD (PCR) of 8 samples of trees (1-8 *Dalbergia,* with Primer GLC-04.

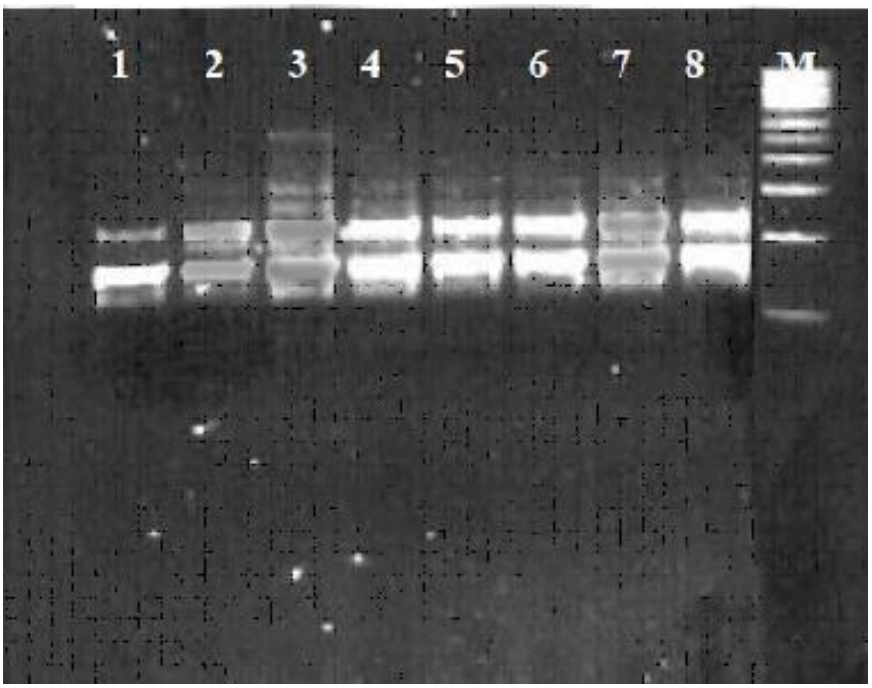


**Figure 4**. RAPD (PCR) of 8 samples of trees (1-8) with Primer GLB-16.

samples. The details are available in (Table 1). The GL Decamer primers used to study PCR and polymorphic bands are given in (Table 2). The samples 1 to 8 treated with 25 GL Decamer primers and DNA fragments bands were studied under agarose gel electrophoresis. The results are shown in Figures 1 to 4. The GL Decamer A-19 showed excellent DNA fragmentation and selected to study the apparent size (bp), actual size (bp) and

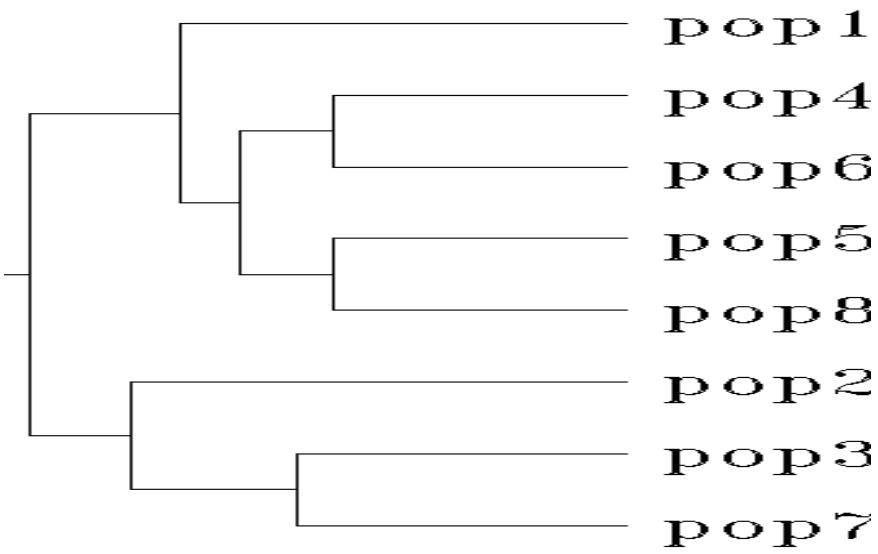


**Figure 5.** Cluster analysis of eight *Dalbergia sissoo* germplasm based on Nei's similarity matrices.

**Table 2.** RAPD primers with their sequence and number of polymorphic bands.

| Sr # | Primer Name | Sequence | NPB | Sr # | Primer Name | Sequence | NPB |
|---|---|---|---|---|---|---|---|
| 1 | GL DecamerA-07 | GAAACGGGTG | 6 | 13 | GL DecamerC- 15 | GGAGGAGGGA | 3 |
| 2 | GL DecamerA-11 | GTGACGTAGG | 3 | 14 | GL DecamerC-17 | AGGCTGGAGG | 3 |
| 3 | GL DecamerA-15 | GGGTAACGCC | 5 | 15 | GL DecamerC-18 | CGAAGGCTTG | 5 |
| 4 | GL DecamerA-17 | GGTGACGCAG | 5 | 16 | GL DecamerC-19 | ACCCC TGCGT | 5 |
| 5 | GL DecamerA-18 | TTCCCGACGG | 2 | 17 | GL DecamerC-20 | GTCGGATCAG | 5 |
| 6 | GL DecamerA-19 | GGAGGGTGTT | 6 | 18 | GL DecamerD-04 | GTGTTACGAG | 4 |
| 7 | GL DecamerB-07 | ACCCCGTTGC | 4 | 19 | GL DecamerD-06 | ACCCCCGAAG | 5 |
| 8 | GL DecamerB-12 | TGCGCCCTTC | 5 | 20 | GL DecamerD-08 | CAAACGTCGG | 5 |
| 9 | GL DecamerB-16 | TGCGTGCTTG | 3 | 21 | GL DecamerD-10 | GTTGCCAGCC | 3 |
| 10 | GL DecamerC-02 | CACACTCCAG | 4 | 22 | GL Decamer D-13 | GTGATCGCAG | 4 |
| 11 | GL DecamerC-04 | TGAGTGGGTG | 3 | 23 | GL Decamer D-14 | CAATCGCCGT | 5 |
| 12 | GL DecamerC- 12 | GATGACCGCC | 5 | 24 | GL Decamer D-18 | TCTGTGCTGG | 4 |
| | | | | 25 | GL Decamer D-19 | TACGAAACGT | 3 |

**Table 3.** Size and quantity of each DNA band of *Dalbergia sissoo* Germplasm 1.

| DNA band No. | Apparent size (bp) | Actual size (bp) | Quantity (ng) |
|---|---|---|---|
| 1 | 4200 | 4250 | 25 |
| 2 | 2000 | 2100 | 20 |
| 3 | 1900 | 1900 | 22 |
| 4 | 1700 | 1700 | 7 |
| 5 | 700 | 750 | 3 |
| 6 | 475 | 475 | 23 |

quantification (ng) of each DNA bands on agarose gel. These results are shown in Figure 1 and Tables 3 to 8. Interestingly, agarose gel electrophoresis images with reference to one Kb DNA ladder produced excellent

**Table 4.** Size and quantity of each DNA band of *Dalbergia sissoo* Germplasm 2.

| DNA band No. | Apparent size (bp) | Actual size (bp) | Quantity (ng) |
|---|---|---|---|
| 1 | 4250 | 4250 | 12 |
| 2 | 2050 | 2060 | 13 |
| 3 | 1930 | 1950 | 15 |
| 4 | 1760 | 1750 | 7 |
| 5 | 780 | 790 | 8 |
| 6 | 500 | 525 | 45 |

**Table 5.** Size and quantity of each DNA band of *Dalbergia sissoo* Germplasm 3.

| DNA band No. | Apparent size (bp) | Actual size (bp) | Quantity (ng) |
|---|---|---|---|
| 1 | 4300 | 4310 | 25 |
| 2 | 2100 | 2150 | 27 |
| 3 | 1950 | 1970 | 10 |
| 4 | 1800 | 1810 | 8 |
| 5 | 800 | 820 | 10 |
| 6 | 550 | 550 | 20 |

**Table 6.** Size and quantity of each DNA band of *Dalbergia sissoo* Germplasm 4.

| DNA band No. | Apparent size (bp) | Actual size (bp) | Quantity (ng) |
|---|---|---|---|
| 1 | 4300 | 4310 | 25 |
| 2 | 2100 | 2150 | 27 |
| 3 | 1950 | 1970 | 27 |
| 4 | 1800 | 1810 | 8 |
| 5 | 800 | 820 | 3 |
| 6 | 550 | 550 | 20 |

**Table 7.** Size and quantity of each DNA band of *Dalbergia sissoo* Germplasm 5.

| DNA band No. | Apparent size (bp) | Actual size (bp) | Quantity (ng) |
|---|---|---|---|
| 1 | 4315 | 4325 | 23 |
| 2 | 2150 | 2160 | 25 |
| 3 | 1960 | 1980 | 26 |
| 4 | 1810 | 1815 | 2 |
| 5 | 815 | 820 | 3 |
| 6 | 550 | 550 | 22 |

*samples 6, 8 showed similar behavior.

**Table 8.** Size and quantity of each DNA band of *Dalbergia sissoo* Germplasm 7.

| DNA band No. | Apparent size (bp) | Actual size (bp) | Quantity (ng) |
|---|---|---|---|
| 1 | 4315 | 4325 | 17 |
| 2 | 2150 | 2160 | 10 |
| 3 | 1960 | 1980 | 18 |
| 4 | 1810 | 1815 | 19 |
| 5 | 815 | 820 | 23 |
| 6 | 550 | 550 | 13 |

**Table 9.** Nei's genetic identity of *Dalbergia sissoo* germplasm.

| POP ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| 1 | **** | **** | **** | **** | **** | **** | **** | **** |
| 2 | 0.7297 | **** | **** | **** | **** | **** | **** | **** |
| 3 | 0.7770 | 0.8176 | **** | **** | **** | **** | **** | **** |
| 4 | 0.8176 | 0.7230 | 0.8243 | **** | **** | **** | **** | **** |
| 5 | 0.8784 | 0.7432 | 0.8446 | 0.8716 | **** | **** | **** | **** |
| 6 | 0.8108 | 0.7568 | 0.7905 | 0.8851 | 0.8646 | **** | **** | **** |
| 7 | 0.7703 | 0.8108 | 0.8716 | 0.8311 | 0.8108 | 0.8514 | **** | **** |
| 8 | 0.8176 | 0.6824 | 0.8243 | 0.8378 | 0.8851 | 0.8311 | 0.777 | **** |

**Table 10.** Nei's genetic distance of Dalbergia sissoo germplasm.

| POP ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| 1 | **** | **** | **** | **** | **** | **** | **** | **** |
| 2 | 0.3151 | **** | **** | **** | **** | **** | **** | **** |
| 3 | 0.2523 | 0.2014 | **** | **** | **** | **** | **** | **** |
| 4 | 0.2014 | 0.3244 | 0.1932 | **** | **** | **** | **** | **** |
| 5 | 0.1297 | 0.2967 | 0.1689 | 0.1374 | **** | **** | **** | **** |
| 6 | 0.2097 | 0.2787 | 0.2350 | 0.1220 | 0.1452 | **** | **** | **** |
| 7 | 0.2610 | 0.2097 | 0.1374 | 0.1850 | 0.2097 | 0.1609 | **** | **** |
| 8 | 0.2014 | 0.3821 | 0.1932 | 0.1769 | 0.1220 | 0.1850 | 0.2523 | **** |

distinct bands for healthy and diseased sissoo samples. Apparently, healthy samples (1, 4, 5, 6, and 8) showed strong and bright bands at 4200 bp with 25 ng to 500 bp with 50 ng DNA quantities. While diseased samples showed weak/faint bands at 4200 bp with 3 ng and 500 bp are only 8 ng quantities, which is clear indication of changes in a genomic sequence of DNA in the both healthy and diseased plants.

It is very good evidence that genetic makeup of diseased plants has been changed due to some unknown reasons like environmental effects, adaptation, soil fertility, edaphic conditions, conservation, phylogenic relationship, lowering of water level or mutation effects like tautomerism, depurination and deamination etc. Such types of diseases like Fusarium wilt, Ganoderma Root Rot, Phellinus Root and Butt Rot, reported by Mehrotra and Sharma (1992). The other GL Decamer primers studied in the same fashion and obtained similar results (Figures 2 to 4).On the bases of cluster, analysis (Nei and Li, 1979) was used to find out genetic identity and genetic distance. The results are given in Tables 9, 10 and Figure 5. Maximum genetic identity was found in set one (healthy), the population 4 and 6 are (88%), population 5 and 8 are 85% similar while population 1 is 76% genetically similar with population 4, 6 and 5, 8. Our studies are in accordance with the findings of Ahuja et al. (1994), Aide et al. (1998) and Binelli and Bucci (1994).

The set second (diseased) population 3 and 7 are 81% genetically similar while population 2 is 73% similar with 3, 7 population. Maximum genetic distance was found in set one (healthy), the population 4 and 6 are (22%), and population 5 and 8 are 15% dissimilar while population 1 is 24% genetically dissimilar with population 4, 6 and 5, 8. The set second (diseased) population 3 and 7 are 19% genetically dissimilar while population 2 is 27% dissimilar with 3, 7 population. The healthy set is 68% genetically identical with the diseased set while the 32% genetic distance is found in the both sets. Similar findings have been reported by Freemark et al. (2002), Gillies et al. (1997) and Singh et al. (1999). Our study would be helpful to control disease and provide important input for conservation of energy and economic status of nation.

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