Pak. J. Bot., 46(5): 1573-1576, 2014.

ISSR ANALYSIS OF GENETIC DIVERSITY IN *DALBERGIA SISSOO* IN PUNJAB, PAKISTAN

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Abstract

Variation in different phenotypic characteristics in shisham (*Dalbergia sissoo* Roxb.) reported in previous studies reveals that a large amount of genetic diversity is available. In the present study, genetic diversity was assessed in *D. sissoo* vegetations from Lahore, Gujranwala and Sialkot districts. Leaf samples from 34 shisham trees showing variation in phenotypic characters such as shape and size of leaflets, pod length, number of seeds per pod, branching pattern, overall physical appearance of the trees etc., were collected from shisham plantations growing on road-sides and BRB canal-bank. A total of 10 inter-simple sequence repeat (ISSR) primers were used. Out of these, 7 primers amplified the genomic DNA of *D. sissoo* yielding 114 fragments, out of that 78 were polymorphic. There were 9 to 25 amplified fragments with different primers with size ranging from 100 bp to 2 kbp. Polymorphism ranged from 27.27% to 89.50%. Genotypic diversity in *D. sissoo* confirmed in the present study can be best exploited for plant breeding and conservation, especially in saving this precious tree species from dieback and wilt diseases.

Keywords: *Dalbergia sissoo*, Genetic diversity, ISSR analysis, Pakistan, Shisham.

Introduction

Shisham (*Dalbergia sissoo* Roxb.) is an economically important multipurpose deciduous tree species of family Papilionaceae (Singh *et al.*, 2011). Its wood is very hard and is best suitable for furniture (Hossain & Martin, 2013). In addition, different parts of this plant have medicinal importance (Pooja *et al*., 2010; Asif & Kumar, 2011). Naturally this plant grows in Sub-Himalayan Tarai tract ranging from Bangladesh to Afghanistan (Khan, 2000). Presently, it is cultivated throughout the South Asian subtropical regions, and widely so in Pakistan, India and Nepal. In Pakistan, *D. sissoo* was introduced in mid 1800s. Irrigated state forests plantations were established at various sites in Punjab, mostly for fuel wood production for steam engines. All of these plantations are located along main railway lines, major ones are Changa Manga, Perowal, Daphar, Kundian, Bahawalpur, Kamalia and Chichawatni. In addition, it is widely planted along roads, rail sides and canals and also around the fields (Sah *et al.*, 2003).

In 1998, dieback was reported as an epidemic in central irrigated tract of Punjab province (Naz, 2002) and incidence was also reported in the Tarai tract of Nepal (Joshi & Baral, 2000), India (Sharma *et al.*, 2000) and Bangladesh (Webb & Hossain, 2005). Bajwa *et al*. (2003) reported that dieback and wilt diseases are responsible for shisham decline. Various fungal pathogens, namely *Fusarium solani*, *Fusarium oxysporum*, *Botryodiplodia theobromae*, *Ganoderma lucidum* and *Phytopthora cinnamomi* have been found as causal agents for dieback and wilt diseases of shisham (Bajwa *et al.*, 2003; Dayaram *et al.*, 2003; Harsh *et al*., 2010; Ahmad *et al.*, 2013). Various seminars have been conducted to highlight the problem of dieback in the country (Javaid, 2008). Sah *et al*., (2001) studied various aspects of soil characteristics of 30 shisham strands in Nepal and found that soil was not the sole factor responsible for decline of this plant. Various research organizations including Punjab Forest Research Institute (PFRI), Ayub Agricultural Research Institute (AARI), Institute of Agricultural Sciences, University of the Punjab Lahore, and University of Agriculture Faisalabad have been engaged in research against shisham decline (Afzal *et al.*, 2006; Javaid, 2008). So far, millions of trees have been vanished due to these epidemic diseases and unfortunately no reliable solution is available to combat the menace. Javaid *et al*., (2003) identified 9 phenotypically different shisham varieties on the bases of physical appearance of the plant, branching pattern, leaf and leaflet size and shape, pod characters, branching and leaf density and stem surface characteristics. Later on, Javaid *et al*., (2004) identified 9 more varieties on the bases of these characters. They also reported that these phenotypically different varieties varied in their response to wilt and dieback diseases in the shisham plantations.

In previous studies, DNA-based molecular markers have been used for phylogenetic analysis and study of genetic diversity in plants (Nybom, 2004). Inter-simple sequence repeat (ISSR) is a PCR-based technique generally used to identify individuals, especially plant species, on the bases of variation occur in the regions between microsatellites. ISSR markers detect polymorphisms in microsatellite and inter-microsatellite loci (Zietkiewicz *et al.*, 1994), and have been widely used to evaluate genetic diversity and population structure (Esselman *et al.*, 1999). ISSRs have been used to estimate the extent of genetic diversity in a wide range of plant species including *Eleusine coracana*, *Vigna* spp., *Ipomoea batatas* and *Plantago major* (Mukherjee *et al.*, 2013). Since earlier studies regarding the shisham diversity were based on phenotypic characteristics, the present study was carried out to investigate the genetic diversity in shisham using ISSR markers. This study would be helpful in future plant breeding programs which possibly lead to save this precocious tree species from decline diseases.

Materials and Methods

Collection of plant materials: Survey of different areas of Lahore, Gujranwala and Sialkot districts were conducted during October 2012 to collect the leaf samples of different shisham varieties. A total of 34 samples were collected. Shisham trees were selected for sampling on the bases of differences in various morphological characters viz. physical appearance of the plant, branching pattern, leaf and leaflet size and shape, pod characters, branching and leaf density and stem surface characteristics (Javaid *et al.*, 2003, 2004). Samples 1–25 were collected from Lahore, 26–28 from Gujranwala and 29–34 from Sialkot.

Isolation of DNA: DNA was isolated from leaves of different shisham varieties following CTAB extraction procedure. One hundred milligrams of leaves of each variety were grinded in liquid nitrogen with pestle and mortar. The ground tissues were transferred to polypropylene tube containing 1.0 mL extraction buffer (2% w/v CTAB, 100 mM Tris HCl pH 8, 1.4 M NaCl, 20 mM EDTA, 0.03% b-mercapta ethanol) pre-heated to 65°C. These samples were incubated for 30 minutes with intermittent shaking at every 15 minutes. Equivalent volume of chloroform isoamyl alcohol (24:1 v/v) was poured in the sample tubes and gently agitated for 10 min to form an emulsion. Sample tubes were centrifuged at room temperature for 10 minutes at 6000 rpm. Supernatant was poured into sterile tubes and 10 mL of chilled isoproponol was added to each tube, mixed by inverting and put at –20°C for 10 minutes. Contents were centrifuged again for 20 minutes (5000 rpm) at 4°C. Pellets were retained and supernatants were discarded. DNA pellets obtained were washed with 70% ethanol and tubes were inverted on blotting paper to dry the pellet. Afterward, DNA was suspended in 50 µL TE buffer (1 mM EDTA, 10 mM Tris HCl) and stored at –20°C. Quality of DNA was tested by running the samples on 1% agarose gel in 1x TAE buffer stain containing 3 µL of 0.1% ethidium bromide solution.

ISSR amplification: Ten ISSR primers were used in this study. PCR reaction mixture 2X *n*Taq provided by Enzynomics® Korea was used. Amplification reactions were conducted in a 25 µL volume containing 1 µL primer, 0.3 µL *Taq* DNA polymerase, 1 µL template DNA, 2 µL dNTP, 2 µL $MgCl_2$, 2.5 µL reaction buffer and rest deionized water. PCR reaction was carried out in a 96-well Asco PCR System. Amplification conditions were an initial denaturing at 94°C for 4 min, and 36 cycles at 30s denaturing at 94°C, annealing at 45-52°C for 45 s, and extension at 72°C for 120 s. Thereafter, a final extension step was carried out at 72°C for 7 min. From each primer, amplified bands were studied on agarose gel and scored as absent (0) or present (1). Dendrogram was constructed by using Single Linkage Euclidean Distance method with the help of MYSTAT® program.

Results and Discussion

In the present study, 10 ISSR primers were used. Among these, 7 primers provided transferability among 34 evaluated shisham varieties. These primers gave variable number of DNA fragments depending on their sequence repeat motifs (Fig. 1). Each of these seven primers provided fine detectable bands with variable intensity. The seven primers amplified a total of 114 loci, out of which 78 were polymorphic. Total fragments amplified with various primers ranged from 9 to 25 with an average of 16. Polymorphic bands varied from 3 to 19 in number in case of different primers with an average of 10. Size of different bands ranged from 100 bp to 2 kbp. Likewise, polymorphism ranged from 27.27% to 89.50% in case of different primers with an average of 58.06%. In the present investigation, maximum polymorphism was exhibited by primer 841 (Table 1). In a previous study, Arif *et al*., (2009) used 15 ISSR primers and recorded 117 bands across 22 shisham genotypes collected from different regions of India. Out of 117 amplified bands, 64 bands were polymorphic. Average polymorphism in their study was 54.7% that was very close to that found in the present study. Likewise, polymorphism range in their study was 0–87.5% that is comparable with maximum polymorphism (89.50%) with ISSR primer 841 in the present study.

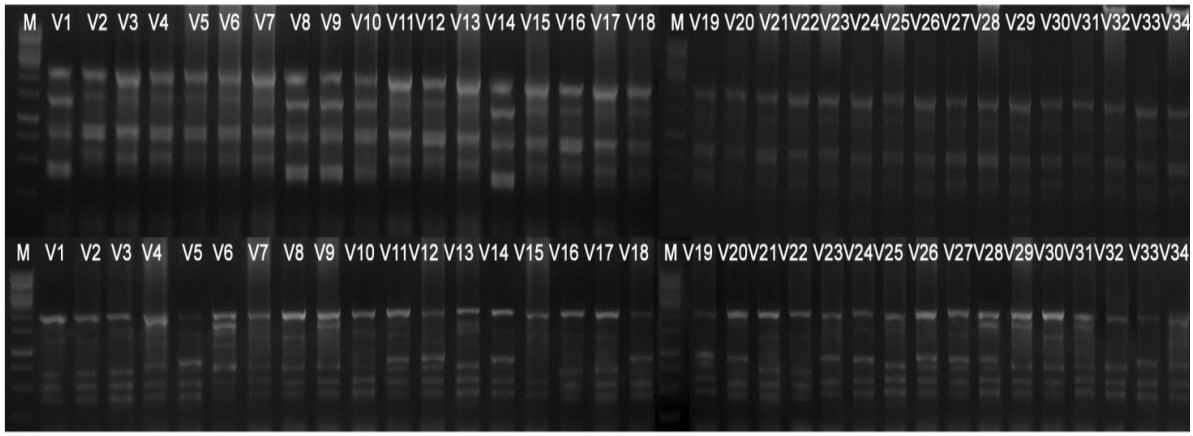

Fig. 1. PCR product of 34 shisham verities generated from ISSR primers 810 (above) and 856 (below). Lane M is ladder and lanes V1 to V34 show different shisham genotypes.

Table 1. Primer sequences, annealing temperature and number of loci generated from ISSR primers.

Primer	Sequence (5'-3')	Annealing temperature (°C)	Total no of bands	Polymorphic bands	Polymorphism (%)
810	GAGAGAGAGAGAGAGAT	50	17	11	64.70
823	TCTCTCTCTCTCTCTCC	50	09	04	44.44
826	ACACACACACACACACC	51	25	19	76.00
841	GAGAGAGAGAGAGAGAYC	52	16	14	89.50
845	CTCTCTCTCTCTCTCTAGG	52	11	03	27.27
855	ACACACACACACACACYT	50	15	08	53.33
856	ACACACACACACACACCTA	52	21	13	61.90
Total			**114**	**78**	**82.03**

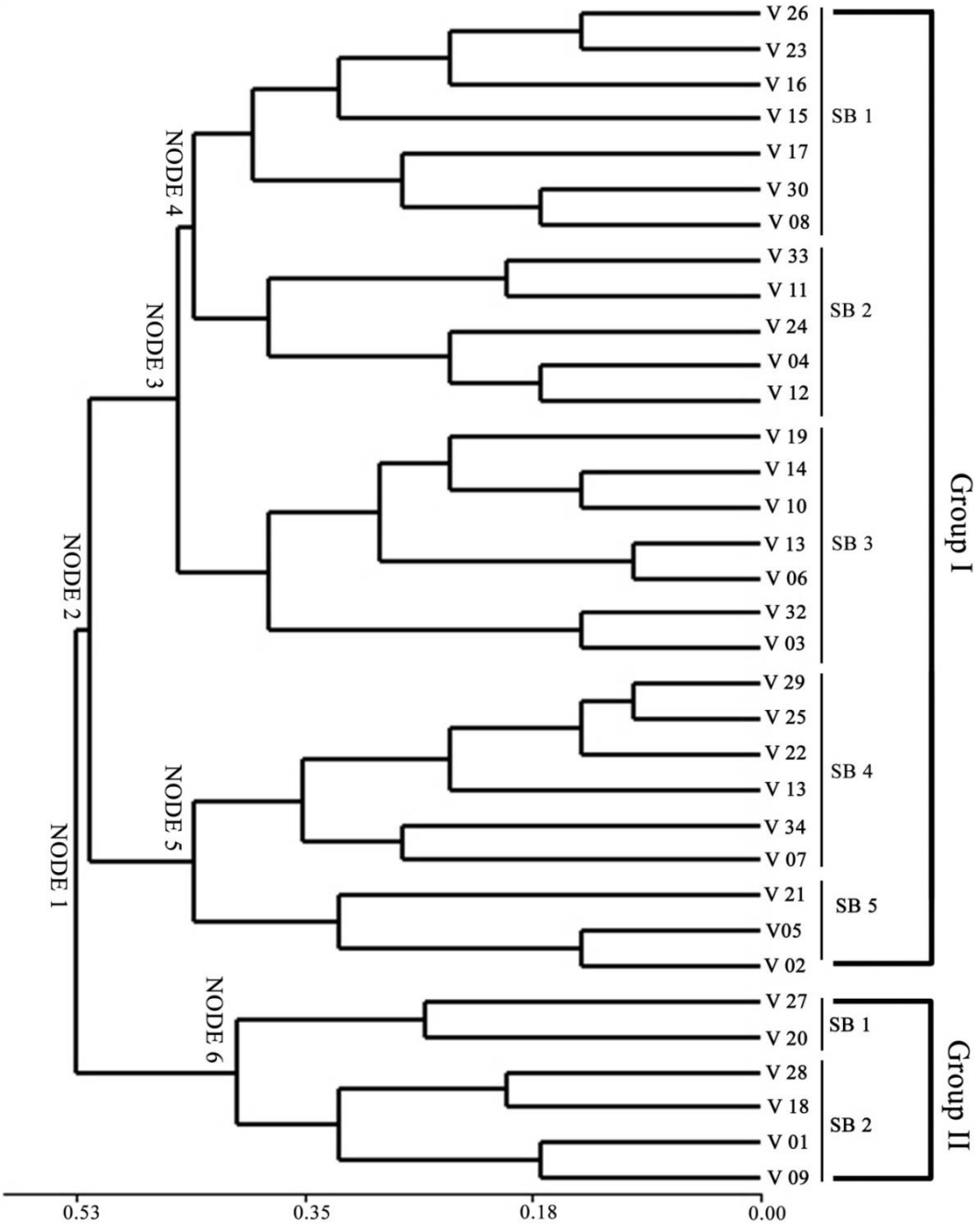

Fig. 2. Dendrogram presenting genetic divergence among 34 shisham varieties constructed by using Single Linkage Euclidean Distance method.

The Single Linkage Euclidean Distance cluster analysis provided two distinct groups *viz*., Group I and Group II. Group I consisted of 28 shisham varieties. This group was further divided into five subgroups namely SB 1 to SB 5 from node 2 to 5. Here subgroup 1 contained 7 varieties. Subgroups 2, 3, 4 and 5 contained 5, 7, 4 and 3 shisham varieties, respectively. Clustering in subgroup 1 was more diverse. Group II contained only two subgroups. Here subgroup 1 contained only two shisham varieties *viz*., V 27 and V 20 whereas subgroup 2 contained four shisham varieties (Fig. 1). Dandrogram do not show any clustering pattern on the basis of collection area of the samples. Similarly, no location specificity in shisham germplasm was reported in India (Arif *et al.,* 2009). Similar findings have also been obtained in Azukibean and groundnut (Yee *et al.,* 1999; Dwivedi *et al.,* 2001) (Fig. 2).

Present study concludes that shisham plantations in the studied areas have considerable polymorphisms. This information would be useful for the breeders to select resistant genotypes for saving shisham from being extinct due to decline diseases.

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(Received for publication 3 May 2013)