INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 13, No. 3, p. 209-222, 2018

RESEARCH PAPER OPEN ACCESS

Assessment of population genetics of shisham (*Dalbergia sissoo*) based on genetic structure and diversity analyses

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Key words: Shisham, ISSR markers, Genomics, Population genetics, Genetic diversity.

http://dx.doi.org/10.12692/ijb/13.3.209-222 Article published on September 30, 2018

Abstract

Population genetic study is an important strategy to shape up a scattered population, for its characterization and improvement. Marker assisted approaches have been allied to computation biology for unraveling and estimating precise genetic pattern of a population. Therefore, this research study was designed by keeping in view the lack of genetic information of shisham (*Dalbergia sissoo*) regarding genetic pattern and diversity of population. ISSR (21) markers were used to investigate 60 genotypes collected across the Punjab (Pakistan) with the aid of different bioinformatics analyses. Genetic diversity analysis showed, a total of 373 loci were amplified with percent polymorphic loci, 99.46. Maximum similarity (96%) was found between genotypes MBP2 & MBP3. However, genotype OP4 was more distantly related to genotype LP1 (98% dissimilar). Principle coordinate analysis (PCoA) revealed first II coords accounted for 30.06% total genetic variation in 2-Dplot.Population genetic pattern showed three subpopulations or genepools with diverse genetic pattern, which were arrayed as distinct and even sharing maximum genetics. These three subpopulations collectively, were yielded genetic diversity tree with hierarchical clustering exhibiting four distinct clusters that sat in population in a range or degree from close to diverse. These results were proved to be helpful for tagging and organizing scattered shisham population and would be an step for finding dieback disease resistant shisham genotypes.

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Introduction

Dalbergia sissoo Roxb. ex DC. (shisham or tali) is an economical perennial tree of family Leguminosae, sub-family Papilionaceae (Singh *et al.*,2011). It is an extensively planted deciduous tree of South Asia predominantly in Indo-Pak. Its natural distribution found in the Himalyas of Indo-Pak, Indo-Gangetic basin and Nepal (Ginwal and Marya, 2009). This tree is widespread due to its valuable timber (furniture mart, fuel wood and agroforestry), high growth rate, nitrogen fixing, pesticidal properties, drought tolerating ability, easy propagation and numerous medicinal values (Arif *et al.*, 2009; Orwa *et al.*, 2009; Shah *et al.*, 2010). It grows up to 30m in height and 80cm dbh in strip plantations (1.8 x 1.8m to 4 x 4 m) but closer spacing is well known planting layout for good quality timber (Orwa *et al.*, 2009). It grows well in alluvial and muddy soil, with plantations along river, roadside, canal, highways and in farmer's field. In Pakistan, shisham was introduced in mid 1800sand plantation prevailed on an area of approximately 10000 ha. However, 154886 ha is occupied by Punjab with 28000 m^3 production per annum (Khan and Khan, 2000).

Shisham is adversely affected by many disease predominantly dieback. Shisham decline due to dieback was reported from Bangladesh and India (Baksha and Basak, 2000; Sharma *et al.*, 2000). In 1998, shisham dieback was declared as an epidemic throughout the Pakistan, most likely in Punjab province (Naz, 2002; Bajwa *et al.*, 2003). Mycopathogens are considered to be the causative agent of dieback (Bajwa and Javaid, 2007). The effective method to overcome this devastating situation is by using resistant source of shisham germplasm.

Genetic characterization would facilitate scientists to acquire knowledge regarding species and population level discriminations, topographical distribution and morphological attributes. Genetic variation can be studied using various DNA markers i.e. ISSR (Inter-Simple Sequence Repeats), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism) and SSR (Simple Sequence Repeat).ISSR markers are being used as most reliable DNA markers for diversity study and population genetics (Zeitkiewicz *et al.* 1994; Wolf and Randle, 2001; Li and Ge, 2001).

Therefor for population genetic study of shisham (collected from Punjab, Pakistan), ISSR markers were used to estimate population structure pattern, genetic differentiation in population in terms of genetic distance and genetic relatedness. On the basis of this study, diverse healthy shisham germplasm were tagged and organized, which would be helpful to identify dieback disease resistant shisham genotypes.

Materials and methods

Germplasm collection

Healthy shisham germplasm was collected by conducting surveys across the Punjab(Pakistan). For collecting germplasm Punjab survey was split into three zones viz., Central Punjab and Northern Punjab and Southern Punjab (Fig. 1).From each zones 5 locations were selected to collect plant material and four trees were selected from each location which made shisham population size, 60 (Table 1). Surveyed areas including farmer fields, nurseries, along road sides and water channels. Branch and root cuttings were taken from healthy shisham and from each tree 3-5 cuttings were taken and placed in different sampling bags and were tagged.

Macropropagation

Mixture of soil and sand (3:1 ratio) was sterilized using 37% formalin soln. in water with ratio 1:49 and filled in polythene bags. Cuttings from shisham trees, about 7-9cm in length with 3-5 nodes, were used and planted in soil filled polythene bags. These bags were kept in green house for nursery raising (Fig. 2). Nursery was maintained by applying fertilizers, timely irrigation, proper drainage and strict observation regarding their vigor as well as health of plants.

DNA extraction

DNA of 60 samples was isolated from young leaves of macro propagated plant material using modified CTAB method. Leaves were ground into preheated CTAB buffer, by adding 2-3 drops of β-marcaptoethanol, followed by incubation at65°C for 30 minutes. Centrifuged at 8000rpm for 4min was done. Supernatant was taken and Phenol Chloroform Isoamyl Alcohol (equal volume) was added and then centrifuged.

The same way was adopted for Phenol and Iso-Propanol treatment. Supernatant was discarded and pellet was washed with 70% ethanol, air dried and dissolved in TE buffer. DNA concentration was measured by UV visible NANODROP (8000 Spectrophotometer, Thermo SCIENTIFIC) and working dilution of 50ng/µL was made.

PCR (ISSR) analysis

For PCR analysis, a total of 21 ISSR primers (Table 2) were used. PCR thermal profile was maintained as described by Arif *et al.* (2009). The polymerase chain reaction (PCR) was performed on 96 well thermal cycler (peq STAR) using 25µLPCR reaction mixture comprising of10X *Taq* PCR buffer, dNTPs, primer, DNA template (50ng/µL), *Taq* DNA polymerase and deionized water. PCR products were resolved on 2.5% agarose gel, visualized under UV using gel documentation system (Bio Rad, USA) and pictures were saved.

Data analysis

DNA bands were counted and scored in the form of binary matrix on excel (MS-toolkit), as 1 (presence) and 0 (absence).

The collected data were aligned for analysis. STRUCTURE software (Version 2.3.4) was used for assessing pattern of genetic structure in a population and similarity matrix was generated using Pop Gen `32 (Version 1.32) based on Nei's original measure. Cluster analysis was performed using softwares; PAST (Version 3.16) and DARwin6 (version 6.0). Principle coordinate analysis (PCoA) and polymorphic information content (PIC) were computed using DARwin6 and Power Marker (Version 3.25) respectively.

Results

PCR (ISSR) analysis

The reproducible and reliable amplifications were obtained in ISSR based PCR analysis, which produced different numbers of DNA bands depending upon their SSR (Simple Sequence Repeats) motifs (Fig. 3). The 21 primers amplified 373 loci across 60 genotypes, of them 371 were shown to be polymorphic with percent value, 99.46 (Table 2). A total of 11554 alleles were identified and number of alleles produced by each primer ranging from 110 to 999 with an average of 550.19 alleles per primer. While average number of alleles identified at single locus ranged from 19.64 (ISSR-3) to 45.41 (ISSR-8).

Table 1. List of shisham germplasm collected from different location of Punjab (Pakistan) for population genetic study.

Sr.No.	Plant code used in this study	Location Punjab/pakistan	Geograph-ical Zone	Sr.No.	Plant code used in this study	Location Punjab/pakistan	Geograph-ical Zone
1	CP1	Chichawatni	Central Punjab	31	LP3	Lahore	Northern Punjab
2	CP2	Chichawatni		32	LP4	Lahore	
3	CP3	Chichawatni		33	SP1	Sialkot	
4	CP4	Chichawatni		34	SP2	Sialkot	
5	FP1	Faisalabad		35	SP3	Sialkot	
6	FP2	AARI*, Faisalabad		36	SP4	Sialkot	
7	FP3	UAF**, Faisalabad		37	MBP1	Mandi Bahauddin	
8	FP4	Faisalabad		38	MBP2	Mandi Bahauddin	
9	OP1	Okara		39	MBP3	Mandi Bahauddin	
10	OP2	Okara		40	MBP4	Mandi Bahauddin	

11	OP3	Okara		41	BP1	Bahawalpur	
12	OP4	Okara		42	BP2	Bahawalpur	
13	ShP1	Shorkot		43	BP3	Bahawalpur	
14	ShP2	Shorkot		44	BP4	Bahawalpur	
15	ShP3	Shorkot		45	BNP1	Bahawalnagar	
16	ShP4	Shorkot		46	BNP2	Bahawalnagar	
17	TP1	Toba Tek Singh		47	BNP3	Bahawalnagar	
18	TP2	Toba Tek Singh		48	BNP4	Bahawalnagar	
19	TP3	Toba Tek Singh		49	KWP1	Khanewal	Southern Punjab
20	TP4	Toba Tek Singh		50	KWP2	Khanewal	
21	GP1	Gujranwala		51	KWP3	Khanewal	
22	GP2	Gujranwala		52	KWP4	Khanewal	
23	GP3	Gujranwala		53	MP1	Muzaffargarh	
24	GP4	Gujranwala		54	MP2	Muzaffargarh	
25	KP1	Khushab		55	MP3	Muzaffargarh	
26	KP2	Khushab		56	MP4	Muzaffargarh	
27	KP3	Khushab		57	RKP1	Rahim Yar Khan	
28	KP4	Khushab		58	RKP2	Rahim Yar Khan	
29	LP1	Lahore		59	RKP3	Rahim Yar Khan	
30	LP2	Lahore		60	RKP4	Rahim Yar Khan	

Northern Punjab

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The ISSR markers revealed high level of polymorphism arrayed from 21.12% (ISSR-8) to 159.72% (ISSR-3). The PIC (polymorphic information content) was calculated for determining marker in formativeness. PIC values sat in from 0.1455 (ISSR-8) to 0.3549(P-49) with mean value of 0.2830. Whereas expected gene diversity was from 0.1706 (ISSR-8) to 0.4638 (P-49) having average value of 0.3559 (Table 2).

Table 2. The nucleotide sequence of 21 selected ISSR primers, and the number of bands scored.

Sr. No.	Primer code	Primer sequence (5'-3')	No. of loci	Total no. of bands/allele	No. of alleles per locus	Total polym-orphic bands/ allele	Polymor-phism (%)	PIC value	Expected gene diversity
1	P-46	$(AG)_8T$	19	439	23.11	625	142.36	0.3186	0.4022
2	P-49	$(GA)_8T$	19	530	27.89	535	100.94	0.3549	0.4638
3	ISSR-1	$(GA)_8A$	17	404	23.76	397	98.26	0.3391	0.4370
4	ISSR-2	$(CT)_8G$	9	229	25.44	284	124.01	0.3447	0.4447
5	ISSR-3	$(TC)_8G$	11	216	19.64	345	159.72	0.3190	0.4026
6	ISSR-4	$(AC)_8T$	13	357	27.46	371	103.92	0.3158	0.3993
7	ISSR-5	$(AC)_8G$	17	375	22.06	401	106.93	0.3363	0.4345
8	ISSR-6	$(GA)_8TT$	19	452	23.79	384	84.95	0.3255	0.4156
9	ISSR-7	$(AC)_8YG$	22	669	30.41	585	87.44	0.3276	0.4179
10	ISSR-8	$CGA(AG)_7$	22	999	45.41	211	21.12	0.1455	0.1706
11	ISSR-9	$GAC(GA)_7$	24	988	41.17	260	26.31	0.1801	0.2148
12	ISSR-10	$GCA(GT)_7$	25	946	37.84	380	40.16	0.2426	0.2995
13	ISSR-11	$GCA(TG)_7$	20	700	35.00	300	42.85	0.2590	0.3144
14	ISSR-12	$(ATG)_6$	21	507	21.14	564	111.24	0.2873	0.3676
15	ISSR-13	$GGG(TGGGG)_2TG$	25	596	23.84	679	113.92	0.3182	0.4029
16	ISSR-14	$(TC)_8C$	14	464	33.14	292	62.93	0.2835	0.3526
17	1SSR-15	$(AC)_8C$	17	756	44.47	162	21.42	0.1682	0.1972

18	1SSR-16	$(GA)_8CC$	16	543	33.94	257	47.32	0.2603	0.3194
19	ISSR-17	$(CT)_8AGG$	5	110	22	130	118.18	0.2499	0.2989
20	ISSR-18	$(AC)_8TT$	17	601	35.35	385	64.05	0.2342	0.2920
21	ISSR-19	$(AC)_8CTA$	21	673	32.05	482	71.61	0.3330	0.4277
		Total	373	11554	628.9	8029	---	5.9433	7.4752
		Average	---	550.19	---	---	83.32	0.2830	0.3559

Genetic diversity analyses

For unbiased genetic diversity analysis, distance matrix based clustering analyses i-e., unweighted pair group method with arithmetic mean (UPGMA) and Unweighted Neighbor-Joining (NJ) were used to construct dendrogram. UPGMA based dendrogram was generated in PAST v.3.16. The similarity index obtained from Pop Gen 32 v. 1.31 were also compared with this dendrogram analysis. Nei's genetic identity ranged from 0.0000 to 0.9598 and showed maximum similarity value of 96% between MBP2 and MBP3 genotypes.

Table 3. Evanno table showing maximum ΔK at K=3.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
2	3	-9287.300000	4.214262	—	—	—
3	3	-8230.933333	8.590887	1056.366667	257.766667	30.004664
4	3	-7432.333333	57.263455	798.600000	40.400000	0.705511
5	3	-6674.133333	16.785212	758.200000	—	—

The hierarchical clustering of dendrogram generated with PAST software, showed four major clusters (I, II, III & IV) (Fig. 4). Cluster I comprised of MBP1, MBP2, MBP3, MBP4 (genotypes of Mandi Bahauddin region); CP2, CP4 (genotypes of Chichawatni region); FP1, FP2 (genotypes of Faisalabad region); ShP1, ShP2, ShP3, ShP4 (genotypes of Shorkot region); TP2, TP3, TP4 (genotypes of Toba Tek Singh region); OP3 (genotype of Okara region); GP1, GP2, GP3, GP4 (genotypes of Gujranwala region); LP2, LP3, LP4 (genotypes of Lahore region); KP2, KP3 (genotypes of Khushab region). These results revealed that cluster I depicted shisham population of two geographical zones (Central and Northern Punjab). In this dendrogram pattern, cluster I, was subdivided into three sub-clusters (IA, IB & IC) which gave genetic diversity of shisham population of these regions. The sub-cluster, IA showed two subgroups of genotypes in which 1st group was showedFP1 & CP4 (82% similar) as sister genotypes but also closely related to CP2& FP2. However, in sub cluster IA, ShP1, ShP2 and ShP4, TP3showed similarity of 89% and 90% respectively. The sub-cluster, IB showed genotypes GP1, GP4 (87% similar) and LP3, LP4 (93% similar) were sister genotypes while KP2 was out group genotype of this sub-cluster.

The genotypes collected from Mandi Bahauddin region were grouped into separate sub-cluster, IC, in which MBP2 & MBP3 were sister genotypes (96% similar) and closely related to MBP1 followed by MBP4.

Similarly, cluster II was comprised of BP1, BP2, BP3, BP4 (genotypes of Bahawalpur region); RKP1, RKP3, RKP2, RKP4 (genotypes of Rahim Yar Khan region); KWP1, KWP2, KWP3, KWP4 (genotypes of Khanewal region); MP1, MP2, MP3, MP4 (genotypes of Muzaffargarh region); BNP1, BNP2, BNP3, BNP4 (genotypes of Bahawalnagar region); CP1 (genotypes of Chichawatni region).

These results revealed that cluster II depicted pre-dominance of shisham population of Southern Punjab. This cluster was subdivided into three sub-clusters (IIA, IIB & IIC). Sub-cluster IIA depicted, shisham genotypes BP1, BP3 (80% similar), KWP1, MP3 (76% similar), MP2, RKPI (74% similar)

andBP2, KWP3 (78% similar) were sister genotypes. Similarly, BNP1 and BNP4 (82% similar) were shown as sister genotypes but closely related to BNP2 and KWP4 genotypes, while MP4 was out group genotype closely related to BP4.This showed these plants of Bahawalnagar, Khanewal and Muzaffargarh were closely related. In sub-cluster IIB, BNP3 & MP1 (78% similar) were sister genotypes but also closely related to KWP2 and CP1 genotype. Rahim Yar Khan genotypes were formed a separate sub-cluster, IIC, in which RKP3 & RKP4 (80% similar) were sister while RKP2 was close to them (72-77% similar).

Fig. 1. Healthy and diseased (dieback) shisham plantation of Punjab a) Dieback affected trees b) Healthy shisham trees.

Cluster III was comprised of CP3 (genotypes of Chichawatni region); FP3, FP4 (genotypes of Faisalabad region); OP2 (genotype of Okara region); TP1 (genotypes of Toba Tek Singh region); KP1 (genotype of Khushab region).Among these, CP3 and OP2 (68% similar) were shown as sister genotypes. Similarly, FP3 & FP4 (72% similar) were depicted as sister genotypes but were close toKP1 while TP1 genotype was root this cluster. This small Cluster III revealed pre-dominance of shisham population of Central Punjab.

Fig. 2. Macro propagated shisham germplasm at green house of Fungal Molecular Biology Laboratory (FMB Lab.), Department of Plant Pathology, University of Agriculture Faisalabad, Punjab, Pakistan.

However, Cluster IV comprised of OP1, OP4 (genotypes of Okara region), KP4 (genotypes of Khushab region), SP1 (genotype of Sialkot region).Among these OP1 & OP4were sister genotypes while closely related to KP4& SP1 genotypes. This cluster was found as highly distinct one as it root the whole dendrogram.

However, in this dendrogram OP4 showed high degree of dissimilarity with LP1 (98% dissimilar) while having 97% dissimilarity with ShP1, ShP3, ShP4, TP3, TP4, OP3 & BP3. But this genotype showed maximum similarity of 32% with SP1. Similarly, OP1 genotype showed genetic distance of 84% with BP1 while 83% dissimilarity was found with GP1 & LP3. This genotype showed maximum similarity of 33% with OP2.

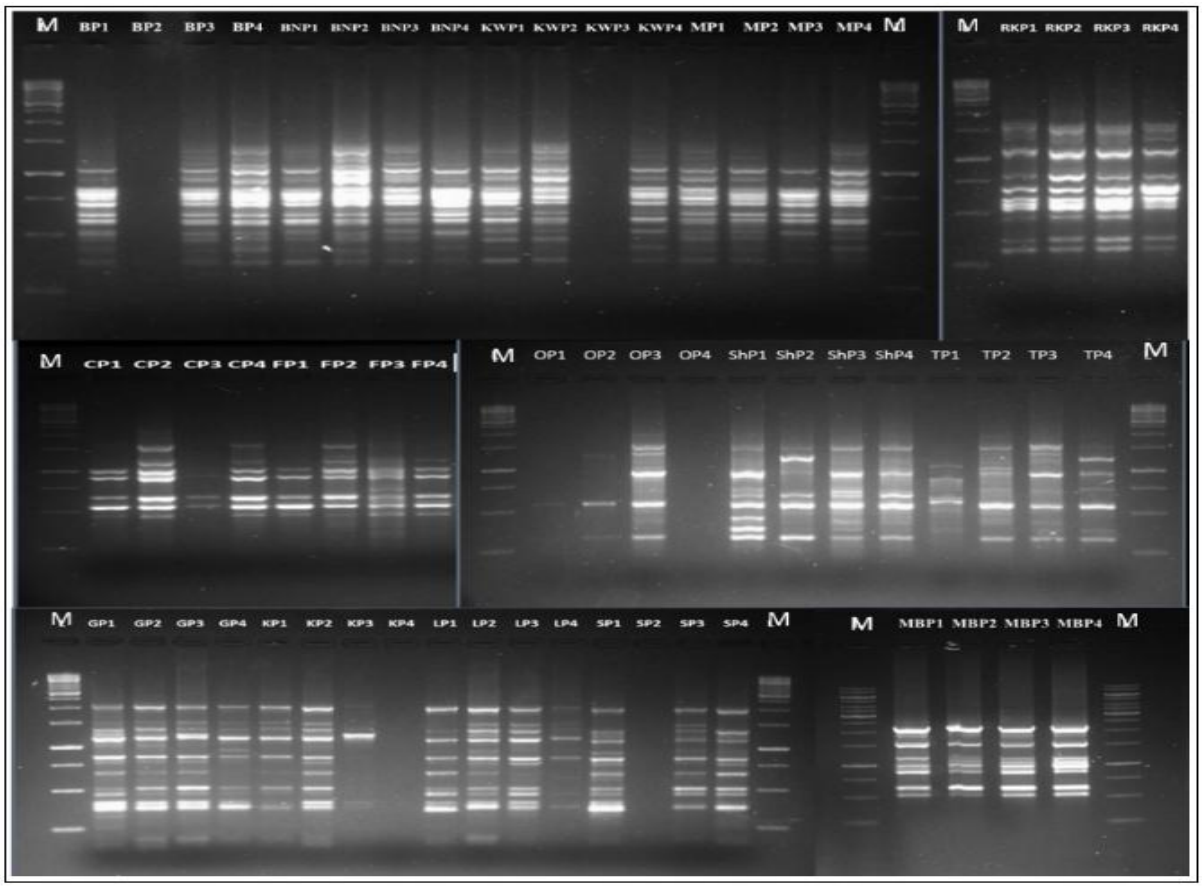

Fig. 3. PCR amplification of 60 genotypes of shisham germplasm listed in Table 1, using ISSR primers. *M=1kb DNA ladder.

Despite another dendrogram was constructed using DARwin6 program package to validate the results. In which hierarchical clustering of selected genotypes (15 × 4 = 60) by Unweighted Neighbor-Joining (NJ) method also divided them into four main clusters (Fig. 5).

This dendrogram clustering pattern supported the similar results of dendrogram generated using UPGMA method in PAST program. The variation was due to the different algorithms used for each software and of two different distance matrices. The observed estimate of total genetic variation (H_t) value was ranged from 0 (ISSR10& ISSR-18) to 0.5000 (P-49 & ISSR-6) with mean value of 0.3521 and standard deviation 0.0205. The observed number of alleles (na), effective number of alleles (ne) Nei's gene diversity (h) and Shannon's information index (I) were recorded as 1.9946, 1.6114, 0.3521 and 0.5230 on an average with standard deviation values of 0.0731, 0.3135, 0.1433 and 0.1787, respectively.

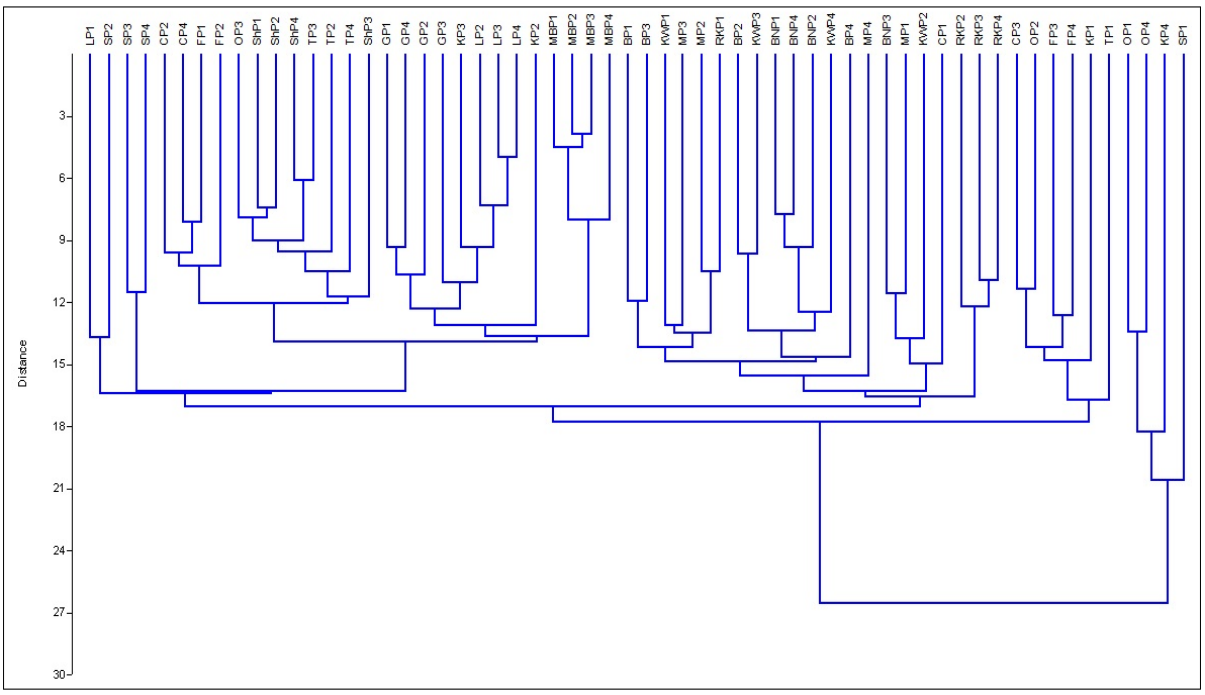

Fig. 4. Overall dendrogram based on Nei's genetic distance using unweighted pair group method arithmetic mean average (UPGMA), showing 60 Shisham samples collected across the Punjab (Pakistan).

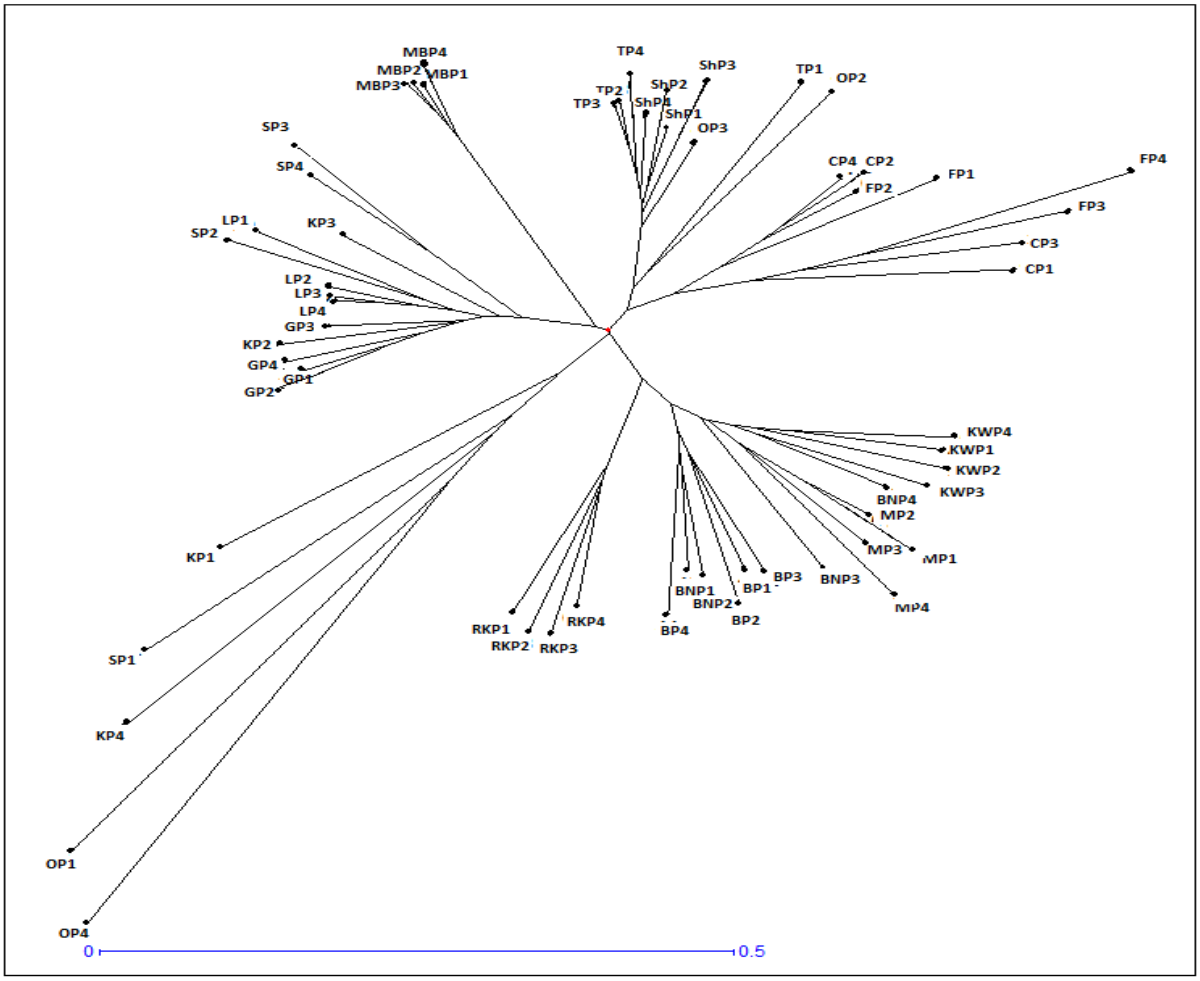

Fig. 5. A dendrogram generated by DARwin6 software showing an unweighted neighbor joining (NJ) hierarchical clustering of 60 representative shisham genotypes from Punjab, Pakistan.

The values of I and h was ranged from 0 (ISSR10 & ISSR-18) to 0.6931(P-49 & ISSR-6) and 0 (ISSR10 & ISSR-18) to 0.5000 (P-49).

Principal Coordinate Analysis (PCoA) was performed using DARwin6 software for determining spatial representation of genetic distance among genotypes of shisham population of Punjab (Pakistan) and to evaluate the consistency of genetic differentiation in population as well (Fig. 6).

The two dimensional (2-D) plot of Principal Coordinate analysis (PCoA) revealed that the first coordinate (Coord I) and second coordinate (Coord II) accounting for 16.23% and 13.83% total genetic variation, respectively.

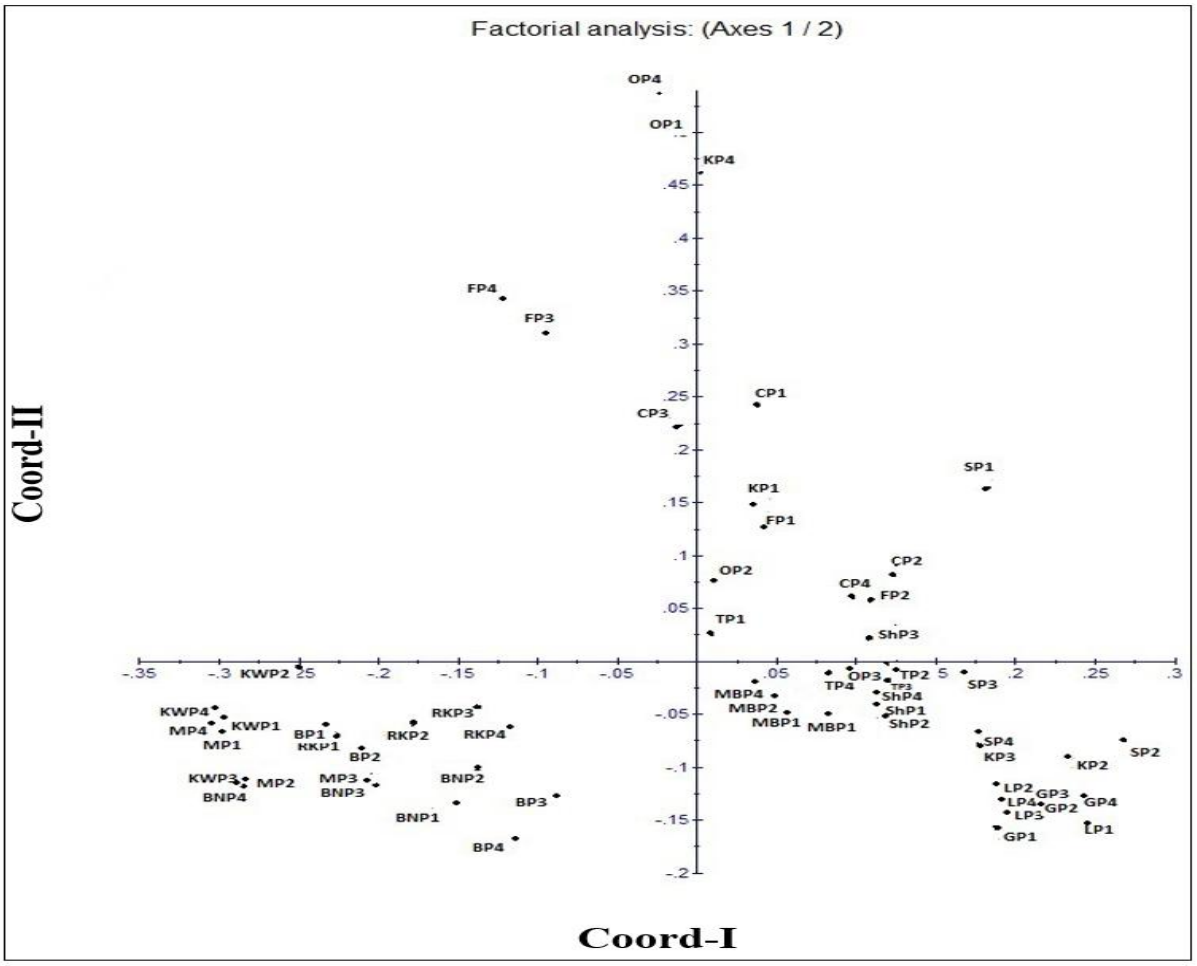

Fig. 6. Two-dimensional plot of 60 shisham genotypes of Punjab, Pakistan using Principal Coordinate analysis (PCoA).

These shisham genotypes of Punjab, Pakistan, distributed in plot were in accordance to the results of UPGMA and NJ based cluster analyses.

According to PCoA, genetic differentiation in 60 genotypes was spatially represented as group viz., Central & Northern as mixed while Southern as separate group. In which some genotypes shown to be scattered while some were more or less closely related or intermixed.

Population genetic structure

Population structure analysis based on Bayesian method using STRUCTURE software was used for analyzing and assessing the pattern of genetic structure of shisham population in Punjab, Pakistan in order to classify the genotypes into appropriate subpopulations.

The number of K was set from 2 to 5 with 10,000 burn-in period and 10,000 MCMC (Markov Chain

Monte Carlo). Identification of subpopulations were estimated by delta K values obtained through STRUCTURE HARVESTER (Table 3). Highest ΔK was found at K=3, which depicted that shisham genotypes of Punjab have three subpopulations (Fig. 7).

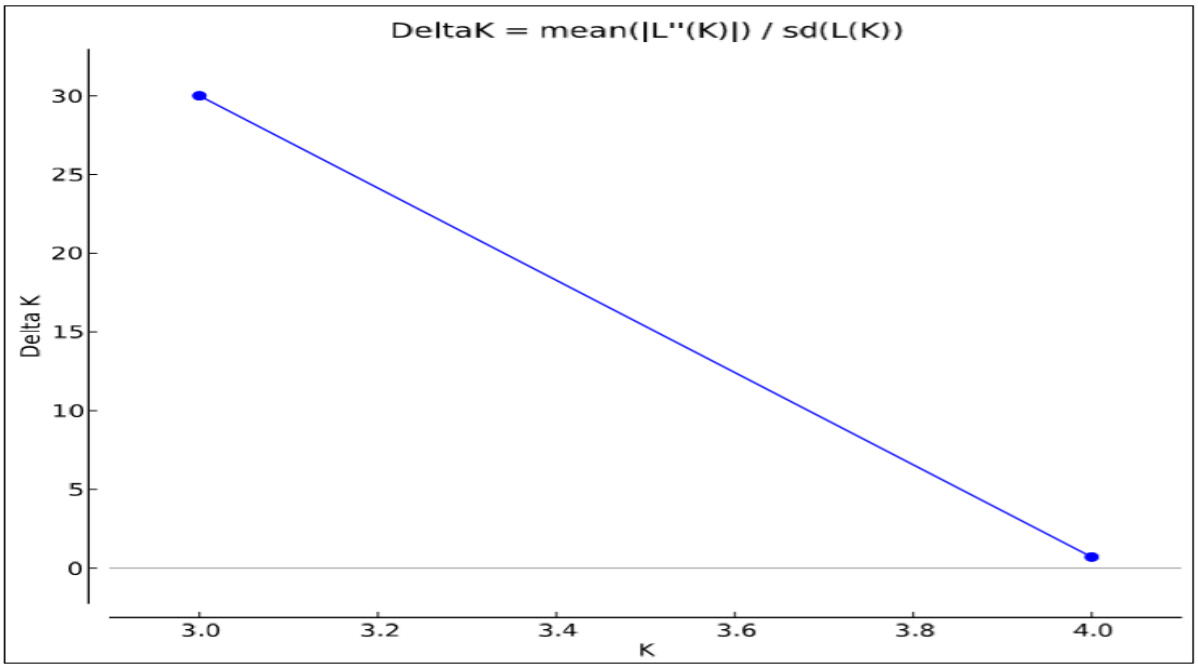

Fig. 7. Result of STRUCTUTE HARVESTER showing most likely number of K cluster as delta K was highest at K=3.

This indicated that these 60 genotypes shared three gene pools and mixed ancestry as shown in structure diagram (Fig. 8).

Discussion

Genetic distinction is imperative for any species to survive under various environments and for its conservation (Falk and Holsinger, 1991). The characterization of genetic diversity within a closely related shisham germplasm is an important tool to understand its genetic basis. ISSR molecular markers have the ability to confirm specific DNA fragment within a species and one being used in the population genetic study and detecting clonal diversity (Li and Ge, 2001). ISSR ascertains the individual through distinction present between microsatellite regions which help to assessing the level of genetic diversity in plant species. In the present study, 21 ISSR primers were used to produce 373 total bands (all were polymorphic) which unambiguously classified 60 shisham genotypes in to four major clusters on the bases of distance based grouping (UPGMA). Results indicated 83.32% polymorphism on an average and show wide range of genetic variability among different genotypes of shisham. Dendrogram clustering pattern indicates that the geographical regions, from where shisham germplasm were collected, may not be the clear index of genetic diversity evaluation and similar results were studied in azuki bean, groundnut and shisham (Yee *et el.*, 1999; Dwivedi *et al.*, 2001; Arif *et al.*, 2009; Bakshi and Sharma, 2011). Among 60 genotypes of present study, four shisham genotypes (OP1, OP4, KP4 & SP1) of cluster IV of dendrogram pattern were found to be highly distinct and could be considered as diverse member of shisham population. The similarity index indicated a maximum of 96% similarity among genotypes of Mandi Bahauddin.

Average PIC values for 21 ISSR markers were calculated as 0.2830 that were similar to the several other plant species (Saleh, 2011; Verma and Rana, 2011; Kumar *et al.,* 2011; Kalpana *et al.*, 2012; Rocha *et al.*, 2014; Araujo *et al.* 2016).

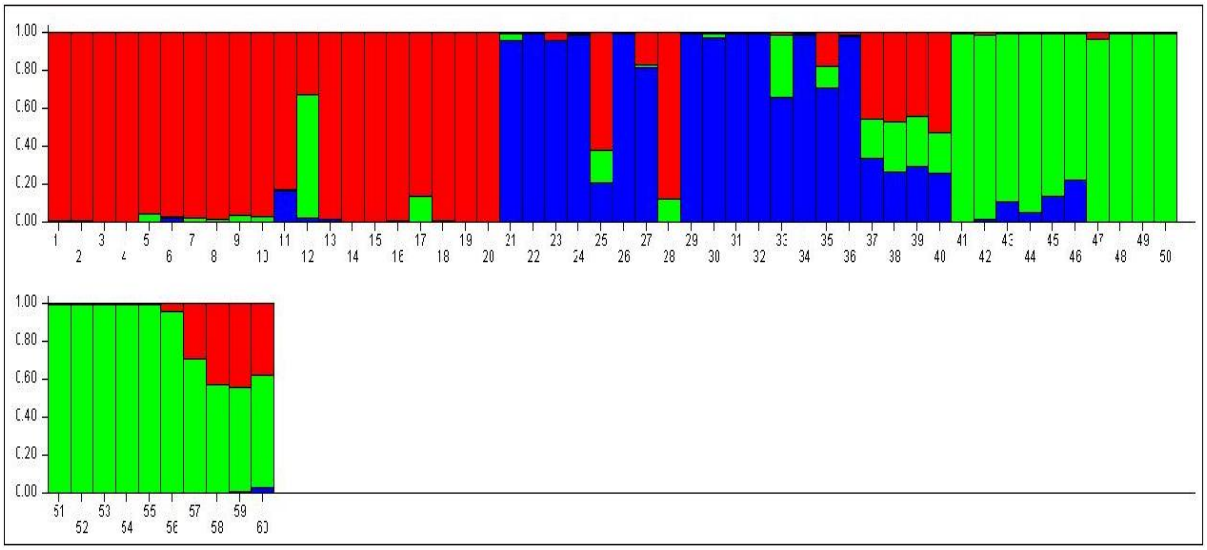

Fig. 8. Structure analysis represents the population structure for K3, coded in different colors in which each color shows the location of genotypes within Subgroups. Number on horizontal axis show the individuals belongs to shisham populations and on vertical axis show the membership coefficient to sub populations.

The two dimensional PCoA plot finding computed 16.23% and 13.83% genetic variation among 60 shisham genotypes these results were comparable to results of Nikanta *et al.*(2017) for bamboo tree. The highest delta K value was observed as K=3 obtained from STRUCTURE HARVESTER which showed 60 shisham genotypes sharing three genetic pool, similar results were studied in bamboo tree (Nikanta *et al.*, 2017). Therefore, this study proved to be an important milestone for tagging the diverse and scattered population of shisham. This study will bring key steps to find the dieback resistant genotypes of shisham.

Acknowledgement

We highly acknowledge "US-Pak Centre for Advance studies for Agriculture and Food Security (USPCAS-AFS)" for providing funds under the project #7(#116) "Resistance gene analogues based molecular identification for dieback disease resistance in Shisham (*Dalbergia sissoo)*" to conduct this research work.

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