Research Article

Genetic Diversity Within and Among Populations of *Picea smithiana* (Wall.) Boiss. from Khyber Pakhtunkhwa, Pakistan Revealed by RAPD Markers

Tanvir Hussain[1,2*], Kafeel Ahmad[2], Muhammad Bilal Zia[1], Hammad Uddin[1], Khalid Hussain[1], Muhammad Inamulla Khan[3] and Said Akhtar Khan[1]

[1]Pakistan Forest Institute, Peshawar, Khyber Pakhtunkhwa, Pakistan; [2]Centre of Biotechnology and Microbiology, University of Peshawar, Peshawar, Khyber Pakhtunkhwa, Pakistan; [3]Department of Environmental Sciences, The University of Haripur, Khyber Pakhtunkhwa, Pakistan.

Abstract | This study was carried out to explore the genetic diversity both within and across the populations of Himalayan Spruce growing in the dry temperate and moist temperate forests of Khyber Pakhtunkhwa (KP), Pakistan using random amplified polymorphic (RAPD) DNA method. Five study sites were selected and 20 samples (needles) from each site were collected. Six RAPD markers produced 92 amplicons with sizes range 250-2000bp. Among all studied populations 100% polymorphism was observed. The highest percentage of polymorphic bands (*PPB*=82.61%), observed number of alleles (n_a=1.82), effective number of alleles (N_e=1.34), Shannon index (*S*=0.33) and Nei's genetic diversity (H_e=0.21) were scored for ANP (Ayubia National Park) population whereas the lowest (*PPB*=60.87%; n_a= 1.60; N_e=1.214; *S*=0.2290; H_e=0.1413) were found in BOR (Bomborate) population. This revealed that the greatest genetic diversity existed in moist temperate forests compared to dry temperate forests. The average geneflow (*Nm*) among populations was 5.45. According to Nei's genetic distances the most genetically close (0.0131) were NAR (Naran) and BOR populations whereas the most apart (0.033) ANP and MLJ (Malam Jabba) populations while cluster analysis among the populations clearly showed two groups i.e., BOR with NAR and BAR with MLJ. The population of ANP was grouped separately with the cluster of BAR and MLJ. The AMOVA analysis revealed high genetic variation (90%) within and low (10%) among populations. Further, Mantel's test explored that geographic distribution was not correlated to genetic distances among the populations. The investigation explored that RAPD approach can detect the genetic diversity among five populations of Himalayan Spruce and high variations indicating extensive gene flow. This adaptive genetic variability has significant promise for the management and conservation of this species in the future.

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***Correspondence** | Tanvir Hussain, Pakistan Forest Institute, Peshawar, Khyber Pakhtunkhwa, Pakistan; **Email:** awt@pfi.gov.pk, thkhan75@gmail.com
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Introduction

Genetic diversity plays several roles in the field of forestry e.g., proper evaluation of genetic diversity of the tree populations is extremely important to prevent their depletion and ensure long-term conservation, planning and management (Sinha *et al.*, 2013). Additionally, it guarantees that forest trees can endure, adapt, and change in response to environmental changes (Xue *et al.*, 2007). Moreover, it is helpful in the construction of the genetic map, study molecular ecology and plant taxonomy (Sinha *et al.*, 2013).

The PCR-based technology RAPD has been demonstrated to be one of the most effective ways to get a large number of genetic markers in a variety of species (Hadrys *et al.*, 1992). Among other techniques like RFLP (Restriction Fragment Length Polymorphism) analysis, PCR (Polymerase Chain Reaction) analysis, STR (Short Tandem Repeat) analysis and AFPL (Amplified Fragment length Polymorphism) etc., it is the most frequently used method for genetic diversity of a species because it generates polymorphic DNA fragment that can be scored rapidly. Further, unlike other DNA markers, no cloning of DNA fragment, radioactivity or DNA sequencing information is required for this technique (Yermagambetova, 2023). This approach has been successfully applied to study genetic variations in many tree species such as Norway spruce (*Picea abies*) (Bucci and Menozzi, 1995), Black spruce (*Picea mariana)* (Perron *et al.*, 1995), White-engelmann spruce (*Picea gluaca*) (Khasa and Dancik, 1996), (Xue *et al.*, 2007), Black (*Picea asperata)* and red Spruce (*Picea rubens*) (Xue *et al.*, 2007; Nkongolo *et al.*, 2003), Sitka spruce (*Picea sitchensis*) (Van de Ven and McNicol, 1995), Chir pine *(Pinus roxburghii)* (Ginwal *et al.*, 2010; Sinha *et al.*, 2013), Red pine (*Pinus resinosa)* (Mosseler *et al.*, 1992), *Pinus gerardiana* (Gul *et al.*, 2021), *Taxus wallichana* (Saikia *et al.*, 2000), *Cedrus deodara* (Rai *et al.*, 2020), Eucalyptus (Keil and Griffin, 1994), *Populus* spp., (Castiglione *et al.*, 1993; Rajagopal *et al.*, 2000) , Mango (Mansour *et al.*, 2014), and many more. RAPD also offers a wide range of applications in the fields of population genetics, gene mapping, plant and animal breeding, and evolutionary genetics due to the method's simplicity and low cost (Nandani and Thakur, 2014).

Himalayyan spruce (*Picea smithiana* (Wall.) Boiss.) is an important species of coniferous biome in the mountainous areas of Pakistan. The tree is endemic to Pakistan, India, Afghanistan, Nepal, and Tibet's Himalayas. In Pakistan, it is fairly common at a high elevation from 2500-3300 m in the dry temperate and moist temperate forests of Azad Kashmir, Murree Hills, Hazara, Swat, Dir, Chitral and Kurram agency (Sheikh, 1993; Schmid, 1997; Ahmed and Naqvi, 2005). It provides important economic and ecological services as its wood is used in constructional work, railway sleeper, packing cases and in pulp and mulch (Siddiqui *et al.*, 1996; Khan and Khatoon, 2007). This species has been declared as endangered or vulnerable in view of climate change and anthropogenic activities in the region (Shah *et al.*, 2008).

The aim of this study was to investigate the genetic diversity of Himalayan Spruce growing in the dry and moist temperate forest of Khyber Pakhtunkhwa, Pakistan to initiate conservation and management efforts for this species.

Materials and Methods

Study sites and samples collection

For the execution of research work, sampling of fresh needles was carried out from five sites i.e., Ayubia National Park (ANP), Malam Jabba (MLJ), Barkan (BAR), Bomborate (BOR), and Naran (NAR) of Khyber Pakhtunkhwa province, Pakistan (Table 1 and Figure 1). Twenty (20) trees were selected in each study site by following the simple random sampling method. Before use for DNA extraction, the needle samples were kept at -20°C.

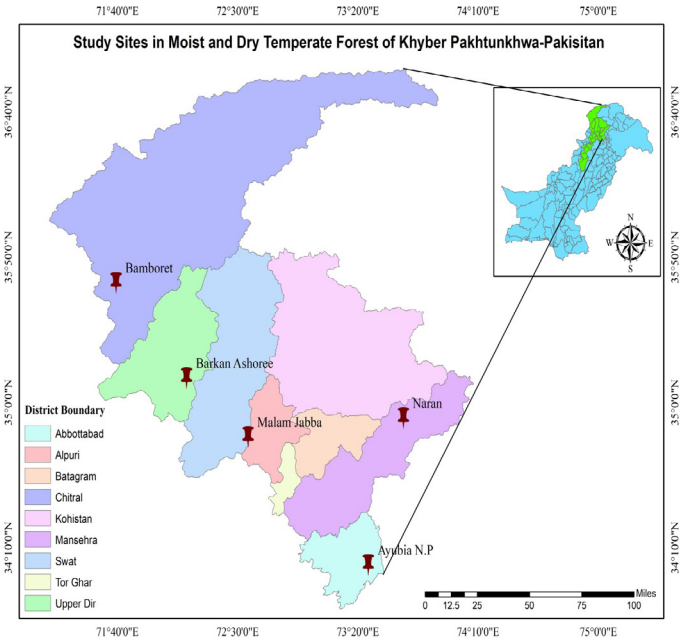

Figure 1: *Map Showing study sites in Khyber Pakhtunkhwa.*

Table 1: *Study sites of Khyber Pakhtunkhwa, Pakistan.*

Forest type	Populations	Abbreviation	Sample size	Co-ordinates	
				Latitude (N)	Longitude (E)
Moist temperate forests	Ayubia Nation Park	ANP	20	34.0409 N	73.322550 E
	Malam Jabba	MLJ	20	34.783213889N	72.572647222E
	Barkan	BAR	20	35.265986111N	71.908905556E
Dry temperate forests	Naran	NAR	20	34.899816667N	73.652294444E
	Bomborate	BOR	20	35.684808333N	71.654088889E

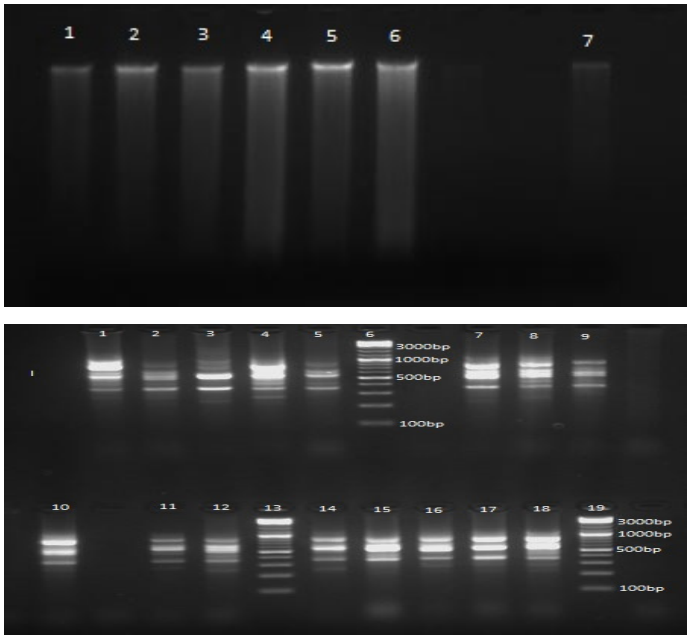

Figure 2: *(a) Total genomic DNA extracted (b) RAPD profile with primer SC10-09 of Picea smithiana collected from Naran. Lanes 6, 13 and19=100bp DNA ladder.*

Extraction and detection of DNA

The CTAB-method was used to extract genomic DNA from leaf samples (Bousquet *et al.*, 1990). During the sample preparation step, 100 mg of fresh needles of Himalayan Spruce were taken, cut into small pieces with sterilized scissors, and then put into the mortar for grinding with a pestle. Before grinding, 1000 µl extraction buffer (2% CTAB, 1.4 M NaCl,100 mM Tris-HCL,20 mM EDTA, 1% polyvinylpyrrolidone (PVP), 0.2% mercaptoethanol, pH 9.5) was added. The sample material was well-grounded. Fine extract (0.75 ml) was transferred into a 1.5 ml tube. The homogenate was incubated at 65 °C for 40 minutes in a hot water bath. After incubation, purification of the extract was done with an equal volume of Chloroform followed by centrifugation at 1500 rpm for 15 minutes. The aqueous phase was transferred into new tubes, and the DNA was precipitated by gently adding 1 volume of cool isopropanol and 1 volume of 3M sodium acetate into the aqueous phase. The mixture was centrifuged at 15000 rpm for 15 minutes. To remove more impurities, the pellet of DNA was washed with 70% ethanol twice followed by centrifuging at 15000 rpm for 1 minute and then the DNA pellet was air-dried for 15 minutes. The DNA pellet was re-suspended in 200 µl low TE buffer. It is then stored at -20 °C for future use to check the quality of DNA through gel electrolysis and RAPD-PCR analysis. The quantification of genomic DNA was performed using 1% agarose gel and visualize under UV light using gel documentation system (SYNGEN, Serial #DYDR/2138) (Figure 2a).

DNA amplification

Six 10-mer RAPD primers (OPA-08, OPA-09, OPA-18 and OPA-19, SC10-02 and SC10-09) were commercially synthesized (Macrogen, Humanizing Genomics, Country name) as reported previously (Van de Ven and McNicol, 1995). Table 2 shows the sequences of primers used in this study and table 3 represents the optimized conditions for PCR amplification. For PCR amplification, 20 µl reaction mixture was taken which consisted of 1µl genomic DNA template, 4 µl of 5x master mix (Solis BioDyne, Estonia; Cat. N0.04-12-00125, 12.5 mM $MgCl_2$ 5X FIREPol Master Mix Ready to Load), 1µl primer, and 14 µl of molecular grade water (Sigma-Aldrich, USA). The amplified products were subjected to gel electrophoresis using agarose gel (1.5%) and 100 bp DNA ladder (Thermo Scientific O, GeneRuler; Lot. No.00445751) as size marker. The amplified bands from each sample were scored and recorded.

Table 2: *Details of primers used for genomic DNA amplification.*

S. No	Primer names	Sequence 5' to 3'
1	OPA-08	GTGACGTAGG
2	OPA-09	GGGTAACGCG
3	OPA-18	AGGTGACCGT
4	OPA-19	CAAACGTCGG
5	SC10-02	GGTCCTCAGG
6	SC10-09	CAGTTCTGGC

Statistical analysis

PCR amplified products for all the six primers were scored for all populations. An allele's existence in a certain genotype was given a score of 1, while the band's absence was given a value of 0. Only bands between 250 bp and 2000 bp that were distinct, unambiguous, consistent, and reproducible were taken into account when grading the binary data. The bi-variate data obtained from RAPD analysis was tabulated for each primer and each population. Bivariate data was analyzed with POPGENE version 1.32 software (Yeh *et al*., 1999) to determine the number of polymorphic loci, observed number of alleles (n_a), effective number of alleles (N_e)(Nei, 1972) percentage of polymorphic loci and (Nei, 1972) (Kimura and Crow, 1964) genetic identity and genetic distance matrices. Further, utilizing the same software, three more genetic diversity indices i.e., (i) Shannon-wienen index (S) (Lewontin, 1972) (ii) genetic differentiation between populations (G_{st}) (McDermott and McDonald, 1993) and (iii) estimated gene flow (N_m) were generated. In order to find correlation between physical distance and genetic distance, Mantel, s test was conducted using GenAlEx 6.5 software (Peakall and Smouse, 2006). Using mega X software, a phylogenetic tree was created based on Nei's genetic distance matrix using the unweighted pair-group method with arithmetic average (UPGMA) (Sneath and Sokal, 1973). An AMOVA (analysis of molecular variance) test was applied to assess the percentage of variation within and among the populations using GeneAlEx 6.5.

Table 3: *PCR conditions for determination of genetic diversity.*

Primer names	Polymerase chain reaction (PCR) conditions	
OPA-08	Initial denaturation	95 °C for 5 minutes
OPA-09	Denaturation	92 °C for 1 minutes
OPA-18	Annealing	35 °C for 1.5 minutes
OPA-19	Extension	72 °C for 2 minutes
SC10-02	Total cycle	45
SC10-09	Final extension	72 °C for 5 minutes

Results and Discussion

RAPD analysis

All the six primers gave reproducible banding patterns with the help of which five populations of *Picea smithiana* were efficiently distinguished. The number of amplicons produced by these primers among five populations ranged from 14 to 18 with an average of 15.3 bands per primer and the range of amplification products sizes were from 250 bp to 2000 bp (Figure 2 and Table 4). The contribution of all the primers and their percentage of polymorphic loci are given in Table 4. All primers showed 100% polymorphism.

Table 4: *Attributes of molecular markers used for RAPD analysis of 100 individuals of Himalayan spruce sampled from five populations.*

S. No	Primer names	No. of amplified bands	No. of polymorphic bands	%Age of polymorphism	Approximate band size (bp)
1	OPA-08	18	18	100	250-2000
2	OPA-09	14	14	100	250-950
3	OPA-18	16	16	100	250-1000
4	OPA-19	15	15	100	300-1000
5	SC10-02	14	14	100	250-1100
6	SC10-09	15	15	100	250-1000
Total		92	92	100	

Genetic diversity within and among populations

The ANP population scored the highest levels of N_a (1.82) and N_e (1.43), while the BOR population captured the lowest levels (1.60 and 1.214, respectively). Similarly, among five populations, the highest level of variability was seen in ANP (*PPB*=82.61%; *Ne*=1.8261; *He*=0.2142 and *S*= 0.3354) while lowest variability was found in BOR (*PPB*=60.87%; *Ne*= 1.2143; *He*=0.1413 and *S*=0.2290). The effective number of alleles per locus at population level and species level were 1.296 and 1.280, respectively. The mean percentage of polymorphic bands (*PPB*) at population level was 72.40% ranging from 60.87 (BOR) to 82.61% (ANP) (Table 5). Under the hardy-Weinberg equilibrium assumption, the average genetic diversity was calculated to be 0.177 (He) among populations and (Ht) 0.194 at the species level (Table 5). The Shannon index (S) value for populations and at species level was estimated as 0.2827 and 0.3177 accordingly.

Nei's (1972) genetic distances (Table 6) among studied Himalayan spruce populations was calculated and found varied from 0.008 to 0.0272. The populations of ANP and NAR were found the most genetically closest whereas the most distant were ANP and NLJ. The UPGMA cluster algorithm based on Nei's genetic distances (Figure 3) assembled the five populationof Himalayan Spruce into two major clusters i.e. Cluster "A" and cluster "B". The cluster "A" was

Table 5: *Genetic diversity within populations of Himalayan spruce captured by RAPD analysis.*

Populations	n	$N_a \pm SD$	$N_e \pm SD$	$H_e \pm SD$	S± SD	PPB (%)	G_{st}	Nm	F_{st}
ANP	20	1.8261(0.38110	1.3491(0.3440)	0.2142(0.1792)	0.3354(0.2461)	82.61			
MLJ	20	1.6522(0.4789)	1.2656(0.3196)	0.1669(0.1747)	0.2639(0.2503)	65.22			
BAR	20	1.7500(0.4354)	1.3015(0.3269)	0.1886(0.1759)	0.2981(0.2470)	75			
NAR	20	1.7826(0.4147)	1.2726(0.2957)	0.1775(0.1623)	0.2875(0.2294)	78.26			
BOR	20	1.6087(0.4907)	1.2143(0.2760)	0.1413(0.1589)	0.2290(0.2340)	60.87			
Mean	20	1.7239(0.4401)	1.2806(0.3124)	0.1777(0.1702)	0.2827(0.2413)	72.40			
Average species level		2.000(0.0000)	1.296 (0.2947)	0.1940(0.1558)	0.3177(0.2115)	100	0.0840	5.4509	0.102

N= population size; N_a= observed number of alleles; Ne= effective number of alleles per locus; He= Nei genetic diversity; S= Shannon index; PPB= percentage of polymorphic bands; G_{st}= genetic differentiation between populations estimated using POPGENE 1.32; Nm= estimated gene flow; F_{st}= genetic differentiation between population estimated by using AMOVA; standard deviation shown in parentheses.

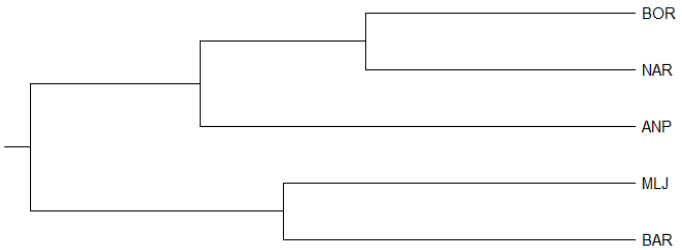

Figure 3: *UPGMA tree using Nei's genetic distances among the populations of Picea smithiana.*

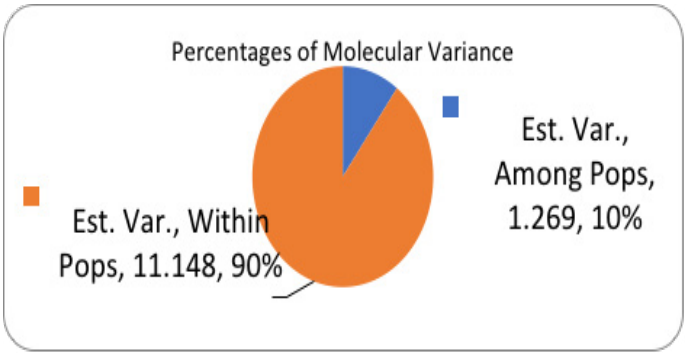

Figure 4: *Analysis of molecular variance (AMOVA).*

further branched into two subgroups of populations "a" and "b". In cluster "A", the populations from BOR and NAR were found to be identical to each other with similarity coefficient value of 0.97 while the population of *Picea smithiana* from ANP was reasonably found different from other two populations with highest genetic distance. In cluster "B" the population of Himalayan Spruce from MLJ and BAR were bunched together as both possessed identical coefficient value of 0.96. According to the Mantel, s test results (Table 6 and Figure 5; Mantel, s test r=-0.016; p=0.56), the genetic divergence of populations was not substantially connected with geographic distances. To quantify genetic differentiation of populations (G_{st}) and AMOVA were calculated. The results showed that the population differentiation amount 0.084 (Table 5) that meant most of the total genetic variation (90.35%) was within the population while 9.65% of the genetic variations was because of among population differences. Same results reflected by AMOVA (Table 7 and Figure 4). The average number of migrants per generation (*Nm*) among the population was 5.45.

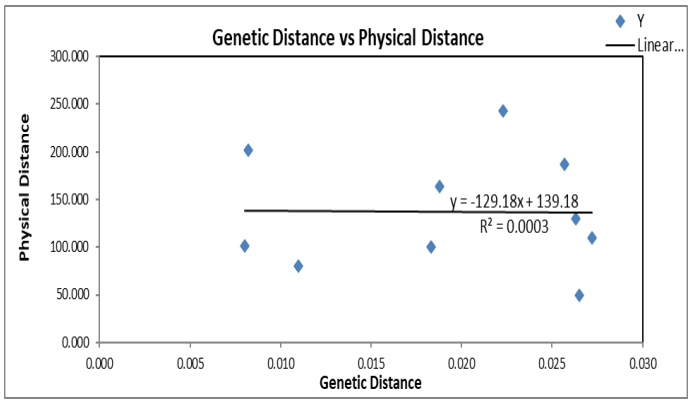

Figure 5: *Resultts of mantels test for relationship between genetic distances and physical distances.*

Table 6: *Nei's (1972) genetic identity (above diagonal) and genetic distance (below diagonal among populations of Himalayan Spruce. The values in parentheses represent Geographic distance in Km.*

Pop ID	ANP	MLJ	BAR	NAR	BOR
ANP	****	0.9672 (110)	0.9684(187)	0.9858 (101)	0.9724(243)
MLJ	0.0333	****	0.9831 (80)	0.9766 (100)	0.9695(130)
BAR	0.0321	0.0171	****	0.9758 (164)	0.9689(50)
NAR	0.0143	0.0237	0.0245	****	0.9870(202)
BOR	0.0280	0.0310	0.0316	0.0131	****

The selection of RAPD markers to study genetic diversity among tree species is being favored over conventional morphological and biochemical markers because these are devoid of any disturbance from

environmental effects and growth stages, which make them highly reliable (Doyle, 1990). This technique has been successfully applied to study genetic variations in many coniferous and broad-leaved tree species. RAPD-PCR analysis with six primers scored a larger number (92) of amplification products. This high number of PCR products might be due to the larger genomic size of this species. These results followed the findings of (Bucci and Menozzi, 1993; Van de Ven and McNicol, 1995) who discovered a larger genomic size and number of PCR products in Norway Spruce (*Picea abies*) and Sitka Spruce (*Picea sitchensis*), respectively. According to Pancoro *et al.* (2016), the more bands a locus has, the more information about polymorphism it provides. Furthermore, polymorphism is a reflection of amplification caused by various DNA changes (Syafaruddin and Nasution, 2012).

Table 7: *Analysis of molecular variance (AMOVA) within and among Himalayan Spruce populations using six RAPD markers.*

Source of variation	df	SS	MS	Est. Var.	%
Among populations	4	146.140	36.535	1.269	10
Within populations	95	1059.050	11.148	11.148	90

df= degree of freedom; SS= sum of square; MS= mean square; Est. Var.= estimation of variance.

The RAPD analysis of 100 individuals within the five populations of Himalayan Spruce revealed a high genetic polymorphism (100%) which reflected a high level of genetic diversity within this species (Annisa *et al.*, 2019). It is commonly accepted that conifers are genetically most variable group of tree species and being long-lived, present a high level of adaptation to different types of environmental conditions (Hamarick *et al.*, 1981). The majority of conifer research have revealed modest genetic difference among populations and substantial levels of genetic diversity within populations (Kim *et al.*, 1994; Müller-Starck, 1995; Agundez *et al.*, 1997; Sharma *et al.*, 2002; Renau-Morata *et al.*, 2005; Fu *et al.*, 2019; Mei *et al.*, 2021). We have also reported the same results in case of Himalayan Spruce (G_{st} = 0.084 and AMOVA= 90% genetic diversity within population and 10% among the populations). According to Hamrick *et al.* (1992), Geographical distribution and a species' evolutionary history, largely explain the genetic difference within and among populations and Bakshi and Konnert (2011) reported that species with bigger and more evenly spaced populations are likely to have more variation within populations and less between populations. The Himalayan Spruce found to follow this trend and hence our results are in accordance with previous studies on conifers. Another important factor that causes genetic variation within and among populations is the mating system. According to reports, wind pollinated tree species exhibit low differentiation between populations due to extensive movement of pollens miles away from the source, high levels of outcrossing, and extensive gene flow by pollen and seed dispersal (Hamrick *et al.*, 1992; Collignon and Favre, 2000). The high value of gene flow (Nm= 5.45) reported in this study also follows this rule and supports the previous results in *Picea* family (Areškevičienė *et al.*, 2005). The high genetic diversity of this species also indicated that Himalayan Spruce is well adopting and combating the changing environmental conditions of the surroundings and also showing a good range of resistance to drought, emerging diseases and epidemics etc. (Xue *et al.*, 2007).

The phylogenetic tree constructed for studying genetic diversity and relationship among the five populations showed that, the climatic conditions and close vicinity played vital role in the distribution and genetic diversity of this species (Ginwal *et al.*, 2010). The populations of BOR and NAR belong to dry temperate forest areas, therefore, these populations clustered together, similarly, the populations of BAR and MLJ grouped together as they grew under the moist temperate conditions. The population of ANP belongs to the moist temperate region but it grouped separately with the cluster of NAR and BOR due to close geographic distance and same elevation (Ginwal *et al.*, 2010). Similar findings were reported by Gul *et al.* (2021) while studying the genetic diversity of *Pinus gerardiana* along the environmental gradient from Zhob, Quetta, Pakistan and by Sinha *et al.* (2013) who investigated the genetic diversity of *Pinus roxburghii* Sarg., which was gathered from various Indian Himalayan locations.

Conclusions and Recommendations

We came to the conclusion that RAPD markers worked well and effectively to identify genetic diversity within and across populations of Himalayan Spruce growing in various climatic conditions in Khyber Pakhtunkhwa, Pakistan. The use of different indices indicated high levels of genetic diversity, which reflected considerable gene flow as a result of

long-distance pollen dissemination. The populations of Himalayan Spruce were grouped into two clusters according to their geographic affiliations. These genetic characteristics provided important mile stone for the conservation and long-term expansion of this species' genetic resources. Further, the results of this study also facilitated research on the genetic diversity of Himalayan Spruce including the samples from entire range of India, Afghanistan, Nepal, and Tibet for understanding the complete genetic makeup of this species.

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Novelty Statement

This work, to the best of our knowledge, is the first attempt to quantify genetic diversity using molecular markers for the populations of Himalayan Spruce growing in different climatic conditions of the region.

Author's Contribution

Tanvir Hussain: This is a part of his PhD, planned, executed and drafted the manuscript.
Dr. Kafeel Ahmad: Supervised the PhD research, input valuable comments, corrected manuscript.
Mr. Muhammad Bilal Zia: Helped in data analysis using statistical software.
Mr. Hammad Uddin: Helped in manuscript drafting and writing.
Mr. Khalid Hussain Sulangi: Supported in data collection, compilation.
Mr. Said Akhtar Khan: Supported in sample preparation and processing in lab.
Mr. M.Inamullah Khan: Assisted in field data, and tree cores sample collection.

Conflict of interest

The authors have declared no conflict of interest.

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