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Pattern and magnitude of genetic diversity in *Pinus gerardiana* wall. Populations

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Abstract
Twelve populations of *Pinus gerardiana* in North- west Himalayas of India were analysed for isozyme variation based on the seven enzyme systems. In isozyme analysis, of the 16 gene loci identified for the assayed seven enzyme, six loci under six enzyme systems were polymorphic. Only one enzyme system i.e. GDH does not show any polymorphic loci. The mean number of alleles per locus was 1.48 and mean expected heterozygosity was 15%. The gene pool allelic differentiation among the populations varied from 38.2% to 45. 2% whereas gene pool genotypic differentiation varied from 52% to 60%. For all possible pairs of populations Nei's genetic distance values averaged 0.0021.The value of differentiation observed in the study could be attributed to the adaptive mechanism to the microenvironment shows high level of polymorphism.

Keywords: Isozymes, gene pool, heterozygosity, populations, polymorphism

Introduction
Pinus gerardiana Wall. (Chilgoza) is most important species among all the pine species found in N-W Himalaya, mainly confined to arid regions where rainfall is less than 500mm annually between an altitude of 1600m to 3000m above mean sea level. Forests in this area are grouped under Himalayan Dry Temperate Forests (Group 13) of the Forest Types of India (Champion and Seth, 1968) [2]. This pine species occurs as pure and mixed forests in dry temperate zone of Himachal Pradesh in Kinnaur (inner Sutlej), Pangi (inner Chenab) and Ravi valleys.
The isozyme analysis fulfill the aims of the geneticists to detect genetic differences as close as possible to the DNA level in the sense that differences in the electrophoretic mobility of isozymes are, in the simplest case, direct reflections of differences among the coding genes (Bergmann, 1991) [1]. In isozyme analysis, the estimates of genetic variation are usually quantified in terms of number of polymorphic loci per species, the effective number of alleles per locus and the number of heterozygotes loci per individual (Hamrick, 1983) [6]. Electrophoresis techniques have come to be used routinely in the study of variations in enzyme systems, and they have been instrumental in determining the origin of populations of unknown ancestry. The isozyme variation expressed as the differences found in the allelic frequencies, is used to characterize the different populations.
Chilgoza is an important social forestry species yielding highly valuable edible nuts, which fetches high price ranging from Rs. 750- 900/kg in the open market. The larger proportion of nut production (180 tonnes per year) comes from Kinnaur alone and remaining requirement of this nut is met through import from Afghanistan (Karwaskara, 1981) [7]. Because of its high price and demand, each and every cone is collected (about 95%), leaving very little for natural seeding. Nevertheless, chilgoza is now categorized as an endangered conifer of India and listed so in the Red Data Book of IUCN (Sehgal and Sharma, 1989) [8]. There is thus, an urgent need to standardize best harvesting date and size of tree for getting quality stock for large scale planting in the species.

Material and Methods
Fourteen geographically distinct populations formed the study material for the present investigation (Table 1). Open pollinated seeds were harvested from 10 trees from each population and kept separately maintaining their identity for isozyme analysis. Ten each of megagametophyte and embryo samples were essayed for 7 enzyme systems.

Horizontal starch gel electrophoresis

Methods of Shaw and Prasad (1970) [9], Conkle *et al.* (1982) [4] and Cheliak and Pitel (1984) [3] were slightly modified for isozyme analysis. Megagametophyte tissues and embryos were isolated separately from the seeds and homogenized in 0.1 M Tris HCL buffer (pH 7.5) containing Tris- 1.211 g; Ascorbic acid- 0.106 g; Saccharose- 17.165 g; Tween-10- 10ml; PVP- 8.0 g; NAD- 0.029 g; Bovine albumin- 0.1 g; Dirhiothreitol- 0.015 g; EDTA- 0.015 g and Tergitol- 1.0 ml. Immediately prior to use 1 per cent (v/v) mercaptoethanol was added. Homogenates were subjected to horizontal starch gel electrophoresis with composition of starch (15%), saccharose and gel buffer. All the isozyme systems were analysed using Tris- Citrate System.

Table 1: Details of various populations of *Pinus gerardiana.*

Population code *	Location	Altitude(m)	Latitude	Longitude
Populations located at Chamba				
1	Pangi (Chamba)	2710	31^{0} 58’ N	78 ^{0}27’ E
2	Bharmour (Chamba)	2889	32^{0} 44’ N	76 ^{0}53’ E
Populations located at Kinnaur				
3	Skibba	2670	31^{0} 83’ N	77^{0} 16’ E
4	Tangling	2200	31^{0} 52’ N	78^{0} 29’ E
5	Morang	2438	31^{0} 60’ N	78^{0} 44’ E
6	Dubling	2981	31^{0} 74’ N	78^{0} 63’ E
7	Kannam	2670	31^{0} 67’ N	78^{0} 45’ E
8	Purbani	2821	31^{0} 83’ N	77^{0} 16’ E
9	Nesang	2675	31 ^{0} 64’ N	78^{0} 52’ E
10	Rarang	2591	31^{0} 60’ N	78^{0} 35’ E
11	Thangi	2550	31^{0} 55’ N	78^{0} 48’ E
12	Pangi	2556	31^{0} 53’ N	78^{0} 25’ E
13	Akpa	2662	31^{0} 58’ N	78^{0} 38’ E
14	Jangi	2556	31^{0} 60’ N	78^{0} 42’ E

*The code for each population corresponds to the codes in tables.

Statistical analysis

The data generated through isozyme analysis were analysed with the help of computer software programme GSED (Gillet, 2010) [5] and POPGENE (Yeh *et al*., 1999) [10]. The measures of genetic diversity included percentage of polymorphic loci, average number of alleles per locus, average number of alleles per polymorphic locus, allele and genotype frequencies, heterozygosity, allelic diversity and differentiation and genetic distance. Tests of homogeneity (G- test and chi square) were used for calculating allele and genotype frequencies.

Results

Isozyme analysis: A total of 7 enzyme systems using Tris – citrate buffer system were analysed for populations of *Pinus gerardiana* using mega gametophytes and embryos separately. Therefore, based on the above presented observations it can be said that for 7 enzyme systems 16 gene loci were identified. Of these 16 gene loci, 6 loci (MDH- A, 6PGDH- A, SKDH-A, IDH-B, MNR- B, ADH- A) were polymorphic (Table 2), whereas 10 isozyme gene loci (MDH-B, MDH- C, MDH-D, GDH- A, 6PGDH-B, SKDH-B, IDH-A, MNR-A, MNR-C, ADH- B) showed no variation.

Table 2: Enzymes, gene loci and no. of alleles per locus

Enzymes	Gene loci	No. of alleles per locus
Malate dehydrogenase E.C.1.1.1.37	MDH- A	2
	MDH- B	1
	MDH- C	1
	MDH- D	1
Glutamate dehydrogenase E.C.1.4.1.2	GDH- A	1
Shikimic acid dehydrogenase E.C.1.1.1.25	SKDH- A	2
	SKDH- B	1
6-Phosphogluconate dehydrogenase E.C.1.1.1.41	6PGDH- A	4
	6PGDH- B	1
Menadione reductase E.C.1.6.99.2	MNR- A	1
	MNR- B	2
	MNR-C	1
Isocitrate dehydrogenase E.C.1.1.1.42	IDH- A	1
	IDH- B	2
Alcohol dehdrogenase E. C. 1.1.1	ADH- A	2
	ADH- B	1

Genetic diversity parameters

The average number of alleles per locus showed a maximum value of 1.500 associated with ten populations (1, 2, 3, 4, 5, 6, 8, 9, 11, 12) whereas lowest value 1.438 was found in four populations (7, 10, 13 and 14). The mean values of average and effective number of alleles per locus for all the populations were found to be 1.482 and 1.281 respectively. The mean observed and expected heterozygosities ranged from 0.090 to 0.109 and 0.143 to 0.170 respectively.

Table 3: Values of genetic parameters per population

Populations	N_a	N_e	H_o	H_e
1	1.500	1.277	0.101	0.153
2	1.500	1.304	0.096	0.154
3	1.500	1.322	0.107	0.170
4	1.500	1.273	0.098	0.147
5	1.500	1.307	0.103	0.164
6	1.500	1.257	0.097	0.143
7	1.438	1.268	0.104	0.150
8	1.500	1.286	0.100	0.150
9	1.500	1.275	0.093	0.151
10	1.438	1.272	0.107	0.149
11	1.500	1.295	0.109	0.156
12	1.500	1.260	0.090	0.145
13	1.438	1.260	0.095	0.146
14	1.438	1.278	0.106	0.147
Mean	1.482	1.281	0.100	0.151

Note: N_a – average number of alleles, N_e- Effective number of alleles, H_o- Observed heterozygosity, H_e- expected heterozygosity

The maximum observed and expected heterozygosities (0.109 and 0.170) were recorded for population 11 and 3 respectively whereas minimum observed and expected heterozygosities (0.090 and 0.143) were found in populations 12 and 6 respectively.

Table 4: Allelic differentiation (values in parenthesis) and diversity in 14 populations

Gene loci	Populations														Mean (δ)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
MDH-A	2.027 (0.508)	1.997 (0.501)	1.997 (0.501)	1.978 (0.496)	1.902 (0.476)	1.933 (0.484)	1.983 (0.497)	1.994 (0.500)	1.989 (0.499)	1.989 (0.499)	1.962 (0.492)	1.997 (0.501)	1.973 (0.495)	1.998 (0.501)	1.979 (0.496)
6PGDH- A	2.246 (0.557)	2.787 (0.643)	2.331 (0.573)	2.406 (0.586)	2.503 (0.603)	2.227 (0.553)	2.164 (0.540)	2.465 (0.596)	2.368 (0.580)	2.259 (0.559)	2.579 (0.614)	2.236 (0.555)	2.240 (0.555)	2.419 (0.589)	2.374 (0.579)
SKDH- A	1.471 (0.321)	1.436 (0.305)	1.777 (0.439)	1.342 (0.256)	1.609 (0.380)	1.376 (0.274)	1.591 (0.373)	1.292 (0.227)	1.351 (0.261)	1.479 (0.325)	1.708 (0.416)	1.301 (0.232)	1.334 (0.251)	1.566 (0.363)	1.474 (0.316)
IDH- B	1.326 (0.246)	1.301 (0.232)	1.523 (0.344)	1.196 (0.164)	1.591 (0.373)	1.196 (0.164)	1.243 (0.196)	1.252 (0.202)	1.540 (0.352)	1.212 (0.175)	1.309 (0.237)	1.317 (0.242)	1.326 (0.246)	1.076 (0.071)	1.315 (0.232)
MNR- B	1.600 (0.376)	1.902 (0.476)	1.937 (0.485)	1.778 (0.439)	1.763 (0.434)	1.763 (0.434)	1.617 (0.383)	1.800 (0.446)	1.732 (0.424)	1.700 (0.413)	1.651 (0.396)	1.642 (0.392)	1.642 (0.392)	1.748 (0.429)	1.734 (0.423)
ADH- A	1.835 (0.457)	1.445 (0.309)	1.583 (0.369)	1.659 (0.399)	1.531 (0.348)	1.617 (0.383)	1.684 (0.407)	1.778 (0.439)	1.419 (0.296)	1.708 (0.416)	1.514 (0.341)	1.659 (0.399)	1.659 (0.399)	1.642 (0.392)	1.624 (0.382)
Gene Pool	1.751 (0.411)	1.811 (0.411)	1.858 (0.452)	1.727 (0.390)	1.817 (0.436)	1.685 (0.382)	1.714 (0.399)	1.764 (0.402)	1.733 (0.402)	1.725 (0.398)	1.787 (0.416)	1.692 (0.387)	1.696 (0.390)	1.742 (0.391)	1.750 (0.405)

Table 5: Genotypic differentiation (values in parenthesis) and diversity in 14 populations

Gene loci	Populations														Mean (δ)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
MDH-A	3.071 (0.679)	2.990 (0.670)	2.983 (0.669)	2.947 (0.665)	2.785 (0.645)	2.832 (0.651)	2.959 (0.666)	2.978 (0.669)	2.961 (0.667)	2.971 (0.668)	2.900 (0.660)	2.983 (0.669)	2.918 (0.662)	2.980 (0.669)	2.947 (0.665)
6PGDH- A	3.389 (0.710)	4.655 (0.790)	3.755 (0.739)	3.875 (0.747)	4.083 (0.760)	3.530 (0.722)	3.485 (0.718)	3.939 (0.751)	3.817 (0.743)	3.626 (0.729)	4.369 (0.776)	3.460 (0.716)	3.612 (0.728)	4.011 (0.756)	3.829 (0.742)
SKDH- A	1.719 (0.421)	1.696 (0.413)	2.183 (0.546)	1.515 (0.342)	2.052 (0.516)	1.602 (0.378)	1.591 (0.373)	1.432 (0.304)	1.558 (0.360)	1.815 (0.452)	2.156 (0.540)	1.472 (0.323)	1.472 (0.323)	1.971 (0.496)	1.731 (0.413)
IDH- B	1.571 (0.366)	1.432 (0.304)	1.818 (0.453)	1.243 (0.197)	2.000 (0.503)	1.347 (0.260)	1.408 (0.292)	1.391 (0.283)	1.895 (0.475)	1.367 (0.271)	1.512 (0.341)	1.473 (0.323)	1.494 (0.333)	1.129 (0.115)	1.506 (0.323)
MNR- B	2.166 (0.542)	2.767 (0.643)	2.856 (0.654)	2.476 (0.600)	2.486 (0.602)	2.476 (0.600)	2.198 (0.549)	2.570 (0.615)	2.370 (0.582)	2.372 (0.582)	2.277 (0.565)	2.236 (0.557)	2.261 (0.562)	2.462 (0.598)	2.427 (0.590)
ADH- A	2.632 (0.624)	1.891 (0.474)	2.147 (0.538)	2.293 (0.568)	2.049 (0.515)	2.198 (0.549)	2.323 (0.573)	2.523 (0.608)	1.844 (0.461)	2.364 (0.581)	2.022 (0.509)	2.281 (0.565)	2.292 (0.567)	2.260 (0.561)	2.222 (0.550)
Gene Pool	2.425 (0.557)	2.572 (0.549)	2.624 (0.600)	2.392 (0.520)	2.576 (0.590)	2.330 (0.527)	2.327 (0.529)	2.472 (0.538)	2.408 (0.548)	2.419 (0.547)	2.539 (0.565)	2.318 (0.526)	2.342 (0.529)	2.469 (0.533)	2.444 (0.547)

Population parameters

Allelic differentiation

In addition to allelic differentiation for each population, Table 4 also shows the allelic differentiation at individual loci and overall means gene pool differentiation which reflects on an average the proportion of the effective number of alleles by which a population differed from the remaining populations over the set of six gene loci.

The gene pool differentiation among the populations varied from 38.2% to 45.2%. Population 3 was the most differentiated followed by population 5. Population 6 was the least differentiated population with a value of 38.2%.

The mean differentiated (δ) values among 6 gene loci varied from 0.23 (IDH- B) to 0.57 (6PGDH-A). Wide range of differentiation values were recorded for different populations within gene loci e.g., IDH-B (0.07 for population 14 to 0.37 for population 5), SKDH-A (0.22 for population 8 to 0.43 for population 3). Overall, a high amount of allelic differentiation among populations was reflected by the mean gene pool value (δ= 40.5), which means on an average, in the gene pool of the six loci, populations differed from their complement populations by 40.5% of the effective number of alleles.

Genotypic differentiation
The gene pool genotypic differentiation for the populations (Table 5) was found to follow gene pool allelic differentiation and it varied from 0.52 (Population 4) to 0.60 (Population 3). The mean genotypic differentiation (δ) values among the 6 gene loci were found to vary for IDH-B (0.32) to 6PGDH-A (0.74) and the mean gene pool value was found 0.54.

Test of Homogeneity for distribution of allele and genotypic frequencies
Significant differences were observed among the allelic frequencies of the fourteen populations. G-test and χ^2- test were found significant for all the polymorphic loci at 0.1% level of significance, as is evident from their values presented in Table 6. Similar to that of allelic frequency distributions, the values for G-test and χ^2- test for genotypic frequency distributions among fourteen populations were also found significant for all the gene loci at 0.1% level of significance.

Table 6: The homogeneity tests for allelic and genotypic frequencies

Sl. No.	Locus	Allelic frequencies		Genotypic frequencies	
		G-Test	Chi square	G-Test	Chi square
1	MDH-A	80.548***	116.151***	74.458***	110.303***
2	6PGDH-A	136.984***	135.634***	137.487***	129.187***
3	SKDH-A	87.527***	89.336***	64.780***	67.262***
4	IDH-B	113.712***	112.371***	93.920***	89.035***
5	MNR-B	39.599***	40.244***	41.911***	41.803***
6	ADH-A	45.853***	45.402***	53.315***	51.132***

*** Significant at 0.1%

Genetic distance
Nei's genetic distance coefficient was used to estimate genetic differentiation amongst all the fourteen populations studied. The populations 12 and 13 had most similar genetic structure, the genetic distance between them was 0, means no genetic differentiation occurred between these two populations, which was followed by 0.0001 found between populations 7 and 10 and the same coefficient value was found between populations 4 and 8 and between populations 4 and 6. The major difference in genetic structure occurred between populations 5 and 6, the genetic distance between them being 0.0057 which is followed by populations 5 and 8, populations 4 and 5 and populations 3 and 12 having same genetic distance of 0.0047. For all possible pairs of populations the genetic distance values averaged 0.0021.

Table 7: Nei's genetic distances between the 14 analysed populations of *Pinus gerardiana*

Population No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	-													
2	0.0042	-												
3	0.0046	0.0025	-											
4	0.0023	0.0016	0.0035	-										
5	0.0030	0.0022	0.0024	0.0047	-									
6	0.0031	0.0022	0.0036	0.0001	0.0057	-								
7	0.0005	0.0032	0.0031	0.0016	0.0028	0.0021	-							
8	0.0017	0.0021	0.0042	0.0001	0.0047	0.0007	0.0019	-						
9	0.0031	0.0013	0.0032	0.0024	0.0011	0.0029	0.0028	0.0029	-					
10	0.0004	0.0020	0.0032	0.0008	0.0027	0.0013	0.0001	0.0008	0.0022	-				
11	0.0022	0.0022	0.0026	0.0029	0.0014	0.0038	0.0007	0.0036	0.0024	0.0013	-			
12	0.0011	0.0021	0.0047	0.0012	0.0028	0.0014	0.0016	0.0011	0.0012	0.0007	0.0030	-		
13	0.0006	0.0022	0.0046	0.0016	0.0020	0.0022	0.0010	0.0014	0.0011	0.0004	0.0021	0.0000	-	
14	0.0021	0.0021	0.0037	0.0010	0.0044	0.0016	0.0006	0.0014	0.0040	0.0004	0.0012	0.0025	0.0021	-

Thus most of genetic variation parameters showed a range of variation in different populations. The low intrapopulation variation in some of the populations was also indicated by genetic variation parameters and intrapopulation similarity coefficient.

Conclusion
The findings revealed ample diversity in population 3 (N_a = 1.56, H_o = 0.107) and population 11 (N_a = 1.50, H_o = 0.109) belonging to Skiba and Thangi respectively. Therefore, it is recommended to conserve these forests as biogenetic resources. Populations 5 (Morang), 2 (Bharmour), 14 (Jangi) and 1 (Pangi) have different allelic architecture, hence may be a potential source for future breeding strategies. Such a wide diversity explored through isozyme analysis in *Pinus gerardiana* will be beneficial for future breeding programmes and improvement of the species.

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