Evaluation of Genetic Diversity in *Pinus gerardiana* Wall. Using Isozymes

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Pinus gerardiana or chilogoza pine is a very important conifer species which is distributed very sparsely and is threatened due to reckless cone harvest leading to almost complete lack of natural regeneration. The present study was carried out to assess genetic variation among ten different populations of the species in Himachal Pradesh using isozymes. A total of nine isozyme loci were recorded for four enzyme systems and fifteen alleles were assigned for different isozymes. The isozyme loci such as PER-I, EST-II and AAT-II were the most variable in terms of allele frequency. For each locus one allele was most common, not only with respect to its frequency, in the population but also with respect to the number of populations which contained it. The isozyme loci PER-II and NADH-I appeared heterozygous. Most of the genetic variation parameters showed a range of variation in different population and also revealed low variation in some of the isolated populations of *P. gerardiana* and perhaps is result of inbreeding in small populations. On the basis of similarity coefficient and genetic distances it is concluded that various populations of *P. gerardiana* differ substantially i.e. upto 7 per cent of total variation can be attributed to the population differences which is generally high for any conifer. It is also concluded that species is highly heterozygous in nature as indicated by negative F-statistics.

Key Words: Genetic diversity, Isozymes, *Pinus gerardiana*.

Pinus gerardiana Wall. (chilgoza pine) is distributed very sparsely in the world. It is confined to the mountains of eastern Afghanistan, parts of Pakistan and scattered patches in dry inner Himalayas. In India, the chilgoza pine is restricted to the dry inner valleys of North-west Himalayas where it grows at altitude of 1600 to 3300 m above mean sea level. As a timber tree, *P. gerardiana* is of little importance and used only where other timber species are not available. However, there is no other conifer species, which can match *P. gerardiana* in terms of its nutritional nuts that have carbohydrates, proteins and fats. Tribals of Kinnaur are largely dependent on the domestic and foreign sale of its nuts and it constitutes the backbone of their economy. Besides, this species is a good soil binder, and thus checks soil erosion. Though chilgoza pine has not been tapped commercially for oleoresin owing to its limited distribution and to avoid destruction of the trees, in order to get more valuable nuts, yet there is great scope for resin extraction. The species is facing a threat due to reckless cone collection by tribals which adversely effects its natural regeneration. Thus, *P. gerardiana* deserves immediate attention for genetic conservation and improvement. The basic pre-requisite for genetic improvement and conservation of genetic resources is the study of genetic variability in existing populations. In order to identify the level of diversity, marker systems have almost become indispensable. During the last two decades, isozymes have been widely used to study genotypic variation in populations (Hamrick and Godt 1989). Unlike quantitative traits, isozymes are not generally influenced by environmental variation, ontogenetically stable and conditionally expressed. The technique fulfils the aim of tree geneticist to detect genetic differences as close as possible to the DNA level because these are direct gene products.

Material and Methods

Seeds of *P. gerardiana* were collected from ten different sites delimited by natural features, such as streams and mountains. These sites lie on both sides of river Sutlej, which divides the chilgoza forests into two mountainous ranges. These sites were Akpa, Rispa, Skiba, Shongthong, Boktu, Kalpa, Dubling, Kanam, Spilow and Morang. The sites Akpa, Skiba, Boktu, Rispa were at the centre of distribution range while Dubling and Morang at the extremes and most isolated. Ten trees were sampled from each site. Megagametophytic tissue from 10-15 seeds was used for enzyme extraction from each tree sampled. Seeds were soaked in 1% hydrogen peroxide for three days and then germinated on moist filter paper at 25°C. When radical emerged out of seed coat by 2 to 5 mm, the megagametophytic tissue was separated and

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homogenised in pre-chilled pestle and mortar using extraction buffer (1:5 w/v). The extraction buffer contained 0.05 M tris base, 0.008 M citric acid (pH 8.3), 0.1% ascorbic acid, 0.1 % cystein hydrochloride and 1 % polyethyelene glycol (PEG). The homogenates thus obtained were centrifuged at 15,000 rpm for 20 minutes at 4°C and stored in a refrigerator in tightly capped tubes till use. Horizontal polyacrylamide gel electrophoresis was carried out to separate the various enzyme systems. The isozymes were fractioned at 4°C at a constant current of 50mA in polyacrylamide gel in electrophoresis tank containing lithium borate buffer (pH 8.3) (Scandalios, 1969). The staining procedure followed for various enzyme systems were according to Conckle *et al.* (1984) with slight modifications. Locations of isozymes were specified by relative mobility (Rm) values, which were calculated according to Kuhn and Fretz (1978). The isozymes of each enzyme system obtained on the gel were named according to Rajora and Zesuffa (1988). The fastest anodal zone was designated as locus 1 for enzyme encoded by multiple loci. Further, the isozymes at a locus were denoted by alphabets A, B, C etc. where A represents the fastest, and B, C, progressively, slower forms. Various genetic parameters such as allele frequency, proportion of polymorphic loci (Nei, 1978), polymorphic index (Allard *et al.*, 1978), observed and expected heterozygosity (Nei, 1978), average allele per locus, effective number of allele per locus (Crow and Kimura, 1970), Wright's F statistic, similarity index (Pontikis *et al.*, 1980) and genetic distance (Nei, 1978) were calculated for each population.

Resuits and Discussion

Only four enzyme systems out of twelve tested were resolved with sufficient activity and clarity, hence were selected for final analysis. These were peroxidase (PER), esterase (EST), nicotinamide adenine dinucleotide dehydrogenase (NADH) and aspartate amino transferase (AAT).

Peroxidase-The investigation revealed that peroxidase isozymes showed two loci and two bands for each locus. Neale *et al.* (1984) also found two zones of activity for peroxidase in Douglas fir. Similarly, Mowrey *et al.* (1990) and Gibson and Hamrick (1991) found two peroxidase loci in *Prunus* spp. and *Pinus pungens*, respectively. Thus, peroxidase seems to be a diamer in *Pinus gerardiana*. The locus PER-I shows a lot of polymorphism while locus PER-II showed a more consistent banding pattern as evidenced from their allele frequencies in various populations.

Esterase-Three different loci were observed for this enzyme system in the sampled genotypes of *P. gerardiana*. The loci EST-I and EST-II had only one band whereas, EST-II had two bands and showed polymorphism. Huang *et al.* (1994) observed three single band zones for esterase in Masson pine.

Nicotinamide adenine dinuleotide dehydrogenase-For this enzyme system two loci were observed and both were polymorphic with two alleles. The allele frequencies at NADH-I did not vary much among the populations. The allele frequencies at locus NADH-II showed variation, NADH-IIA being the most common allele.

Aspartate amino transferase-Two loci were detected for the enzyme aspartate amino transferase activity. The locus AAT-I had only one allele and was monomorphic over all the populations while AAT-II had two alleles, AAT-IIA being the most common allele in all the populations. The result are in agreement with those of Myburg and Harris (1997) who also recorded two loci for AAT activity with one allele at each locus in *P. kesia* and with Yeh and O'Malley (1980) who recorded two loci with two alleles at each locus in Douglas fir. However, some other workers such as Goncharenko *et al.* (1993), Timerjanov (1997), Scaltsoyiznnes *et al.* (1994) and Beaulien and Simon (1994) have recorded three loci for AAT activity in various conifer species.

Genetic interpretations-A total of nine isozyme loci were recorded for four enzyme systems and fifteen alleles were assigned for different isozymes. The allele frequencies for fifteen alleles at nine loci for different populations are listed in Table 1. The loci such as PER-I, EST-II, and AAT-II were most the variable and most of populations did not differ much at loci PER-II, EST-I, EST-III, NADH-I, NADH-II and AAT-I in terms of allele frequencies. It can also be seen that for every locus, there is a most common allele not only with respect to its frequency in a population, but also with respect to number of populations contained it. In the present investigation the per cent polymorphism varied from 33.33 to 66.67 per cent. It is interesting to note that populations which lie in the centre of the distributional range such as Akpa, Rispa, Shongthong, Spilow except Skiba depicted a high per cent polymorphism as compared to the isolated populations such as Dubling, Moorang and Boktu. In pines a wide variation in percentage of polymorphic loci have been reported in literature ranging from 5 per cent in *P. palustris*

Table 1. Probable genotype, allele(s), allele frequency, of *Pinus gerardiana* at different sites based on isozymes

Loci	Sites/ Parameters		Dubling	Moorang	Skiba	Akpa	Rispa	Kanam	Spilow	Shongthong	Boktu	Kalpa
PER–I	Genotype		BB	BB&AB	BB	BB&AB	BB&AB	BB&AB	BB&AB	BB&AB	BB	BB&AB
	Allele frequency	A	0	0.33	0	0.43	0.20	0.20	0.33	0.20	0	0.33
		B	1	0.67	1	0.57	0.80	0.80	0.67	0.80	1	0.67
PER–II	Genotype		AB	AB	AB	AB	AB	AB	AB	AB&AA	AB	AB
	Allele frequency	A	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.67	0.50	0.50
		B	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.33	0.50	0.50
EST–I	Genotype		BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
	Allele frequency	A	0	0	0	0	0	0	0	0	0	0
		B	1	1	1	1	1	1	1	1	1	1
EST–II	Genotype		AA	AA	AA	AA&AB	AA&AB	AA&AB	AB	AA&AB	AA&AB	AA&AB
	Allele frequency	A	1	1	1	0.67	0.67	0.67	0.50	0.80	0.57	0.80
		B	0	0	0	0.33	0.33	0.33	0.50	0.20	0.43	0.20
EST–III	Genotype		AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
	Allele frequency	A	1	1	1	1	1	1	1	1	1	1
		B	0	0	0	0	0	0	0	0	0	0
NADH–I	Genotype		BB&AB	AB	AB	AB	AB	AB	AB	AB	AB	AB
	Allele frequency	A	0.33	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
		B	0.67	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
NADH–II	Genotype		AA&Nil	AA&AB	AB	AA&AB	AA&AB	AB	AB	AB	AB&Nil	Nil
	Allele frequency	A	1	0.80	0.50	0.80	0.57	0.50	0.50	0.50	0.50	
		B	0	0.20	0.50	0.20	0.43	0.50	0.50	0.50	0.50	
AAT–I	Genotype		AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
	Allele frequency	A	1	1	1	1	1	1	1	1	1	1
		B	0	0	0	0	0	0	0	0	0	0
AAT–II	Genotype		AA	AA&AB	AA&AB	AA&AB	AA&AB	AA&AB	AA&AB	AB	AA&AB	AB
	Allele frequency	A	1	0.57	0.57	0.57	0.67	0.57	0.67	0.50	0.67	0.50
		B	0	0.43	0.43	0.43	0.33	0.43	0.33	0.50	0.33	0.50

(Synder and Hamaker, 1978) to 100 per cent in *P. heplensis* (Agundez *et al.*, 1999), *P. henryi* (Guo *et al.*, 1998) and *P. taeda* (Schmidtting *et al.*, 1999). The average number of alleles per locus varied from 1.22 to 1.67, which also followed the same trend i.e. lower number of alleles per locus was observed in isolated populations. In 20 species of *Pinus*, the mean of average number of alleles per locus was observed to be 2.29 (Ledig, 1986). Thus in present studies less number of alleles per locus was observed than the average reported in the literature.

The expected mean heterozygosity for various populations in the present study ranged from 0.10 to 0.32, again some of the isolated populations being on the lower side in this parameter also (Table 2). The expected heterozygosity for the species as a whole was recorded to be 0.30, which is on the higher when compared with expected heterozygosities reported in 37 *Pinus* species, which ranged between 0-0.362 with a mean of 0.174 (Ledig, 1986). Direct comparison with other studies may not however be reliable as type of the enzymes, the number of enzymes and the total loci studied vary from study to study. In many cases workers take into account all the loci including the monomorphic ones for calculation of the genetic parameters, whereas, others exclude them. This may lead to differences in the calculated values. The estimate of the fixation index was negative for all the populations studied except for the population at Dubling. The negative values of fixation index reflect the predominance of heterozygotes in these populations. In conifers excess of heterozygotes is an important factor, which can be ascribed to large differences in male and female allelic frequencies. Significant predominance of heterozygotes is common in mature stands of conifers (Muller-Strack *et al.*, 1983; Morgante *et al.*, 1993).

Similarity index-The similarity index values (Pontikis *et al.*, 1980) between and within various populations are directly based on isozyme phenotypes. The maximum similarity coefficient value was found between the populations of Rispa and Kanam followed by that between Kanam and Spilow, which lie at central part of distributional range of *Pinus gerardiana* (Table 3). Minimum similarity index value was recorded between Dubling and Spilow, Dubling being an isolated population. For the populations such as Dubling, Moorang, the within population similarity coefficient was high which indicates higher relatedness.

Table 2. Genetic variation parameters of different populations of *Pinus gerardiana*

Site Parameters	Dubling	Moorang	Skiba	Akpa	Rispa	Kanam	Spilow	Shongthong	Boktu	Kalpa	In total in the species
Total number of loci	9	9	9	9	9	9	9	9	9	8	9
Total number of allele in all loci	11	14	13	15	15	15	15	15	14	13	15
Per cent polymorphic loci	33.33	55.56	44.44	66.67	66.67	66.67	66.67	66.67	55.56	62.50	66.67
Polymorphic index	0.11	0.25	0.22	0.30	0.26	0.30	0.32	0.29	0.27	0.28	0.30
Average number of allele/locus	1.22	1.56	1.44	1.67	1.67	1.67	1.67	1.67	1.56	1.63	1.67
Effective average number of allele/locus	0.12	0.15	0.14	0.20	0.16	0.16	0.16	0.19	0.15	0.17	0.30
Mean observed heterozygosity	0.17	0.39	0.42	0.47	0.44	0.50	0.56	0.44	0.47	0.47	0.42
Mean expected heterozygosity	0.10	0.25	0.22	0.30	0.30	0.30	0.32	0.29	0.27	0.28	0.30
F-statistics	0.32	-0.15	-0.17	-0.32	-0.28	-0.38	-0.48	-0.28	-0.30	-0.36	-0.40

Table3. Intra- and inter-population similarity matrix between ten different populations of *Pinus gerardiana* based on isozymes

	Dubling	Moorang	Skiba	Akpa	Rispa	Kanam	Spilow	Shongthong	Boktu	Kalpa
Dubling	0.96									
Moorang	0.81	0.94								
Skiba	0.89	0.84	0.86							
Akpa	0.78	0.89	0.84	0.85						
Rispa	0.88	0.85	0.72	0.83	0.84					
Kanam	0.76	0.86	0.80	0.85	0.90	0.85				
Spilow	0.61	0.81	0.86	0.85	0.88	0.89	0.90			
Shongthong	0.72	0.86	0.84	0.72	0.84	0.86	0.85	0.90		
Boktu	0.78	0.80	0.84	0.71	0.84	0.88	0.84	0.79	0.82	
Kalpa	0.78	0.83	0.75	0.81	0.77	0.79	0.74	0.74	0.81	0.91

Table 4. Genetic distance matrix between ten different populations of *Pinus gerardiana* based on isozymes

	Dubling	Moorang	Skiba	Akpa	Rispa	Kanam	Spilow	Shongthong	Boktu	Kalpa
Dubling										
Moorang	0.046									
Skiba	0.060	0.029								
Akpa	0.072	0.018	0.058							
Rispa	0.079	0.021	0.182	0.011						
Kanam	0.156	0.031	0.189	0.023	0.001					
Spilow	0.102	0.054	0.056	0.023	0.004	0.009				
Shongthong	0.086	0.060	0.028	0.209	0.064	0.019	0.026			
Boktu	0.076	0.061	0.029	0.224	0.018	0.009	0.017	0.024		
Kalpa	0.130	0.060	0.060	0.060	0.053	0.048	0.068	0.024	0.072	

The mean values of within population similarity coefficient and between population similarity coefficients were 0.883 and 0.813, respectively. This indicates that about 7 per cent variation is due to their interpopulation differences. This means that genetic variation in *P. gerardiana* is high in local populations and the same allele tends to be distributed throughout the whole range of the species. The results are in agreement with those of Hamrick and Godt (1989) who concluded that conifer species have approximately 6.8 per cent of their variation among populations.

Genetic distance-The Nei's genetic distances are also estimation of the inter-population genetic variability. The genetic distance almost followed the same trend as that of similarity coefficient (Table 4). The mean genetic distance for all possible pairs of populations was 0.06 showing that differentiation among populations was substantially high.

Although average inter-population genetic distances within populations for most of the conifers is rather low viz., 0.003 in Russian population of *P. koraiensis* (Potenko and Velikov, 1998), 0.013 in *Larix sukaczewii* (Timerjanov, 1997), 0.008 in *P. thunbergii* (Kim *et al.*, 1997), 0.017 in *P. sylvestris* (Goncharennko *et al.*, 1994) and 0.0037

in Doublas fir (Yeh and O'Malley, 1980). However, Scaltsoyiannes (1999) observed very high genetic distance between population of *Cedrus libani* (upto 0.487).

References

Agundez D, B Degen, GV Wuelisch and R Alia (1999) Multilocus analysis of Pinus helepensis Mill. from Spain: Genetic diversity and clinal variation. *Silvae Genetica.* **48:** 173-178.

Allard RW, RD Miller and AL Kalher (1978) The relationship between degree of environmental heterogenity and genetic polymorphism. In: Freysen AJ and J Woldendrop (eds) *Structure and functioning of plant populations.* New York, North Holland Publishing house, pp. 49-43.

Beaulien T and JP Simon (1994) Inheritance of linkage of allozymes in *Pinus storbus* L. *Silvae Genetica.* **43:** 253-261.

Conkle MT, PD Hodgskin, LB Nunnally and SC Hunter (1984) Starch gel electrophoresis of conifer seeds: A laboratory manual USDA. *For. Serv. Gen. Tech. Rep.* PSW-64 pp. 18.

Crow JF and M Kimura (1970) An Introduction to Population Genetics Theory. Harper and Row, New York, Evanston, London.

Gibson JP and JL Hamrick (1991) Genetic diversity and structure in Pinus pungens (Table mountain Pine) populations. *Canadian J. For. Res.* **21:** 635-642.

Gonchararenko GG, VE Pandutov and AE Silin (1993) Allozyme variation in natural populations of Eurasian pines II. genetic variation, diversity differentiation and gene flow in Pinus sibirica Du Tour in some low land and mountain populations. *Silvae Genetica.* **42:** 246-253.

Gonchararenko GG, AE Silin and VE Pandutor (1994) Allozyme variation in natural populations of Eurasian pines. III populations structure, diversity differentiation and gene flow in central and isolated populations of *Pinus sylvestis* L. in Eastern Europe and Siberia. *Silvae Genetica.* **43:** 119-132.

Guo J.Z, CX Bi and SM Len, (1998) Genetic variation of isoenzymes in natural population of *Pinus henryi Mast. J. Northwest For. College.* **13:** 44-49.

Hamrick JL and MJW Godt (1989) Allozyme diversity in plant species. In: Weir ADH (eds) *Plant Population Genetics, Breeding and Genetic Resources.* Sinauer Associates Inc. Sunderland. 43-63.

Huang QQ, LH Wang and K Ohba (1994) Genetic control of isozyme variation in masson pine, *Pinus massoniana* Lamb. *Silvae Genetica.* **43:** 285-291.

Kim ZS, SW Lee and JW Hwang (1997) Genetic diversity and structure of natural populations of *Pinus thunbergii* in Korea. *Silvae Genetica.* **46:** 120-124.

Kuhn LJ and TA Fretz (1978) Distinguishing rose cultivars by polyacrylamide gel electrophoresis. I. Extraction and storage of proteins and enzymes from rose leaves. *J. Amer. Soc. For. Horti. Sci.* **103:** 503-508.

Ledig FT (1986) Heterozygosity, heterosis and fitness in outbreeding plants. In: Soutle ME (ed) *Conservation Biology: The Science of Scarcity and Diversity.* Sinauer Associates Sunderland, Mass. pp. 77-104.

Morgante M, GG Vendramin, P Rossi and AM Olivieri (1993) Selection against inbreed in early lifecycle phase of in *Pinus leucoderinis Ant. Heredity.* **70:** 622-627.

Mowrey BD, DJ Werner and DH Byrne (1990) Isoenzyme survey of various species of *Prunus* in the subgenus Amygdalus. *Scientia Hortic.* **44:** 251-260.

Muller-Strack G, M Ziene and H Hattemer (1983) Reproductive systems in conifer seed orchards. 2. Reproductive selection monitored at LAP gene locus in *Pinus sylvestris* L. Theor. Appl. Genet. **65:** 309-316.

Myburg H and SA Harris (1997) Genetic variation across the natural distribution of the South East Asian pine, *Pinus Kesiya* Gordon. *Silvae Genetica.* **46:** 295-301.

Neale DB, JC Weber and WT Adams (1984) Inheritance of needle tissue isozymes in Douglas fir. *Canadian J. Genet. Cytogenet.* **26:** 459-468.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics.* **89:** 583-590.

Ponteko VV and AV Velikov (1998) Genetic diversity and differentiation of natural population of *Pinus koraiensis* in Russia. *Silvae Genetica.* **47:** 202-208.

Pontikis CA, M Loukas and G Kousounis (1980) The use of biochemical markers to distinguish olive cultivars. *J. Hort. Sci.* **55:** 333-343.

Rajora OP and L Zesuffa (1988) Multi locus genetic structure, characterization and relationships of Populus x candensis cultivars. *Genome.* **32:** 99-108.

Scaltsoyiannes A (1999) Allozyme differentiation in phylogeny of cedar species. *Silvae Genetica.* **48:** 61-68.

Scaltsoyiannes A, R Rohar and KP Panetsos (1994) Allozyme frequency distribution in five European population of black pine(Pinus nigra Arnold).I Estimation of genetic variation within and among populations II) contribution of isozyme analysis to the taxonomic status of the species. *Silvae Genetica.* **43:** 20-30.

Scandalios JG (1969) Genetic control of multiple molecular form of enzyme in plants: A Review. *Biochem. Genet.* **3:** 37-39.

Schmidtling RC E Carroll and T Lafarge (1999) Allozyme diversity of selected and natural loblloly pine population. *Silvae Genetica.* **48:** 35-45.

Synder EB and JM Hamaker (1978) Inheritance of peroxidase isozymes in needles of loblolly and long leaf pines. *Silvae Genetica.* **28:** 125-129.

Timerjanov AS (1997) Lack of Allozyme variation in Larix sukaezewii Dyl. from the Southern urals. *Silvae Genetica.* **46:** 61-64.

Yeh FCH and D O'Malley (1980) Enzyme variations in natural populations of Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco, from British Columbia I. Genetic variation pattern in coastal populations. *Silvae Genetica.* **29:** 83-92.