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Study of different isozymes in *Pinus gerardiana* Wall.

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Abstract
Pinus gerardiana (Chilgoza) is a small to medium sized evergreen tree yielding a highly valuable edible nut. The present investigations were undertaken to assess the different isozymes in Chilgoza pine. Seven isozymes viz., MDH (Malate dehydrogenase), GDH (Glutamate dehydrogenase), SKDH (Shikimic acid dehydrogenase), 6PGDH (6-Phosphogluconate dehydrogenase), MNR (Menadione reductase), IDH (Isocitrate dehydrogenase) and ADH (Alcohol dehdrogenase) were studied. A total of sixteen gene loci were recorded for seven enzyme systems out of which 6 loci namely MDH- A, 6PGDH- A, SKDH- A, IDH- B, MNR- B and ADH- A were polymorphic whereas the remaining ten gene loci i.e. MDH- B, MDH- C, MDH- D, GDH- A, 6PGDH- B, SKDH- B, IDH- A, MNR- A, MNR- B and ADH- B showed no variation. The percentage of polymorphic loci and average number of alleles per locus are one of the useful criterions for comparing species of populations for genetic diversity.

Keywords: *Pinus gerardiana*, Isozymes, Alleles, Loci

INTRODUCTION

Knowledge of genetic diversity among and within species is needed for all conservation purposes. Genetic diversity is characterized by measuring the genetic variability within a species. Forest trees have high rate of genetic diversity within population (White *et al*., 2007). Variation within populations of tree species has been exploited in the selection of superior seed source/ provenance for a given site and evolving conservation strategies of genetic diversity within populations of tree species (Rawat *et al*., 2006). To study the genetic variation within and between populations, the selection of appropriate genetic markers assumes great significance.It is worth to add quick and cheap marker system and good alternative to assay and identify level of genetic variation in populations (Bakshi and Konnert, 2011). Analysis of structurally different primary products of allelic genes, i.e. isozyme analysis is one of these. Isozymes are especially useful when several taxa, accessions and individuals are to be compared which can also serve as convenient and reliable gene markers allowing for observation of allelic variation at individual gene loci and thus, facilitating a reliable estimation of genetic variation in populations and species.
Furthermore, the data generated from isozyme analysis can be used to establish correlations between genetic parameters and environmental factors as well as phenotypic characters of the trees, which facilitating conservation biology activities e.g. gene bank- enables choosing of proper sample for long time conservation(Bednorz *et al*., 2006) could be helpful in screening of suitable reproductive material. 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. Therefore, the present work was done to assess the different isozymes in *Pinus gerardiana*.

MATERIALS AND METHODS

Surveys and selection of the trees: The distribution of the species is confined to arid regions where rainfall is less than 500 mm annually between an altitude of 1800 m to 3350 m above mean sea level. The survey of the populations was carried out in the areas of Kinnaur and Chamba district of Himachal Pradesh namely Dubling, Nesang, Morang, Thangi, Kannam, Skibba, Akpa, Rarang, Jangi, Purbani, Tangling, Pangi in Kinnaur district and Pangi and Bharmour areas of Chamba district. Ten trees were selected from

each population to harvest the open pollinated seeds.

Isozyme analysis: Megagametophyte tissues and embryos of seeds of chilgoza pine were isolated separately from the seeds and homogenized in the extraction buffer 0.1 M Tris- HCL pH 7.5 containing Tris- 1.211 g; Ascorbic acid- 0.106 g; Saccharose- 17.165 g; Tween-10- 10ml; polyvinyl pyrrolidone-8.0 g; Nicotinamide adenine dinucleotide- 0.029 g; Bovine albumin- 0.1 g; Ethylene diamine tetra acetic acid- 0.015 g; Dirhiothreitol- 0.015 g; and Tergitol- 1.0 ml. Immediately prior to use 1% (v/v) mercaptoethanol was added.

Preparation of gel: An appropriate gel buffer was used to prepare starch gel for specific enzymes. A solution of starch (15%), Saccharose and gel buffer was carefully homogenized in one litre conical flask and heated inside a microwave oven till boiling solution was obtained. The solution was then de- aerated with membrane vacuum pump and poured into gel plates. Before the gel was allowed to cool and set for approximately 30 minutes at room temperature, the solid particles and air bubbles were removed quickly with the help of forceps. Placed the gel for ½ hr in a refrigerator for final cooling before use.

Sample preparation: The sample comprised of half of the endosperm and full embryo of the seed put in separate labeled wells of grinding block, which is kept on ice tray. The sample material was homogenized by using 90 µl of the extraction buffer. Homogenization of the sample was done with almost care to avoid contamination in the samples. Whatman filter papers (wicks) which were dipped in the extraction solution and stored in deep freeze were placed on spot positions after gel was cooled. A wick with bromophenol blue solution was inserted to serve as a tracking dye for comparing the migration distances among the bands.

Electrophoresis: Enzyme was separated into discrete bands by horizontal starch gel electrophoresis. The gels were run in the Horizontal Electrophoresis unit, Tris buffer (pH- 7.4) was used as Electrode buffer. Electric current (250 V with 130 mA) was applied for 5 $\frac{1}{2}$ hours. Initially tried for six hours but standardized at 5 $\frac{1}{2}$ hr.

Slicing: After Electrophoresis, slicing of the gel was done. The gel was cut into 2mm slices horizontally by a wire. This procedure was repeated until the desired number of slices was prepared. In one gel 4 slices of 2 mm thickness were produced.

Staining: The gel slice was immersed into a staining assay for revealing the zones of enzymatic activities. 100ml of solutions was used to stain a slice. The staining solution was specific for the individual enzyme. Seven isoenzymes (ADH, MDH, IDH, SKDH, 6- PGDH, GDH, MNR) were stained using specific substrates and additives (Liensgri, 1990) with modification. The bands were observed after deepen the slice in specific staining solution for few minutes (30 minutes, 45 minutes, 60 minutes or overnight). The gel images were photographed immediately using white light trans-illuminator.

Scoring: In the description of the isozyme patterns obtained from different isozyme systems, the zone migrating at highest migrating rate towards the anode is designated “A” and those that migrate less far are designated in successive order, “B”, “C”, etc. In the same order, the alleles within each locus are designated “1”, “2”, “3” etc.

RESULTS AND DISCUSSION

Description and genetic interpretation of zymograms: A total of 7 enzyme systems using Tris – citrate buffer were analysed for populations of *Pinus gerardiana* using megagametophytes and embryos separately. The isozymes were numbered in order of decreasing mobility from the anode. The locus that specifies the isozyme with the least anodic migration was labelled as 1, the next as 2, etc. At each locus alleles of different isozymes were also designated in order of decreasing mobility from the anode. The results obtained for the various isozyme systems with only representative zymograms for each system are described and discussed as under:

Malate dehydrogenase (MDH): Four zones of activity were observed on gels for MDH, but only one locus (MDH- A) was interpreted. Other 3 zones of activity were either monomorphic or not clearly visible. MDH- A was found polymorphic with 2 alleles (Fig. 1). This result is in line with Kurt *et al.* (2008) where four locus of MDH was identified but only one locus (MDH- A) was interpreted in *Cedrus libani* because remaining three locus were either monomorphic or not clearly re-

Table 1. Enzymes with their abbreviations and enzyme commission numbers (Anonymous, 1992).

Enzymes	Abbreviation used	Enzyme commission Number
Malate dehydrogenase	MDH	E.C.1.1.1.37
Glutamate dehydrogenase	GDH	E.C.1.4.1.2
Shikimic acid dehydrogenase	SKDH	E.C.1.1.1.25
6-Phosphogluconate dehydrogenase	6PGDH	E.C.1.1.1.41
Menadione reductase	MNR	E.C.1.6.99.2
Isocitrate dehydrogenase	IDH	E.C.1.1.1.42
Alcohol dehydrogenase	ADH	E. C. 1.1.1

Table 2. Enzyme analysed, number of gene loci observed and polymorphic gene loci under each enzyme in *Pinus gerardiana* populations.

S. N.	Enzymes	Number of loci observed	Number of polymorphic loci	Per cent polymorphic loci
1	Malate dehydrogenase (MDH)	4	1	25
2	Glutamate dehydrogenase (GDH)	1	-	-
3	6- Phosphogluconate dehydrogenase (6PGDH)	2	1	50
4	Shikimic acid dehydrogenase (SKDH)	2	1	50
5	Isocitrate dehydrogenase (IDH)	2	1	50
6	Menadione reductase (MNR)	3	1	33.3
7	Alcohol dehydrogenase (ADH)	2	1	50
	Total	16	6	

Table 3. Number of alleles observed for 7 enzyme systems.

S. N.	Enzymes	Number of alleles per locus
1	MDH- A	2
	MDH- B	1
	MDH- C	1
	MDH- D	1
2	GDH- A	1
3	6PGDH- A	4
	6PGDH- B	1
4	SKDH- A	2
	SKDH- B	1
5	IDH- A	1
	IDH- B	2
6	MNR- A	1
	MNR- B	2
	MNR-C	1
7	ADH- A	2
	ADH- B	1

solved. Sharma *et al.* (2008) also observed four gene loci in MDH in *Pinus roxburghii*.

Glutamate dehydrogenase (GDH): Only one zone of activity was observed on the gels stained for GDH. In all megagametophytes and embryo extracts, no variation was found as only a single allelic variant encoded by a sharp band was noticed for the both (Fig. 2). Not even a single population was found with variation and it was not included in the analysis. Kurt *et al.* (2008) observed and identified 1 polymorphic locus with 2 alleles in *Cedrus libani* whereas Gülbaba and Özkurt (2002) also found similar results. This study also gets support from a single allelic variant encoded by a sharp band noticed by Sharma *et al.* (2008) in *Pinus roxburghii*.

6- Phosphogluconate dehydrogenase (6- PGDH): Two zones of activity for 6PGDH with almost similar staining intensities were observed. The presence of single bands in endosperms and three bands (middle one as intralocus hybrid band) in heterozygous embryos suggested the dimeric structure of 6 PGDH as shown in Fig. 3. The four detected alleles were found to be distributed in the populations differentially. The band encoded by allele 6 PGDH- A4 was found close to the slower migrating zone 6 PGDH- B, which was monomorphic. The single band of 6 PGDH- B was

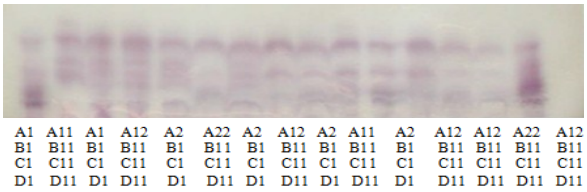

Fig 1. *Banding patterns of Malate dehydrogenase in Pinus gerardiana.*

Fig 2. *Banding patterns of Glutamate dehydrogenase in Pinus gerardiana.*

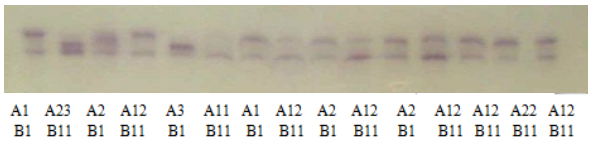

Fig 3. *Banding patterns of 6- Phosphogluconate dehydrogenase in Pinus gerardiana.*

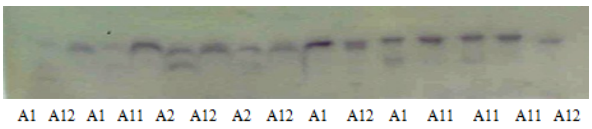

Fig 4. *Banding patterns of Shikimic acid dehydrogenase in Pinus gerardiana.*

observed in all the endosperms and embryos investigated. Allele 6PGDH- A2 was present in maximum samples followed by 6 PGDH- A1. Alleles 6 PGDH- A3 with 9.0% mean allele frequency and 6 PGDH- A4 with 1.4% mean allele frequency were observed in all the populations. Kurt *et al.* (2008) stained this enzyme in *Pinus resinosa* megagametophytes, but were not able to stain it in *Cedrus libani* megagametophytes on the same gels. Four alleles for 6 PGDH at locus A in *Pinus roxburghii* were reported by Sharma *et al.* (2008).

Shikimic acid dehydrogenase (SKDH): Two zones of activity were observed on the gels stained for SKDH. SKDH- A was polymorphic which showed two alleles. Out of two alleles, allele 1 occurred frequently than allele 2. However, a weakly staining monomeric second locus SKDH- B was also observed (Fig. 4). This study also gets

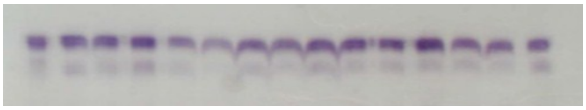

Fig 5. *Banding patterns of Isocitrate dehydrogenase in Pinus gerardiana.*

Fig 6. *Banding patterns of Menadione reductase in Pinus gerardiana.*

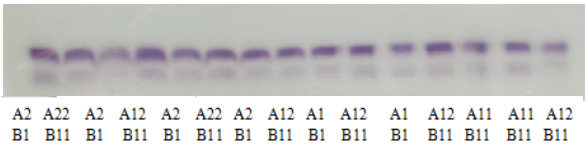

Fig 7. *Banding patterns of Alcohol dehydrogenase in Pinus gerardiana.*

support from the report of a weakly staining monomeric second locus by Sharma *et al.* (2008) in *Pinus roxburghii*. Two single banded phenotypes were identified for endosperms and embryos. In addition, three double banded phenotypes were also noticed in embryos. Genotypes A11, A12 and A22 were inferred from the single bands. The two bands of the heterozygous embryos were separated clearly. Appearance of two banded phenotypes in heterozygous embryos and only single banded phenotype in endosperms indicated the monomeric structure of SKDH.

Isocitrate dehydrogenase (IDH): Two zones of activity were observed for the gels stained for IDH. The faster migrating zone (IDH- A) was stained heavily in comparison to the slower one (IDH- B) and the former (IDH- A) showed no variation in endosperms and embryos. At locus IDH- B, two phenotypes encoded with two faint bands were found for the endosperm tissue, while in embryos, in addition to the phenotypes observed for endosperms a third phenotypes with four bands was also observed (Fig. 5). This was found only in heterozygous individuals. The results are in accordance with Kurt *et al.* (2008) observed 1 monomorphic locus with 2 alleles. Sharma *et al.* (2008) observed the two zones of activity in *Pinus roxburghii,* IDH- A stained heavily in comparison to IDH- B.

Menadione reductase (MNR): Three zones of activity was observed after staining the gel for MNR. MNR- A was intensively stained as compared to MNR- B and MNR-C. There was considerable difference between the migration distances of the three zones. MNR- A and MNR- C were found monomorphic whereas MNR- B was found to have two phenotypes for megagametophytes, which appeared likewise under homozygous embryo (Fig. 6). The heterozygous embryos showed third phenotypes with a thick or broader band. At locus MNR- B, allele 1 was found more frequently in all the populations as compared to allele 2. Kurt *et al.* (2008) observed 3 zones of activity: the MNR- A locus with 2 alleles, the MNR- B locus with 4 alleles and the MNR-C locus with 2 alleles

Alcohol dehydrogenase (ADH): Two zones of activity were observed on the gels stained for ADH. ADH- A locus was found polymorphic with two alleles whereas locus ADH- B locus was found monomorphic with no variations as shown in Fig. 7. ADH- A1 allele was found with higher mean allelic frequency (74%) for all the populations as compared to mean allelic frequency (26%) of ADH- A2. Kurt *et al.* (2008) observed and identified 1 locus with 2 single-banded alleles in *Cedrus libani* whereas Fallour *et al.* (2001) were unable to resolve this enzyme system because of its weak or non-detectable activity.

Isozyme summary: 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.

A total of 24 alleles were observed for different enzymes as shown in Table 3. A maximum of 4 alleles were observed for 6PGDH and MDH whereas minimum of 1 allele were found for GDH.

Conclusion

A prerequisite of any genetic analysis of the isoenzyme is the presence of different banding patterns on the gel after staining. Banding patterns of seven isoenzymes i.e. MDH, GDH, 6PGDH, SKDH, IDH, MNR and ADH showed different polymorphic and monomorphic gene loci. Assuming the band mobility that occurred in both haploid and diploid tissues from the same seed, represented an allele transmitted by the female gamete, it followed that the remaining allele present in embryo tissue was contributed by the male gamete (pollen). The percentage of polymorphic loci and average number of alleles per locus are one of the useful criterions for comparing species of populations for genetic diversity.

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