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Isozymatic Characterization of Accessions of *Arundinaria falcata* (Nees)

Chandrakant Tiwari* and Meena Bakshi

Plant Physiology Discipline, Botany Division, Forest Research Institute, Dehradun, India

Abstract

Bamboos are arborescent grasses belonging to the family *Poaceae*. To study the genetics of bamboo specially, one has to face enormous difficulties such as rare flowering and we cannot make hybridizations as easily as like other plant taxa. Unlike other tropical bamboos, studies on hill bamboos are scarce. *Arundinaria falcata*, an important hill bamboo, acts as soil stabilizer in hilly terrain. This study assessed the genetic diversity of 10 accessions of *A. falcata* collected from different localities of Garhwal Himalayas (India) and established at Hill bamboo Germplasm at Khirsu (India), using isozyme marker with four enzyme system (peroxidase, esterase, malate dehydrogenase and malic enzyme). Isozymatic analyses were performed with polyacrylamide gels (one system), bands were scored as binary data. Cluster analyses were conducted, using Jaccard´s similarity coefficient and UPGMA method. Very high degree of similarity was reported i.e. 63- 94% among different accessions. Dendrogram revealed two major clusters with three (A8- A10) and seven (A1-A7) accessions respectively. The results obtained inferred low genetic diversity in the species and urgent need of the *in situ* conservation of the natural genetic resources of the *A. falcata* species.

Keywords: Genetic diversity; dendrogram; Polyacrylamide; Jaccard's coefficient

Introduction

Genetic conservation programmes are directed towards the long-term preservation of genetic resources either *in situ* or *ex situ* so that the potential for continuing evolution or improvement could be sustained. *In situ* conservation includes the organization and/or servicing of natural supplies where species are permitted to stay in maximum environments with the lowest of management. On the other hand, *ex situ* conservation includes the use of botanic landscapes, field farms, seeds shops and gene financial banks and germplasm.

The characterization of germplasm is required to maintain identity and purity for proper conservation and management. Various breeding strategies of plants are based on the knowledge of germplasm. Germplasm characterization is thus an important link between the conservation and utilization of plant genetic resources for improvement.

Arundinaria falcata vs. Gol Ringal, is socio- economically and ecologically, an important member of hill bamboo, present in Uttarakhand Garhwal Himalayas (India). Like other bamboo species, Ringal (hill bamboo) also has erratic and long flowering cycles. *Arundinaria falcata* flowers irregularly at times, gregarious over large areas, while few culms may be found in flowers almost every year. After seeding the clumps died [1].

Due to over usage of genetic resources and heavy extraction of the material from natural forests, the species are depleting at an alarming rate, gregarious flowering in turn intensifying the depletion of Ringal resources since flowering result into death of entire clumps following seeding. If a single regeneration event coincides with unsuitable conditions, the entire population might lose its chance to reproduce. Heavy forest degradation and agricultural encroachment, forest fires, human impact, heavy grazing pressures, etc. narrows the genetic base and eventually degradation of the gene pool of these species.

Isozymes are commonly used as biochemical markers as detectably different enzymes, which catalyze the same reaction [2]. Enzymatic analyses are added tools for detecting diversity [3]. The relationship between observed phenotypes and unobserved genotypes is simpler and better understood for electrophoretic evidence. Allozymes are the biochemical consequence of the substitution, deletion, or addition of amino acids in the polypeptides that comprise the enzymes and they can be distinguished if these changes affect their electrophoretic migration [4]. Isozyme electrophoresis is used to describe population structure, breeding structure and gene flow; to know species boundaries, and to document adaptive differences in allozymes; and to investigate phylogenetic relationships, rates of evolution, origin of polyploid plants and ploidy levels [5].

The present work was therefore, undertaken to assess variability among accessions of *A. falcata* growing in a Germplasm at Khirsu (Pauri) through morphological and genetic tools with the objective i.e. to study the genetic polymorphism in four hill bamboo species on the basis of morphological traits.

Materials and Methods

Sample collection

The young leaves of selected accessions were collected from Ringal Germplasm, Khirsu, tagged properly in polybags and stored in ice bucket till they were brought to Plant Physiology Laboratory (FRI, Dehradun). The leaves were stored at -20°C in freezer (vest frost DFS 345) till use.

Extraction of enzymes

Two grams of young leaves were macerated to powder with

***Corresponding authors:** Chandrakant Tiwari, Plant Physiology Discipline, Botany Division, Forest Research Institute, Dehradun, India, Tel: +91 135-2752673; E-mail: tiwari.chandrakant08@gmail.com

Weimin Xi, Department of Biological and Health Sciences,Texas A&M University, Tel: 1-361-593-2758; E-mail: weimin.xi@tamuk.edu

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liquid Nitrogen in a mortar – pestle and then 0.1 gm PVP and 5 ml of extraction buffer was added. The extraction buffer consisted of 1 M Sucrose, 0.2 M Tris and 0.056 M β - Marcaptoethanol. The volume was made up to 100 ml with distilled water and pH was adjusted at 8.5. The macerates were centrifuged at 1500 rpm for 20 minutes at 4^0C to collect the supernatants.

Casting of polyacrylamide gels

Separating gel: Two slab gel plates were assembled in a casting tub using 1.5 mm thick spacers. Required amount of stock solutions were mixed thoroughly excepting freshly prepared Ammonium Per Sulphate (APS) and TEMED (N'N'N'N' Tetra Methyl Ethylene Diamine), which were mixed just prior of pouring the mixture into the slab sandwich. The mixture was pipetted into the glass up to a level about 4 cm from the top. The gel solution was allowed to polymerize at room temperature.

Staking gel: Measured amount of stock solutions were mixed for stacking gel. The staking gel mixture was gently poured into the glass slab over the polymerized separating gel. A comb was inserted into the glass slab to make wells in the staking gel for loading the sample materials before it polymerization. The staking gel was allowed to polymerize for half an hour.

Electrophoresis of the enzyme extracts: After the complete polymerization of the staking gel, the comb was removed from the staking gel. Both the upper and lower tanks of the electrophoretic apparatus were filled with the Tris- Glycine (pH 8.3) running buffer and the lower surface of the separating gel should remain immersed in the running buffer.

25 µl of the enzyme extracts of the different accessions were loaded in the wells of the staking gel. A constant current of 30 mA was applied across 1.5 mm thick gel. Initially the current applied was 20 mA, but increased to 30 mA when the dye front entered into the separating gel. The electrophoresis was performed in a cold chamber having a constant temperature of 4^0 C. With the completion of electrophoresis, dye approached the end of the gel, the gel was removed and stained as per staining protocol of different enzymes.

Staining of the gel

Peroxidase (PRX, EC 1.11.1.7): For the staining of peroxidase isozymes, staining solution was prepared with 100 ml of redistilled water and 1 ml solution of acetone with 0.1 gm of the Orthodiacidine. Immediately after putting the gel into the above solution, 0.4 ml of H_2O_2 was added over the gel. The gel was agitated gently till brown colored bands were visible.

Esterase (EST, EC 1.1.1.1): D' α- Napthyl Ester, α- Napthol + Salt, α- Napthol + Fast Blue RR, Colour Precipitate.

Gel was kept in 50 ml of Phosphate A Buffer; pH 7.0 (Add 3.12 gm of $NaH_2PO_4.2H_2O$ and 2.8 4gm of Na_2HPO_4 in 1000 ml of distilled water). Further, 1 ml of α- Napthyl Acetate and 20 mg of Fast Blue RR stain was added in the gel. The gel was incubated at room temperature until bands appear. The staining solution was replaced with fixative quickly.

Malate dehydrogenase (MDH, EC 1.1.1.37): Gel was kept in 50 ml of Tris Buffer; pH 8.5. Further, 70 mg of L- malic acid, mono-Na salt, 0.5 ml of 1% NAD solution, 0.5 ml of 0.6% PMS solution (6 mg/ml) and 1 ml of 0.6% MTT solution (6 mg/ml) (10 mg/ml)and 20 mg of Fast Blue RR stain was added in the gel. The gel was incubated at room temperature until bands appear. The staining solution was replaced with fixative quickly. The gel was incubated at room temperature until bands appear. The staining solution was replaced with fixative quickly.

Malic enzyme (ME, EC 1.1.1.40): Gel was kept in 50 ml of Tris Buffer; pH 8.0. Further, 100 mg of L- malic acid, mono-Na salt, 1.0 ml of 1% NADP solution (10 mg/ml), 0.5 ml of 10% $MgCl_2$ solution (100 mg/ml), 0.4 ml of 0.6% PMS solution (6 mg/ml) and 1.5 ml of 0.6% MTT solution (6 mg/ml) was added as a staining solution in the above gel. The gel was incubated at room temperature until bands appear. The staining solution was replaced with fixative quickly. The gel was incubated at room temperature until bands appear. The staining solution was replaced with fixative quickly.

Fixation and storage of the gel

After appearance of the bands, the gel was transferred to a fixative solution that contained 100 ml of 50% methanol, 20 ml of 10% acetic acid and 40 ml of distilled water. The gel was stored at 4^0C in refrigerator.

Statistical analysis of isozyme profile data

Each accession was considered a taxonomical operational unit (OTU) and isozyme bands were analysed as binary characteristics. The presence (coded as 1)/ absence (coded as 0) data matrix was prepared by scoring the gel. Relationships among the taxa were inferred with UPGMA clustering from Jaccard's similarity matrix of the bands. Data were analysed using the SAHN (Sequential Agglomerative Hierarchical and Nested) module of NTSYS-PC 2.11 (Numerical Taxonomy and Multivariate Analysis System) software program and dendrogram with bootstrap values was constructed by Darwin (version 5.0) software program [6].

Results

A total of 31 bands were recovered for esterases (EST), in 10 accessions with six loci viz. EST-1, EST-2, EST-3, EST-4, EST-5 and EST-6. Esterase loci (E1-E6) were numbered in sequence from the anode according to their decrease in negative charge. The locus EST-4 was monomorphic for all OTU's whereas EST- 5 was present in only accession A8. Locus EST-3 was present in three accessions viz. A2, A8 and A9.

With Peroxidase (PRX) resolution, a total of twenty two bands were resolved with maximum (three) loci viz. PRX-1, PRX-2 and PRX-3 for accessions A3 and A5. Loci PRX-1 and PRX-2 were monomorphic for all accessions. Locus PRX- 3 was found to be polymorphic and represented by the accessions A3 and A5.

Malate Dehydrogenase (MDH) enzyme system was monomorphic for all the accessions showing single locus for each. Overall, 10 bands were recovered in all the accessions.

Malic enzyme was represented with thirty seven bands with four loci viz. ME- 1, ME- 2, ME- 3 and ME- 4. Locus ME-2 was found to be polymorphic whereas remaining three loci i.e. ME-1, ME-2 and ME-3 were monomorphic for all accessions of *A. falcata*.

A total of 100 bands with fourteen putative loci in the four different enzyme assays were resolved with sufficient consistency and clarity. The number of polymorphic loci was 7. The polymorphism ranged from 25 - 83.33%. Maximum polymorphism (83.33%) was achieved through Esterase followed by Peroxidase (33.33%). and minimum for Malic enzyme (25%). MDH revealed only monomorphic bands (Table 1). Overall 50% polymorphism was depicted by ten accessions of *A. falcata* with four enzyme systems (Table 1).

Cluster analysis

Data scored for 10 accessions of *A. falcate* (Table 2) with four enzyme systems, were used to generate similarity coefficients. The

genetic relatedness among the accessions revealed by unweighted pair group methods with arithmetic mean (UPGMA) cluster analysis is presented in dendrogram as below (Figure 1).

Cluster analysis was performed using isozyme data and it produced stable and consistent patterns. At 73% similarity level, the dendrogram revealed two major clusters; First cluster consisted of seven accessions (A1 to A7) representing different localities. The second cluster consisted of only three accessions viz. A8, A9 and A10 (Figure 2A-2D) with overall similarity of 82%, which was further sub divided into two sub clusters at overall similarity of 88%, consisting of A10 and A9 as one sub cluster and A8 as second sub cluster. Similarity between different accessions was laid between 63 to 94%. Maximum of 94% similarity was found between accessions A4- A5 and A4- A6 (Table 3).

Discussion

Isozymes electrophoresis is a powerful tool for population genetics [7] and the Sodium Dodecyl Sulphate-Native Poly Acrylamide Gel Electrophoresis technique is particularly considered as a reliable way because storage proteins are largely independent of environmental fluctuations [8,9]. The International Union for Protection of New Varieties of Plants (UPOV) has harmonized and adopted test guidelines and procedures for the use of isozyme electrophoresis as a characteristic for establishing uniqueness of plants [10]. Due to its simplicity and validity for describing genetic structure of groups of plants [11] resolving systematic relationships and inter and intra specific studies [12].

Isozyme profiling of four enzyme systems viz. Esterases, Peroxidases, Malate dehydrogenase and Malic enzyme were exploited to find out the diversity within and among accessions of Ringal species. The isozyme profiling revealed significant diversity among and within accessions of Ringal species. Isozyme analysis of limited selection of bamboos from five genera was reported by [13].

In present study, Esterase and Peroxidase were found to be more polymorphic than other two systems i.e. Malate dehydrogenase and Malic enzyme. The Esterase fingerprints were the most distinguishing of the 4 enzymes analysed and indicated that the species constitute a relatively heterogenous group. Esterase is useful as a diagnostic tool for cultivar identification in view of the extensive polymorphism for this enzyme [14]. Esterase banding patterns in different species had shown variable loci (isoforms) with high polymorphism [14] among all four enzyme system. In *A. falcata*, total six isoforms were resolved on the gel whereas. The Esterase (EST), isozyme pattern recorded a total of 31 bands produced from six loci viz. EST-1, EST-2, EST-3, EST-4, EST-5 and EST-6. Esterase loci (E1-E6) were numbered in sequence from the anode according to their decrease in negative charge. Accessions A8 and A9 showed maximum of five loci. The locus EST-4 was monomorphic for all OUT's whereas EST- 5 was present in only accession A8. EST-3 was present in three accessions viz. A2, A8 and A9. A total of 7 isoforms were observed for this particular enzyme. Esterase has also been studied in different plants such as *Nicotiana* [15], *Musa* [14], *Allium* [16], *Pinus* [17] and Cotton [18]. Along with esterase, peroxidase was also appreciably polymorphic with maximum number of isoforms i.e. three. Various numbers of loci from one locus (*Citrullus*) to thirteen loci (maize) was reported [19].

A biochemical study was done using esterase and Peroxidase and phenolics to study the different banding patterns in genera *Arundinaria, Pseudosasa, Semiarundinaria, Shibataea, Sinobambusa*and *Yushania* [20]. Phylogenetic study among 16 species of eight genera were conducted by Li Shengfeng [21] by using peroxidase and esteraseand concluded that isozymes are suitable markers to distinguish among species and genera.

The mitochondrial MDH isozyme (Malate Dehydrogenase, MDH) pattern were more conservative and uniform with one band (monomorphic) for all species studied hence, less differentiating. Overall, 10 bands were recovered in all the accessions. In the study on *Lythrumsalicaria* [22], eight bands were reported.

Malic enzyme was represented with thirty seven bands with four loci viz. ME- 1, ME- 2, ME- 3 and ME- 4. ME-2 was found to be polymorphic whereas remaining three loci were monomorphic for all accessions of *A. falcata*. In *Pinushalepensis* [23], this enzyme is expressed by one locus only.

Malic enzyme showed four to five isoforms but Malate dehydrogenase was totally failed to induce polymorphism among accessions. Similar banding pattern of MDH was reported by Siddiquee et al. [24].

Results of isozyme analysis revealed very high percentage of similarity due to less discriminatory power of the isozyme marker [25]. Cluster analysis revealed very close proximity among the accessions of individual species, since dendrogram of each individual species inferred grouping of accessions into two major clusters with very high similarity percent in marvel grass [25].

Results and findings of the study revealed high percentage of similarity or low variability which is the reflectance of deterioration of natural resources of *A. falcata*. Therefore, there is a need to develop *ex situ* as well as *in situ* approach for conservation and enrichment of genetic resources of the species and unscientific harvesting should be kept under control.

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Locus name	Total no. of loci	No. of polymorphic loci	No. of monomorphic loci	Polymorphism (%)
EST	6	5	1	83.33
PRX	3	1	2	33.33
MDH	1	0	1	0
ME	4	1	3	25.0
Total	**14**	**7**	**7**	**50**

Table 1: Isozyme loci and the properties of resolved loci per accession of *A. falcate.*

Accession. No.	Place of Collection	Lattitude	Longitude	Altitude (m)
	Arundinaria falcata			
A1.	Hanumanchatti, Uttarkashi	30° 55.923 ' N	78° 23.951 ' E	2072.0
A2.	PithondiKhirsu. Pauri	30° 8.827' N	78° 46.472' E	1685.5
A3.	Maroda Village, Khirsu, Pauri	30° 8.827' N	78° 46.472' E	1685.5
A4.	Mandal I, Chamoli.	30° 27.593' N	79° 16.483' E	1499.5
A5.	Solan, H.P.	30° 54.238' N	77° 5.816' E	1446.0
A6.	Netwar, Uttarkashi	31° 3.789 ' N	78° 6.393 ' E	1524.0
A7.	Dewal, Chamoli	30° 3.665 ' N	79° 34.632 ' E	1488.9
A8.	Mandal II, Chamoli.	30° 27.593' N	79° 16.483' E	1499.5
A9.	Mandal III, Chamoli.	30° 27.593' N	79° 16.483' E	1499.5
A10.	Jarmola, Uttarkashi	30° 43.998 ' N	78° 26.394 ' E	1457.6

Table 2: Geographical details of accessions of *A. falcate.*

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
A1	1.00									
A2	0.75	1.00								
A3	0.88	0.88	1.00							
A4	0.81	0.69	0.81	1.00						
A5	0.75	0.75	0.88	0.94	1.00					
A6	0.75	0.63	0.75	0.94	0.88	1.00				
A7	0.81	0.81	0.94	0.88	0.94	0.81	1.00			
A8	0.63	0.75	0.63	0.69	0.63	0.75	0.69	1.00		
A9	0.81	0.81	0.81	0.75	0.69	0.69	0.75	0.81	1.00	
A10	0.81	0.69	0.81	0.75	0.69	0.81	0.75	0.81	0.88	1.00

Table 3: Similarity matrix index showing relatedness among the accessions of *A. falcata* using Isozyme marker.

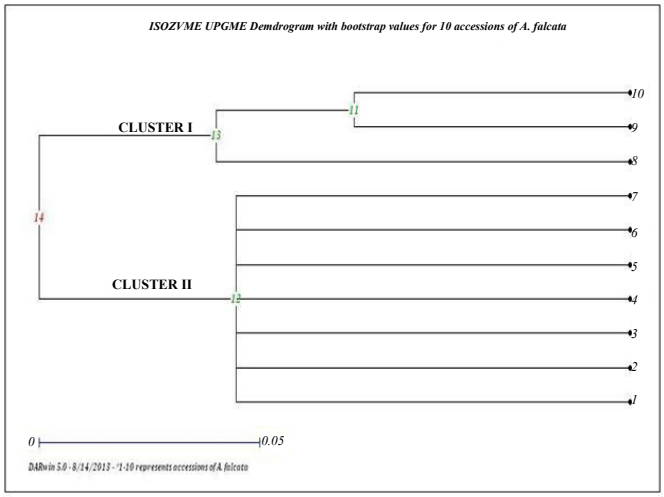

Figure 1: Illustration of UPGMA based dendrogram exhibiting genetic relationships among the accessions of *A. falcata* using Isozyme markers.

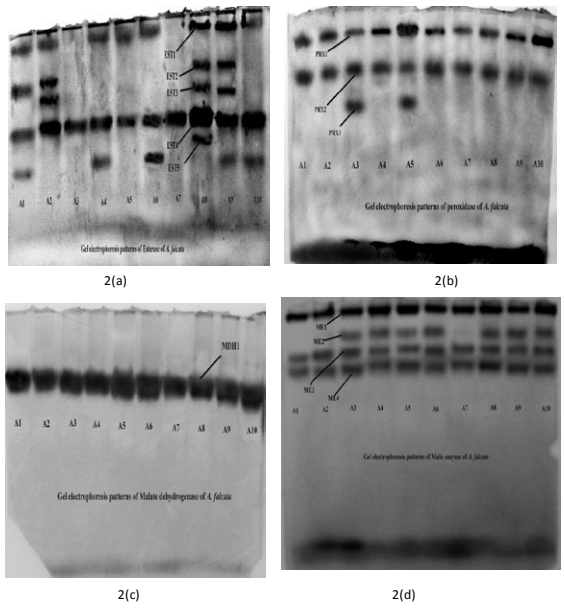

Figure 2A-D: Isozyme profile of accessions of *A. falcate*: represent isozyme profiles of esterase, peroxidase, malate dehydrogenase and malic enzyme respectively of 10 accessions of *A. falcata* (A1- A10)

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