Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; SP1: 515-519

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(Special Issue- 1)
2nd International Conference
"Food Security, Nutrition and Sustainable Agriculture - Emerging Technologies"
(February 14-16, 2019)

Genetic diversity assessment of *Sinarundinaria anceps* (Mitf.) Chao and Renvoize accessions through Isozyme markers

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Abstract
Hill bamboo (Ringal) is socio-economically and ecologically important bamboo in Himalayan range. *Sinarundinaria anceps* have wide variety of uses ranging from household to commercial purposes. Due to over use of the species, serious deterioration of natural resources have been recorded. In light of these facts, as attempt has been made to assess genetic diversity through isozyme marker with four enzyme system (peroxidase, esterase, malate dehydrogenase and malic enzyme) prevailed in the genotypes of *S. anceps* collected from different localities of Garhwal Himalayas (India) and established at Hill bamboo Germplasm at Khirsu (India). Native PAGE was performed for the resolution of isozymes and bands were scored as binary data (0 or 1). Data obtained was analyzed under cluster analyses by using Jaccard´s similarity coefficient and UPGMA method. Very high degree of similarity was reported i.e. 54 to 100% among different accessions. Dendrogram constructed revealed two major cluster with nine accessions in first cluster and one accession in second cluster. First cluster was further divided in three minor clusters. The results recorded reflected very low degree of genetic diversity which necessitates *in situ* conservation of the natural genetic resources of the species.

Keywords: Genetic diversity; dendrogram; polyacrylamide; Jaccard's coefficient; cluster analysis

Introduction
Hill bamboo (commonly known as Ringal) is socio- economically and ecologically important group of hill bamboo in Himalaya. *Sinarundinaria anceps* (Mitf.) Chao and Renvoize (old name *Sinarundinaria jaunsarensis,* Gamble) commonly known as Saura Ringal have wide variety of uses in the form of roofing, flooring material for houses, vegetables, medicines, fuel, fodder in periods of scarcity, stakes for cash crops, handicrafts, walking sticks, fishing rods, hookah pipes and baskets especially kiltas. These bamboos are widely used in Basketry that contributes significantly to the livelihood of poor rurals/artisans in this state. The species are restricted to cooler, damper sites and thrive at altitudes of 1800m- 2300m The species flowers irregualry at times, gregarious over large areas with flowering cycle of 45-55 years (Bahadur & Naithani, 1978) [18]. The study on genetic evaluation of this species is very limited and that is why it necessitates to explore genetic diversity prevailing in the natural reservoir of the species for the conservation of its genetic resources, broadening of the genetic base and practical applications in breeding programs. Safflower possesses considerable diversity across different regions of the world (Knowles, 1989). In order to design an appropriate breeding program, it is important to know how much the phenotypic variation of a trait is heritable (Kearsey and Pooni, 1996), since the efficiency of a selection program is mainly dependent on the magnitude of genetic variation and heritability of a trait (Falconer and Mackay, 1996). Genetic diversity of a crop species has been investigated using either agro-morphological traits or molecular markers. Genetic diversity is of considerable practical interest in any crop improvement programme. Precise information on the nature and degree of genetic divergence would help the plant breeder in choosing the right type of parents for different breeding programmes. Therefore, the present investigation was undertaken to study the nature and magnitude of genetic divergence in 150 germplasm accessions of safflower.

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.
Sinarundinaria anceps ver. Saura 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. *S. anceps* flowers irregularly at times, gregarious over large areas, while few culms may be found in flowers almost every year. After seeding the clumps died (Naithani *et al.* 2003) [18].
The whole set of genetic material of a species of plant is known as Germplasm of the organism. 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.
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 (Hamrick *et al.*, 1992) [9]. Enzymatic analyses are added tools for detecting diversity (Zeidler, 2000) [26]. 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 (Gottlieb, 1977) [8]. 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 (Murphy *et al*., 1996) [17].
The present work was therefore, undertaken to assess variability among accessions of *S. anceps* 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.

Material and Method

Sample Collection: The young leaves of selected accessions (Table.1)were collected from Hill Bamboo Germplasm, Khirsu (India), 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.

Table 1: Geographical details of accessions of *S. anceps*

Accession. No.	ID. No.	Place of Collection
C1.	18	Musk deer farm I, Chopta, Chamoli
C2.	20	Musk deer farm II, Chopta, Chamoli
C3.	21	Musk deer farm III, Chopta, Chamoli
C4.	24	Joshi math Chandi I, Chamoli
C5.	64	Van I, Chamoli
C6.	21	Chopta I, Chamoli
C7.	68	Van II, Chamoli
C8.	72	Van III, Chamoli
C9.	26	Joshi math Chandi II, Chamoli
C10.	22	Chopta II, Chamoli

Extraction of Enzymes: Two grams of young leaves were macerated to powder with liquid Nitrogen in a mortar – pestle and then 0.1 gm PVP and 5ml 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 4^0 C to collect the supernatants (Das and Mukherjee, 1997).

Electrophoresis of the Enzyme Extracts: Each sample was applied to a native discontinuous polyacrylamide gel (4% stacking, 10% separating gel) and the runs were performed on a mini gel apparatus in Tris- Glycine (pH 8.3) buffer. Four enzyme systems were examined in this study. They are peroxidase (PRX, E.C.1.11.1.7), esterase (EST, E.C. 3.1.1.1), malate dehydrogenase (MDH, E.C.1.1.1.37) and Malic enzyme (ME, EC 1.1.1.40).

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 40ml 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 (Sharifi *et al*., 2006) and dendrogram with bootstrap values was constructed by Darwin (version 5.0) software program.

Results: For Esterases (EST), a total of 17 bands were recovered in 10 accessions with three loci viz. EST-1, EST-2 and EST-3. The locus EST-3 was monomorphic for all OTU's. Locus EST-1 was present in C6, C8, C9 and C10 accessions whereas locus EST- 2 was present in only three accessions i.e. C6, C8 and C10.

With Peroxidase (PRX) resolution, a total of eighteen (18) bands were resolved with maximum (three) loci viz. PRX-1, PRX-2 and PRX-3. All three loci were polymorphic. Locus PRX-1 was found in all accessions except C4, C5 and C7 whereas locus PRX-2 was not found in the accession C7. Locus PRX-3 was recorded for C7 only.

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 three (33) bands with five loci viz. ME- 1, ME- 2, ME- 3, ME- 4 and ME- 5. All loci were found to be polymorphic except ME-1. Locus ME- 2 was found in only C7 whereas ME- 3 was absent in C10.

A total of 87 bands with twelve putative loci in the four different enzyme assays were resolved with sufficient consistency and clarity. The number of polymorphic loci was 8. The polymorphism ranged from 60 - 100%. Maximum polymorphism was achieved through Peroxidase (100%) followed by Esterase (66.67%) and minimum for Malic enzyme (60%). MDH revealed only monomorphic bands. Overall 75.56% polymorphism was depicted by ten accessions of *S. anceps* with four enzyme systems (Table 02).

Table 2: Isozyme loci and the properties of resolve loci per accession of *S. anceps*

Locus name	Total no. of loci	No. of polymorphic loci	No. of monomorphic loci	Polymorphism (%)
EST	3	2	1	66.67
PRX	3	3	0	100.0
MDH	1	0	1	0
ME	5	3	2	60.0
Total	12	8	4	75.56

Cluster analysis

Data scored from 10 accessions of S. anceps 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 Fig. 01.

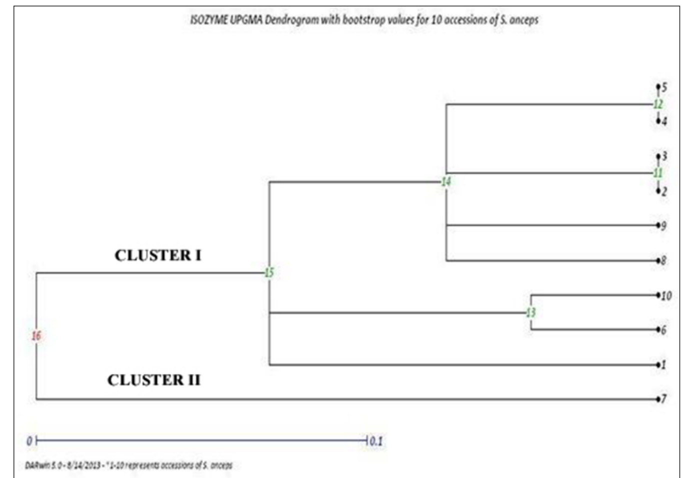

Fig 1: Illustration of UPGMA based dendrogram exhibiting genetic relationships among the accessions of *S. anceps using* Isozyme markers

Taxonomic Classification	
Clusters	**Accessions**
Cluster I	C5, C4, C3, C2, C9, C8, C10, C6, C1
Cluster II	C7

Cluster analysis was performed using isozyme data and it produced stable and consistent patterns. At 62% similarity level, the dendrogram revealed two major clusters; one cluster consisted of nine accessions. Second cluster contained only one accession i.e. C7. Cluster one was further divided in to three sub- clusters having 6, 2 and 1 accessions respectively at 77% similarity. Similarity between different accessions was laid between 54 to 100%. Accession pairs C4- C5 and C2- C3 were grouped with 100% similarity. Most dissimilarity (46%) was obtained among the accessions C9 and C10 with C7. (Table 03)

Table 3: Similarity matrix index is showing relatedness among the accessions of *S. anceps* using Isozyme marker

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
C1	1.00									
C2	0.85	1.00								
C3	0.85	1.00	1.00							
C4	0.77	0.92	0.92	1.00						
C5	0.77	0.92	0.92	1.00	1.00					
C6	0.77	0.77	0.77	0.69	0.69	1.00				
C7	0.69	0.69	0.69	0.62	0.62	0.62	1.00			
C8	0.77	0.77	0.77	0.85	0.85	0.85	0.62	1.00		
C9	0.69	0.85	0.85	0.92	0.92	0.77	0.54	0.92	1.00	
C10	0.69	0.85	0.85	0.77	0.77	0.92	0.54	0.77	0.85	1.00

Discussion

Isozymes electrophoresis is a powerful tool for population genetics (Parker *et al.*, 1998) [19] and the SDS-Native PAGE technique is particularly considered as a reliable way because storage proteins are largely independent of environmental fluctuations (Javid *et al.*, 2004 and Iqbal *et al.*, 2005) [12, 11]. 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 (UPOV, 1997) [25]. Due to its simplicity and validity for describing genetic structure of groups of plants (Sher *et al.*, 2010) [22], resolving systematic relationships and inter and intra specific studies (Karihaloo *et al.*, 2002) [14].

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 Heng *et al.* (1996) [14].

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 (Jarret and Litz, 1986) [13]. Esterase banding patterns in different species had shown variable loci (isoforms) with high polymorphism (Jarret and Litz, 1986) [13] 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* (Bhatiya *et al.*, 1967) [2], *Musa* (Jarret and Litz, 1986) [13], *Allium* (Abdul Wahab, 1994), *Pinus* (Gonzalez-Andres *et al.*, 1999) [7] and Cotton (Farooq *et al.*, 1999) [6]. 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 (Brewbaker *et al.*, 1985) [3].

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* (Chou *et al.*, 1986) [4]. Phylogenetic study among 16 species of eight genera were conducted by Li Shengfeng (1989) by using peroxidase and esterase and 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 *Lythrum salicaria* (Strefeler *et al.,* 1996) [24], 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 *Pinus halepensis* (Loukas *et al.*, 1982) [16], 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.* (2010) [23].

Results of isozyme analysis revealed very high percentage of similarity due to less discriminatory power of the isozyme marker (Saxena and Chandra, 2010) [20]. 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 (Saxena and Chandra, 2010) [20].

Acknowledgment

Our profound thanks to Forest Research Institute (FRI), Dehradun, India for providing the facilities. Authors are also thankful to the staff of Plant Physiology Discipline, Forest Research Institute (FRI), Dehradun, India for providing necessary assistance.

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