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Study of biochemical variability in the populations of *Aconitum balfourii* by soluble protein and isoenzyme electrophoretic patterns

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ABSTRACT
Biochemical variability between four populations of Aconitum balfourii (Ranunculaceae) was studied by seed protein and isozymes electrophoretic patterns. Electrophoregram of SDS-PAGE showed a total of 20 bands with molecular weight ranging from 12 kDa to 82 kDa with difference in number of bands, band width and intensity between different populations. In the isozyme analysis four enzyme systems viz. Esterase, Malate dehydrogenase, Superoxide dismutase and Catalase were studied. 7 out of 8 resolved loci (87.5%) were polymorphic. Based on presence and absence of bands Nei and Lei's similarity index was calculated. Similarity ranged from 64.1% to 74.4%. Matrix derived from both SDS-PAGE and Native-PAGE data were used to construct UPGMA dendrogram. The dendrogram showed two main clusters.

Key words: Isozymes, Aconitum, Catalase, Malate dehydrogenase, Esterase, Superoxide dismutase.

INTRODUCTION

Aconitum atrox (Bruhl) Muk. Syn. *A. balfourii* Stapf (Ranunculaceae), is distributed in central sub Himalaya from 2400 to 4500m. Tubers of *A. balfourii* contain a crystalline toxic alkaloid called pseudo aconitine (0.4-0.5%) and a small quantity of aconitine, picroaconitine, aconine, benzyl aconine and hemonapalline [2]. It is medicinally used as anti-inflammatory, as an analgesic, vermifuge, anti-rheumatic, dries up serious fluid, against all types of pain and inflammation from gout or arthritis, all disorders due to worms, micro organisms, amnesia, loss of heat, leprosy and paralysis [16,13]. Like any other medicinal plant species, *Aconitum atrox* have also become critically endangered due to over exploitation of the underground tubers.

Over the long term, the ability of a population to respond adaptively to environmental changes depends on the level of genetic variability or diversity it contains [8]. During the process of evolution, genetic differentiation by natural selection to facilitate reproductive isolation involves the presupposition of the origin of geographic races, subspecies and species [9]. A species without enough genetic diversity is thought to be unable to cope with changing environments or evolving competitors and parasites [4].

Therefore studies of population genetic diversity and the structure of population within a species may not only illustrate the evolutionary process and mechanism but also provide information useful for biological conservation of *Aconitum balfourii* an endangered medicinal herb grows wild in the Himalayan ranges of India.

Electrophoretic analysis of protein and isoenzymes offers an efficient and cost effective method towards evaluation of geographical and taxonomic distribution of genetic variation for sampling strategies in germplasm conservation [1].

In the present work polypeptide pattern and isozyme markers are used to locate genetic variations and relationships between four populations of *Aconitum balfourii*.

EXPERIMENTAL SECTION

Plant material:

Seed of *Aconitum balfourii* used for the analysis were collected from four alpine and subalpine habitats namely Panwali Kantha (3120m), Yamnotri (3200m), Madhya Maheshwar (4000m) and Kilpur (3500m) in Garhwal Himalaya (Table: 1).

Tables 1: Location of *Aconitum balfourii* populations analysed in the study.

Populations	Abbreviation	Altitude(m asl)	Latitude(N)	Longitude(E)
Panwali Kantha	**PK**	**3120**	30°34'798"	76°52'634"
Yamnotri	**YM**	**3200**	30°59'917"	78°27'806"
Madhya Maheshwar	**MD**	**4000**	30°39'022"	79°14'826"
Kilpur	**KP**	**3500**	-	-

Protein extraction and estimation:

50 mg seeds of each population were soaked in water for 24 hours. Total soluble proteins were extracted by homogenization of the seeds with 0.1 M tris- HCl buffer (pH 7.2; containing 1% PVP, 0.5% β mercaptoethanol and 0.1μM PMSF) at 4^0C in a prechilled mortar and pestel. Homogenates were centrifuged at 10,000xg for 10 min. Supernatants were collected and stored at 4^0C. Total soluble protein contents in the seeds were estimated according to dye binding principle of Bradford [15].

SDS-Polyacrylamide Gel Electrophoresis

The protein extracts were mixed with equal volumes of Lammili buffer [19] containing 0.065 M Tris-HCl buffer (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.5% bromophenol blue. The mixture was heated in boiling water bath for 5 minutes and then centrifuged at 10,000xg for 10 minutes. SDS-Polyacrylamide gel electrophoresis was performed under discontinuous gel system – 5% stacking gel (0.125M Tris- HCl, pH 6.8) and 10% separating gel (0.375 M Tris-HCl, 8.8) initially at 20 milli ampere (mA) till the sample was in stacking gel and after that at 60mA. Polypeptides were visualised by staining the gel with coomassie brilliant blue R-250. Destained gels were scanned by ALPHA – IMAGER.

Native-Polyacrylamide Gel Electrophoresis

The protein extracts were mixed with equal volumes of 0.065 M Tris HCl buffer containing 10% glycerol and 0.5 % bromophenol blue and loaded in Polyacrylamide gel electrophoresis. Superoxide Dismutase, Esterase and Malate Dehydrogenase were resolved in 10% separating gel and Catalase was resolved in 8% separating gel. Staining recipe for each isozyme is given in Table: 2. After staining, the gels were washed properly and visualized by ALPHA – IMAGER.

Table 2: The enzyme analysed and their staining recipes

Enzyme	IUBMB(1)	Staining recipe	Reference	Section
Esterase	E.C. 3.1.1.(2)	0.1 M Na – Phosphate buffer pH 6.2, 0.1 % Fast Blue RR salt, 0.05% α- naphthyl acetate	[1, 2]	Hydrolase
Malate Dehydrogenase	E.C.1.1.1.37			Oxido-reductase
Superoxide Dismutase	E.C.1.15.1.1	50mM potassium phosphate pH 7.8, 0.1 mM EDTA , 0.2 % TEMED , 1% riboflavin, 100 mg NBT for 100 ml staining solution	[3]	Oxido-reductase
Catalase	E.C. 1.11.1.6	0.01% H_2O_2, 1% $FeCl_3$, 1% $K_3Fe(CN)6$ in distill water	[4]	Oxido-reductase

Cluster Analysis:

Protein bands were scored depending on their presence (1) and absence (0) of bands. Nei and Lei's similarity indices determined and hierarchical clustering was constructed by Unweighted Paired Group Method with Arithmetic average (UPGMA). The computer softwere NTSYSpc-2.02e was used for this purpose.

RESULTS AND DISCUSSION

SDS-PAGE resolved a total of 69 bands in four populations of *A. balfourii* (Figure: 1). These SDS protein bands belonged to different molecular weight ranging from 12 to 82 kDa. The relative mobilities of these bands varied from 0.17 to 0.95 in the studied populations. Eleven polypeptide bands exhibiting Rm of 0.17, 0.25, 0.33, 0.36, 0.37, 0.59, 0.70, 0.85, 0.86, 0.89 and 0.95 representing molecular weight of 82, 60, 55, 50, 49, 29, 22, 15, 14.8, 14 and 12kDa were present in all populations (Table: 3). Populations of Kilpur (KP) and Panwali Kantha (PK) exhibit maximum number of bands followed by populations of Yamnotri (YM) Madhya Maheshwar (MD). Polypeptide bands of Rm 0.31, 0.55 and 0.65 were unique to the populations of Panwali Kantha and a polypeptide band of Rm 0.38 was unique to the population of Kilpur.

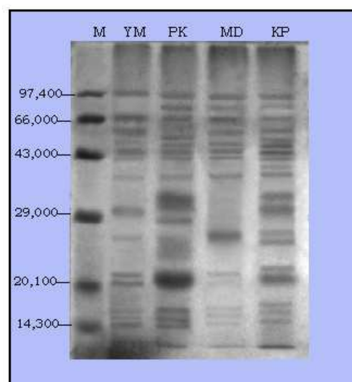

Figure: 1. Protein profiling of Aconitum balfourii Stapf. Populations

(M: protein molecular weight marker in Dalton; YM: Population from Yamnotri; PK: Population from Panwalikantha; MD: Population from Madhyamaheshwar; KP: Population from Kilpur)

Table 3: SDS – PAGE Seed Protein Profile of *Aconitum balfourii*

Popul.	+/- of protein bands(Rm values)																							total bands
.	0.17	0.22	0.25	0.27	0.28	0.31	0.33	0.36	0.37	0.38	0.45	0.51	0.54	0.55	0.59	0.62	0.65	0.70	0.77	0.85	0.86	0.89	0.95	
KP	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	**20**
MD	+	+	+	+	-	-	+	+	+	-	+	-	-	-	+	-	-	+	-	+	+	+	+	**14**
PK	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	**20**
YM	+	-	+	-	+	-	+	+	+	-	+	-	+	-	+	-	-	+	+	+	+	+	+	**15**
Mol. Wt 69 *(K Da)*	81.28	70.79	60.07	63.1	61.66	57.54	54.95	50.12	48.98	47.86	40.74	35.48	32.36	31.62	28.84	26.3	25.12	21.88	18.62	15.14	14.79	13.8	12.02	

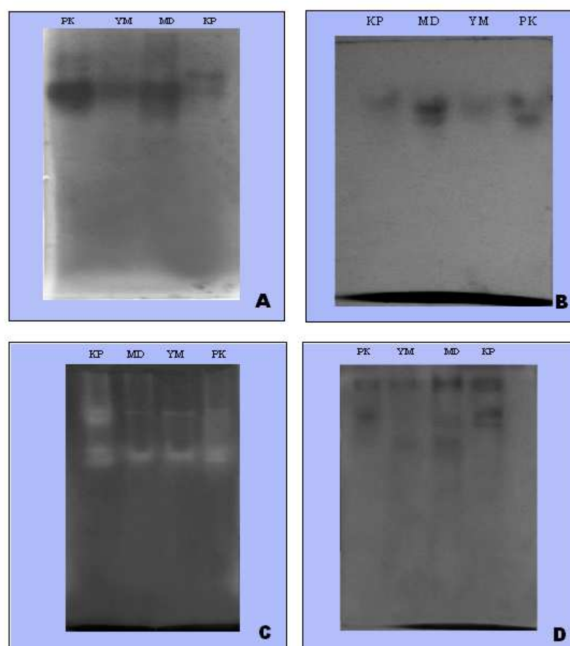

Figure: 2. Isozyme (Esterase (A); Malate Dehydrogenase (B); Superoxide dismutase (C) and Catalse (D))pattern of Aconitum balfourii Stapf. Populations (M: protein molecular weight marker in Dalton; YM: Population from Yamnotri; PK: Population from Panwalikantha; MD: Population from Madhyamaheshwar; KP: Population from Kilpur)

Table 4: Banding patterns and relative mobilities (Rm) of different isozymes for four populations of *Aconitum balfourii*

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Isozymes	Rm	populations				Total bands
		KP	MD	PK	YM	
Esterase	0.12	-	+	+	-	**5**
	0.15	-	+	+	-	
	0.18	+	-	-	-	
	0.24	-	+	+	+	
	0.27	+	+	+	+	
Malate Dehydrogenase	0.25	+	+	+	+	**2**
	0.32	-	+	+	-	
Superoxide dismutase	0.21	+	+	+	+	**4**
	0.22	+	-	-	-	
	0.32	+	+	+	+	
	0.36	+	-	+	-	
Catalase	0.07	+	+	+	+	**5**
	0.09	+	+	+	+	
	0.19	+	+	+	-	
	0.23	+	+	-	-	
	0.35	-	+	-	+	

The banding pattern of isozymes Esterase, Malate dehydrogenase, Superoxide dismutase and Catalase showed comparable patterns in the studied populations of *A. balfourii* (Table: 4; Figure: 2). They displayed 7 polymorphic loci (87.5%) and 2.14 alleles expressed per polymorphic loci according to the interpretation of the produced banding patterns (Table: 5) .

Table5: The number of enzyme loci, polymophic loci and alleles expressed for each of isozyme in studied populations of *A. balfourii*

Enzymes	Number of		
	Total loci	Polymorphic loci	Alleles expressed by polymorphic loci
Esterase	2	2	5
Malate dehydrogenase	1	1	2
Superoxide dismutase	2	2	4
Catalase	3	2	4
Total	**8**	**7**	**15**
% of polymorphic loci = 87.5 %			

The genetic similarity values estimated based on scored data ranged from 64.1% to 74.4% (Table: 6). The dendrogram revealed two major cluster A and B. cluster B contained population of Panwali Kantha. The C subgroup of cluster A contained populations of Yamnotri and Madhya Maheshwar (Figure: 3). The populations of Yamnotri and Madhya Maheshwar were found to be most similar in terms of Nei and Lei's similarity coefficient (74.4%).

Table 6: Similarity index for the Electreophoretic profile of SDS – PAGE and Native PAGE

	KP	MD	PK	YM
KP	**1.0**			
MD	**0.641**	**1.0**		
PK	**0.641**	**0.692**	**1.0**	
YM	**0.692**	**0.744**	**0.641**	**1.0**

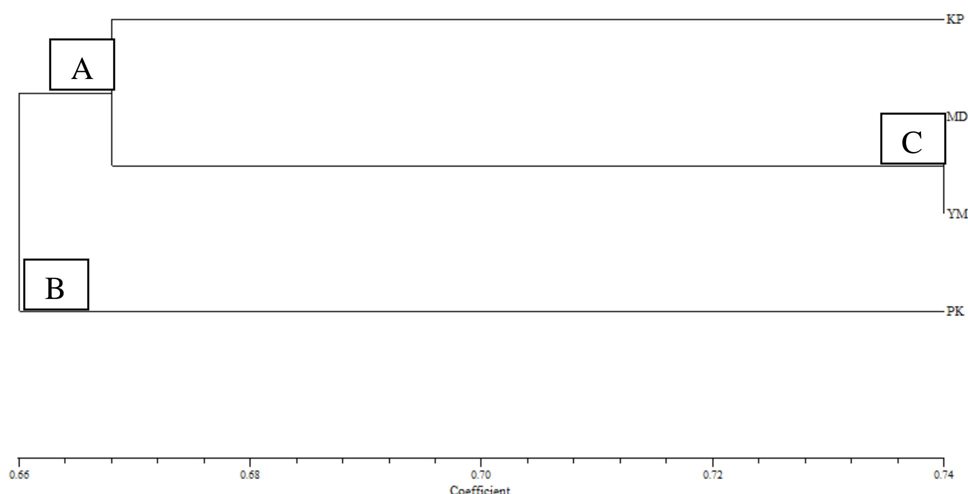

Fig 3. Dendrogram demonstrating relationship between four populations of A. balfourii based on compiled data set of SDS-PAGE and Native- PAGE

Polypeptides of high, intermediate and low molecular weight were resolved from the seed protein of different populations of *A. balfourii* [6]. They also found considerable Esterase isozyme variability between studied populations. Considerable variation in polypeptides profile of the leaves of three *Polygonum* species when grown in different altitudes [20]. At the end of one month after transplantation polypeptides of low molecular weight dominated at lower and middle altitudes. Considerable variability was found in the seed polypeptide patterns of different populations of *A. heterophyllum* [17].

Biochemical variability between thirteen European and five Czech potato (*Solanum tuberosum* L.) cultivars grown in the Czech Republic was found using soluble protein, isoesterase, and isoperoxidase electrophoretic patterns [10]. It was confirmed that cultivar difference in protein polymorphism can be revealed by applied electrophoretic pattern.

During the process of evolution, genetic differentiation by natural selection to facilitate reproductive isolation involves the presupposition of the origin of geographic races, subspecies and species [9]. A species without enough genetic diversity is thought to be unable to cope with changing environments or evolving competitors and parasites [4]. Therefore studies of population genetic diversity and the structure of population within a species may not only illustrate the evolutionary process and mechanism but also provide information useful for biological conservation.

As predicted by population genetic theory, loss of genetic variation is a major threat to endangered species with small populations or located in narrow geographic areas. A low level of genetic variability often results in minor fitness of individuals [11, 14], reduces the viability or adaptability of populations in changing environments [3], and in extreme cases causes the extinction of species. These effects may be most pronounced in species that are self compatible and/or have limited seed dispersal ability.

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REFERENCES

[1] AHD Brown; Isoenzymes, *Theor. Appl. Genet.*, **1978**, 52, 145-157

[2] Anonymous. Wealth of India, Revised. Vol. 1. Publication and Information Directorate, CSIR, New Delhi. **1985**, 429

[3] A Young; T Boyle; T Brown; *Trends in Ecology and Evolution.*, **1996**, 11, 413- 418

[4] BA Schaal; WJ Leverich; SH Rogstad; Comparison of methods for assessing genetic variation in plant conservation biology. In: Falk DA, Holsinger KE (Eds.), Genetics and Conservation of Rare Plants. Oxford University Press, New York, **1991**, 123-134

[5] CP Kuniyal; H Lata; C Rajesekaran; R Bahuguna; P Prasad; SK Bhadula; *Physiol. Mol. Biol. Plants*, **2001**, 7(2), 145-148

[6] FJ Ayala, JA Kiger. Modern Genetics, 2nd Edition, Benjamin/ Cummings, Menlo Park USA. **1984**.

[7] GL Stebbins. *Am. J. Bot.,* **1999**, 86, 1207-1208

[8] J Batra; V Curn; J Divis; *Plant soil science,* **2003,** 49, 230-236.

[9] JGB Oostermeijer; R Van't Veer; JCM den Nijs; *Journal of Applied Ecology,* **1994**, 31, 428- 438
[10] K R Kirtikar, B D Basu. Indian medicinal Plants. Vol I. Bishen Singh, Mahendra pal Singh, Dehradun, India. **1935**.
[11] M Fischer; D Matthies. *Am. J. Bot.,* **1998**, 85, 811- 819.
[12] M M Bradford. *Anal. Biochem.*, **1976**, 72, 248-254
[13] O Stapf**.** *Ann. Roy. Bot. Gard. Calcutta*. **1905**, 10 (2), 115-181
[14] R Bahuguna; MC Purohit; BP Nautiyal; V Prakash; MC Nautiyal; AN Purohit, *Physiol. Mol. Biol. Plants.,* **2003**, 9(1), 95-100
[15] UK Laemilli, *Nature*, **1970**, 227, 680- 685
[16] V Prakash; P Prasad; MC Nautiyal, *Physiol. Mol. Biol. Plants.,* **2002**, 8(2), 267-277