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Assessment of genetic diversity of *Indigofera pulchella* in Himachal Pradesh using isozyme markers

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ABSTRACT

***Indigofera pulchella*, a well known medicinal plant, has recently gained the attention of floriculturists due to its bright violet flowers. For efficient conservation and management of medicinal plant diversity, the genetic composition of species collected from different phyto-geographical areas need to be assessed. Isozyme analysis was conducted for the purpose and nine enzyme systems were investigated for 30 *Indigofera* genotypes collected from ten sites in five districts of Himachal Pradesh. The five enzyme markers, *viz.* PER I, CAT I, MDH I, ADH Ib and PPH I at RM values 0.60, 0.30, 0.50, 0.58 and 0.45 respectively, successfully demarcated the six sites of Mandi, Hamirpur and Bilaspur districts from four sites of Solan and Sirmour districts. Seven enzyme systems were found to be polymorphic, whereas two enzymes systems were monomorphic. Allele frequency in the present study ranged from 0.5 to 1.0. Polymorphism in the selected *Indigofera* genotype was estimated to be 30 per cent.**

Key words: *Indigofera pulchella*, genetic marker, isozymes, polyacrylamide gel electrophoresis.

INTRODUCTION

Genus *Indigofera* belongs to family Fabaceae and consists of about 700 different species all over the world. It is commonly named as "*kathi*". It grows naturally in tropical and temperate Asia, as well as in parts of Africa. Few species are distributed throughout India and *Indigofera pulchella* has spread in the Himalayas and other hills of India (Chauhan, 2). *Indigofera* spp. has multiple purposes, it has numerous medicinal and cosmetic use and also produces a blue dye material known as indigo. Due to indiscriminate use of the resources overtime and fragmentation of habitat, many of medicinal plant species are increasingly threatened and face the risk of becoming genetically impoverished. It is imperative that viable strategies be developed to conserve the surviving populations and thus assessment of genetic diversity is necessary (Singh *et al*., 10).

Molecular methods provide valuable tools for precise identification of plants at any stage of their growth and development. Isozymes analysis offers the most reliable single gene markers (Arulsekar and Parfitt, 1) and polymorphism at enzyme loci were shown to be stable under a number of environmental conditions. Moreover, results obtained with isozymes are easily repeatable and can be carried out without field experiments thereby saving money, time and space (Udupa *et al*., 12). In the present study, variability of *Indigofera pulchella* germplasm was evaluated using isozyme analysis with genotypes of various geographic locations of Himachal Pradesh.

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MATERIALS AND METHODS

Thirty *Indigofera pulchella* genotypes were collected from ten sites in five districts, *viz*., Mandi, Hamirpur, Bilaspur, Solan and Sirmour of Himachal Pradesh, which have been assigned code (Table 1). Nine enzyme systems investigated were, *viz*., peroxidase (PER), acid phosphatase (ACP), aspartate amino transferase (AAT), esterase (EST), catalase (CAT), malate dehydrogenase (MDH), alcohol dehydrogenase (ADH), phosphorylase (PPH) and alkaline phosphatase (ALP). Fresh, young and green leaves were excised from each genotype and then stored in liquid nitrogen till further use. Three hundred milligrams of leaves were washed thoroughly and ground in pre-chilled pestle and mortar containing 1.5 ml of Tris-citrate buffer, pH 8.3 (Trizma base 6.2 g/l and citric acid 1.46 g/l) and 500 mg of PVP (polyvinypyrrolidone). The homogenate was filtered through two folds of muslin cloth and then centrifuged at 20,000 rpm for 20 min. at 4°C. The supernatant was taken and stored at -20°C till further study.

Electrophoresis was carried out for nine enzyme systems using miniVE electrophoretic and electrotransfer unit (Amersham Biosciences). The electrophoretic tank buffer was pre-chilled to 4°C to provide passive cooling. Two tray buffer systems were used for resolution of nine enzyme systems. First, tray buffer used was lithium borate (lithium hydroxide 12 g, boric acid 118.9 g, distilled water 10.0 l and pH 8.3) and gel buffer used was Tris citrate

Table 1. Geographical location of *Indigofera pulchella* genotypes.

District	Site	Site code	Sample code	Altitude (m)
Mandi	Harabagh	S_1	I_1	1100 to 1369
			I_2	
			I_3	
	Jogindernagar	S_2	I_4	
			I_5	
			I_6	
Hamirpur	Annu	S_3	I_7	785
			I_8	
			I_9	
	Awah Devi	S_4	I_{10}	
			I_{11}	
			I_{12}	
Bilaspur	Ghumarwin	S_5	I_{13}	350 to 670
			I_{14}	
			I_{15}	
	Bhararighat	S_6	I_{16}	
			I_{17}	
			I_{18}	
Solan	Jatoli	S_7	I_{19}	1365 to1400
			I_{20}	
			I_{21}	
	Kandaghat	S_8	I_{22}	
			I_{23}	
			I_{24}	
Sirmour	Rajgarh	S_9	I_{25}	900 to 3994
			I_{26}	
			I_{27}	
	Dhanech	S_{10}	I_{28}	
			I_{29}	
			I_{30}	

(Trizma base 62.0 g, citric acid 14.6 g, distilled water 10.0 l and pH 8.3) and this system was used for PER, EST, AAT, MDH and ADH assay. Second tray buffer used was sodium borate (sodium hydroxide 20.0 g, boric acid 185.5 g, distilled water 10.0 l and pH 8.0) and gel buffer was Tris-citrate (Trizma base 121.1 g, citric acid 0.2 M, distilled water 10.0 l and pH 8.8). This system was used for ACP, CAT, ALP and PPH. Polyacrylamide gel electrophoresis was carried out to separate various isozymes due to its high resolving power, transparency and chemical inertness. Anionic system was used for different enzyme systems. The enzyme extracts were loaded in each well with the help of a micropipette alongwith a drop of tracking dye (0.2 per cent bromophenol blue) in one well to work out the relative mobility (RM) values. Electrophoresis was carried at a constant current of 25 mA and 250 V till the marker dye reached the other end.

The bands of the enzyme activity were revealed by immersing the gels in specific histochemical stains. Staining recepies for all nine isozymes were performed as per the methods described by (Tanksely and Orton, 11). The genotypes were determined from the banding patterns obtained for each isozyme. In enzyme systems with multiple loci, the locus with greatest anodal migration was designated as I. Similarly, the fastest isozyme at a locus was represented by 'a' being controlled by allele A. The numbering of the additional loci and alleles within an enzyme system progressed

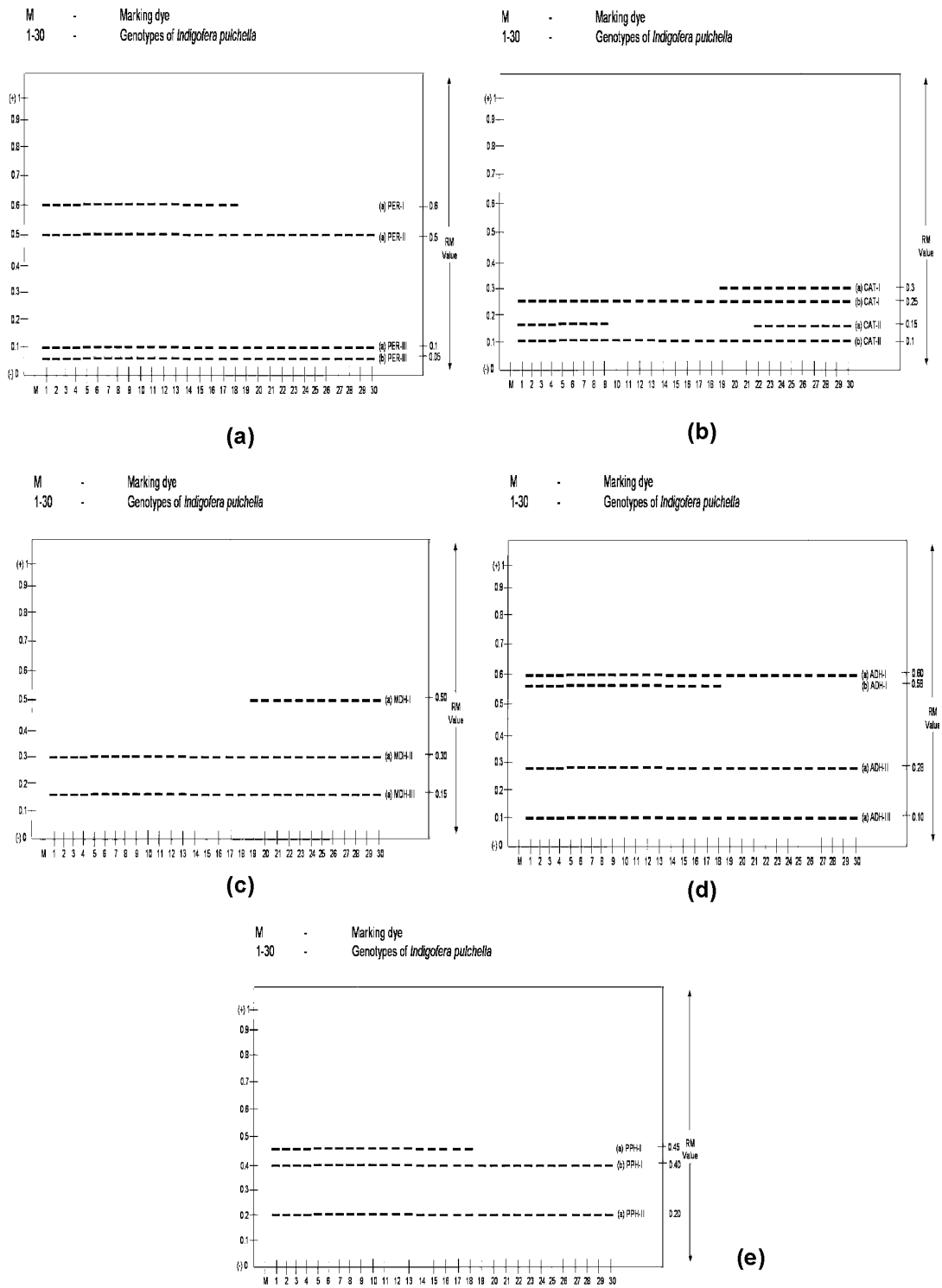

Fig. 1. Isozymic patterns of (a) Peroxidase, (b) Catalase, (c) Malate dehydrogenase, (d) Alcohol dehydrogenase, and (e) Phosphorylase. 1-3 = Harabagh,10-12 = Awah Devi,19-21 = Jatoli, 4-6 = Jogindernagar,13-15 = Ghumarwin, 22-24 = Kandaghat, 7-9 = Annu, 6-18 = Bhararighat, 25-27 = Rajgarh, and 28-30 = Dhanech.

sequentially in cathodal direction. The genotypes on the basis of isozymes were compared for each locus to assess genetic variability.

Different genetic parameters were obtained by analysing the data. The allele frequency was calculated as the proportion of a particular allele present at a given locus. Other genetic parameters were studied to differentiate the genotypes included the proportion of polymorphic loci, the polymorphic index, the average and effective number of alleles per locus and the observed (h_o) and expected heterozygosity (h_e). The observed and expected heterozygote frequencies were compared using F-statistics. The inter-relationships of genotypes could be found on the basis of the following parameters.

Similarity index was calculated according to Hunter and Kannenberg (Hunter and Kannenberg, 3) on the basis of presence or absence of bands. The identical bands in the particular enzyme system were rated as zero. Where two individuals differed for the presence or absence of a band within the same locus, these were given a rating of one. Thus, a diversity index comprised of cumulative ratings obtained in all the enzyme systems for each pair of the genotypes. Dendrogram was constructed from genetic distance values by the unweighted pair group arithmetic average (UPGMA) method and NTSYS-pc software version 2.0 to analyse the patterns of genotype relatedness.

RESULTS AND DISCUSSION

In the present study with ten sites were studied for *Indigofera pulchella* diversity in different geographical locations of Himachal Pradesh (Table 1). Out of nine enzyme systems studied, seven were found to be polymorphic in nature and only five (PER, CAT, MDH, ADH, PPH) were with informative markers and, were able to differentiate among the ten sites. The five markers: PER I, CAT I, MDH I, ADH Ib, PPH I at positions of RM values 0.60, 0.30, 0.50, 0.58, 0.45, respectively produced significant polymorphism and successfully demarcated the six sites of Mandi, Hamirpur and Bilaspur districts from other four sites of Solan and Sirmour districts. However, the two enzyme systems ALP and ACP were found to be monomorphic. Similar findings were reported with seven cultivars of *Actinidia deliciosa* (Shirkot *et al.*, 9) . The qualitative description of five enzyme systems which successfully differentiated the above six sites from other four sites were depicted in the form of zymograms.

Alcohol dehydrogenase (EC 1.1.1.1) produced three enzyme activity zones in thirty genotypes of *Indigofera pulchella* plant species. The band ADH IIb with RM value 0.58 was present in genotypes of site Harabagh and Jogindernagar of Mandi district, Annu and Awah Devi of Hamirpur district and Ghumarwin and Bhararighat of Bilaspur districts. The bands ADH Ia, ADH IIa and ADH IIIa with RM values 0.60, 0.28 and 0.10 respectively were found to be monomorphic in nature.

Catalase (EC 1.11.1.6) system displayed a banding pattern with two zones of enzyme activity. The anodal zone having locus CAT I showed two bands. CAT Ia with RM value 0.30, was present in genotypes of site Jatoli, Kandaghat, Rajgarh and Dhanech of Solan and Sirmour district. The band at position CAT IIa with RM value 0.15 was present in genotypes of site Harabagh and Jogindenagar of Mandi district and Annu of Hamirpur district. The CAT Ib with RM value 0.25 and CAT IIb with RM value 0.1 were found to be monomorphic.

Peroxidase (EC 1.11.1.7) enzyme resolved to produce three zones of enzyme activity. The anodal zone with locus PER I possessed a single band PER Ia with RM value of 0.60 in genotypes of Mandi, Hamirpur and Bilaspur districts only. The bands at positions PER IIa with RM value 0.50, PER IIIa and PER IIIb with RM value 0.10 and 0.05 respectively were smonomorphic. Malate dehydrogenase (EC 1.1.1.37) system produced three enzyme activity zones. The anodal zone, having MDH I showed single band MDH Ia with RM value 0.50 observed in genotypes of site Jatoli and Kandaghat of Solan district and Rajgarh and Dhanech of Sirmour districts. The bands at position MDH IIa and MDH IIIa with RM values 0.30 and 0.15 respectively were again monomorphic. Phosphorylase (EC 2.4.1.1) system produced two zones of enzyme activity. First most anodal zone having locus PPH I showed two bands, PPH Ia with RM value 0.45 present in genotypes of sites Harabagh, Jogindernagar, Annu, Awah devi, Ghumarwin and Bhararighat. Second band PPH Ib with RM value 0.40 was present in all genotypes. The second zone showed single locus, PPH II and single band PPH IIa with RM value 0.20 in all 30 *Indigofera pulchella* genotypes.

Genetic interpretation studies revealed that in total 22 isozyme loci were scored and the number of loci coding for individual enzyme ranged from 1 to 3 with identification of 26 alleles (Table 2). Allele frequency varied between 0.5 to 1.0 depending upon whether a locus displayed heterozygous or homozygous conditions. In thirty genotypes various monomorphic loci were identified as; PER II, PER IIIa, PER IIIb, ACP I, ACP II, AAT IIa, EST II, EST III, CAT Ib, CAT IIb, MDH II, MDH III, ADH Ia, ADH II, ADH III, PPH Ib, PPH IIa, ALP Ia, ALP Ib and ALP II. All the genotypes showed single band at these loci and were referred as homozygous for respective alleles. In the present investigation, polymorphic loci identified were PER I, AAT I, EST I, CAT Ia, CAT II a, MDH Ia, ADH Ib and PPH Ia. A locus was considered polymorphic if the frequency of the most common allele was not more than

Table 2. Genetic variation for different parameters between 30 *Indigofera pulchella* genotypes.

Parameter	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Total No. of loci	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22
Total No. of alleles	26	26	26	26	26	26	25	25	25	24	24	24	25	25	25	25	25	25	23	23	23	25	25	25	24	24	24	25	25	25
Proportion of polymorphic loci	0.272	0.272	0.272	0.272	0.272	0.272	0.227	0.227	0.227	0.181	0.181	0.181	0.181	0.181	0.181	0.227	0.227	0.227	0.136	0.136	0.136	0.181	0.181	0.181	0.181	0.181	0.181	0.181	0.181	0.181
Polymorphic index	0.136	0.136	0.136	0.136	0.136	0.136	0.113	0.113	0.113	0.090	0.090	0.090	0.090	0.090	0.090	0.113	0.113	0.113	0.068	0.068	0.068	0.090	0.090	0.090	0.090	0.090	0.090	0.090	0.090	0.090
Average No. of alleles per locus	1.181	1.181	1.181	1.181	1.181	1.181	1.136	1.136	1.136	1.090	1.090	1.090	1.136	1.136	1.136	1.136	1.136	1.136	1.045	1.045	1.045	1.136	1.136	1.136	1.090	1.090	1.090	1.136	1.136	1.136
Effective No. of alleles per locus	0.058	0.058	0.058	0.058	0.058	0.058	0.057	0.057	0.057	0.055	0.055	0.055	0.052	0.052	0.052	0.057	0.057	0.057	0.054	0.054	0.054	0.052	0.052	0.052	0.055	0.055	0.055	0.052	0.052	0.052
Mean observed heterozygosity (h_o)	0.272	0.272	0.272	0.272	0.272	0.272	0.227	0.227	0.227	0.181	0.181	0.181	0.181	0.181	0.181	0.227	0.227	0.227	0.136	0.136	0.136	0.181	0.181	0.181	0.181	0.181	0.181	0.181	0.181	0.181
Expected heterozygosity (h_e)	0.136	0.136	0.136	0.136	0.136	0.136	0.113	0.113	0.113	0.090	0.090	0.090	0.090	0.090	0.090	0.113	0.113	0.113	0.068	0.068	0.068	0/090	0/090	0.090	0.090	0.090	0.090	0.090	0.090	0.090
F-statistics	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

0.99 (Nei, 5). The proportion of polymorphic loci varied from 0.181 to 0.272 and the average number of alleles per locus showed values 1.181 in the samples of Mandi district, 1.136 in Annu, Bilaspur district, Kandaghat and Dhanech and 1.090 in the samples of Awah Devi, and Rajgarh. Effective number of alleles per locus values was found to be 0.058, 0.057.0.055 and 0.052. The mean observed heterozygosity was found to be 0.136, 0.113, 0,090 and 0.068. F-statistics showed negative values suggesting more heterozygosity than depicted (Table 2) This suggests that populations are not completely inbred. However, such observation may be the result of very small sample size. Nei reported that in order to reduce the sampling error heterozygosity, and to examine alleles frequency distribution a large number of loci rather than large number of individuals per locus must be examined (Nei, 5).

Isozyme polymorphism data was analysed considering all the bands obtained from nine enzymes. The similarity index revealed all the thirty varieties in the range of 0.72 to 1.00 (Table 3). According to the dendrogram thirty genotypes were divided into two major clusters (Fig. 2) The six sites of Mandi, Hamirpur and Bilaspur were grouped into one cluster showing 90.8% similarity whereas the four sites of Solan and Sirmour district were differentiated into another cluster with 93% similarity. The six genotypes from Harabagh and Jogindernagar sites of Mandi district depicted 100% similarity. Other eight sites also depicted 100% intrasite similarity except Kandaghat with three genotypes showing 95.6% similarity. Maximum dissimilarity was observed in I_1 genotype of Harabagh site in Mandi district and I_{24} genotype of Kandaghat site of Solan district. Thus, there was no intrasite variation in ten sites, whereas significant intrersite variation has been observed. All thirty genotypes showed 77% similarity and 23% dissimilarity using a set of nine isozyme systems.

Isozymes polymorphism and analysis has been successfully applied for cultivar identification in various plants (Sanchez *et al*., 7), but there have been no attempts to study isozyme variation in *Indigofera pulchella*. Isozymes polymorphism has been investigated in few medicinal plants such as *Podophyllum hexandrum* Royle by Sharma *et all*., (8), studied the population from Chamba and Kullu districts of Himachal Pradesh and observed 65 per cent level of similarity. In *Phyllanthus emblica* (Umashankar and Ganeshaiah, 13) genetic diversity was mapped for *in situ* conservation of genetic resources in the southern states of Karnataka, Tamilnadu and Kerela. Rogge *et al.* (6) characterized 70 clone families of *Picea abies*, prior to establishment of seed orchard using isozymes. Ivanek (4) studied isozyme polymorphism of Norway spruce populations on the plots with various site conditions. In the present study five markers, *viz*., PER I, CAT I, MDH I, ADH Ib and PPH I produced significant polymorphism and successfully demarcated the six sites of Mandi, Hamirpur, Bilaspur districts from four sites of Solan and Sirmour districts.

Therefore, from the present study it is concluded that thirty genotypes of *Indigofera pulchella* studied from ten sites of various geographic locations of Himachal Pradesh had 30% polymorphism with low genetic variability.

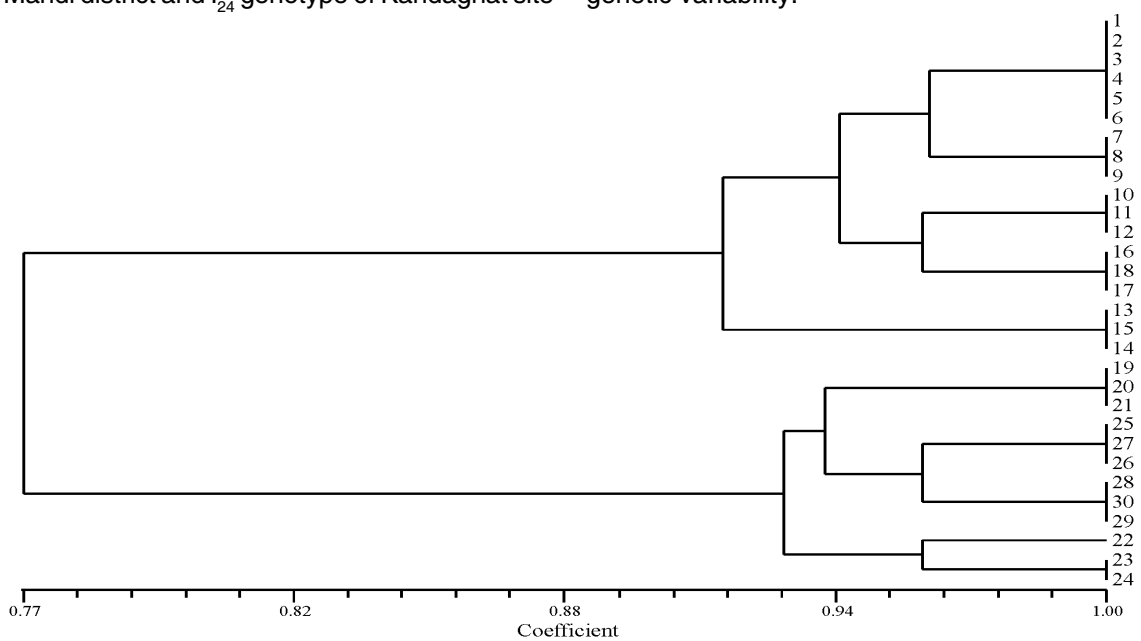

Fig. 2. Dendrogram showing genetic relatedness among 30 *Indigofera puchella* genotypes.

Table 3. Similarity index among the 30 *Indigofera pulchella* genotypes.

	I_1	I_2	I_3	I_4	I_5	I_6	I_7	I_8	I_9	I_{10}	I_{11}	I_{12}	I_{13}	I_{14}	I_{15}	I_{16}	I_{17}	I_{18}	I_{19}	I_{20}	I_{21}	I_{22}	I_{23}	I_{24}	I_{25}	I_{26}	I_{27}	I_{28}	I_{29}	I_{30}
I_1	1.00																													
I_2	1.00	1.00																												
I_3	1.00	1.00	1.00																											
I_4	1.00	1.00	1.00	1.00																										
I_5	1.00	1.00	1.00	1.00	1.00																									
I_6	1.00	1.00	1.00	1.00	1.00	1.00																								
I_7	0.96	0.96	0.96	0.96	0.96	0.96	1.00																							
I_8	0.96	0.96	0.96	0.96	0.96	0.96	1.00	1.00																						
I_9	0.96	0.96	0.96	0.96	0.96	0.96	1.00	1.00	1.00																					
I_{10}	0.92	0.92	0.92	0.92	0.92	0.92	0.96	0.96	0.96	1.00																				
I_{11}	0.92	0.92	0.92	0.92	0.92	0.92	0.96	0.96	0.96	1.00	1.00																			
I_{12}	0.92	0.92	0.92	0.92	0.92	0.92	0.96	0.96	0.96	1.00	1.00	1.00																		
I_{13}	0.88	0.88	0.88	0.88	0.88	0.88	0.92	0.92	0.92	0.96	0.96	0.96	1.00																	
I_{14}	0.88	0.88	0.88	0.88	0.88	0.88	0.92	0.92	0.92	0.96	0.96	0.96	1.00	1.00																
I_{15}	0.88	0.88	0.88	0.88	0.88	0.88	0.92	0.92	0.92	0.96	0.96	0.96	1.00	1.00	1.00															
I_{16}	0.96	0.96	0.96	0.96	0.96	0.96	0.92	0.92	0.92	0.96	0.96	0.96	0.92	0.92	0.92	1.00														
I_{17}	0.96	0.96	0.96	0.96	0.96	0.96	0.92	0.92	0.92	0.96	0.96	0.96	0.92	0.92	0.92	1.00	1.00													
I_{18}	0.96	0.96	0.96	0.96	0.96	0.96	0.92	0.92	0.92	0.96	0.96	0.96	0.92	0.92	0.92	1.00	1.00	1.00												
I_{19}	0.75	0.75	0.75	0.75	0.75	0.75	0.77	0.77	0.77	0.80	0.80	0.80	0.77	0.77	0.77	0.77	0.77	0.77	1.00											
I_{20}	0.75	0.75	0.75	0.75	0.75	0.75	0.77	0.77	0.77	0.80	0.80	0.80	0.77	0.77	0.77	0.77	0.77	0.77	1.00	1.00										
I_{21}	0.75	0.75	0.75	0.75	0.75	0.75	0.77	0.77	0.77	0.80	0.80	0.80	0.77	0.77	0.77	0.77	0.77	0.77	1.00	1.00	1.00									
I_{22}	0.72	0.72	0.72	0.72	0.72	0.72	0.75	0.75	0.75	0.77	0.77	0.77	0.75	0.75	0.75	0.75	0.75	0.75	0.95	0.95	0.95	1.00								
I_{23}	0.75	0.75	0.75	0.75	0.75	0.75	0.78	0.78	0.78	0.75	0.75	0.75	0.72	0.72	0.72	0.72	0.72	0.72	0.92	0.92	0.92	0.96	1.00							
I_{24}	0.75	0.75	0.75	0.75	0.75	0.75	0.78	0.78	0.78	0.75	0.75	0.75	0.72	0.72	0.72	0.72	0.72	0.72	0.92	0.92	0.92	0.96	1.00	1.00						
I_{25}	0.78	0.78	0.78	0.78	0.78	0.78	0.81	0.81	0.81	0.77	0.77	0.77	0.75	0.75	0.75	0.75	0.75	0.75	0.95	0.95	0.95	0.92	0.96	0.96	1.00					
I_{26}	0.78	0.78	0.78	0.78	0.78	0.78	0.81	0.81	0.81	0.77	0.77	0.77	0.75	0.75	0.75	0.75	0.75	0.75	0.95	0.95	0.95	0.92	0.96	0.96	1.00	1.00				
I_{27}	0.78	0.78	0.78	0.78	0.78	0.78	0.81	0.81	0.81	0.77	0.77	0.77	0.75	0.75	0.75	0.75	0.75	0.75	0.95	0.95	0.95	0.92	0.96	0.96	1.00	1.00	1.00			
I_{28}	0.75	0.75	0.75	0.75	0.75	0.75	0.78	0.78	0.78	0.75	0.75	0.75	0.78	0.78	0.78	0.72	0.72	0.72	0.92	0.92	0.92	0.88	0.92	0.92	0.96	0.96	0.96	1.00		
I_{29}	0.75	0.75	0.75	0.75	0.75	0.75	0.78	0.78	0.78	0.75	0.75	0.75	0.78	0.78	0.78	0.72	0.72	0.72	0.92	0.92	0.92	0.88	0.92	0.92	0.96	0.96	0.96	1.00	1.00	
I_{30}	0.75	0.75	0.75	0.75	0.75	0.75	0.78	0.78	0.78	0.75	0.75	0.75	0.78	0.78	0.78	0.72	0.72	0.72	0.92	0.92	0.92	0.88	0.92	0.92	0.96	0.96	0.96	1.00	1.00	1.00

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