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Kusum
Assistant Professor, Department of Botany, St. Bede's College, Navbahar, Shimla, Himachal Pradesh, India

Verma Jagdeep
Assistant Professor, Department of Botany, Sardar Patel University, Mandi, Himachal Pradesh, India

Sembi Jaspreet Kaur
Assistant Professor, Department of Botany, Punjab University, Chandigarh, India

Corresponding Author:
Kusum
Assistant Professor, Department of Botany, St. Bede's College, Navbahar, Shimla, Himachal Pradesh, India

Isoenzyme studies in a polymorphic orchid species, *Satyrium nepalense* D. Don

Kusum, Verma Jagdeep and Sembi Jaspreet Kaur

Abstract
Isoenzyme studies were carried out in these morphotypes to reveal genetic relationships among them. Five enzyme systems, Esterase (EST), Glutamate oxaloacetate transaminase (GOT), Peroxidase (Per), Acid phosphatase (ACP) and Leucine amino peptidase (LAP) were studied for this purpose using native-Page. Out of these, only three enzyme systems (EST, GOT, PER) produced reproducible and consistent bands and were further analyzed. The analysis of eight isozyme loci revealed that these morphotypes exhibit low genetic variability (P=15.1%, A=1.15, Ho=0.015, He=0.088). Two morphotypes (pinkish white and white) have negative fixation index values i.e. F_{IS} = -1 which showed more heterozygosity than observed. The pink flowered morphotype showed more observed heterozygosity than expected, and hence having F_{IS} = +1. The dendrogram constructed based on cluster analysis (UPGMA) suggested that morphotypes; pinkish white and white were closely related (63% similarity). While pink flowered morphotype showed only 26% similarity and was distantly related to others.

Keywords: Isoenzymes, polymorphic species, *Satyrium nepalense* & Himalaya

Introduction
Satyrium nepalense D. Don is a threatened orchid known for therapeutic properties of its tubers. It is a highly polymorphic species that exhibits variations both in floral and vegetative characteristics. Based primarily on flower colour, three (pink, pinkish white, white) sympatrically distributed morphotypes of this taxon were identified in Himachal Pradesh (Northwest Himalaya). *Satyrium* Sw. is an orchid genus of about 90 species majority of which are South African and South Asian in distribution (Xinqi *et al.*, 2009) [71]. Himachal Pradesh is an Indian province located in Northwest part of the Himalayas. *Satyrium nepalense* D. Don is widely distributed across the state between 1450-3000 m altitudes. In addition to continuous vegetative variations (size of plant, size of tubers, size and shape of leaves), three strikingly distinct flower colours (pink, pinkish white, white) were observed in this species. Interestingly, all of these morphotypes were distributed sympatrically. Table 1 summarizes the vegetative and floral details, flowering and fruiting periods, and distribution range of these three morphotypes.

Polymorphism in any taxon occurs due to certain genetic or environmental differences or both. However, when different morphotypes are sympatrically distributed, it hints towards the possible genetic variations among them. Variations in morphology among the members of the species indicate the physiological attributes that are of ecological significance (Karimi *et al*., 2009) [36]. Electrophoretic analysis of isoenzymes (multiple molecular forms of a protein that usually have similar enzymatic properties, but differ in their physical characteristics such as molecular mass, electrical charge, shape, structures, etc.) has been used successfully to provide rapid and quantitative estimation of the extent of genetic variation between and within species of fungi (Micales *et al.*, 1988; Sariah, 1988; Bonde *et al.*, 1993; Aly *et al.*, 1996, 2003; Dez *et al*., 2011; Padmanaban *et al*., 2013; Haliem and Al-Huqail, 2014; Ivanova *et al*., 2015; Maslahat and Nurhayati, 2016) [44, 55, 8, 4, 6, 27, 47, 29, 33, 43]. Isoenzymes can be identified from different tissues and they offer one of the most reliable single-gene markers (Weeden and Wendel, 1989) [69]. The polymorphism at enzyme loci is stable under varied environmental conditions (Arulsekar and Parfitt, 1986) [6]. They have been successfully used to investigate genetic relationships among various orchid taxa such as *Cypripedium* spp. (Case, 1994) [19], *Epipactis youngiana* (Stephen and Abbott, 1997) [63], *Cymbidium* spp.

(Obara-Okeyo *et al*., 1997, 1998) [45, 46], *Pleurothallis* (Borba and Semir, 2001) [9], *Laelia speciosa* (Avila-Diaz and Oyama, 2007) [67], *Pogonia minor* and *Goodyera rosulacea* (Chung and Chung, 2008, 2010) [21, 22], *Oreorchis patens* and *Oreorchis coreana* (Chung *et al.*, 2012) [23] and According to Chauhan *et al.*, (2014) [20] the variations on the basis of morphological, biochemical, and isoenzyme pattern in of *Dactylorhiza hatagirea* may be used as markers for genetic improvement program. More over isoenzyme analysis can be used as a marker for Somoclonal variations *in vitro* cultivation (Ivanova *et al*. 2015) [33] of the plant species. Isoenzyme characteristics have been proved useful in elucidating genetic relationships among taxa and for solving taxonomic and phylogenetic hurdles (Karaca, 2013) [35]. Even knowledge of population genetic variation have been proved very important for the development of conservation strategies for the protection of endangered species *i.e*. *Cypripedium japonicum* (Qian *et al*., 2014) [51]. Present study was therefore carried out to investigate the genetic variations among three morphotypes (pink flowered, pinkish white flowered and white flowered) of *Satyrium nepalense* D. Don (Fig.1). (GOT), Peroxidase (PER), Acid phosphatase (ACP), Leucine amino peptidase (LAP)] were studied using Polyacrylamide gel electrophoresis (Page). Out of these, only three (EST, GOT, PER) that produced reproducible and consistent bands were further analysed.

Objective of the Study

The main objective of the study was to know the *Satyrium Nepalense* D. Don (Orchidaceae): A Therapeutic Species Exhibiting Polymorphism.

Design of the Study

Field surveys were conducted in Himachal Pradesh (Northwest Himalaya) and based primarily on flower colour, three morphotypes of *Satyrium nepalense* [pink (S1), pinkish white (S2), white (S3)] were identified. A total of nine accessions (three of each morphotypes S1, S2 and S3) were collected from following three localities where they were sympatrically distributed: a) Didag (30°82´N, 77°26´E, 1880 m), b) Fagu (26°57´N, 88°42´E, 2530 m) and c) Kasauli (30°53´N, 76°57´E 1820 m). The accessions were named after their flower colour and place of occurrence (e.g. 'S1a' refers to pink flowered morphotype collected from Didag). Healthy mature leaves of the selected plants were harvested in field, wrapped in aluminium foil, placed in chill packs and brought to the laboratory. The material so collected was either processed immediately or stored at 4°C to process within 48 hrs. of collection. Leaf sample (1g) was crushed in 2.5 ml of grinding buffer [100ml Tris-HCl (0.1mol/L, pH 7.0), 6.846g Saccharose, 0.6 g PVP (Polyvinylpyrrolidone), 0.0372g EDTA (Ethylenediaminetetraacetic acid), 0.6 g Borax, 100 µL β-mercaptoethanol] modified from Sun and Ganders (1990) [65]. The homogenate was centrifuged at 15,000 rpm for 20 min, and the supernatant immediately used or stored in a deep freezer at -20 °C until needed. Acrylamide gel (7.5%) was prepared by using Tris Borate gel buffer (Tris 0.1M, EDTA 0.10025M, Boric Acid 0.04M) of pH 8.0. An electrode buffer system (Boric acid 0.3mol/L, NaOH 0.06 mol/L) of pH 8.0 as modified from Shaw and Prasad (1970) was used. This method provided good resolution of esterase (EST), glutamate oxaloacetate transaminase (GOT) and peroxidase (PER) enzyme systems.

For the statistical analysis, bands across both enzyme systems were compared and entries were coded as present (1) or absent (0). Genetic similarities and distances were calculated between all pairs based on the method of Sneath and Sokal (1973) [62]. Isoenzyme genotypes of different morphotypes were compared at each locus to find out genetic variability. Relationships between entries were quantified using the complementary similarity indices. A dendrogram was constructed based on the genetic distance matrix data applying the unweighted pair group method with arithmetic averages (UPGMA) cluster analysis, using numerical taxonomy and multivariate analysis system computer program package (NTSYS version 2.0).

Results

Three morphotypes of *Satyrium nepalense* (S1, S2, S3) with different flower colours and some other morphological differences were studied for five isoenzyme systems (ACP, EST, GOT, LAP and PER). Out of these only three (EST, GOT, PER) produced slight disparity in banding pattern. The remaining two (ACP, LAP), on the other hand, produced inconsistent and complex bands and therefore were excluded from further analysis. Based on qualitative and quantitative banding patterns of EST, GOT and PER, following results were obtained.

Esterase (EST)

Electrophoretic pattern of esterase showed the presence of 27 bands under four loci (Fig. 2a). Their Rm values ranged between 0.20 and 0.61. These were grouped into five loci *viz*. EST I, EST II, EST III, EST IVa and EST IVb and had Rm values 0.20, 0.32, 0.55, 0.59 and 0.61 respectively. A monomorphic band with varied intensities was observed near the origin (EST I); it was lighter for morphotypes S2 and S3 but was light for S1. Locus EST II was represented by a unique band of lighter intensity only in case of S1. Locus EST III was of medium intensity for S2 and S3 and was negative for S1 irrespective of the locality. EST IV locus was represented by two allelic forms (EST IVa, EST IVb). EST IVa was of dark intensity and was observed in case of morphotype S1 only. On the other hand, EST IVb represented the most anodal band with medium colour intensity for S2 and S3; it was negative for morphotype S1. Therefore out of total four zones of esterase enzyme activity, only three (EST II, EST III, and EST IV) can be considered as effective Isoenzyme markers due to quantitative as well qualitative differences in their banding pattern.

Glutamate oxaloacetate transaminase (GOT)

This enzyme showed only a single zone of activity i.e. GOT I. It was expressed as all enzymic forms, GOT Ia and GOT Ib that were resolved at Rm values 0.30 and 0.32 respectively. A total of nine darkly stained thick bands were observed (Fig. 2b). GOT Ia allele was represented in morphotypes S1 and S2 whereas GOT Ib in morphotype S3 only. Since all bands were of equal intensity, no quantitative differences were reflected. The qualitative difference, however, makes GOT as an effective Isoenzyme marker in presently studied morphotypes

Peroxidase (PER)

Isoenzymic pattern for peroxidase with a total of 21 bands produced three main zones of activity with Rm values ranging from 0.27 to 0.72 (Fig. 2c). Four PER isozyme phenotypes *viz*. PER I, PER II, PER IIIA and PER IIIb were observed 0.27, 0.60, 0.70 and 0.72 respectively. PER I zone was represented by a lightly stained unique band in morphotype S3 only. A monomorphic band with varied intensities was

observed at PER II locus; it was lighter for morphotype S1 and medium for other two. PER III locus was represented by two allelic forms. Where one allele (PER IIIa) with lighter band intensity was observed in morphotype S1, PER IIIb phenotype represented the most anodal band with medium intensity in case of morphotypes S2 and S3. So three PER phenotypes *viz*. PER I, PER IIIA AND PER IIIb can be considered effective for quantitative as well as qualitative differences in presently studied morphotypes. PER II locus, on the other hand, showed only quantitative differences and cannot be considered very effective for differentiating the morphotypes.

Statistical analysis and interpretations of the data

Table 1: Table showing the Vegetative and floral characteristics of different morphotypes of *Satyrium Nepalense* D. Don

S. No.	Character	Morphotype-S1 (Pink flowers)	Morphotype-S2 (Pinkish white flowers)	Morphotype-S3 (White flowers)
1.	Stem height (cm)	21.15±2.93*	27.61±1.87	25.57±1.66
2.	Tuber length × width (cm)	4.33±0.26 × 2.05±0.20	5.11±0.35 × 2.21±0.43	4.66±0.34 × 2.29±0.28
3.	Leaf length × width (cm)	10.36±0.61 × 6.83±0.38	16.50±0.62 × 6.93±0.27	14.67±0.53 × 6.56±0.34
4.	Leaf shape	Oblong-lanceolate to narrowly ovate	Broadly ovate to ovate-lanceolate	Broadly ovate to ovate-lanceolate
5.	Inflorescence length (cm)	13.32±0.56	12.03±1.05	9.14±0.40
6.	Floral bract length × width (cm)	2.11±0.07 × 0.60±0.06	1.97±0.11 × 0.56±0.12	2.64±0.10 × 0.63±0.15
7.	Flower arrangement	Dense/ rarely lax	Dense/ rarely lax	Lax/ rarely dense
8.	No. of flowers/ inflorescence	23.28±1.74	16.50±1.20	15.40±2.68
9.	Flower length × width (cm)	1.75±0.25 × 1.16±0.08	1.62±0.15 × 0.86±0.05	1.69±0.10 × 1.10±0.10
10.	Sepal length × width (cm)	0.50±0.06 × 0.14±0.03	0.35±0.07 × 0.13±0.06	0.40±0.04 × 0.15±0.05
11.	Petal length × width (cm)	0.46±0.05 × 0.14±0.07	0.34±0.04 × 0.12±0.05	0.36±0.06 × 0.13±0.05
12.	Lip length × width (cm)	0.41±0.07 × 0.42±0.06	0.48±0.04 × 0.47±0.06	0.44±0.05 × 0.35±0.06
13.	Ovary length (cm)	0.81±0.29	0.87±0.16	0.94±0.09
14.	Spur length (cm)	0.89±0.08	0.91±0.05	1.03±0.12
15.	Distribution range (m)	1500-3000	1500-3000	1450-2500
16.	Flowering	August-September	August-September	August-September
17.	Fruiting	September-October	September-October	September-October

The values represent average of fifteen random observations (five from each locality) ± S.D

Table 2: Table showing the electrophoretic similarity matrix between nine accessions of three *Satyrium nepalense* morphotypes

	S1a	S2a	S3a	S1b	S2b	S3b	S1c	S2c	S3c
S1a	1.0000								
S2a	0.3333	1.0000							
S3a	0.1818	0.6250	1.0000						
S1b	1.0000	0.3333	0.1818	1.0000					
S2b	0.3333	1.0000	0.6250	0.3333	1.0000				
S3b	0.1818	0.6250	1.0000	0.1818	0.6250	1.0000			
S1c	1.0000	0.3333	0.1818	1.0000	0.3333	0.1818	1.0000		
S2c	0.3333	1.0000	0.6250	0.3333	1.0000	0.6250	0.3333	1.0000	
S3c	0.1818	0.6250	1.0000	0.1818	0.6250	1.0000	0.1818	0.6250	1.0000

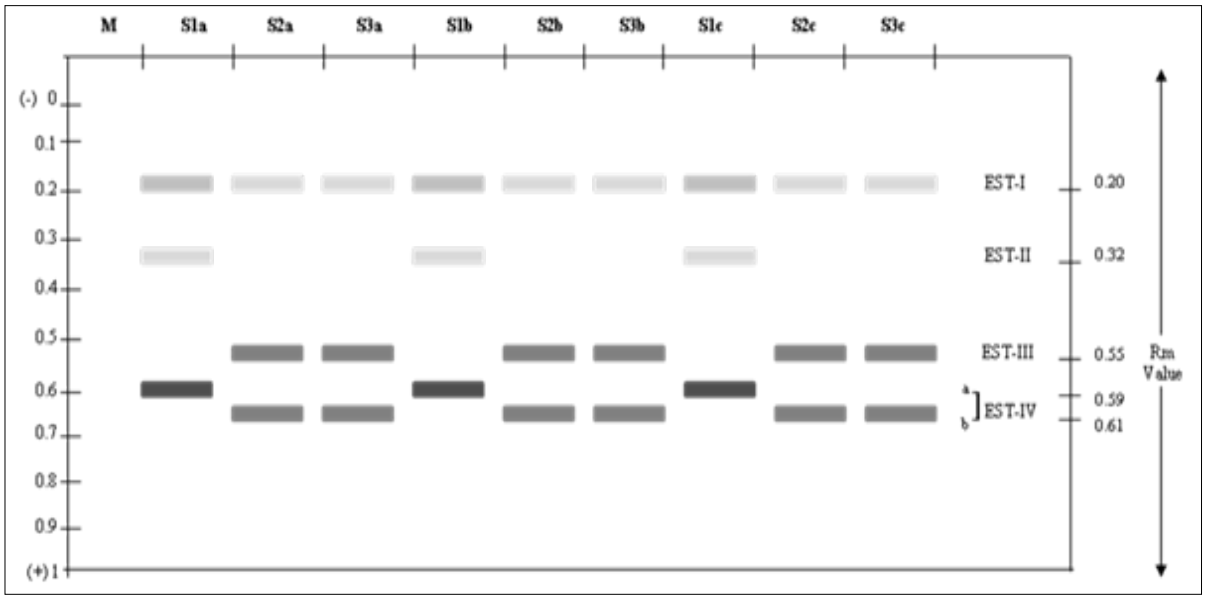

Fig 2a: Morphotypes of *Satyrium nepalense* D. Don selected for isozyme analysis. S1, Pink flowered; S2, Pinkish white flowered; S3 White flowered

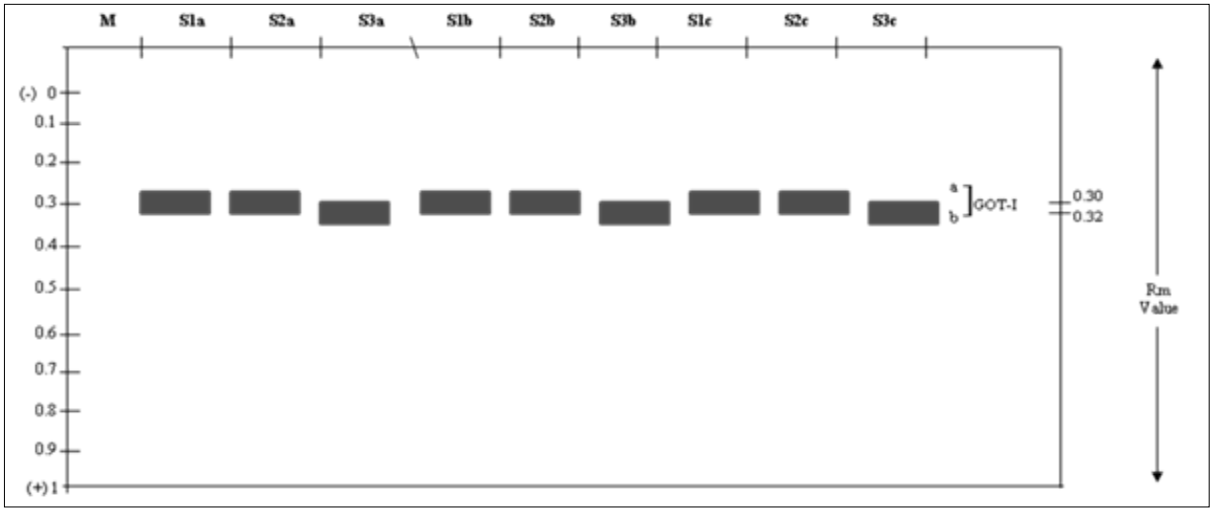

Fig 2b: Zymogram pattern for Esterase (EST) enzyme system in nine accessions of *Satyrium nepalense*

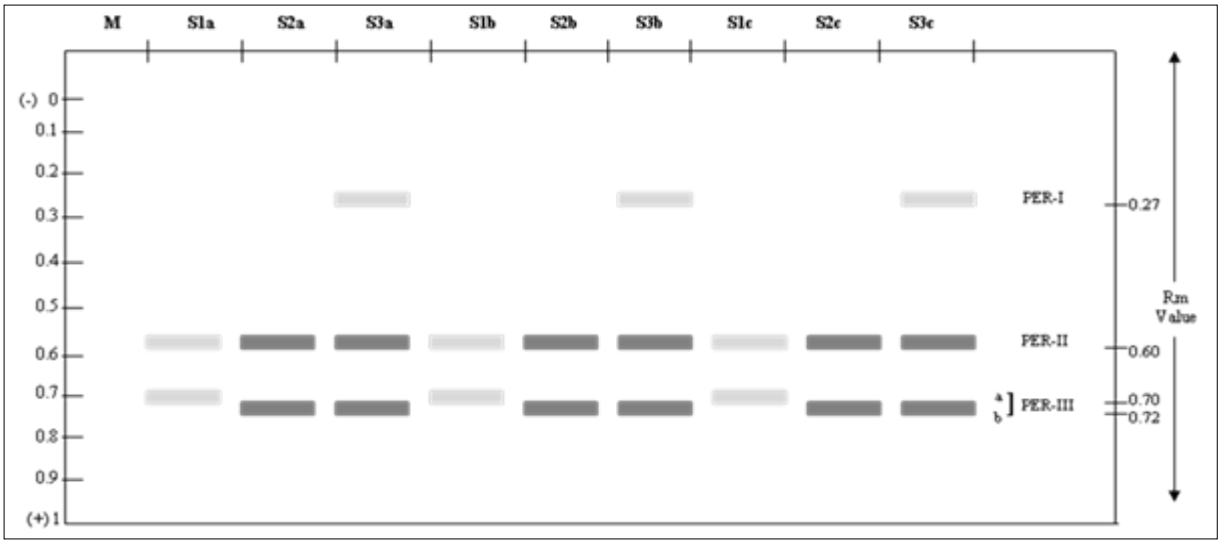

Fig 2c: Zymogram pattern for Glutamate oxaloacetate transaminase (GOT) enzyme system in nine accessions of *Satyrium nepalense*

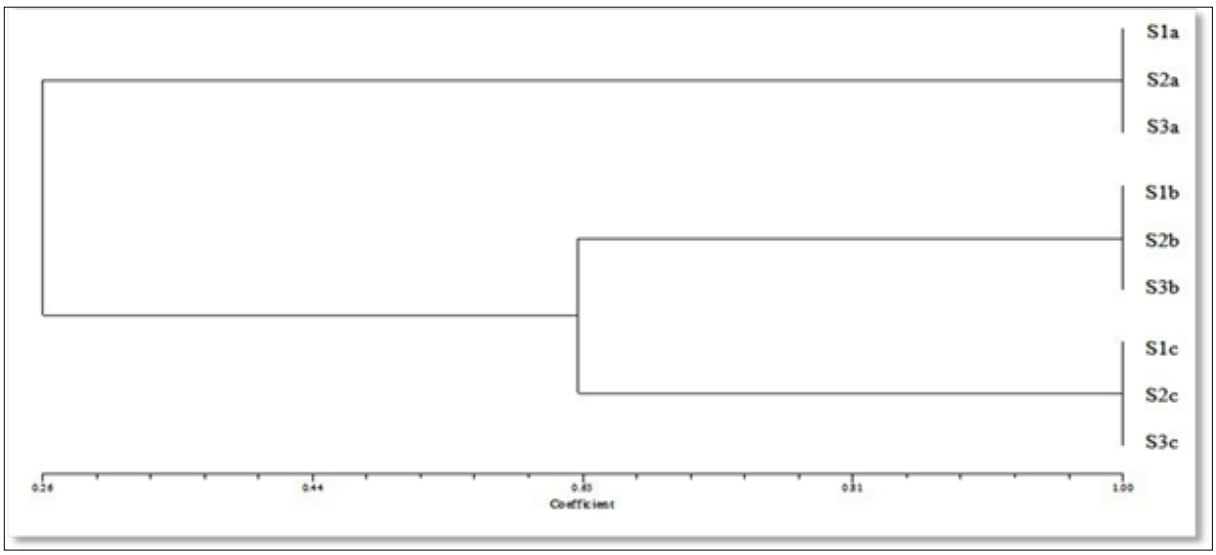

Fig 2d: Zymogram pattern for Peroxidase (PER) enzyme system in nine accessions of *Satyrium nepalense*

Fig 3: Dendrogram showing clustering of nine accessions of *Satyrium nepalense* D. Don, Morphotypes

Discussions

Out of total loci studied for the above enzyme systems, two were monomorphic (EST I, PER II), three (EST IV, GOT I, PER III) were polymorphic. EST IV was the resultant of double homozygous genotypes (AA, BB) and the other two of heterozygous genotype (AB). All other loci except these three polymorphic loci were the resultant of homozygous genotypes (either AA or BB). Allele frequency was found to range from 0.5 to 1.0 at different loci, only two alleles could identify. The mean for allele per locus (A) was 1.15 that was quite near to 1.1allele/ locus as described by Byrne (1989). Polymorphism (P) was 15.1% as observed in present investigation. Mean observed heterozygosity (H_o) value was 1.50%. These parameters have also been studied in several orchid species and a low genetic variability has been observed Ackerman and Ward, 1999; Wallace and Case, 2000; Brzosko, 2002a, 2002b; Brzosko, *et al*. 2004, 2006, 2011, 2013; Qian, *et al.*, 2014) [2, 68, 11, 12, 15, 14, 16 51]. Very low values for these parameters have been observed in *Listera ovata* (P = 9.4%, A = 1.09). For *Satyrium nepalense* the results for all the parameters are in line with these results. Where in *Cypripedium arietinum* and *Cephalanthera damasonium* no genetic diversity (P = 0%) was documented (Case, 1994) [19], in *Goodyra repens* its value was as high as 50% (Brzosko *et al*., 2013) [16]. Present estimates of genetic variation in *Satyrium nepalense* revealed the occurrence of low intra-specific variation in them.

The value of mean observed heterozygosis was observed low for *Satyrium nepalense* (H_o = 0.15). This value was observed very low (H_o = 0.058) for *Listera ovata* and higher for *Goodyra repens* (H_o =0.21; Brzosko, *et al*., 2013) [16]. The low value of observed heterozygosis (H_o) is therefore related to the low genetic diversity among different morphotypes of the species. F_{IS} value (a parameter of F-statistics) ranged between -1 to 1 for presently studied species. The F_{IS} value = -1 was found in S2 and S3 represent the overabundance of homozygotes, while F_{IS} value = +1 in S1 (Pink) represents the predominance of heterozygotes.

Genetic similarity coefficient derived from pairwise comparison among three morphotypes of *Satyrium nepalense* the morphotypes are summarized in Table 2. Phylogenetic relationships among the morphotypes were analyzed, according to isozyme banding patterns. The dendrogram trees shows, two morphotypes i.e. pinkish white (S2) and white (S3) are closely related showing 63% similarity; the genetic distance between them being very small. Pink (S1) morphotype showing only 26% similarity with the pinkish-white (S2) and white (S3) morphotypes. Dendrogram trees (Fig. 3), based on isozyme banding patterns have been used to investigate genetic distances among the species.

Conclusions

Following conclusions were drawn after analyzing the experimental results

Present investigation suggests that different morphotypes of *Satyrium nepalense* exhibited variations for three Isoenzyme systems studied; pinkish white and white flowered morphotypes were found genetically closer to each other as compared to the pink flowered one. Since all of the three morphotypes are sympatric in distribution along the mountain ranges of Himachal Pradesh and they can freely share their gene pool, they should be treated under a single species, *Satyrium nepalense* D. Don. Probably the pinkish white is the hybridization product of pink of and white.

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