Research Article

Morphobiochemical Variability and Selection Strategies for the Germplasm of *Dactylorhiza hatagirea* (D. Don) Soo: An Endangered Medicinal Orchid

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Dactylorhiza hatagirea (D. Don) Soo (Orchidaceae) is an important endangered medicinal herb, distributed in subalpine to alpine regions of the Himalayas. Its tubers are important constituents of many medicines and health tonics. Overexploitation for medicinal uses has decreased availability in natural habitats and this species has been enlisted as endangered, making conservation and cultivation studies necessary. Variability studies may serve as an important tool for effective conservation and for a crop improvement program. Therefore, natural populations of *D. hatagirea* were analyzed for variability on the basis of morphological, biochemical, and isoenzyme patterns. The studied populations were grouped into two clusters. Existing variability among different populations opens up new areas for conservation and perspectives for a genetic improvement program for *D. hatagirea*.

1. Introduction

The genus *Dactylorhiza* (Orchidaceae) is represented by approximately 75 species, distributed in most parts of Northern temperate zone. *Dactylorhiza hatagirea* (D. Don) Soo is a perennial orchid, native to the Himalayan region [1]. This species is distributed in India, Pakistan, Afghanistan, Nepal, Tibet, and Bhutan. In India, it is distributed in Jammu and Kashmir [2], Uttarakhand [3–5], and Himachal Pradesh [6]. Tubers of this species are supposed as a potent medicine in Leucorrhea in Traditional System of Medicine (TSM) rich source of mucilage and are used to make Salep, as food and medicine [7], useful as expectorant and astringent [8]. Recently herbal health beverages have also been developed from this species [9]. The species has been categorized as endangered [10], due to overexploitation of tubers for medicinal value, habitat degradations, and other biotic interferences in its distribution ranges. The market demand of this species is increasing while supply is gradually decreasing [11] that call for conservation as well as cultivation.

Effective conservation, management, and recovery of rare and endangered species can be achieved through variability

analysis. Variations in morphological appearance among the members of a species become important to the biologist solely because they indicate the presence or absence of physiological attributes that are of ecological significance. Statistical methods including principle components or cluster analysis can be used as useful tools for screening of such populations [12]. Adequate information is available on the distribution pattern [4, 5], medicinal properties [7–9] and propagation methods [13] of *D. hatagirea* but there is a lack of knowledge about morphological, biochemical and genetic variability. In view of above, present paper is focused on detail investigation on morpho-biochemical variability among different populations of *D. hatagirea*. Such studies will be helpful for effective conservation management and genetic improvement of *D. hatagirea*.

2. Materials and Methods

Field survey was conducted in several parts of Uttarakhand, India known for occurrence of *D. hatageria*, and nine different populations were selected for detail investigations.

FIGURE 1: Map showing locations of selected sites of *D. hatagirea* (1: DH1, 2: DH2, 3- DH3, 4: DH4, 5: DH5, 6: DH6, 7: DH7, 8: DH8, 9: DH9).

TABLE 1: General characteristic of selected natural habitats of *D. hatagirea*.

Study sites	Code	Distribution range (msl)	Habitat	Latitude	Longitude
Dayara (Uttarkashi)	DH1	3000–3400	Open meadow	30°50'N	78°33'E
Dronagiri (Chamoli)	DH2	3200–3500	Open meadow	30°30'N	79°52'E
Kedarnath (Rudraprayag)	DH3	2900–3500	Open meadow	30°44'N	79°03'E
Kunwari Pass (Chamoli)	DH4	3000–3400	Open meadow	30°07'N	79°58'E
Madhyameshwar (Rudraprayag)	DH5	3500–3800	Open meadow	30°39'N	79°14'E
Panwalikantha (Tehri)	DH6	3300–3500	Open meadow	30°34'N	78°52'E
Rudranath (Chamoli)	DH7	3000–3200	Open meadow	30°11'N	79°28'E
Tungnath (Rudraprayag)	DH8	3200–3600	Open meadow	30°14'N	79°22'E
The Valley of Flowers (Chamoli)	DH9	3000–4200	Open meadow	30°10'N	79°57'E

A detailed description of selected study sites is presented (Figure 1, Table 1). Considering endangered status of the species in Uttarakhand [10], experiments were designed in such a way that minimum number of samples may provide optimum information. Ten mature plants from each study site were sampled randomly for morphological details (plant height, number of flower/plant, inflorescence length, and economic yield) during the month of September. These plants were dug out for the estimation of economic yield (tubers). The tubers were separated, washed with running water, and dried at 40°C temperature until constant weight. One set of fresh tubers was immediately crushed in liquid nitrogen to seize enzymatic activities and then stored at (–80°C) in a deep freezer until analysis.

These samples were used to estimate variations in soluble sugars, protein content, and isoenzymes. Soluble sugar content was estimated using the Anthrone method [14] and soluble protein content was determined using the Bradford method [15]. The absorbance of the reaction mixture was read in a spectrophotometer (Beckman DU-640). Isoenzyme variation was analyzed on 10% polyacrylamide slab gels in

a discontinuous gel electrophoretic system at a constant current of 20 mA [16]. Esterase (EC 3.1.1.1) and peroxidase (EC 3.4.1.1) isoenzymes were detected in gels using the methods of Bhadula and Sawhney [17] and Welter [18], respectively, among different populations. Collected data were subjected to analysis of variance (ANOVA) and only significant variations were used for multivariate analysis of Mahalanobis distance [19]. The analysis was performed using indostat statistical package (<http://www.windostat.org>) and the cluster formation was confirmed by the Tocher method [20].

3. Results

The germplasm collected from different populations showed considerable variation in plant height, number of flowers, length of inflorescence, economic yield, and biochemical parameters (Table 2). The variability range was between 13.2 and 54.3 cm in plant height, 14.9 and 29.4 in number of flowers per plant, 3.9 and 11.7 cm in inflorescence length, and

TABLE 2: Mean growth performance of *D. hatagirea* at different natural populations.

Study sites	Economic yield (g/plant)	Plant height (cm)	Number of flowers/plants	Length of Inflorescence (cm)	Soluble sugar (mg/g)	Starch (mg/g)	Soluble protein (mg/g)
DH1	0.5	45.0	22.0	10.8	20.6	45.6	10.6
DH2	0.6	35.5	22.3	10.0	34.0	54.8	26.8
DH3	0.7	13.8	14.9	3.9	34.5	57.1	11.5
DH4	0.5	13.2	24.5	6.5	35.6	56.0	10.4
DH5	0.6	37.2	26.5	9.5	28.0	59.1	14.3
DH6	0.5	54.3	26.6	9.6	33.5	57.0	11.8
DH7	0.5	51.3	29.4	11.7	40.7	68.1	10.4
DH8	0.4	41.0	23.4	10.7	21.7	53.2	15.6
DH9	0.4	29.0	22.0	5.7	23.0	74.3	26.9
Mean	0.5	35.6	23.5	8.6	30.2	58.4	15.4
SD*	0.1	14.6	4.1	2.6	7.1	8.4	6.8
Range	0.4–0.7	13.2–54.3	14.9–29.4	3.9–11.7	20.6–40.7	45.6–74.3	10.4–26.9
CD 5%**	0.18	9.99	5.72	4.73	9.58	8.55	4.87

*SD: standard deviation; **CD: critical difference.

FIGURE 2: Dendrogram of *D. hatagirea* by Tocher method (1: DH1, 2: DH2, 3: DH3, 4: DH4, 5: DH5, 6: DH6, 7: DH7, and 8: DH8, 9: DH9).

0.4 and 0.7 g per plant in economic yield. Biochemical parameters also showed great variability among studied populations. The variability range was between 20.6 and 40.7 mg/g in soluble sugar, 45.6 and 74.3 mg/g in starch, and 10.4 and 26.9 mg/g in soluble protein content. Such variations could be due to edaphic, microclimate, environmental factors, and so forth. In view of considerable amount of variability, D^2 values were compared for all possible pairs of populations. Based on D^2 values these populations could be grouped into two clusters (Figure 2). The higher contribution of characters regarding variability was shown by soluble protein (72.22%), plant height (13.89%), and starch content (8.33%), whereas other quantitative characters, that is, number of flowers and soluble sugar showed similar share of 2.78%. Mean growth performance of both clusters is presented separately in Table 3. Cluster one contains maximum 6 populations, that is, DH1, DH8, DH5, DH6, DH2, and DH9, whereas cluster two contains 3 population DH3, DH4, and DH7. The average intracluster distance ranged from 7 to 8 whereas the intercluster distance ranged from 8 to 13 (Table 4).

There were significant differences among the parameters measured, indicating variability among the populations

(Figure 3(a)). Total number, intensity, and position of bands varied from population to population. DH8 and DH4 populations emerged as a distinct cluster by showing specific banding pattern of esterase isoenzyme as compared to the rest of the populations. These two populations showed more compact and dark stained band. The peroxidase pattern in DH3 and DH7 populations emerged as a distinct cluster by showing specific banding pattern. Intensity of bands was very dark in DH3 followed by DH7 populations, whereas bands of light intensity were resolved in DH1 and DH2 population (Figure 3(b)).

4. Discussion

Germplasm of *D. hatageria* collected from different geographical locations of the Garhwal Himalaya was grouped into two clusters based on their similarities and variations. They varied considerably in the morphology, biochemical parameters, and isoenzyme pattern. 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 [21]. Variations in plant height were related to the soil and climatic factors [22]. Variation in the morphological characters is possible due to variation in topography, elevation, soil, rainfall, and other climatic conditions, peculiar to Himalaya [23]. In addition, available soil nutrient level also plays an important role in determining morphological variations in plants [24, 25] and climatic factors can be considered as indicators to alter assimilate investment pattern [26].

Biochemical variations suggested polymorphic nature of the species and it is argued that they can be used as an indicators to the impact of microclimatic conditions on plant life [25] and therefore, reflects an adaptive feature of the species [27]. Morphological variability in plants and differences in protein and sugar levels were important parameters to study natural populations of rare plants; these

TABLE 3: Cluster mean of seven characters of *D. hatagirea*.

Clusters	Number of locations	Economic yield (g/plant)	Plant height (cm)	Number of flowers/plant	Inflorescence length (cm)	Soluble sugar (mg/g)	Starch (mg/g)	Soluble protein (mg/g)
1	6	0.5	40.32	23.81	9.27	26.8	57.31	17.66
2	3	0.55	26.09	22.91	7.37	36.94	60.4	10.78

TABLE 4: Intra- and intercluster divergence (D^2) among three clusters of *D. hatagirea*.

Clusters	1	2
1	7.52	13.40
		8.51

(a)

(b)

FIGURE 3: Heterozygosity in isoenzyme banding pattern. (a) Esterase and (b) peroxidase among ten different populations of *D. hatagirea*.

parameters also reflect a variation due to environmental conditions. Average economic yield in DH2 population is positively correlated with the number of flowers, soluble sugar, starch, and soluble protein content. Such information on morphometric, primary metabolites and isoenzyme pattern suggests that secondary metabolites may also vary in these populations. Increase in primary metabolites showed significant increase in active constituents in adiantoid ferns [28]. Isoenzyme studies also supported variability among studied populations. Germplasm diversity based on standard morphological marker has proved to be inadequate because

of wide spectrum of phenotypic variation and their interaction with environment [29], whereas esterase isoenzyme is an excellent marker in variability studies which indicates intrapopulation variations [30].

5. Conclusions

This study revealed significant differences among the parameters measured, indicating variability among these populations. Variability existing among different populations opens a new area for conservation and genetic improvement of *D. hatagirea*. These variations may be used as markers for crop improvement program. DH2 (Dronagiri) population showed better performance among the studied populations, which can be used as elite germplasm source for multiplication of *D. hatagirea*. On the basis of such multifaceted information on specific species, future conservation strategies and cultivation of wild medicinal species can be initiated [31].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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