BIODIVERSITY AND CONSERVATION OF *DACTYLORHIZA HATAGIREA* (D. DON) SOO, FROM TRANS-HIMALAYAN LADAKH REGION OF INDIA

A thesis submitted to

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

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By

ASHISH R. WARGHAT

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ASHISH R. WARGHAT

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR

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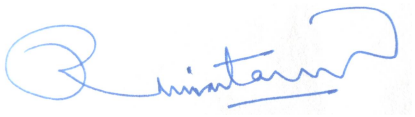
This is to certify that the thesis entitled, **"Biodiversity and conservation of *Dactylorhiza hatagirea* (D.Don) Soo, from Trans-Himalayan Ladakh region of India"** which is being submitted by **Ashish R. Warghat** in fulfillment for the award of degree of Doctor of Philosophy in Biotechnology by the Jaypee University of Information Technology, is the record of candidate's own work carried out by him under our supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.



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(Ashish R. Warghat)

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LIST OF ABBREVIATIONS

Abbreviations	Full form	Abbreviations	Full form
AMOVA	Analysis of Molecular Variance	ISSR	Inter Simple Sequence Repeat
ANOVA	Analysis of Variance	LnP(D)	Most likelihood
AUC	Area Under Curve	MCMC	Markov Chain Monte Carlo
CA	Correspondence analysis	MVSP	Multivariate Statistical Package
CAMP	Conservation Assessment and Management Plan	MDS	Multidimensional scaling
CVA	Canonical variates analysis	NPL	Number of Polymorphic Loci
CITES	Convention of International Trade in Endangered Species	PLBs	Protocorm like bodies
CTAB	Cetyltrimethylammonium bromide	PPL	Percentage Polymorphic Loci
EVI	Enhanced Vegetation Index	PHYLIP	Phylogeny Inference Package
ENM	Ecological Niche Modelling	RAPD	Random Amplified Polymorphic DNA
FDA	Fluorescein diacetate	RD	Relative density
GIS	Geographic Information System	RCN	Research Coordination Network
GPS	Global positioning system	SPSS	Statistical Package for Social Sciences
IUCN	International Union for Conservation of Nature	TTC	Triphenol Tetrazolium Chloride
IBD	Isolation by distance	UPGMA	Unweighted Pair Group Method with Averages
ICBN	International Code of Botanical Nomenclature		
ITS	Internal transcribed spacer		

ABSTRACT

The morphometric study was conducted during 2009 to 2010. About 28 morphological characters were measured under 13 natural locations of *D. hatagirea* (D. Don) Soo. Geographic variation in morphology reflects phenotypic responses to environmental gradients and evolutionary history of populations and species. At points, beside its broad geographic range (Nubra, Suru and Indus valley) characterization of *Dactylorhiza* phenotype was normally accomplished by use of morphological descriptors, hence as a first step, phenotype collection and its morphometric analysis was assessed for the first time. However, plant height, leaf length, lowermost leaf length, length of second leaf from base and mean length from lowest bract to the top of inflorescence were presented to account for the remarkable variation in morphological traits. Tirith location showed more values of this trait while Skurru showed less value. From this, it was concluded that Tirith showed great morphometric variation as compared to other location. Multivariate morphometric techniques, principal component analysis (PCA), multidimensional scaling (MDS) and cluster analysis were used to determine whether these locations can be reliably considered as morphologically similar or dissimilar. The first two principal components derived more than 75% variation among population. The results of PCA and MDS analysis were comparable to that of cluster analysis, which showed considerable phenotypic variation in morphological and horticultural traits that can be utilized for its genetic improvement. To support this study, further constructive information have been provided in the present study on the status of the populations of *D. hatagirea* which may increase the conservation value of this population and may resolve the taxonomic and nomenclatural controversies related to the suitable areas.

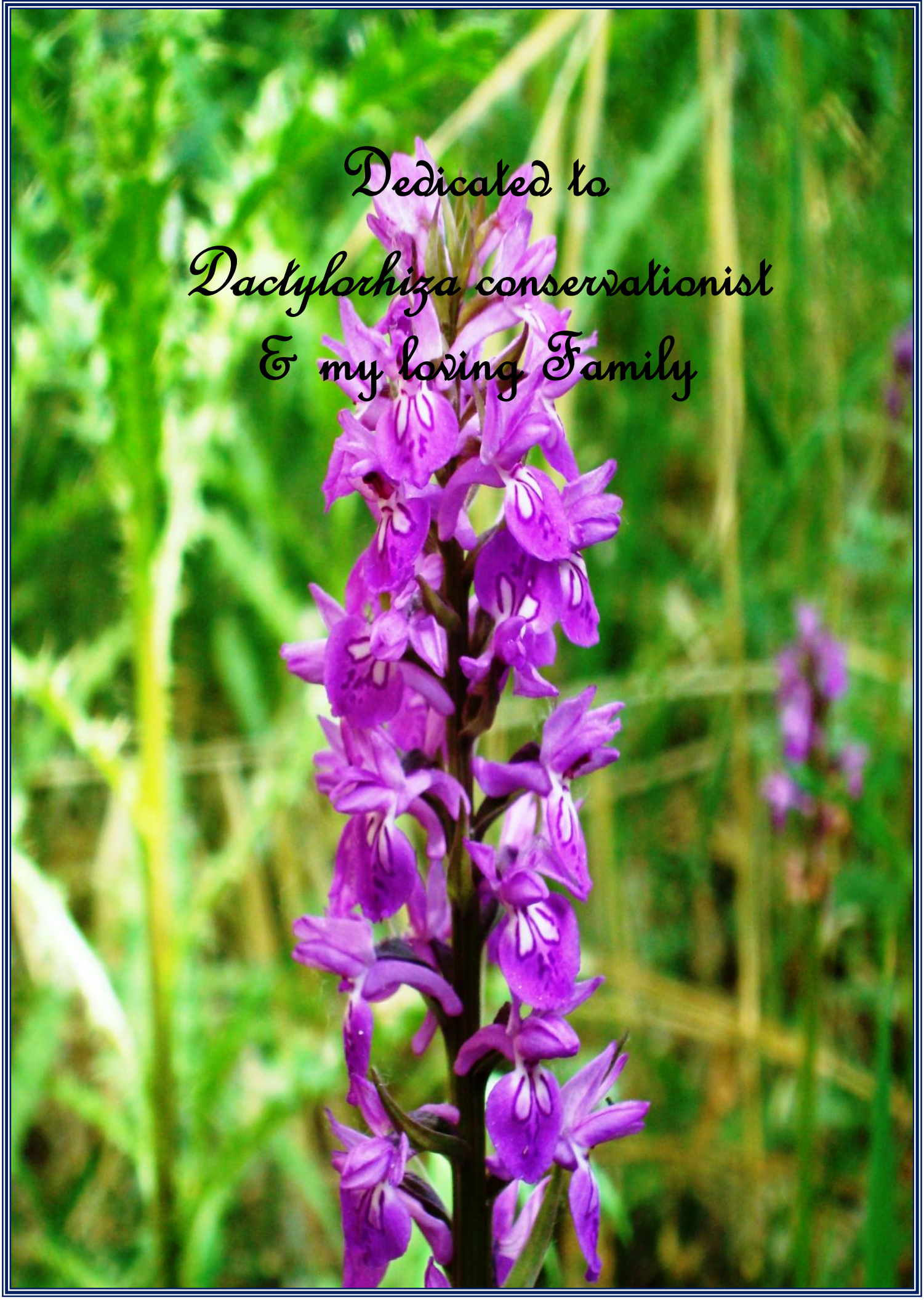
RAPD and ISSR marker analysis have been used for the first time to characterize the population genetic structure and differentiation within and among thirteen locations of *D. hatagirea.* The genetic diversity of *D. hatagirea* has been revealed by Nei's diversity index (H), Shannon's diversity index (I), polymorphic loci and percentage of polymorphic loci (PPL). Pair-wise location genetic distances ranged from 0.05 to 0.48. Although, both the molecular markers revealed high percentage of polymorphism, ISSR marker detected more diversity than RAPD marker. Analysis of molecular variance (AMOVA) revealed that 57% RAPD and 60% ISSR variability was partitioned among population with moderate level of genetic differentiation and gene flow. Both Principal coordinate's analysis (PCoA) and neighbour joining (NJ) cluster analysis supported the grouping of all 136 sample sizes of

thirteen locations into two collection groups. Model based Bayesian clustering, principal coordinate analysis and neighbour-joining analysis highlighted the role of high mountain Ladakh range (6500m amsl) as an important geographic barrier for this species at the studied site. Two main gene pools have been observed one in Nubra- Indus valley and other in Suru valley. The present level and pattern of genetic diversity and structure of *D. hatagirea* are assumed to result largely from its habitat fragmentation, its unique biological traits and evolutionary history. The genetic structure could be attributed to an earlier period of more pronounced gene flow when the species had a more continuous distribution. A Mantel test revealed no significant positive correlation between genetic distances and geographic distances.

The population status of *D. hatagirea* was investigated for the first time which covers Ladakh region. At random 20 quadrates in four habitats of each location were drawn and vegetation was measured accordingly. This study is also supported with soil data for identifying appropriate measures for the vegetation of this endangered orchid. Result showed that 21 species belonging to 16 families were encountered and it was found that 1812 individuals of *D. hatagirea* were present in our surveyed area with low density i.e. 6.9% (Unprotected area). For improving the conservation status of the species; Vegetation analysis, potential area and habitat for reintroduction were predicted using Maximum Entropy (MaxEnt) distribution modelling algorithm. The model was developed using data from 13 locations in the native range of Ladakh region along with 13 environmental parameters including enhanced vegetation index (EVI) and digital elevation data. The model predicted that suitable habitats of *D. hatagirea* were restricted to an area of 485 km in the Indus and Suru valley of Ladakh region. Population status was positively correlated with higher model thresholds in the undisturbed habitats confirming the usefulness of the habitat model in population monitoring, particularly in predicting the successful establishment of the species. The study describes the potential habitats in the elevation of Indus and Suru Valley within the native range where the species can be reintroduced.

The study was carried out where immature seeds cultured on ten different media for germination. Maximum germination was achieved in Lindeman orchid medium (37.12%) within 17 days of culturing. Protocorms with leaf primordia were cultured on BM-2 and seven different modifications of MS media with various hormone combinations (0-3 mg/L IBA and 0-3 mg/L Kin) for plantlets regeneration and mass multiplication. Maximum number of shoots (18.12 ± 2.3), highest shoot length (17.80 cm ± 2.16), maximum root number (8.25 ± 0.69) and highest root length (8.02 cm ± 1.45) were found in MS medium with 3 mg/L IBA

and 1 mg/L Kin. Plantlets with 2-3 shoots were transferred to different potting mixtures for acclimatization to field conditions and further multiplication. 100% survival was obtained in C-8 potting mixture consisting of Cocopeat + Vermiculite + Perlite (1:1:1) which produced 75 number of shoots (25 plantlets) after one month of transplantation in the glass house. The current study reports for the first time a rapid *in vitro* protocorm development and mass multiplication protocol for *D. hatagirea* which holds robust potential for large- scale propagation and metabolite production from the plant.


Dedicated to
Dactylorhiza conservationist
& my loving Family

CHAPTER 1

INTRODUCTION

Dactylorhiza [as named by Necker ex Nevski, 1937], commonly called marsh, orchid or spotted orchid is a genus of Orchidaceae family. The name *Dactylorhiza* is derived from Greek word δάκτυλος "dactylos" (finger) and ρίζα "rhiza" (root), referring to the palmately two to five lobed tubers of this genus. They are hardy tuberous geophytes. In a thickened underground stem, they can store large amount of water to survive in arid conditions. The tuber is flattened and finger-like. The long leaves are lanceolate and in most species, also speckled. They grow along a rather long stem which reaches a height of 70-90 cm. Leaves are higher and shorter on the upper part of stem as compared to basal part. The inflorescence is short as compared to the length of the plant. It consists of a compact raceme with 25-50 flowers. These develop from axillary buds. The dominant colours are white and all shades of pink to red, sprinkled with darker speckles. They occupy a wide range of open habitats from dune slacks to alpine meadows, including swamps and peat bogs.

1.1 Distribution and diversity:

The distribution of *Dactylorhiza,* including *D. viridis* Bateman, Pridgeon and Chase formerly Coeloglossum viride Hartman, covers most of Europe, most of temperate Asia, North Africa, Japan, the Aleutian Islands and northern parts of North America (Figure 1.1). Averyanov [1990] distinguished three centres of diversity: Western Europe (including the British Isles, Germany and southern Scandinavia), the Carpathian Balkan area and Asia Minor (Figure 1.2). According to whom the greatest species richness is found in northwestern Europe. For instance, nine species are endemic to the British Isles according to Delforges classification [2001]. *D. viridis*, the species with the largest range, is the only one to become widespread in the New World [Luer, 1975]. *Dactylorhiza* is thus unusual among European orchid genera, most of which show greatest diversity around the Mediterranean Basin. The subtribe Orchidinae is most diverse in Eurasia encompassing the majority of European orchids. According to Averyanov [1990], there are 75 species of *Dactylorhiza* found worldwide and 58 in Europe, North Africa and the Near East [Delforge, 2001.]

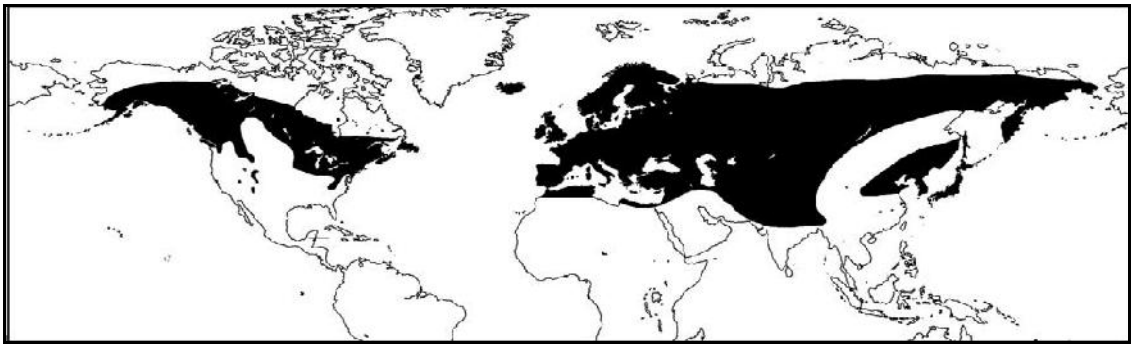


Figure 1.1 World distributions of the genus *Dactylorhiza* (shaded areas), including the former genus *Coeloglossum*, modified from [Pridgeon et al., 2001] and [Luer, 1975].

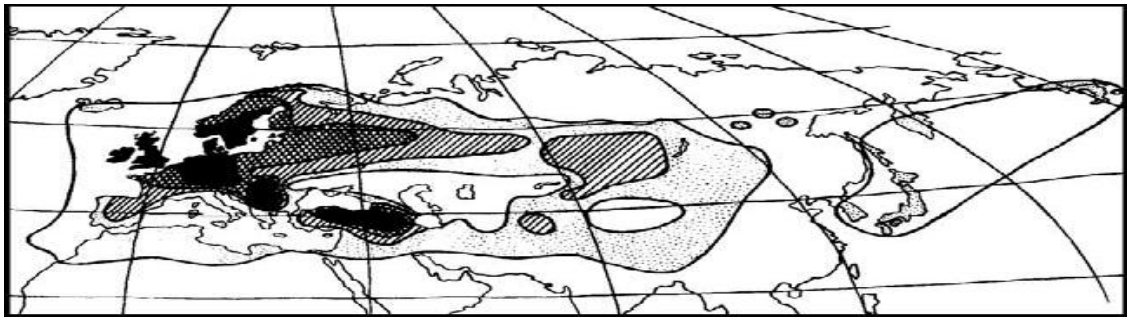


Figure 1.2 Species diversity of *Dactylorhiza* across Eurasia reproduced from [Averyanov, 1990]. Darker areas are said to contain the greatest number of *Dactylorhiza* species

1.2 Taxonomic issues in the genus:

Dactylorhiza is universally recognized as a taxonomically challenging genus [Bourne rias et al., 1998; Pedersen, 1998; Delforge, 2001; Hedren, 2001], as demonstrated by the differences in the number of species recognized by different authors reviewed by [Pedersen, 1998] from 12 to 75 worldwide and from 6 to 58 in Europe. There can even be important differences between treatments by the same author. Delforge [1995] added nine species between his monographs of 1995 and 2001. This taxonomic complexity can largely be explained by the frequency of hybridization and nearly all hybrid combinations are possible [Averyanov, 1990]. Most *Dactylorhiza* species belongs to the *D. incarnata/maculata* polyploid complex, which is composed of three broad groups: *D. incarnata*, *D. maculata* and allotetraploids that are hybrids between the first two groups [Hedren, 2001]. The *D. maculata* group is itself composed of diploid and tetraploid species, delimitation of which is often difficult. *D. hatagirea* is the only one which has no polyploidy complex and found as near to endemic in India.

1.3 Threats and conservation status:

As with many other terrestrial orchids, populations of *Dactylorhiza* have decreased due to habitat loss. Many wetlands in Europe have been drained and changing agricultural practices have led to the degradation of their habitats through use of fertilizers, early haymaking, etc. More recently, the decrease in agricultural pressure has had a counterintuitive effect: abandonment of grassland leads to forest expansion and fewer suitable habitats. However, a few species such as *D. fuchsii* and *D. praetermissa* have shown some ability to colonize human disturbed environments, but generally transiently. Another threat to *Dactylorhiza* is the collection of their tubers to make salep, used as food and medicine. This is a particularly important threat in the Himalayas [Srivastava and Mainera, 1994], where *D. hatagirea* or ‘‘Panch aunle’’ is judged critically endangered [Forest department of Uttar Pradesh, 1998] due to over-collection. Thus, several species of *Dactylorhiza* are declining, and some are already protected at a national scale, e.g., in Belgium, Luxembourg, Nepal, and the UK. Setting conservation priorities in taxonomically complex groups is an essential but especially difficult task because these species tend to be over-represented in red lists [Pilgrim et al., 2004]. Hybridization has often made decision-making difficult in conservation [Rieseberg and Gerber, 1995; Wayne and Gittleman, 1995], and neglecting taxonomy can have disastrous effects on the conservation of a particular group, e.g., the tuatara [Daugherty et al., 1990]. In the case of *Dactylorhiza* such problems have already been encountered; *D. lapponica*, formerly classed as a threatened species in Britain, proved to be indistinguishable from *D. traunsteineri* (a more frequent species) after morphological and molecular investigations (Bateman, 2001). Thus, caution should be applied before setting taxon priorities, and molecular systematics can aid in this task (Soltis and Gitzendanner, 1999).

1.4 *Dactylorhiza hatagirea*:

1.4.1 Taxonomy and Botany

D. hatagirea is a high value medicinal plant belonging to family Orchidaceae. It is commonly known as Salam Panja (Kashmir), Angmo-Lakpa in Ladakh, Hatajari in Uttarakhand and Spotted Heart Orchid in English. It is a terrestrial ground dwelling perennial orchid, up to 76 cm in height. The stem is erect, hollow and obtuse, and bears palmately lobed and lanceolate leaves with sheathing leaf base. The cylindrical and terminal spike bears rosy purple flowers with green bracts. Flowers are 1.7-1.9 cm long

with curved spur. The inflorescence consists of a compact raceme with 25-50 flowers developed from axillary buds. The dark purple spotted lip of the flower is rounded and lobed. Root is tuberous palm shaped (Figure 1.3). Flowering and fruiting occurs in July-September. The special character of this plant is that, it remains erect in excessive snowfall [Warghat et al., 2012b]. The classification of *D. hatagirea* is as follows:

Kingdom: Plantae

Division: Angiosperms

Class: Monocots

Order: Asparagales

Family: Orchidaceae

Subfamily: Orchidoideae

Tribe: Orchideae

Sub tribe: Orchidinae

Genus: *Dactylorhiza*

Species: *hatagirea*

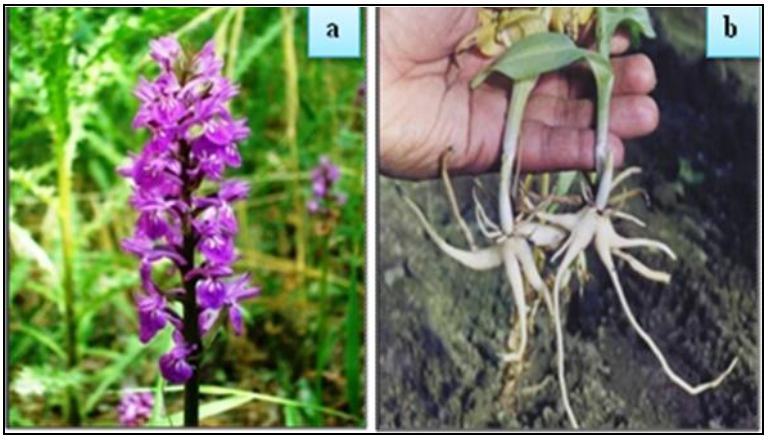


Figure 1.3 Plant morphology of *D. hatagirea* a. Inflorescence b. Tuber

1.4.2 Geographical distribution

The plant is native and near endemic to Indian Himalayan region [Badola and Aitken, 2003; Samant et al., 1998; Ved et al., 2003]. Its distribution extends to Pakistan, Afghanistan, Nepal, Tibet and Bhutan. In India, it is reported from Jammu and Kashmir, Sikkim, Arunachal Pradesh, Uttarakhand and Himachal Pradesh [Samant et al., 2001; Dhar and Kachroo, 1983; Aswal and Mehrotra, 1994; Hajra and Balodi, 1995]. Generally, it is

widely and narrowly distributed at altitudinal ranges between 2500 to 5000 m amsl in open grassy slopes and alpine meadows (Figure 1.4) [Bhatt et al., 2005].

Figure 1.4 Habitat of *D. hatagirea* a. Open grassy slopes b. Alpine meadows

1.4.3 Pharmacological activity

According to Ranpal [2009], rhizomatous part of *D. hatagirea* has shown resistance against all Gram positive and Gram negative bacteria, but its aerial part has shown limited resistance against some bacteria. Zonation of inhibitions (ZOIs) between two parts of *D. hatagirea* indicates that the rhizome part is more effective than the aerial part against all tested organisms, except *E. coli*. Further, it is interesting to note that *E. coli*, one of the very resistant bacteria to synthetic drugs, was found to be very susceptible to the extract of this plant. This finding is distinctive from the folkloric uses of *D. hatagirea*. Hence, this plant can be a potential source for evolving newer antimicrobial compounds for treating dysentery caused by *E. coli*.

As per Thakur and Dixit [2007], *D. hatagirea* showed effectiveness in improving and preventing functionality of sexual organ and may be helpful in improving the sexual behavior and performance also. The results also corroborate the hype that the plant is capable of being nominated as herbal cure for sexual dysfunction.

Bancroft [2005] gave evidence that plant increased testosterone level in adult male rats. Clinical data on testosterone also suggested that slightly increased level of testosterone in adult male's results in increased sexual desire and arousability.

1.4.4. Indigenous Uses

Since time immemorial, this species is used in various Indian medicine systems, that is, Ayurveda, Siddha and Unani, and also, in some Traditional medicinal systems, that is, Amchi medicinal system. It is widely used to cure dysentery, diarrhoea, chronic fever, cough, stomachache, wounds, cuts, burns, fractures and general weakness, particularly in

debilitated women after delivery and to increase regenerative fluids. Tubers of *D. hatagirea* are rich in starch, mucilage, sugar, phosphate, chloride and glucoside-loroglossin [CSIR, 1996]. In Uttarakhand, *D. hatagirea* is also used in bone fracture [Kala et al., 2004]. The tubers of *D. hatagirea* are known to yield a high quality 'Salep' which is extensively used in local medicine as nervine tonic for its astringent and aphrodisiac properties [Vij et al., 1992; Lal et al., 2004; Baral and Kurmi, 2006]. Salep boiled with milk is being used as a rejuvenating tonic in Ladakh and about 2.5 gm powder of tubers is considered as a full day's diet in adverse conditions by local people. Decoction of Salep with sugar and flavoured with spices has got a tremendous nutraceutical value [Vij et al., 1992; Lal et al., 2004; Baral and Kurmi, 2006]. Besides its medicinal importance, salep obtained from the tubers of *D. hatagirea*, is used as a sizing material in silk industry.

1.4.5 Phytochemical composition

The mature tuber contains mucilage (45%), starch, glucoside, loroglossin, albumen, volatile oil, phosphate (2.7%), chloride and ash contains potassium and lime [Dutta and Karn, 2007]. Five new compounds known as dactylorhin A-E and two natural compounds i.e. dactyloses A-B have been reported from the roots of this plant. Dactyloses A and B get synthesized from L-ascorbic acid and 4-hudroxybenzyl alcohol via 2-c-(4-hudroxybenzyl)-α-L-xylo-3-ketohexulofuranosono-1, 4-lactone (Figure 1.5). However**,** Dactylorhin A and Dactylorhin E on enzymatic hydrolysis using almond emulsion give Dactylorhin C (Figure 1.6). Also, Dactylorhin D and Dactylorhin B on enzymatic hydrolysis using cellulose gives 7a compound (-2-3-dihydroxy-2-2-methylpropyl) butanedioic acid which on hydrolysis gives loroglossin. Leaves contain loroglossin compound which help in peristaltic movement of gastrointestinal tract and tuber contains dactylorhin compound which acts as a neuroprotective agents against dementia, Alzheimer's disease, depression, anxiety, and irritable bowel syndrome and significantly improved the memory which is treated with scopolamine, cycloheximide or alcohol.

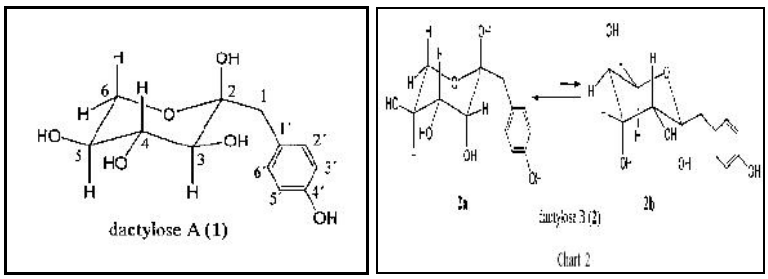


Figure 1.5 Structure of dactylose

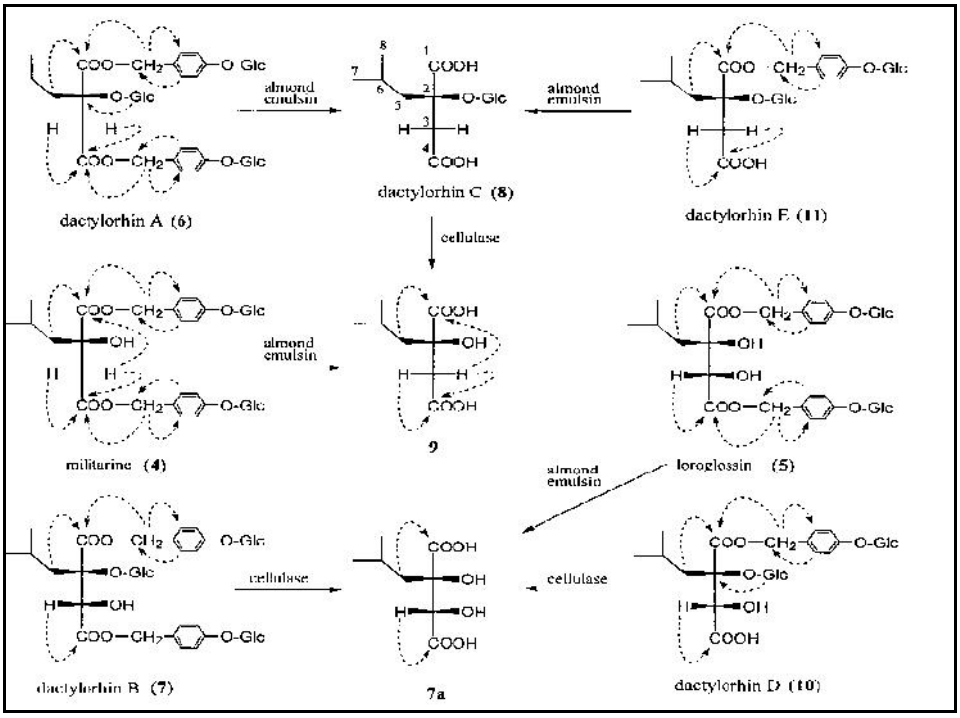


Figure 1.6 Structure of Dactylorhin (A to E)

1.4.6 Agro techniques

Dactylorhiza favours acidic and sandy loamy soil with rich organic manure and sufficient moisture. The healthy plant development and rooting requires 80-90% humidity. The flowering season starts in early June and spreads up to July end. Subsequently the fruiting season starts in August-September. It is generally propagated vegetatively using rhizomes, which is collected after flowering while it may also be propagated through seeds. The mature fruits are collected in September and air dried for 2-3 weeks to extract the seeds and stored at low temperature until used. Being very minute in size, seeds are mixed with sand before sowing. Seeds require symbiosis with mycorrhiza for germination and only 0.2% germination is observed under natural habitat. Tubers may be harvested after seed maturation during mid September to October end. The plants that are developed from the tubers become ready for harvesting in two years. About 3.5 – 4 qt/ ha dry tubers could be harvested from the well maintained field. However, being recognized as a critically endangered plant species it is recommended that only 80% tubers should be harvested as measure of *in situ* conservation [Chaurasia et al., 2007].

1.4.7 Conservation Status

D. hatagirea has been categorized as critically endangered species (CAMP status), critically rare (IUCN status) and is listed under Appendix II of CITES [Kala, 2000; Samant et al., 2001]. Besides these, being an orchid, *Dactylorhiza* can be considered inherently slow growing and poorly regenerating species, because of pollinator specificity and requirement for mycorrhizal association [Bhatt et al., 2005]. Due to its high medicinal and edible value, the species has great demand in national and international market [Badola and Pal, 2002; Olsen and Helles, 1997]. Furthermore, extraction of the raw material from its wild population is the only source for meeting the national and international market demand. According to a report, the annual demand of this species is approximately 5000 tons [Kala, 2004]. This leads to over-exploitation of the species from wild habitats (Figure 1.7). Local inhabitants collect this high value medicinal plant for illegal trading. The local inhabitants could collect Rs. 100 to 200 per kg of dried roots of *D. hatagirea.* For 1 kg of dried roots, 90 to 100 mature plants are exploited [Chaurasia et al., 2007]. As a result, so many areas are there where *D. hatagirea* is present in abundance, but now, a few individuals of this species are seen. This indicates that if the casual factors continue to operate, this species may become extinct within a few years. During the survey, it was also observed that local inhabitants carry their livestock in the higher regions of the valley for grazing. This is another level of disturbance, because due to the grazing and trampling, the under-ground part of the *D. hatagirea* get exposed or removed. These levels of disturbances, like grazing pressure, over exploitation and unawareness of proper procedure of collection and propagation, etc., are the other major factors for declining of this species from its natural habitats.

Figure 1.7 Habitat destruction of *D. hatagirea*

1.5 Review of literature:

1.5.1 Morphological and molecular analysis of *Dactylorhiza*

Shipunov et al [2004] studied 14 morphometric traits and 4 plastid microsatellite for morphological and molecular analysis of *D. maculata*/*incarnata* complex in 78 populations of European Russia. They observed 53.4% variation for all the traits. Both PCA and MDS of the morphological data revealed similar structure that corroborates haplotype distribution and formed four groups. Most samples belong to group I in the upper left (one member of this group falls into group II), which contained *D. maculata*, *D. fuchsii*, and *D. traunsteineri* samples with haplotypes A, Q, and RU1, mostly from the Russian North. Others are group II in the lower left (one member falls into group IV) contained *D. incarnata* with haplotype E; group III with two subgroups in upper right and center of the ordination contained *D. maculata* with haplotypes B and X; and group IV in lower right contained *D. fuchsii*, *D. baltica* and putative allotetraploids with haplotype A, most from central Russia. The highest loadings were: plant height, leaf length, length of lateral lobes of the lip, and position of maximal leaf width for PC1, and leaf spot shape, spike length, lip width, and leaf width for PC2. Heterogeneity within populations was much higher for ITS data and strongly correlated with latitude. They found haplotypes and ITS alleles in Western Europe which were more widely distributed in Russia, whereas some frequent haplotypes from Western Europe were absent.

Shipunov and Bateman [2005] used geometric morphometric tools for understanding *Dactylorhiza* diversity in European Russia. They used lip shape of flowers as trait in 83 populations and found 75.9% PCA variation of the group-based data matrix. Second and third components accounted for similar amounts of variance which served only to separate *D. fuchsii* samples. There was also some overlap of taxa in the centre of the graph, where several samples of *D. incarnata*, *D. fuchsii*, *D. baltica* and *D. purpurella* were indistinguishable. *D. incarnata* specimens overlapped both with *D. praetermissa* samples and *D. russowii*, *D. lapponica* and *D. praetermissa*. Four *D. baltica* populations formed a group located within *D. fuchsii* but far from *D. praetermissa*. In contrast, *D. euxina* sample was placed close to *D. praetermissa*. *D. maculata* and *D. fuchsii* populations from the Russian Arctic occupied a position intermediate between centres of these two species.

Shipunov et al [2005] used four plastid markers, four nuclear markers and 14 morphometric characters to investigate evolution of *D. baltica* (Orchidaceae) in European Russia. They found three overlapping groups consistent with haplotype and ITS allele distributions and species descriptions (*D. fuchsii* + *D. maculata*; *D. baltica* and *D. incarnata*). Plants of the presumed autotetraploid *D. maculata* were not clearly separated from those of *D. fuchsii*. *D. baltica* plants were not located between the two putative parents. Several plants of *D. baltica* overlapped with two parental groups. *D. baltica* plants with haplotypes A and E were distributed closer to *D. fuchsii* (A haplotype) and *D. incarnata* (E haplotype). The percentages of *D. incarnata* ITS allele varied among *D. baltica* individuals; plants possessing more copies of this allele were located closer to *D. incarnata*. Population of *D. incarnata* ssp. *coccinea* (Pugsley) Soó from Wales was marginal to *D. incarnata* group. In both cases, the most important characters (which have relatively high loadings in the first component, PC1) were for individuals, plant heights, all leaf characters and inflorescence lengths, and for populations, bract lengths, stem diameters and leaf lengths. *D. baltica* specimens were divided into two groups, each corresponding to their contrasting haplotypes and consequently, to their putative maternal parents. Some *D. incarnata* samples appeared close to *D. fuchsii* but most have the haplotype of *D. fuchsii* with a low frequency of *D. incarnata* ITS allele.

Hedren and Nordstrom [2007] used 28 morphometric traits, plastid microsatellite, three nuclear microsatellite and vegetation data for understanding polymorphism in *D. incarnata.* They used CVA and found high degree of overlap between populations. Populations from Storsund, Hoburgsmyr and Lojsthajd were placed to the left, Harudden to the right, Agbod to the centre, and Lillmyr to the lower middle. Secondly, *D. ochroleuca* was placed to the right of the other varieties from the same site whereas *D. cruenta* was placed below the other morphs. They used plastid microsatellite and twelve haplotypes were created by combining fragment-length variants at two plastid marker sites. Haplotypes 01 and 02 dominated *D. cruenta*. Haplotype 02 was even more dominant in *D. ochroleuca*, but it had few samples with haplotypes 01, 12, 23 and 33. All haplotypes were found in *D. incarnata*. The fixed twenty-five populations for haplotype 02 were located at a single point in the left part of the diagram and nine populations of *D. ochroleuca*, six populations of *D. cruenta*, seven population of *D. incarnata* and the two populations of *D. incarnata*/*D. ochroleuca* and *D. incarnata*/*D. cruenta* intermediates. Of the remaining

populations, four populations of *D. ochroleuca* were located close to this cluster, five populations of *D. cruenta* were located in the left part of the diagram and the remaining 23 populations of *D. incarnata* were found in other parts of the plot.

Stahlberg and Hedren [2008] used flow cytometry, morphometry and molecular markers [plastid DNA and internal transcribed spacers (ITS) of nuclear ribosomal DNA] to determine taxonomic and phylogeographic patterns in *D. maculata* from 27 populations of Scandinavia. Diploid *D. maculata* ssp. *fuchsii* and autotetraploid *D. maculata* ssp. *maculata* were morphologically differentiated and fragment size variants from 10 plastid DNA loci (seven microsatellite loci and three loci with indel variation) were combined to give 43 haplotypes. They found three major groups of haplotypes. Group I haplotypes were prevalent in the north and the northeast, whereas Group II haplotypes were prevalent in the south and the southwest. Group III was represented by only a single haplotype. Group I and Group II haplotypes have not corresponded with cytologically and morphologically defined *D. maculata* ssp. *fuchsii* or *D. maculata* ssp. *maculata*.

Pedersen and Hedren [2010] studied Allozyme, plastid microsatellite, ITS, nuclear microsatellite and 31 morphological traits in 8 populations from western and Eastern Europe. They used MDS on Allozyme data and clearly separated populations of *D. baltica* from *D. pardalina* along the first axis. The Danish populations were situated fairly close to each other, and separated from all the other populations along the second axis. The shortest distance from a Danish population to the nearest population of *D. baltica* was shorter than the shortest distance from a Danish population to the nearest population of *D. pardalina* and found low stress value (0.06) in the order of distances between populations. Based on variation in plastid haplotype, Danish indeterminate populations only contained haplotype 87, or similar haplotypes differing at highly variable marker site 10b only [Hedren et al., 2008]. This group of haplotypes was also dominant in British populations of *D. praetermissa* /*D. traunsteineri*. A single individual of Danish *D. praetermissa* contained haplotype 59, which was common in *D. praetermissa* from Netherlands and in *D. baltica* from Estonia. The haplotype was rare in *D. praetermissa*/*D. traunsteineri* from Britain and in *D. majalis.* Populations and reference groups were characterized by high frequencies of allele's ITS-III and ITS-V which have been inherited from *D. maculata* subsp. *fuchsii* parent. However, several Danish populations of *D. praetermissa* also had high frequencies of typical *D. incarnata* allele ITS-X and indicated local backcrossing between

allotetraploids and *D. incarnata,* and found allele at high frequencies in Dutch *D. praetermissa* and it was in fact was present at low frequency in all reference groups. At locus ms3, Danish indeterminate populations were dominated by 162 bp allele, which was also dominant in all of the reference groups, except in *D. baltica* where 159 bp allele was equally common. At locus ms8, Danish indeterminate populations were dominated by the 203 bp allele, which was also common or scattered in the reference material. The 206 bp allele, which dominated in *D. praetermissa* from Netherlands and *D. majalis*, was common in *D. praetermissa/D. traunsteineri* from Britain, *D. baltica* and Danish *D. praetermissa*. A UPGMA dendrogram based exclusively on the three characters distinguished successfully *D. baltica* and *D. pardalina*. There are two main clusters. One consists of all populations identified as *D. baltica* and the other consists of all populations identified as *D. pardalina*, together with *D. baltica* and *D. pardalina* turned out to be clearly distinguished genetically and morphologically similar.

Thakur and Kaur [2013] studied RAPD markers for assessing genetic diversity of *D. hatagirea* from Himachal Pradesh, India. They used 40 RAPD primers, 33 were observed to be polymorphic whereas seven did not amplify and found 99% polymorphism among the accessions. Accessions were divided into two clusters namely cluster I which included all the accessions collected from spiti and cluster II contained Sirmour accessions.

But till date, studies related to morphometric analysis of *D. hatagirea* have not been attempted. Molecular markers have been extensively used to characterize *Dactylorhiza* species. But till date, studies related to Genetic diversity and population genetic structure of natural population of *D. hatagirea* from the Trans-Himalayan Ladakh region of India has not been attempted.

1.5.2 Population status of *Dactylorhiza*

Grootjans et al [2005] studied species richness of *D. majalis* in Netherlands. They found 5% frequency of *D. hatagirea* in 19 (1 m × 1m) permanent plot of Netherland.

Bhatt et al [2005] studied quantum of availability of *D. hatagirea* in Garhwal Himalaya. They have drawn fifteen quadrats of 1 m × 1m randomly in studied sites and analysed frequency, density, abundance, relative density, relative abundance and relative frequency. They found density of *D. hatagirea* i.e. 1.13 ind/m^2 (Lata) and 2.19 ind/m^2 (Nagtal). Density of the species in VoF, Nagtal (both protected) and Donidhar (unprotected) was significantly higher ($P < 0.05$) than Lata and Pindari (both unprotected).

However, density of the species in Nagtal was significantly higher ($P < 0.05$) than that at Kedarnath. Contiguous distribution of the species was recorded in all the populations. Species like *A. tetrasepala* (RD – 23.33% in VoF; 9.19% in Nagtal; 12.85% in Lata; 23.54% in Donidhar and 29.22% in Kedarnath), *G. wallichianum* (RD – 21.77% in VoF; 12.07% in Nagtal; 14.28% in Pindari; 22.62% in Lata; 22.49% in Donidhar and 15.06% in Kedarnath), *Heracleum* sp. (RD – 13.03% in VoF), *P. polystachyum* (RD – 9.83% in VoF; 16.63% in Nagtal and 19.36% in Donidhar), *S. tenuifolium* (RD – 23.77% in Pindari), *D. cachmyriana* (RD – 16.23% in Pindari; 11.11% in Lata; 10.44% in Donidhar and 14.55% in Kedarnath), *P. atrosanguinea* (RD – 17.83% in Lata), *A. triplinervis* (RD – 23.67% in Lata) and *M. longifolia* (RD – 18.24% in Kedarnath) were dominant associates of *D. hatagirea*.

Hedren and Nordstrom [2009] used PCA for analysis of habitat data of *D. incarnata* and found releves from Agbod dispersed over central and left parts of the plot, whereas remaining material was aggregated to the right. Plants responsible for separation of Agbod releves included several typical seashore plants, but the presence of *S. nigricans* at the locality demonstrated that it was not an ordinary seashore meadow. Because releves from localities other than Agbod were so poorly separated in analysis of the data, a second analysis was performed from which releves from Agbod were excluded. They found clear overlap of releves from localities in central part of the plot.

Giri et al [2008] studied analysis of associated vegetation and present status of *D. hatagirea* in Garhwal Himalaya, India. He found 24 herb species across the study sites and out of six study sites, only two sites showed its presence. The maximum density was found in *P. alpinum* L. (141.52-201.28 ind/m2) followed by *G. trichophylla* Royle, (14.2-75.0 ind/m2), *D. cachyemyriana* Jaub. and Spach, (8.32-40.32 ind/m2), *P. depressa* Willd.(15.0-58.4 ind/m2) and *A. aptera* DC (2.80- 32.2 ind/m2) and dominant herbs in all study sites. *D. hatagirea* showed minimum density (0.70- 1.8 ind/m^2) in all study sites.

Rinchen et al [2012] studied population census of *D. hatagirea* in Suru valley of Jammu and Kashmir, India. He found 11 associated species across the study sites and out of forty eight sites, only sixteen sites showed its presence. They found least density of *D. hatagirea* (8.00 and 6.1 ind/m^2) among other associates. The maximum density was found in *Equisetum* sp. (3.45 to 17.2 ind/m^2) followed by *Polygonum* sp. (2.6 to 4.35 ind/m^2), *S.*

chrysanthemoides (2.05 to 5.75 ind/m2), *M. lupalina* (1.75 to 5.6 ind/m2), *R. hirtellus* (1.5 to 4.55 ind/m2) and *P. hexandrum* (1.95 to 3.65 ind/m2).

But till date, studies related to Population inventory and vegetation mapping of *D. hatagirea* through Ecological Niche Modelling (ENM) have not been attempted.

1.5.3 Tissue culture of *Dactylorhiza*

Znaniecka and lojkowska [2004] used mature seeds for establishment of *in vitro* collection of endangered European *D. majalis. D. majalis* seeds started to germinate after 6 weeks of culture on Fast medium.

Vaasa and Rosenberg [2004] used semi ripened seeds for preservation of *D. ruthei and D. praetermissa*. *D. ruthei* and *D. praetermissa* seeds started to germinate after four months of culture on Norstog medium.

Rajasekaran et al [2009] studied multiplication and conservation of *Dactylorhiza hatagirea* and found satisfactory results.

Aggarwal and Zettler [2010] used green capsule for reintroduction of *D. hatagirea* symbiotically. They found 100% germination within 10 days of sowing and seedling after 3 months.

Giri and Tamta [2012] used green pod culture for conservation of *D. hatagirea*. They have tried Knudson C (KC), Murashige and Skoog (MS), Vacin and Went (VW) and Vejsadova (VJ) media for seed germination and found better response in MS medium supplemented with peptone (P) (1.0 g/L), morphoinoethane sulphonic acid (MES) (1.0 g/L) and activated charcoal (AC) (0.1%).

But till date, studies related to protocorm formation and mass multiplication of *D. hatagirea* has not been attempted.

1.6 Thrust area-*D. hatagirea* in Trans-Himalayan Ladakh region of India:

There is a lack of information on the morphometry analysis and plant descriptor for exact identification of *D. hatagirea* and *D. kafiriana*. *D. hatagirea* is taxonomically difficult and challenging species. Therefore, it is prerequisite to differentiate and characterize *D. hatagirea* from *D. kafiriana* with the help of morphometry. Being self pollinated and endangered class of the plant, there is a need to study genetic diversity for identifying and evaluating genetic threats. Most *D. hatagirea* are gathered from natural habitat with little attention to preservation of germplasm resources and genetic diversity

and there is lack of information on the genetic diversity of *D. hatagirea*. Therefore, there is a need to carry out preliminary studies to assess the extent of genetic variations between and within natural population. In cold desert Ladakh, continuous removal of plant species for various uses and overgrazing by domestic animals has resulted in desertification and loss of biodiversity. Hence, there is a need to survey the area and checking the present and future status of population of *D. hatagirea*.

D. hatagirea is facing extremely high risk of extinction due to overexploitation of tubers for medicinal purposes. Therefore, it is listed into the Indian Red Data Book. Near about all the species of *Dactylorhiza* spread world-wide are listed in red data book. Therefore, there is urgent need of conservation. *In situ* conservations including complete ban of its collection from its natural habitat must be implemented at an early date. The standardization of agro-technologies and cultivation in new areas will give further boost to its conservation. The orchids are propagated through vegetative means as well as seeds. However, the rate of vegetative propagation of *D. hatagirea* is very slow and seed germination in nature is very poor, i.e. 0.2% [Vij, 2002]. Therefore, *in vitro* germination of seeds can be an important part in orchid multiplication and conservation programmes.

However, detailed survey of *D. hatagirea* in Ladakh region is not carried out by far now and further exploitation on cited problems have not been conducted, so the present study is carried out with the following objectives:

1. Morphological discrimination in geographical populations of *D. hatagirea*.
2. Studies on population genetic structure and differentiation analyses of *D. hatagirea.*
3. Population inventory and vegetation mapping of *D. hatagirea* through Ecological Niche Modelling (ENM).
4. Optimization of tissue culture techniques for *in vitro* multiplication of endangered orchid *D. hatagirea.*

CHAPTER 2

Morphological discrimination in geographical populations of *D. hatagirea*

2.1 Introduction:

Dactylorhiza is a very challenging genus and have always presented taxonomic difficulties [Pedersen, 1998; Hedren, 2001]. Taxonomy of these dactylorchid is widely considered to have been complicated by relatively great morphological variability within species and high frequency of hybridization between species. The mean number of species recognized in published studies has increased progressively through time [reviewed by Pedersen, 1998] and is epitomized by the jump from 49 to 61 species during the first and third editions of Delforge [1993, 2005]. Refining, with justification, the taxonomy of the genus has become increasingly important because many putative *Dactylorhiza* species are declining and others have probably always shown endangered narrow endemics. Reconciling morphologically and genetically circumscribed entities (recognized at taxonomic level) is necessary pre-requisite for a meaningful taxonomic hierarchy, which in turn is needed to accurately characterize their biogeography, ecology and conservation status.

Geographical variation in plant morphology is a function of phenotypic changes in response to local environmental conditions, genetic variation and evolution among populations and biogeographic history of a species [Thompson, 1991; Schlichting and Pigliucci, 1998]. Morphological variation and geographical separation among populations are also prerequisite to the formation of subspecies and variety [Losos and Glor, 2003]. Morphometric analysis can be used to illuminate the interplay of climate, geographical history and evolutionary dynamics in generating new taxa [Avise et al., 1987; Templeton et al., 1995; Arbogast and Kenagy, 2001]. They could also be caused by phenotypic variation using parameters, multiple observations and investigations of local variability. More recently, it has been documented that orchid systematics needs to be based on a much broader establishment of morphometry. In particular, greater attention should be paid to the direct morphometric analysis of living plant [Dressler and Dodson, 1960] and to the characterization of the environment conditions at the same degree as that of the morphological variation in the plants [Sanford, 1974]. Considering the size of Orchidaceae,

there are a relatively small number of studies using morphometry to estimate population variability; mostly, this has been focused on terrestrial temperate orchids [Bateman and Denholm, 1988; Bateman and Farrington, 1989; Dufrene et al., 1991].

D. hatagirea and *D. kafiriana* are terrestrial orchids belonging to family: Orchidaceae and found in valleys of Cold desert Ladakh region of India. Both are phenotypically similar and difficult to distinguish in nature. Some report on other species of *Dactylorhiza*, also faced difficulties in identification. So, earlier morphometric analyses of these problems have often pointed to the form of the lip (= the labellum: the insect landing stage in the flower) as a particularly valuable source of diagnostic characters [Heslop-Harrison, 1954; Bateman and Denholm, 1985; Reinhard, 1990; Tyteca and Gathoye, 2000]. In *Dactylorhiza*, unlike some other orchids, this structure is relatively flat (and can be fully flattened without serious damage), contains no holes and is of macroscopic size (usually around maximum 1 cm in diameter) but this trait is not sufficient for understanding taxonomy of *Dactylorhiza* species [Shipunov et al., 2004]. Therefore, dactylorchid lip and trait from plant is potentially an excellent model for investigations using morphometry. Despite the unexplored plant and level of problem related to taxonomy, morphometric analysis and discrimination on that basis is still unknown.

The objectives of the present study are: (1) Comparison between *D. hatagirea* and *D. kafiriana* (2) Discrimination of *D. hatagirea* among population with the help of plant descriptor (3) Identification of pattern of variation and determining the characters related to these patterns.

2.2 Materials and Methods:

2.2.1 Sampling

The study was based on taxonomy of *D. hatagirea* and separation from *D. kafiriana* and samples were collected from 13 locations of Ladakh region (Figure 2.1). About 20 plants from four different habitats (Herbaceous meadows, Sedge meadow, Riverine scrub and Open slopes) in each location were studied for morphometric analysis (Figure 2.2). Number of samples taken from each location was based on the geographic distribution and area in order to cover, as much as possible, diverse growing habitat (Table 2.1).

2.2.2 Morphometric traits

Morphological measurements were taken from all parts of plant including stem, leaves and flowers [Bateman and Denholm, 1985] but lobe of tuber was described somewhat in

different manner by using single characters according to locations. A total of 28 quantitative and qualitative traits were determined on all 260 individuals (Table 2.2). Individual plants of *D. hatagirea* and *D. kafiriana* have been represented in Figure 2.3 and pencil sketch of flower traits in Figure 2.4. Measured traits included plant height (PLH), length of longest leaf (LFL), width of longest leaf (LFW), position of maximal width, the distance from leaf base to the place of maximal width (MAX W), leaf spot presence (1 = none, 2 = weak and 3 = heavy), leaf spot shape (1 = elongated and 2 = rounded), uppermost leaf length (ULFL), uppermost leaf width (ULFW), lowermost leaf width (LLFW), position from base, of the second leaf greatest width (POSFRB), length of second leaf from base (LSECLF), width of second leaf (WIDOFSEC),

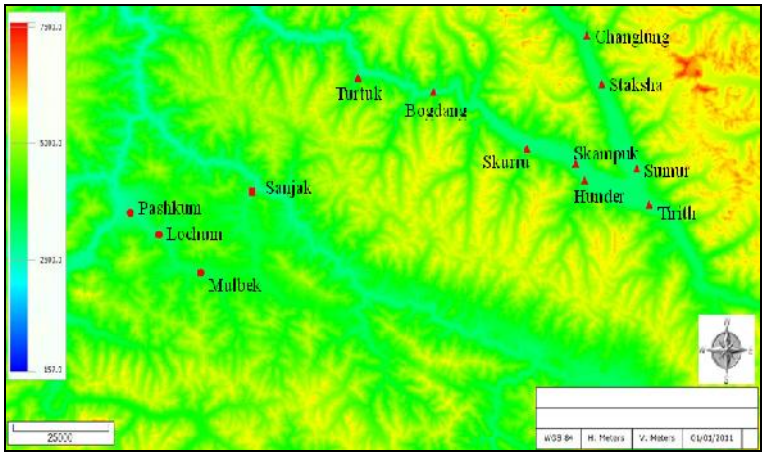


Figure 2.1: Map of Trans-Himalayan Ladakh region of India

Figure 2.2: Habitats of *D. hatagirea* 1: Herbaceous meadows,

2: Sedge meadow, 3: Riverine scrub, 4: Open slopes

number of cauline leaves (CAULF), spur length measured underneath the spur (SPULEN), lip color (1 = pink purple and 2 = dark pink purple), lip width (LIPW), length of middle lobe of lip, from the base to the top of lobe (LIPMID), length of lateral lobe of lip, from the base to the top of lobe (LIPLATE), bract length (BRAL), bract width (BRAW), length from lowest bract to the top of inflorescence (LOWBRA), length of inflorescence axis between the insertion points of first and fifth (INFAXIS), number of flowers (NOFLO), uppermost internodium length (UPPINT), stem diameter under inflorescence (STDI), stem diameter above lowermost leaf (STDLOW) and number of lobe of tubers (NOLOB) (1-5).

Table 2.1: Geographic localities of populations of *D. hatagirea*

Sr. no.	Population	Location	Latitude	Longitude	Altitude (m)
1	Nubra	Tirith	N 34°32'.378	E 77°38'.481	2817.20
2		Sumur	N 34°31'.128	E 77°34'.481	3197.80
3		Changlung	N 34°55'.884	E 77°28'.276	3138.40
4		Staksha	N 34°55'.885	E 77°28'.276	3157.20
5		Turtuk	N 34°50'.849	E 76°49'.720	2817.10
6		Bogdang	N 34°48'.198	E 77°02'.453	3183.70
7		Hunder	N 34° 35'.043	E 77°28'.592	3084.90
8		Skurru	N 34° 40'.229	E 77° 18'.031	3348.30
9		Skampuk	N 34°35'.238	E 77°34'.481	3378.70
10	Indus	Sanjak	N 34°34'.458	E 76°31'.584	2817.20
11	Suru	Mulbek	N 34°35'.437	E 76°32'.673	3197.80
12		Lochum	N 34°28'.064	E 76°15'.337	3138.40
13		Pashkum	N 34°31'.326	E 76°10'.960	3157.20

Table 2.2: List of morphological characters (Bateman and Denholm, 1985)

Sr. No.	Acronym	Plant characters
1	PLH	Plant height
2	LFL	Length of longest leaf
3	LFW	Width of longest leaf
4	MAX W	Position of maximal width, the distance from leaf base to the place of maximal width
5	LFSP	Leaf spot presence (absence, presence)
6	LFSPSH	Leaf spot shape (1 Weak elongated, 2 heavy elongated, 3 Weak rounded, 4 Heavy rounded)
7	ULFL	Uppermost leaf length
8	ULFW	Uppermost leaf width
9	LLFL	Lowermost leaf length
10	LLFW	Lowermost leaf width
11	POSFRB	Position from base, of the second leaf greatest width
12	LSECLF	length of second leaf from base
13	WIDOF	width of second leaf
14	CAULF	Number of cauline leaves
15	SPULEN	Spur length, measured underneath the spur
16	LIPC	Lip color (1 pink purple, 2 dark pink purple)
17	LIPW	Lip width
18	LIP MID	Length of middle lobe of lip, from the base to the top of lobe
19	LIPLATE	Length of lateral lobe of lip, from the base to the top of lobe
20	BRAL	Bract length
21	BRAW	Bract width
22	LOWBRA	Length from lowest bract to the top of inflorescence
23	INFAXIS	Length of inflorescence axis between the insertion points of first and fifth
24	NOFLO	No. of flowers
25	UPPINT	Uppermost internodium length
26	STDI	Stem diameter under inflorescence
27	STDLOW	Stem diameter above lowermost leaf
28	NOLOB	No. of lobe of tubers (1,2,3,4,5)

Figure 2.3: Individual plant of *D. hatagirea* (a) and *D. kafiriana* (b)

Comparison of *D. hatagirea* with *D. kafiriana*

(1) Leaves sub obtuse or acute, broadest in the middle or above. Labellum normally broader than long. Spur straight, 7-9 (11) mm, slightly shorter than ovary
- *Dactylorhiza hatagirea* (Figure 2.3a)

(2) Plant slender leaves usually crowded towards base of stem, often arched and folded, narrowly lanceolate. Inflorescence usually loose labellum narrow when seen from above, rose coloured - *Dactylorhiza kafiriana* (Figure 2.3b)

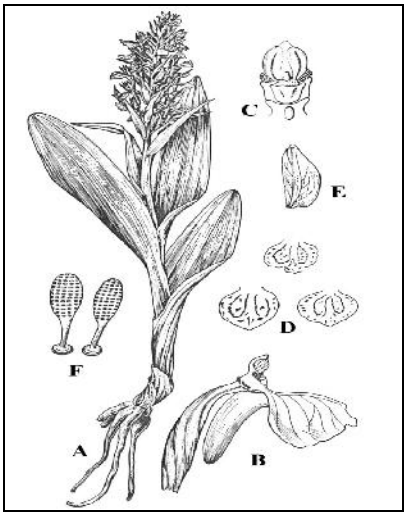


Figure 2.4: A. *D. hatagirea* plant, B. flower with sepals and petals, C. column, D. labellum, E. petal, F. pollinaria

2.2.3 Data and statistical analysis

A range of univariate and multivariate statistical procedures were used to analyze the data. Descriptive analysis of the data was performed using SPSS 19 software. Analysis of variance (ANOVA) was performed and the mean of the results were compared by Duncan's multiple range tests at 5% significance level. To determine the degree of associations among the characters, Pearson's coefficients were used. Principal component analysis (PCA) and multidimensional scaling (MDS) was used to ordinate population means considering variance and covariance among characters within and among populations [Kim, 1975]. Average Euclidean distance was calculated for each location and the resulting distance matrix was used to construct a phenetic dendrogram using average linkage method [Mohammadi and Prasanna, 2003].

2.3 Results:

Mean and standard error comparisons of each trait were based on Duncan's test as presented in Table 2.3. Results showed the mean of plants height (74.60 to 34.2), leaf length (13.4 to 8.4), lowermost leaf length (12.4 to 7.8), length of second leaf from base (13.2 to 10.5) and length from lowest bract to the top of inflorescence (16.5 to 9.7). The highest values of mean of plants height, leaf length, lowermost leaf length, length of second leaf from base and length from lowest bract to the top of inflorescence were found in Tirith, while lowest values were found in Skurru. Therefore, at Tirith location, individuals were superior and significantly different as compared to other location at 5% level. Correlation coefficients of maximum variables were significant positively and negatively at 1 and 5%. Higher significant positive correlation was observed between PLH with LFL (0.945), ULFL (0.944), LFL with ULFL (0.933), ULFL with LLFL (0.911), BRAL (0.927), LOWBRA (0.937), INFAXIS (0.956), UPPINT (0.949), LLFL with LOWBRA (0.925), INFAXIS (0.910), LSECLF with LOWBRA (0.907), BRAL with LOWBRA (0.922), INFAXIS (0.937), UPPINT (0.908), LOWBRA with INFAXIS (0.965), UPPINT (0.908), ULFW with LSECLF (0.956), UPPINT with INFAXIS (0.939), STDLOW with STDI (0.970) at 1 % level. While, CAULF with SPULEN and LIPW, and BRAW with LLFW, LIPLATE, STDI, LOWBRA and NOFLO were non significant at 5 % (Table 2.4). Figures 2.5 and 2.6 showed the characters values plotted against the first two principal component variates from PCA and sample locations with respect to their Euclidean distance from MDS. However, two patterns could be seen regarding the position

of variable mean scores and Euclidean distance. First, traits were positioned relatively close to each other in the axis with respect to their location and secondly, sample location formed two morphometric groups. The most closely related traits from location clustered two groups namely group I contained Skampuk, Lochum, Staksha, Skurru, Turtuk and Bogdang. While, group II contained Tirith, Pashkum, Sanjak, Hunder, Mulbek, Sumur and Changlung (Figure 2.6). The most important morphological traits distinguishing these two groups were reflected in their loadings on the first two principal components. In this case, the first two principal components extracted more than 75% variation among the population. The highest loading characters were: plant height, length of longest leaf, position of maximum width, uppermost leaf length, uppermost leaf width, lowermost leaf length, lowermost leaf width, position from base of the second leaf, greatest width, length of second leaf from base, width of second leaf, number of cauline leaves, length of lateral lobe of lip, from the base to the top of lobe, bract length, length from lowest bract to the top of inflorescence, length of inflorescence axis between the insertion points of first and fifth, number of flowers and uppermost internodium length for PC1, spur length, measured underneath the spur, lip width and bract width for PC2. While, remaining traits were lowest loading characters in PC1 and PC2 axis (Table 2.5). A dendrogram was drawn to display the phenetic relationships among different locations of Ladakh region based on Euclidean distances from the morphological data matrix. All locations were represented into two clusters. Dendrogram was based on average linkage (within group) analysis which grouped the 260 phenotype into population group with two main clusters I and II. Cluster I represented the phenotype of Skampuk, Lochum, Staksha, Skurru, Turtuk and Bogdang while, cluster II represented the phenotype of Tirith, Pashkum, Sanjak, Hunder, Mulbek, Sumur and Changlung (Figure 2.7). The results of PCA and MDS analysis matched with the cluster analysis.

Table 2.3: Duncan's test for mean comparisons of Morphological Characters among *D. hatagirea*

Location	Mean plant height (SE) (cm)	Mean leaf length (SE) (cm)	Mean leaf width (SE) (cm)	Mean Maximum width (SE) (cm)	Mean Uppermost leaf length (SE) (cm)	Mean Uppermost leaf width (SE) (cm)	Mean Lowermost leaf length(SE) (cm)
Tirith	74.6^{e} (0.54)	13.4^{e}(0.10)	3.9^{e}(0.08)	5.7^{e}(0.10)	10.4^{e}(0.08)	2.5^{a}(0.04)	12.4^{e} (0.07)
Sumur	73.6^{e}(0.42)	13.5^{e}(0.06)	4.0^{e}(0.05)	4.9^{c}(0.08)	9.5^{d}(0.04)	1.7^{c} (0.03)	10.5^{d} (0.05)
Changlung	45.2^{c}(0.65)	10.6^{c}(0.06)	3.5^{bc}(0.04)	4.3^{a}(0.03)	8.1^{b}(0.05)	1.4^{b} (0.02)	8.6^{b} (0.45)
Staksha	35.2^{ab}(0.58)	8.4^{a}(0.04)	3.3^{a} (0.05)	4.3^{ab}(0.05)	6.7^{a}(0.02)	1.3^{b}(0.04)	7.8^{a} (0.03)
Turtuk	66.2^{d}(0.59)	12.5^{d}(0.07)	3.2^{a} (0.07)	4.1^{a}(0.05)	8.7^{c}(0.08)	1.2^{a} (0.03)	9.3^{c} (0.17)
Bogdang	65.9^{d}(0.55)	12.3^{d}(0.12)	3.2^{a}(0.06)	4.1^{a}(0.05)	8.6^{c}(0.07)	1.2^{a}(0.04)	9.2^{c} (0.18)
Hunder	65.2^{d}(0.81)	10^{b}(0.06)	3.6^{c} (0.06)	5.1^{d}(0.07)	8.6^{c} (0.04)	1.4^{b}(0.02)	10.2^{d} (0.04)
Skurru	34.2^{a}(0.73)	8.4^{a}(0.05)	3.2^{a} (0.05)	4.2^{a}(0.06)	6.7^{a}(0.02)	1.3^{b}(0.04)	7.8^{a} (0.04)
Skampuk	36.0^{b}(0.52)	8.5^{a}(0.03)	3.4^{ab} (0.04)	4.5^{b}(0.07)	6.7^{a}(0.02)	1.4^{b}(0.03)	7.8^{a} (0.03)
Sanjak	72.8^{e}(0.60)	13.4^{e}(0.10)	3.9^{e} (0.08)	5.7^{e}(0.10)	10.5^{e}(0.08)	2.5^{a}(0.04)	12.4^{e}(0.07)
Mulbek	65.9^{d}(0.55)	12.3^{d}(0.12)	3.2^{a} (0.06)	4.1^{a}(0.05)	8.6^{c}(0.07)	1.2^{a} (0.04)	9.2^{c} (0.18)
Lochum	36.0^{b}(0.52)	8.5^{a}(0.03)	3.4^{ab} (0.04)	4.5^{b}(0.07)	6.7^{a}(0.02)	1.4^{b}(0.03)	7.8^{a}(0.03)
Pashkum	74.3^{e}(0.54)	13.4^{e}(0.10)	3.9^{e} (0.08)	5.7^{e}(0.10)	10.4^{e}(0.08)	2.5^{a}(0.04)	12.4^{e}(0.07)

Location	Mean Lowermost leaf width (SE) (cm)	Mean Length of second leaf from base (SE) (cm)	Mean Position from base of the second leaf greatest width (SE) (cm)	Mean Width of second leaf(SE) (cm)	Mean Number of cauline leaf (SE) (cm)	Mean Spur length measured underneath the spur (SE) (cm)	Mean Length of middle lobe of the lip from the base to the the top of the lobe(SE) (cm)	Mean Length of lateral lobe of the lip from the base to the the top of the lobe(SE) (cm)
Tirith	2.7^{d} (0.06)	13.2^{c} (0.10)	2.6^{c} (0.09)	3.8^{e} (0.05)	9.7^{d} (0.11)	1.31^{d} (0.05)	1.39^{d} (0.04)	1.57^{e} (0.04)
Sumur	3.0^{e} (0.07)	11.4^{b} (0.10)	2.7^{c} (0.05)	3.5^{d} (0.03)	9.5^{d} (0.17)	1.14^{c} (0.04)	1.28^{c} (0.02)	1.43^{d} (0.04)
Changlung	2.4^{c} (0.06)	11.2^{b} (0.08)	2.5^{b} (0.05)	3.5^{d} (0.05)	8.1^{abc} (0.18)	1.38^{d} (0.0)	1.39^{d} (0.05)	1.33^{c} (0.04)
Staksha	1.7^{a} (0.04)	10.4^{a} (0.01)	2.1^{a} (0.02)	2.5^{b} (0.03)	8.0^{a} (0.18)	1.02^{b} (0.05)	1.05^{ab} (0.04)	0.97^{a} (0.04)
Turtuk	2.1^{b} (0.03)	10.5^{a} (0.02)	2.1^{a} (0.03)	2.4^{ab} (0.07)	8.5^{bc} (0.11)	0.75^{a} (0.03)	1.11^{b} (0.02)	1.21^{b} (0.01)
Bogdang	2.1^{b} (0.02)	10.5^{a} (0.04)	2.1^{a} (0.03)	2.4^{a} (0.06)	8.6^{c} (0.11)	0.74^{a} (0.04)	1.12^{b} (0.02)	1.21^{b} (0.00)
Hunder	2.3^{c} (0.02)	10.5^{a} (0.05)	2.4^{b} (0.04)	3.53^{d} (0.02)	9.4^{d} (0.15)	1.12^{c} (0.02)	1.28^{c} (0.01)	1.34^{cd} (0.03)
Skurru	1.7^{a} (0.03)	10.5^{a} (0.06)	2.0^{a} (0.02)	2.6^{c} (0.03)	8.1^{ab} (0.20)	0.89^{b} (0.05)	0.98^{a} (0.03)	0.97^{a} (0.03)
Skampuk	1.7^{a} (0.04)	10.5^{a} (0.03)	2.1^{a} (0.02)	2.5^{c} (0.03)	7.9^{a} (0.18)	1.02^{b} (0.05)	1.05^{ab} (0.04)	0.97^{a} (0.04)
Sanjak	2.7^{d} (0.06)	13.2^{c}(0.14)	2.6^{c}(0.09)	3.8^{c}(0.05)	9.7^{d}(0.11)	1.31^{d}(0.05)	1.40^{d}(0.04)	1.57^{e}(0.04)
Mulbek	2.1^{b}(0.03)	10.5^{a}(0.07)	2.1^{a}(0.03)	2.4^{a}(0.06)	8.6^{c}(0.11)	0.74^{a}(0.04)	1.12^{b}(0.02)	1.22^{b}(0.01)
Lochum	1.7^{a}(0.05)	10.5^{a}(0.06)	2.1^{a}(0.02)	2.5^{a}(0.03)	7.9^{a}(0.18)	1.02^{bc}(0.05)	1.05^{ab}(0.04)	1.03^{a}(0.04)
Pashkum	2.7^{d} (0.06)	13.2^{c}(0.14)	2.6^{c}(0.09)	3.8^{c}(0.05)	9.7^{d}(0.11)	1.31^{d}(0.05)	1.40^{d} (0.04)	1.57^{e}(0.04)

Location	Mean Lip width(SE) (cm)	Mean Bract length (SE) (cm)	Mean Bract width (SE) (cm)	Mean Length from lowest bract to the top of inflorescence (SE) (cm)	Mean Length of inflorescence axis (SE) (cm)	Mean No of flowers (SE) (cm)	Mean Upper internodium length (SE) (cm)	Mean Stem diameter under inflorescence(SE) (cm)	Mean Stem diameter above lowermost leaf (SE) (cm)	Mean No of lobes (SE) (cm)
Tirith	1.22^{c} (0.04)	2.86^{e} (0.04)	0.48^{ab} (0.01)	16.5^{e}(0.24)	6.33^{e}(0.08)	13.2^{e}(0.1)	9.03^{d}(0.1)	1.60^{b}(006)	1.75^{b}(0.01)	3.3^{bc}(0.1)
Sumur	1.2^{c} (0.07)	2.57^{d} (0.04)	0.81^{e} (0.06)	14.2^{d}(0.14)	5.58^{d}(0.06)	10.6^{c}(0.35)	7.68^{c}(0.4)	1.59^{b}(0.06)	1.75^{b}(0.03)	3.4^{bc}(0.1)
Changlung	1.24^{c} (0.06)	2.39^{c} (0.05)	1.14^{f} (0.09)	11.4^{b}(0.1)	4.68^{b}(0.07)	10^{b}(0.16)	7.5^{bc}(0.11)	1.57^{b}(0.04)	1.79^{b}(0.03)	3.1^{b}(0.1)
Staksha	0.99^{b} (0.06)	1.43^{a} (0.03)	0.77^{de} (0.0)	9.7^{a}(0.2)	3.87^{a}(0.05)	9.5^{ab}(0.14)	5.67^{a}(0.0)	1.05^{a}(0.06)	1.25^{a}(0.02)	3.4^{bc}(0.7)
Turtuk	0.77^{a} (0.04)	1.99^{b} (0.04)	0.38^{a} (0.02)	11.2^{b}(0.21)	4.71^{b}(0.05)	9.75^{ab}(0.0)	7.34^{b}(0.04)	1.59^{b}(0.04)	1.77^{b}(0.02)	3.5^{bc}(0.1)
Bogdang	0.78^{a} (0.04)	1.99^{b} (0.04)	0.38^{a} (0.02)	11.1^{b}(0.20)	4.66^{b}(0.06)	9.65^{ab}(0.1)	7.35^{b}(0.03)	1.63^{b}(0.03)	1.79^{b}(0.02)	3.6^{bc}(0.15)
Hunder	1.18^{c} (0.03)	2.55^{d} (0.02)	0.64^{bcd} (0.03)	13.1^{c}(0.03)	5.1^{c}(0.03)	11.4^{d}(0.23)	7.6^{bc}(0.10)	1.88^{b}(0.02)	1.84^{b}(0.01)	3.6^{c}(0.11)
Skurru	0.85^{a} (0.06)	1.47^{a} (0.02)	0.57^{bc} (0.03)	9.7^{a}(0.05)	3.94^{a}(0.07)	9.37^{a}(0.12)	5.66^{a}(0.04)	1.18^{a}(0.07)	1.34^{a}(0.01)	4.1^{d}(0.12)
Skampuk	0.99^{b} (0.06)	1.43^{a} (0.03)	0.77^{de} (0.05)	9.7^{a}(0.03)	3.96^{a}(0.06)	9.5^{ab}(0.14)	5.73^{a}(0.04)	1.15^{a}(0.07)	1.36^{a}(0.07)	2.7^{a}(0.11)
Sanjak	1.22^{c}(0.04)	2.86^{e}(0.04)	0.49^{ab}(0.04)	16.5^{e} (0.24)	6.33^{e}(0.08)	13.0^{e}(0.18)	9.03^{d}(0.18)	1.59^{b}(0.06)	1.75^{b}(0.07)	3.3^{ab}(0.16)
Mulbek	0.79^{a}(0.04)	1.99^{b}(0.04)	0.39^{a}(0.02)	11.1^{b}(0.20)	4.66^{b}(0.06)	9.65^{ab}(0.11)	7.35^{b} (0.03)	1.63^{b}(0.03)	1.79^{b}(0.02)	3.4^{bc}(0.15
Lochum	0.96^{b}(0.06)	1.43^{a}(0.03)	0.78^{de}(0.06)	9.7^{a}(0.03)	3.96^{a}(0.06)	9.5^{ab}(0.14)	5.73^{a} (0.04)	1.16^{a}(0.07)	1.36^{a}(0.07)	2.8^{a} (0.14)
Pashkum	1.22^{c}(0.04)	2.86^{e}(0.04)	0.49^{ab}(0.04)	16.5^{e}(0.24)	6.33^{e}(0.08)	13.0^{e}(0.19)	9.03^{d}(0.18)	1.60^{b}(0.06)	1.75^{b}(0.07)	3.2^{ab}(0.16)

Table 2.4: Correlation of 28 morphological characters with respect to population

	PLH	LFL	LFW	MAXW	ULFL	ULFW	LLFL	LLFW	POSFRB	LSECLF	WIDOF	CAULF	SPULEN
PLH	1	**.945(**)**	.522(**)	.556(**)	**.944(**)**	.563(**)	.821(**)	.784(**)	.569(**)	.603(**)	.548(**)	.646(**)	.245(**)
LFL		1	.509(**)	.474(**)	**.933(**)**	.568(**)	.781(**)	.797(**)	.575(**)	.650(**)	.501(**)	.583(**)	.237(**)
LFW			1	.807(**)	.629(**)	.738(**)	.654(**)	.753(**)	.792(**)	.715(**)	.767(**)	.421(**)	.730(**)
MAX W				1	.696(**)	.876(**)	.768(**)	.661(**)	.704(**)	.814(**)	.803(**)	.581(**)	.622(**)
ULFL					1	.760(**)	**.911(**)**	.859(**)	.706(**)	.804(**)	.723(**)	.686(**)	.408(**)
ULFW						1	**.**814(**)	.673(**)	.703(**)	**.956(**)**	.797(**)	.541(**)	.662(**)
LLFL							1	.742(**)	.654(**)	.814(**)	.751(**)	.649(**)	.437(**)
LLFW								1	.870(**)	.731(**)	.800(**)	.645(**)	.564(**)
POSFRB									1	.741(**)	.818(**)	.541(**)	.676(**)
LSECLF										1	.801(**)	.548(**)	.649(**)
WIDOF											1	.547(**)	.742(**)
CAULF												1	.145(*)
SPULEN													1
LIPW													
LIP MID													
LIPLATE													
BRAL													
BRAW													
LOWBRA													
INFAXIS													
NOFLO													
UPPINT													
STDI													
STDLOW													
NOLOB													

Cont...

	LIPW	LIP MID	LIPLATE	BRAL	BRAW	LOWBRA	INFAXIS	NOFLO	UPPINT	STDI	STDLOW	NOLOB
PLH	.292(**)	.564(**)	.772(**)	.842(**)	-.285(**)	.812(**)	.862(**)	.620(**)	.880(**)	.663(**)	.607(**)	0.075
LFL	.246(**)	.556(**)	.749(**)	.802(**)	-.257(**)	.781(**)	.837(**)	.564(**)	.867(**)	.620(**)	.571(**)	0.047
LFW	.648(**)	.594(**)	.684(**)	.676(**)	.279(**)	.733(**)	.731(**)	.567(**)	.631(**)	.406(**)	.404(**)	-0.077
MAX W	.538(**)	.576(**)	.682(**)	.718(**)	0.013	.835(**)	.798(**)	.764(**)	.692(**)	.295(**)	.275(**)	-0.057
ULFL	.383(**)	.670(**)	.839(**)	**.927(**)**	-.225(**)	**.937(**)**	**.956(**)**	.760(**)	**.949(**)**	.587(**)	.538(**)	0.034
ULFW	.552(**)	.629(**)	.737(**)	.737(**)	-0.018	.889(**)	.863(**)	.801(**)	.758(**)	.305(**)	.286(**)	-0.097
LLFL	.390(**)	.578(**)	.759(**)	.864(**)	-.271(**)	**.925(**)**	**.910(**)**	.787(**)	.864(**)	.457(**)	.409(**)	0.004
LLFW	.486(**)	.764(**)	.796(**)	.868(**)	.124(*)	.850(**)	.853(**)	.652(**)	.832(**)	.544(**)	.534(**)	-0.029
POSFRB	.541(**)	.786(**)	.743(**)	.779(**)	.240(**)	.780(**)	.769(**)	.647(**)	.739(**)	.445(**)	.438(**)	-0.057
LSECLF	.514(**)	.686(**)	.791(**)	.780(**)	-0.023	**.907(**)**	.881(**)	.807(**)	.821(**)	.359(**)	.345(**)	-0.056
WIDOF	.659(**)	.745(**)	.756(**)	.871(**)	.213(**)	.854(**)	.825(**)	.742(**)	.734(**)	.391(**)	.374(**)	-0.04
CAULF	.159(*)	.486(**)	.584(**)	.655(**)	-.274(**)	.698(**)	.646(**)	.624(**)	.641(**)	.345(**)	.295(**)	0.066
SPULEN	.879(**)	.702(**)	.663(**)	.551(**)	.641(**)	.547(**)	.579(**)	.454(**)	.537(**)	.407(**)	.453(**)	-.174(**)
LIPW	1	.605(**)	.639(**)	.523(**)	.626(**)	.470(**)	.533(**)	.399(**)	.484(**)	.460(**)	.499(**)	-.169(**)
LIP MID		1	.814(**)	.742(**)	.366(**)	.701(**)	.727(**)	.643(**)	.755(**)	.537(**)	.527(**)	-0.02
LIPLATE			1	.862(**)	.129(*)	.827(**)	.882(**)	.685(**)	.881(**)	.699(**)	.686(**)	-0.008
BRAL				1	-0.026	**.922(**)**	**.937(**)**	.747(**)	**.919(**)**	.610(**)	.565(**)	0.032
BRAW					1	-.141(*)	-0.075	-.139(*)	-0.093	.148(*)	.227(**)	-.162(**)
LOWBRA						1	**.965(**)**	.855(**)	**.908(**)**	.461(**)	.420(**)	-0.002
INFAXIS							1	.812(**)	**.939(**)**	.598(**)	.552(**)	-0.022
NOFLO								1	.775(**)	.350(**)	.299(**)	-0.029
UPPINT									1	.675(**)	.641(**)	-0.019
STDI										1	**.970(**)**	0.03
STDLOW											1	-0.001
NOLOB												1

** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

Table 2.5: Characters loadings in first two principal components for the analysis of *D. hatagirea* (morphological data only) (high loadings are highlighted in boldface type)

Characters	Characters acronyms	PC1	PC2
		63.49 %	**11.70 % = 75.19**
Plant Height	PLH	**0.911**	-0.165
Length of longest leaf	LFL	**0.883**	-0.163
Width of longest leaf	LFW	0.667	0.519
Position of maximal width, the distance from leaf base to the place of maximal width	MAX W	**0.760**	0.317
Uppermost leaf length	ULFL	**0.983**	-0.029
Uppermost leaf width	ULFW	**0.805**	0.305
Lowermost leaf length	LLFL	**0.930**	-0.005
Lowermost leaf width	LLFW	**0.859**	0.260
Position from base, of the second leaf greatest width	POSFRB	**0.747**	0.451
length of second leaf from base	LSECLF	**0.844**	0.277
width of second leaf	WIDOF	**0.768**	0.475
Number of cauline leaves	CAULF	**0.748**	-0.161
Spur length, measured underneath the spur	SPULEN	0.430	**0.865**
Lip width	LIPW	0.384	**0.814**
Length of middle lobe of lip, from the base to the top of lobe	LIP MID	0.688	0.496
Length of lateral lobe of lip, from the base to the top of lobe	LIPLATE	**0.857**	0.334
Bract length	BRAL	**0.933**	0.179
Bract width	BRAW	-0.238	**0.898**
Length from lowest bract to the top of inflorescence	LOWBRA	**0.964**	0.129
Length of inflorescence axis between the insertion points of first and fifth	INFAXIS	**0.967**	0.169
No. of flowers	NOFLO	**0.816**	0.112
Uppermost internodium length	UPPINT	**0.950**	0.118
Stem diameter under inflorescence	STDI	0.580	0.204
Stem diameter above lowermost leaf	STDLOW	0.527	0.278
No. of lobe of tubers (1,2,3,4,5)	NOLOB	0.060	-0.263

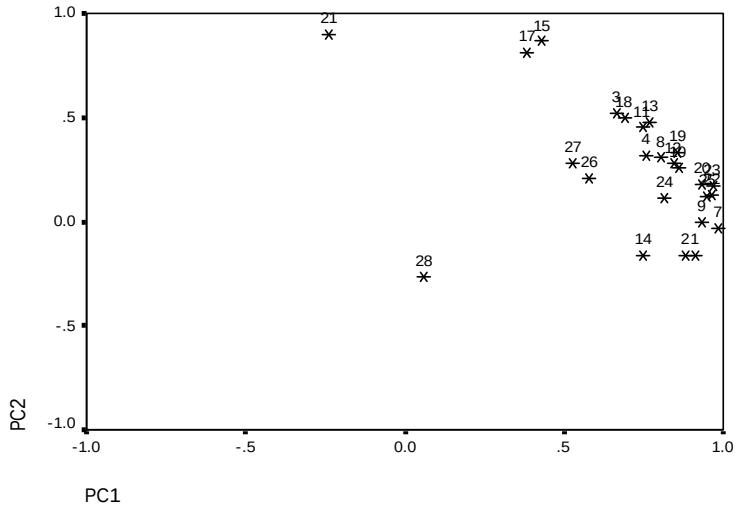


Figure 2.5: Principal component analysis of Morphological data of *D. hatagirea.*

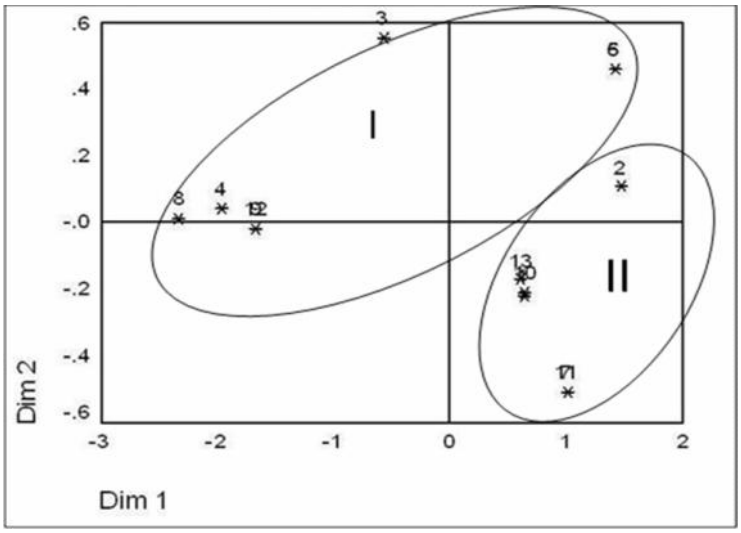


Figure 2.6: Multidimensional Scaling of Morphological data of *D. hatagirea* with their respective locations

1-Tirith, 2-Sumur, 3-Changlung, 4-Staksha, 5-Turtuk, 6-Bogdang, 7-Hunder, 8-Skurru, 9 Skampuk, 10-Sanjak, 11-Mulbek, 12-Lochum, 13-Pashkum

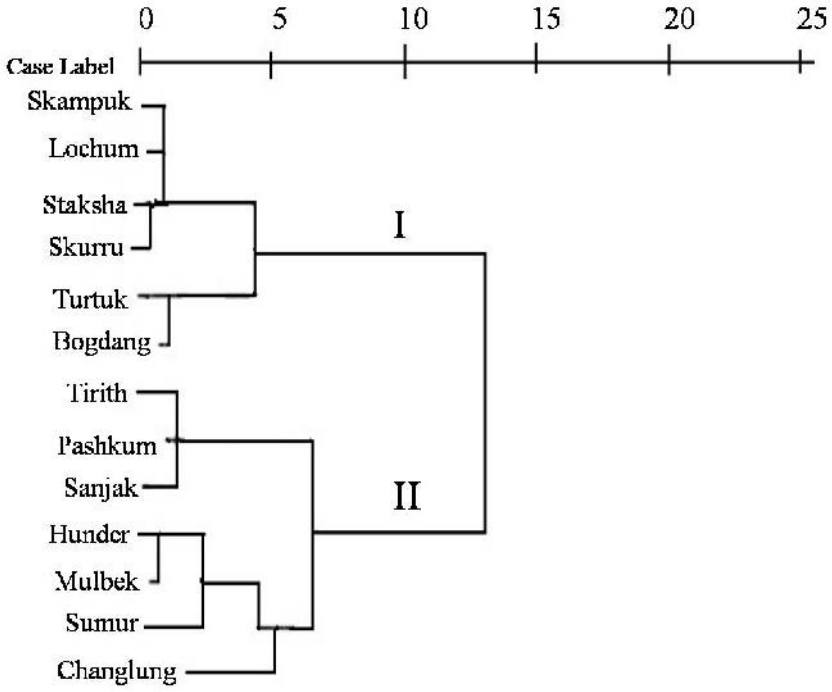


Figure 2.7: Dendrogram showing the phenetic relationships among 13 locations based on Euclidean distances from morphological data matrix

2.4 Discussions:

This is the first ever study on the morphometric analysis of *D. hatagirea.* The aim of the study was to investigate morphological variations in geographic ranges for differentiation of *D. hatagirea*. Morphological traits were selected based on phenotype expression of the population and influenced by different environmental factors [Heywood, 2002]. Significant variation was observed for different morphological traits among population of Ladakh region, but plant height was the only trait that showed great variation. Earlier report of *D. hatagirea* on taxonomy suggested plant height about 40 to 60 cm [Baral and Kurmi, 2006], but our results accomplished that it varied from 34.20 cm in Skurru to 74.60 cm at Tirith location at 5% significant level. In this study, traits such as plants height, leaf length, lowermost leaf length, length of second leaf from base and length from lowest bract to the top of inflorescence have been found varying from location to location. Tirith location showed more values of plants height, leaf length, lowermost leaf length, length of second leaf from base and length from lowest bract to the top of inflorescence while Skurru showed less value of these traits.

From this, it can be concluded that Tirith location phenotype was superior and showed great morphometric variation as compared to other locations. It implied that Nubra valley population showed great morphological dissimilarity with population of Suru and Indus valley. This may be due to wide geographical range, species richness and environmental factors. Therefore, *Dactylorhiza* populations deserve specific conservation attention as regards its habitat fragmentation. Conservation of its population's *ex-situ* and *in-situ* will have greater effects on population richness and on the status of such an endangered orchid species.

CHAPTER 3

Studies on population genetic structure and differentiation analyses of *D. hatagirea*

3.1 Introduction:

Orchidaceae is one of the most diverse families of flowering plants. Terrestrial species account for approximately one-third of the family [Swarts and Dixon, 2009] and these tend to have small and isolated populations placing them at risk of extinction [Chung et al., 2005]. Terrestrial species presently growing in desert islands are especially endangered or vulnerable to decline and extirpation because they often exhibit a relatively low level of genetic variation [Brzosko and Wroblewska, 2003] as a result of founder effects. Investigation on population structure of genetic diversity provides insights into evolutionary and demographic history of the threatened or rare species [Milligan et al., 1994]. Currently, many medicinal plant species are severely threatened by overexploitation, destructive harvesting and habitat deterioration. *Ex-situ* conservation of threatened medicinal plants has become an increasingly important role such as an insurance policy against loss of resources in the wild [Hawkins 2008].

India occupies 2.4% of the world's land area, the second largest country in Asia and seventh in the world which has a total geographical area of about 329 million ha. Of the world's 34 biodiversity hotspots, Ladakh is one of the megacentres of high value endangered plants. *D. hatagirea* is one of them. *Dactylorhiza hatagirea* (D.Don) (Family: Orchidaceae) is a terrestrial ground dwelling perennial orchid. The plant is native and near endemic to Indian Himalayan region. Generally, it is widely and narrowly distributed at altitudes ranging between 2500 to 5000 m amsl in open grassy slopes and alpine meadows [Bhatt et al., 2005]. Due to overexploitation of rhizomes, harvest for medicine and habitat fragmentation or reduction, it has been identified as critically endangered under Conservation Assessment and Management Plan (C.A.M.P.) [Forest Department of Uttar Pradesh, 1998] and is listed under Appendix II of Convention of International Trade in Endangered Species (CITES) [Murkute et al., 2011]. The economic potential of the species could be assessed on the basis of its high demands in national and international markets valuing Rs 2,700-3,200 kg^{-1} (dry tubers) and annual consumption of 'Salep' obtained from the species in India is about 7.38 tonnes (valued at about Rs. 50 lakhs) [Murkute et al., 2011]. Despite the economic importance and the level

of endangerment of *D. hatagirea*, we are not aware of any studies related to its population structure and its conservation management is still unknown. The objectives of the present study are: (1) Assessment of genetic diversity and population structure among and within population of *D. hatagirea*. (2) To develop baseline genetic information suitable for the development of its conservation strategies.

3.2 Materials and Methods:

3.2.1 Study area

Thirteen locations from three valley population (Nubra, Indus and Suru) of Ladakh region were surveyed. Young leaf tissues of 136 sample sizes were selected randomly for molecular analysis. The number of samples taken from each location depended on its geographic distribution (Table 3.1).

Table 3.1: Geographic localities and sample sizes of naturally distributed *D. hatagirea*

Sr. No.	Population	Location	Location ID	Latitude	Longitude	Altitude (m)	Sample Sizes
1	Nubra	Tirith	TIR	N 34° 32'.378	E 77° 38'.481	3183.7	10
2		Sumur	SUM	N 34° 31'.128	E 77° 34'.481	3084.9	10
3		Changlung	CHA	N 34° 55'.884	E 77° 28'.276	3348.3	10
4		Staksha	STA	N 34° 55'.885	E 77° 28'.276	3378.7	6
5		Turtuk	TUR	N 34° 50'.849	E 76° 49'.720	2817.1	10
6		Bogdang	BOG	N 34° 48'.198	E 77° 02'.453	2817.2	10
7		Hunder	HUN	N 34° 35'.043	E 77° 28'.592	3157.2	10
8		Skurru	SKU	N 34° 40'.229	E 77° 18'.031	3138.4	15
9		Skampuk	SKA	N 34° 35'.238	E 77° 34'.481	3197.8	15
10	Indus	Sanjak	SAN	N 34° 34'.458	E 76° 31'.584	2929.5	10
11	Suru	Mulbek	MUL	N 34° 35.437	E 76° 32'.673	2966.8	10
12		Lochum	LOC	N 34° 28'.064	E 76° 15'.337	3058.2	10
13		Pashkum	PAS	N 34° 31'.326	E 76° 10'.960	2888.3	10

3.2.2 DNA extraction Young leaves were collected and transported back to the laboratory and kept in -80^{0}C freezer until DNA extraction. Genomic DNA was extracted from *Dactylorhiza* leaves using CTAB method [Doyle and Doyle, 1990] which included use of 200 mg per sample polyvinylpyrrolidone (PVP). Briefly, 2 g of fresh plant tissues were grinded to a very fine powder in liquid nitrogen using mortar and pestle and transferred into a 50 ml polypropylene centrifuge tube containing 10 ml of pre-warmed (60^{0}C) DNA extraction buffer (100 mM Tris-Cl pH 8.0, 20 mM Na-EDTA salt pH 8.0, 1.4 M NaCl, 2% CTAB, 0.2% β-Mercaptoethanol). After mixing well and incubating at 60^{0}C for one hour with intermittent swirling, an equal volume of chloroform and isoamyl alcohol (24:1 v/v) was added and mixed thoroughly. Upper aqueous phase was collected by centrifuging at 12,000 × g for 15 min and extracted again by an equal volume of phenol, chloroform and isoamyl alcohol (25:24:1 v/v). Genomic DNA was precipitated by adding half volumes of 3M sodium acetate and again 2 volumes of absolute ethanol. After washing with 70% ethanol, the dried genomic DNA pellet was resuspended in TE buffer (10 mm Tris-HCl, 1mM EDTA). The quantity and quality of isolated total genomic DNA were determined using 0.8% agarose gel electrophoresis in 0.5 × TAE buffer for mobility related to known concentrations of lambda DNA.

3.2.3 RAPD analysis

Twenty random decamer primers obtained from integrated device technology (IDT) Tech. USA (Table 3.2) were used for RAPD amplification by following the protocol of Williams et al. [1990]. Amplification reactions were performed in volumes of 17 µL containing 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl2, 50 mM KCl, 200 µM of each deoxynucleotide triphosphates (dNTPs), 0.4 µM primer, 20 ng template DNA and 0.5 unit of Taq polymerase (Sigma-Aldrich, USA) with the following program: initial denaturation at 94°C for 5 min, followed by 1 min denaturation at 94°C, 1 min denaturation at specific annealing temperature (37°C), 1 min extension at 72°C for 39 cycles, and 5 min at 72°C for a final extension. Amplification product were electrophoresed on 2.0% agarose gel (Life Science technologies, USA) and were run at constant voltage (80V) in 1× TAE for 3 h, visualized by staining with ethidium bromide (0.5 µg ml-1) and a total of 2.5 µl loading buffer (6x) was added for each reaction before electrophoresis. After electrophoresis, the gels were documented on a gel documentation system (Alpha Innotech, Alphaimager, USA). Molecular size of the amplicon was estimated using 100 and 500 bp DNA ladders (‘Bangalore Genei, India’)

3.2.4 ISSR analysis

Forty arbitrary primers were obtained from 'Applied Biosciences, India' Out of these 28 primers showed amplification (Table 3.3). Amplification reactions were performed as follows: Initial denaturation at 94°C for 4 min, followed by 1 min denaturation at 94°C, 45s annealing temperature, 1 min extension at 72°C for 39 cycles and 5 min at 72°C for a final extension. Amplification products were electrophoresed on 2.5% agarose gel (Life Science technologies, USA) and were run at constant voltage (80V) in 1× TAE for 3h. Visualized by staining with ethidium bromide (0.5µg ml^{-1}) and a total of 2.5µl loading buffer (6X) was added for each reaction before electrophoresis. After electrophoresis, gels were documented in a gel documentation system (Alpha Innotech, Alphaimager, USA). The molecular size of the amplicon was estimated using 100 bp and 500 bp DNA ladders ('Bangalore Genei. India')

Table 3.2: List of primers used for RAPD amplification

Primer	Primer Sequence (5'⟶3')	GC (%)	Tm (^{0}C)	Total number of loci	Number of polymorphic loci	Percentage of polymorphic loci	Total number of fragments amplified	Resolving power
S21	CAGGCCCTT C	70	36.4	9	9	100	597	12.44
S22	TGCCGAGCT G	70	40.7	11	11	100	545	11.35
S23	AGTCAGCCA C	60	34.3	6	6	100	341	7.10
S24	AATCAGCCA C	50	30.1	9	9	100	582	12.13
S25	AGGGGTCTT G	60	32.6	8	8	100	508	10.58
S26	GGTCCCTGA C	70	35.2	10	10	100	530	11.04
S27	GAAACGGGT G	60	33.2	9	9	100	423	8.81
S28	GTGACGTAG G	60	31.1	10	10	100	507	10.56
S29	GGGTAACGC C	70	37.4	9	9	100	362	7.54
S30	GTGATCGCA G	60	33.1	8	8	100	287	5.98
S31	CAATCGCCG T	60	36.7	8	8	100	485	10.10
S32	TCGGCGATA G	60	34.0	9	9	100	410	8.54
S33	CAGCACCCA C	70	37.7	9	9	100	558	11.63
S34	TCTGTGCTG G	60	34.3	9	9	100	455	9.48
S35	TTCCGAACC C	60	34.2	10	10	100	476	9.92
S36	AGCCAGCGA A	60	38.3	8	8	100	310	6.46
S37	GACCGCTTG T	60	35.7	9	9	100	459	9.56

S38	AGGTGACCG T	60	36.2	9	9	100	511	10.65
S39	CAAACGTCG G	60	34.2	8	8	100	380	7.91
S40	GTTGCGATC C	60	33.5	9	9	100	430	8.96
	Total	-	-	**177**	**177**	**100**	**9156**	-

Table 3.3: List of ISSR primers used with the details of amplification (Where Y = C, T; R = A, G; B=C, G, T; D=AGT)

Primer	Primer Sequence (5' ⟶ 3')	GC (%)	Tm (^{0}C)	Total number of loci	Number of polymorphic loci	Percentage of polymorphic loci	Total number of fragments amplified	Resolving power
ISSR2	$(AG)_8T$	47	47.0	9	9	100	614	12.79
ISSR3	$(GA)_8T$	47	45.4	9	9	100	287	5.98
ISSR4	$(CT)_8T$	47	45.7	10	8	80	311	7.25
ISSR6	$(TC)_8A$	47.0	47.0	7	7	100	294	6.13
ISSR7	$(AC)_8T$	47	51.4	14	14	100	673	14.02
ISSR8	$(TG)_8A$	47	51.3	7	7	100	267	5.56
ISSR10	$(AG)_8YT$	47.2	49.2	11	11	100	681	14.18
ISSR12	$(GA)_8YT$	47.2	47.4	5	5	100	163	3.39
ISSR15	$(ACC)_6$	66.6	60.6	12	10	83.33	608	13.21
ISSR16	$(CCG)_6$	10	76.8	9	9	100	316	6.58
ISSR17	$(GGC)_6$	10	77.3	11	11	100	150	3.13
ISSR21	$(GA)_8C$	52.9	46.8	8	8	100	324	6.75
ISSR22	$(TC)_8C$	52.9	48.1	8	7	87.5	529	11.02
ISSR23	$(AG_8T_2)C$	47.3	51.2	7	6	85.7	390	8.13
ISSR24	$(CT_8G_2)A$	52.6	49.4	11	11	100	681	14.19
ISSR25	$(ATG)_6$	33.3	43.6	11	11	100	744	15.5
ISSR26	$GACA_4$	50	47.4	9	9	100	572	11.92
ISSR27	$(AGT_3)_3C8$	47.8	54.5	8	8	100	525	10.93
ISSR28	$(AG_8)G$	52.9	48.2	4	4	100	206	4.29
ISSR30	$(AC_8)C$	52.9	52.8	6	6	100	283	5.90

ISSR31	$(AG_8)YA$	47.2	48.9	9	9	100	462	9.63
ISSR32	$(GA_8)YC$	52.7	48.5	7	7	100	316	6.58
ISSR33	$(AC_8)YG$	52.7	54.3	12	12	100	744	15.5
ISSR34	$(TG_8)RC$	52.7	54.5	9	9	100	485	10.10
ISSR35	$(G_4T)_3$	80	59.3	10	10	100	405	8.44
ISSR36	BHB(GA)7	50.9	48.3	3	3	100	258	5.38
ISSR37	BDB(CA)7	50.9	52.4	6	6	100	229	4.77
ISSR38	DBD(AC)7	49	50.1	6	6	100	232	4.83
	Total	-	-	**238**	**232**	**97.73**	**11749**	-

3.2.5 Statistical analysis

Amplified fragments were scored for each individual as present (1) or absent (0) of homologous bands. The resulting (presence/absence) binary data matrix was analyzed using POPGENE version 1.31 [Yeh et al., 1999]. According to [Prevost and Wilkinson, 1999] "The resolving power (Rp) of a primer is: Rp = Σ *IB* where *IB* (band informativeness) takes the value of: 1–[2* (0.5–P)], *P* being the proportion of the 136 genotypes containing the band". Nei's genetic diversity (H), Shannon's information index (I), number of polymorphic loci (NPL) and percentage polymorphic loci (PPL) across all the thirteen locations were analyzed. In order to describe genetic variability within and among populations, the non-parametric analysis of molecular variance (AMOVA) was performed using squared Euclidean distances among all samples to partition the variation into two hierarchical levels; individual and population [Excoffier et al., 1992]. GenAlEx software was used to calculate a principal coordinates analysis (PCA) that plots the relationship between distance matrix elements based on their first two principal coordinates [Peakall and Smouse, 2001]. The geographical distances among locations were calculated using the online program Research Coordination Network (RCN) Utilities and Tools. Isolation by distance (IBD) over the distribution area was assessed by correlating the genetic and geographic distances (Km) between all location pairs by using Mantel test [Mantel, 1967]. Nei's analysis of gene diversity among locations (Nei 1978) were carried out by measuring total genetic diversity (Ht), within species diversity (Hs), gene differentiation (Gst), estimation of gene flow *(*Nm) from the parameters and Fst index [Wright, 1951].

3.2.6 Data analysis

By using Bayesian Markov Chain Monte Carlo (MCMC), software STRUCTURE 2.3.2 was used to study the affiliation of individual from locations into K cluster population, to minimize the Hardy-Weinberg disequilibrium between loci within groups, within the option of including prior information on the spatial location of populations and test for admixture [Hubisz et al., 2009]. Number of clusters was set from K=1 to 13 with five simulations and for each simulation we have fixed burnin period of 100,000 steps followed by 250,000 MCMC replicates to check for convergence of likelihood values for each K value. We used these methods simultaneously as they have different prior distributions and assumptions and we wished to compare and evaluate the robustness of the resulting genetic clusters and population structuring. STRUCTURE assigns individuals probabilisticity to populations using marker frequencies, giving probabilities corresponding to the proportion of the genome arising from each parent cluster, qi (where i = 1 - K) and hence does not require knowing priori the assignment of an individual to a species. One thousand bootstrapped Nei's genetic distance matrix was generated using AFLP-SURV [Vekemans et al., 2002] and this matrix was used for constructing Neighbour-Joining (NJ) dendrogram using the NEIGHBOUR and CONSENSE module in the PHYLIP package v3. 69 [Felsenstein, 2006] and the tree was viewed using TREE-VIEW. Genetic differentiation coefficient (Gst) was calculated by POPGENE and Wright's (Fst) averaged over loci was calculated by using AFLP-SURV. Results obtained from STRUCTURE were interpreted by online available tool called STRUCTURE HARVESTER [Earl et al., 2012] which implements Evanno's method [Evanno et al., 2005] for calculation of correct number of clusters (K). CLUMPP indfile obtained from STRUCTURE HARVESTER was used as input for CLUMPP [Jakobsson et al., 2007] program which permutes replicated matrix into one representative matrix, CLUMPP output is visualized graphically by DISTRUST program [Rosenberg, 2004].

3.3 Results:

3.3.1 Genetic diversity

RAPD and ISSR polymorphisms were resolved in gel picture (Figure 3.1 & 3.2). A total of 177 reproducible bands were produced using 20 RAPD primers (8.8 Bands per primer) of which 174 were polymorphic (PPL= 98.30%) and 238 reproducible bands were produced using 28 ISSR primers (8.5 Bands per primer) of which 232 were polymorphic (*PPL*= 97.47%). RAPD genetic diversity analysis revealed the highest values of Nei's genetic

diversity (0.23), Shannon information index (0.36) and polymorphic loci (81.36%) among accession from Skampuk sites and lowest values of Nei's genetic diversity (0.11), Shannon information index (0.18) and polymorphic loci (36.72%) among accession from Bogdang sites (Table 3.4). While, in ISSR highest genetic diversity was found in accession from Skampuk site with Nei's genetic diversity (0.29), Shannon information index 0.44 and polymorphic loci 89.08% and the accession from Staksha site showed lowest with Nei's genetic diversity 0.14, Shannon information index 0.22 and polymorphic loci 39.50% (Table 3.5). [Nei's 1978] classified levels of genetic distance at < 0.05 as low, between 0.05 and 0.15 as medium and > 0.15 as high. In RAPD analysis, Staksha site varied in narrow range while, the Sumur site was more diverse. Pair-wise Nei's distances [Nei's, 1973] were calculated for all locations. The greatest inter-population average distance (0.48) was observed between Sumur and Pashkum. While, the corresponding least distance (0.05) was found between Staksha and Changlung (Table 3.6) and in ISSR analysis, the greatest inter-population average distance (0.45) was between Skampuk and Pashkum. While, the corresponding least distance (0.05) was between in Staksha and Changlung (Table 3.7). AMOVA analysis unravelled that there were significant ($P < 0.001$) genetic differences within and among population of *D. hatagirea* (Nei 1978). In RAPD analysis, the total genetic diversity, 57% was attributed among populations and the rest 43% to within populations and In ISSR analysis, 60% and 40% in among and within populations (Figure 3.3). The result of the Mantel Test of RAPD and ISSR data revealed that no significant positive correlation was detected ($R^2 = 0.024$, $P = 0.290$, RAPD; $R^2 = 0.016$, $P = 0.310$, ISSR) and showed no clear geographic tendency in the distribution (Figure 3.4).

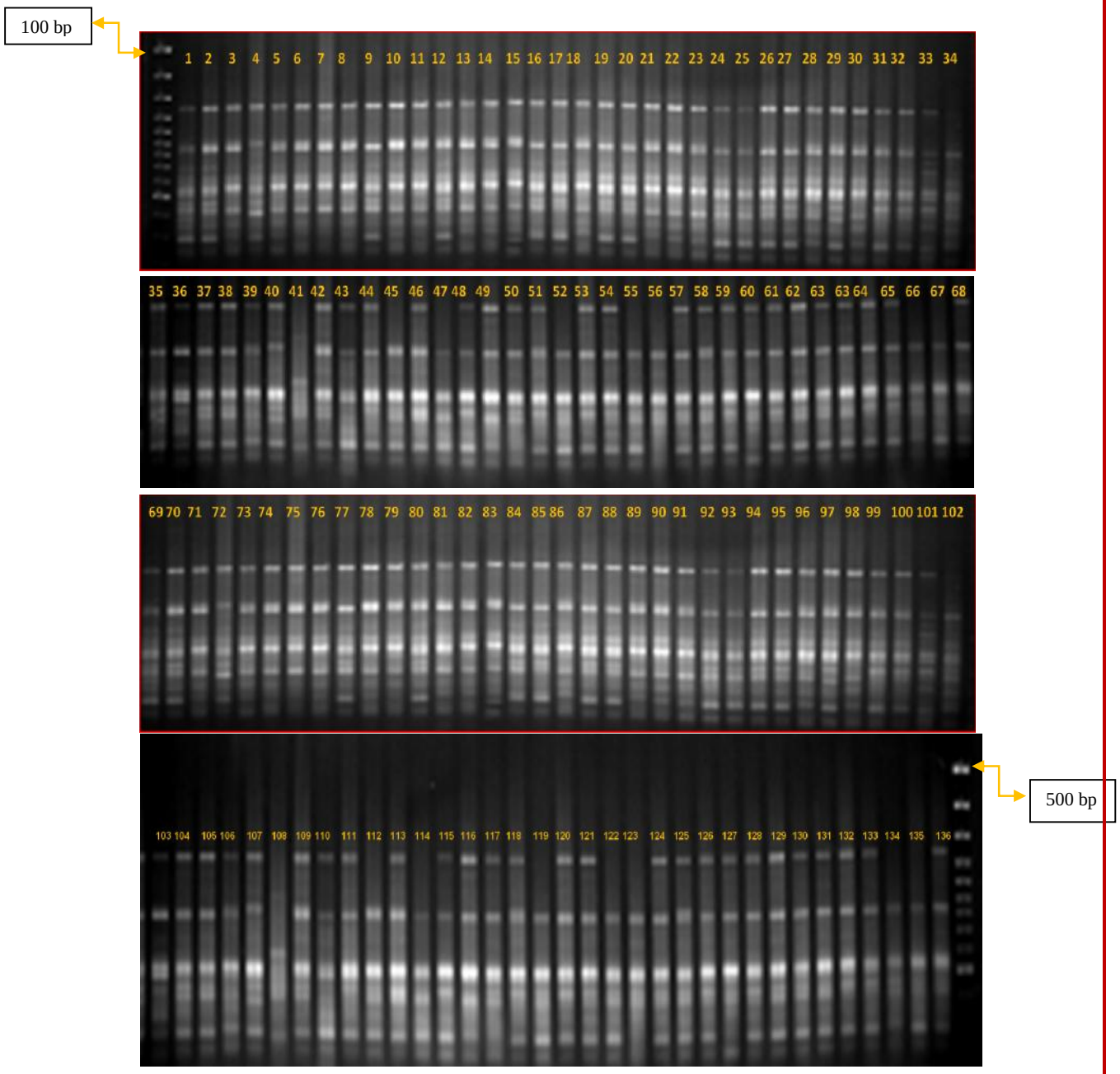


Figure 3.1: RAPD- PCR amplification

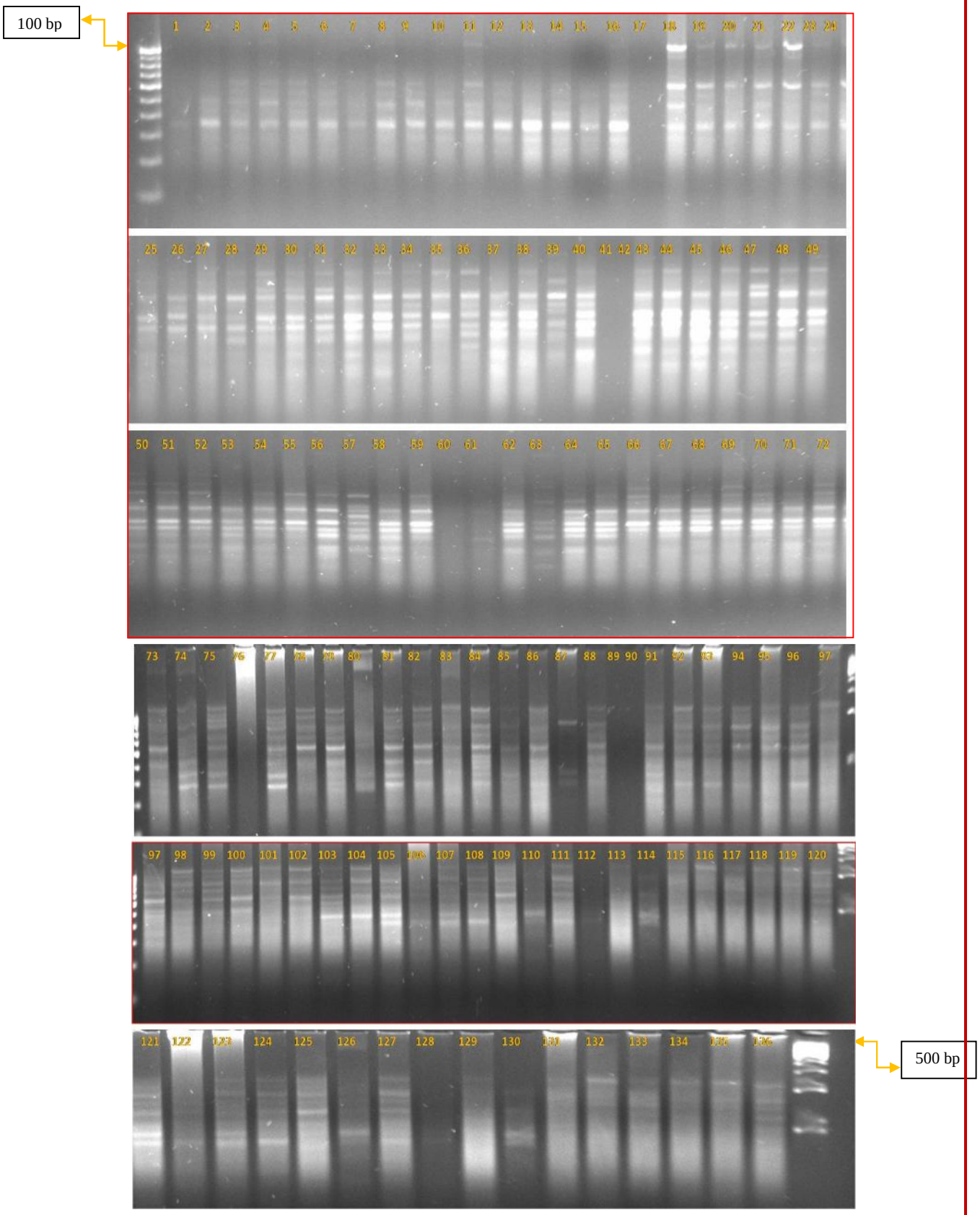


Figure 3.2: ISSR- PCR amplification

Table 3.4: Summary of genetic variation statistics for all loci of RAPD among the *Dactylorhiza* populations with respect to their distribution

Marker	Population	Location	Sample size	H (Mean ± SD)	I (Mean ± SD)	NPL	PPL
RAPD	Nubra	Tirith	10	0.12 ± 0.17	0.18 ± 0.25	65	36.72
		Sumur	10	0.24 ± 0.16	0.37 ± 0.23	141	79.66
		Changlung	10	0.16 ± 0.18	0.25 ± 0.23	90	50.85
		Staksha	6	0.13 ± 0.18	0.19 ± 0.27	60	33.90
		Turtuk	10	0.21 ± 0.20	0.31 ± 0.29	102	57.63
		Bogdang	10	0.20 ± 0.19	0.31 ± 0.27	107	60.45
		Hunder	10	0.26 ± 0.18	0.39 ± 0.26	130	73.45
		Skurru	15	0.21 ± 0.18	0.32 ± 0.26	122	68.93
		Skampuk	15	0.19 ± 0.19	0.30 ± 0.26	114	64.41
	Indus	Sanjak	10	0.16 ± 0.18	0.25 ± 0.26	90	50.85
	Suru	Mulbek	10	0.21 ± 0.16	0.33 ± 0.24	128	72.32
		Lochum	10	0.22 ± 0.16	0.34 ± 0.23	133	75.14
		Pashkum	10	0.21 ± 0.17	0.33 ± 0.24	127	71.75
Mean				**0.19**	**0.30**	-	-

H= Nei's gene diversity, I= Shannon's info. Index, NPL= No. of polymorphic loci, PPL= Percentage of polymorphic loci

Table 3.5 Summary of genetic variation statistics for all loci of ISSR among the *Dactylorhiza* populations with respect to their distribution

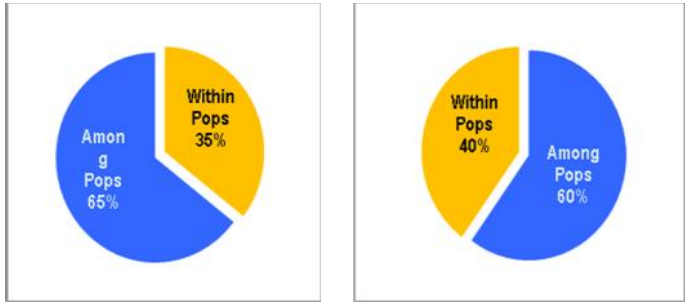
Marker	Valley	Location	Sample size	H (Mean±SD)	I (Mean± SD)	NPL	PPL
ISSR	Nubra	Tirith	10	0.23 ± 0.20	0.34 ± 0.27	153	64.29
		Sumur	10	0.20± 0.19	0.30 ± 0.27	137	57.56
		Changlung	10	0.15 ± 0.14	0.22 ± 0.28	102	42.86
		Staksha	6	0.14 ± 0.18	0.22 ± 0.28	94	39.50
		Turtuk	10	0.27 ± 0.18	0.40 ± 0.26	178	74.79
		Bogdang	10	0.17 ± 0.20	0.25 ± 0.28	114	47.90
		Hunder	10	0.25 ± 0.18	0.37 ± 0.25	179	75.21
		Skurru	15	0.20 ± 0.18	0.31 ± 0.25	156	65.55
		Skampuk	15	0.29 ± 0.17	0.44 ± 0.22	212	89.08
	Indus	Sanjak	10	0.23 ± 0.23	0.39 ± 0.26	165	67.12
	Suru	Mulbek	10	0.18± 0.28	0.24 ± 0.19	125	56.90
		Lochum	10	0.29 ± 0.19	0.24 ± 0.15	224	78.45
		Pashkum	10	0.29 ± 0.13	0.24 ± 0.22	214	77.34
Mean				**0.22**	**0.31**	-	-

H= Nei's gene diversity, I= Shannon's info. Index, NPL= No. of polymorphic loci, PPL= Percentage of polymorphic loci

Table 3.6: Inter-population genetic distances calculated by Nei's method in RAPD data

Location	Pashkum	Lochum	Mulbek	Sanjak	Skampuk	Skurru	Hunder	Bogdang	Turtuk	Staksha	Changlung	Sumur	Tirith
Pashkum	***	0.07	0.08	0.07	0.06	0.06	0.30	0.08	0.14	0.20	0.30	0.28	0.31
Lochum	0.08	***	0.09	0.10	0.11	0.08	0.30	0.10	0.14	0.14	0.31	0.29	0.32
Mulbek	0.09	0.11	***	0.07	0.07	0.07	0.28	0.07	0.12	0.21	0.29	0.25	0.29
Sanjak	0.08	0.07	0.07	***	0.08	0.07	0.21	0.07	0.07	0.16	0.21	0.46	0.22
Skampuk	0.07	0.06	0.07	0.06	***	0.06	0.22	0.06	0.07	0.15	0.22	0.20	0.22
Skurru	0.08	0.18	0.08	0.06	0.09	***	0.29	0.07	0.12	0.17	0.29	0.27	0.30
Hunder	0.31	0.31	0.29	0.23	0.24	0.30	***	0.27	0.28	0.35	0.09	0.06	0.08
Bogdang	0.09	0.11	0.33	0.08	0.07	0.08	0.28	***	0.13	0.22	0.28	0.24	0.29
Turtuk	0.15	0.16	0.13	0.07	0.07	0.13	0.29	0.14	***	0.16	0.26	0.24	0.26
Staksha	0.21	0.15	0.23	0.18	0.17	0.19	0.09	0.24	0.18	***	0.10	0.38	0.43
Changlung	0.31	0.33	0.30	0.23	0.23	0.30	0.11	0.29	0.28	[a]**0.05**	***	0.07	0.06
Sumur	[a]**0.48**	0.30	0.26	0.22	0.22	0.28	0.08	0.26	0.26	0.17	0.29	***	0.08
Tirith	0.32	0.33	0.31	0.24	0.24	0.31	0.10	0.31	0.28	0.44	0.08	0.06	***

Above diagonal values are Nei's unbiased genetic distances, those below the diagonal are Nei's genetic distances. [a]values in bold are maximum or minimum genetic distances.



RAPD ISSR

Figure 3.3: AMOVA analysis among the populations of *Dactylorhiza*

Table 3.7: Inter-population genetic distances calculated by Nei's method in ISSR data

Location	Pashkum	Lochum	Mulbek	Sanjak	Skampuk	Skurru	Hunder	Bogdang	Turtuk	Staksha	Changlung	Sumur	Tirith
Pashkum	***	0.08	0.09	0.07	0.06	0.06	0.30	0.08	0.14	0.20	0.25	0.28	0.29
Lochum	0.10	***	0.10	0.10	0.11	0.08	0.30	0.10	0.14	0.14	0.29	0.29	0.28
Mulbek	0.12	0.11	***	0.07	0.07	0.07	0.28	0.07	0.12	0.21	0.29	0.25	0.32
Sanjak	0.13	0.08	0.12	***	0.08	0.07	0.21	0.07	0.07	0.16	0.21	0.32	0.30
Skampuk	[a]**0.45**	0.06	0.12	0.06	***	0.06	0.22	0.06	0.07	0.34	0.22	0.20	0.28
Skurru	0.06	0.38	0.18	0.06	0.09	***	0.29	0.07	0.12	0.17	0.29	0.28	0.30
Hunder	0.37	0.31	0.29	0.23	0.24	0.30	***	0.27	0.28	0.28	0.09	0.06	0.08
Bogdang	0.15	0.11	0.33	0.08	0.07	0.08	0.28	***	0.13	0.22	0.26	0.24	0.29
Turtuk	0.25	0.26	0.13	0.07	0.07	0.13	0.29	0.14	***	0.16	0.26	0.26	0.26
Staksha	0.21	0.13	0.13	0.18	0.17	0.19	0.09	0.24	0.18	***	0.10	0.29	0.35
Changlung	0.21	0.13	0.31	0.23	0.23	0.30	0.11	0.29	0.28	[a]**0.05**	***	0.07	0.06
Sumur	0.26	0.31	0.26	0.22	0.22	0.28	0.08	0.26	0.26	0.17	0.28	***	0.09
Tirith	0.34	0.23	0.31	0.24	0.24	0.31	0.10	0.31	0.28	0.34	0.08	0.06	***

Above diagonal values are Nei's unbiased genetic distances, those below the diagonal are Nei's genetic distances. [a]values in bold are maximum or minimum genetic distances.

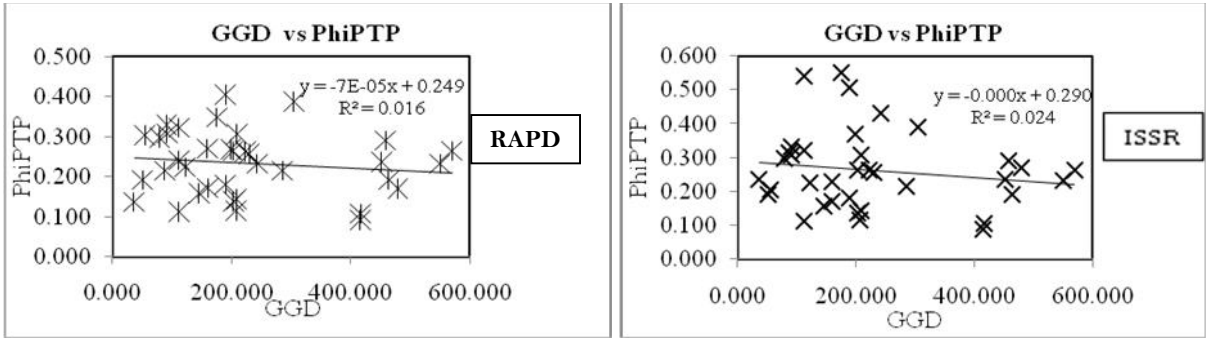


Figure 3.4: Graph showing the correlation between geographic (X-axis) and genetic distance (Y-axis)

3.3.2 Population genetic structure and differentiation

RAPD and ISSR population genetic diversity based on 174 and 232 polymorphic loci revealed that a moderate level of genetic differentiation and gene flow (RAPD: Gst = 0.235; Fst = 0.132, P < 0.001, PhiPT = 0.570, P= 0.001, Nm = 1.4703); (ISSR: Gst = 0.258; Fst = 0.135, P < 0.001, PhiPT = 0.595, P= 0.001, Nm = 1.4324) occurred among populations of *D. hatagirea* (Table 3.8). Genetic analysis of RAPD marker showed that the highest genetic identity (0.62) existed between location of Changlung and Staksha. While, the lowest (1.02) was between the location of Sanjak and Skampuk (Table 3.9) and in ISSR marker analysis showed that highest genetic identity (1.02) existed between locations Pashkum and Lochum. While, the lowest (0.81) occurred between the location of Staksha and Skampuk (Table 3.10). STRUCTURE analyses the proportion of membership of individual samples within assigned groups. The modal value of the distribution of true K identified two clusters or groups; DK when graphed against K showed a peak at K = 2, dropping down to near zero at K = 3. STRUCTURE commonly plateau near the true value of K; each subsequent value of delta K consistently returned a clear peak at the true value of K under a variety of migration models including a hierarchical island model [Evanno et al., 2005]. The specific model assumed in this case correlated with allele frequencies and admixture. From this data set, we determined the assignment of individuals clusterwise; further how well these clusters matched population assignment and the possible levels of introgression in each individual. The admixture assigned by STRUCTURE, corresponded in part with the population groupings. In RAPD STRUCTURE analysis, Orange color cluster covered Sanjak, Turtuk, Staksha, Hunder, Sumur, Tirith and Changlung. While, green color cluster covered Skampuk, Lochum, Mulbek, Bogdang, Pashkum and Skurru (Figure 3.5) and in ISSR STRUCTURE analysis, blue color cluster covered Pashkum, Bogdang, Changlung, Sumur, Mulbek, Staksha and Tirith. While, orange color cluster covered Hunder, Turtuk, Lochum, Skurru, Sanjak and Skampuk (Figure 3.6). This result is identical to the splitting in the Neighbor-Joining (NJ) dendrogram using PHYLIP package v3.69 and TREE-VIEW with 1000 boot strapping. Overall, the cluster analysis of RAPD and ISSR data strongly suggested that the 13 locations can be divided into two clusters (Figure 3.7 & 3.8). The Principal component analysis (PCA) of RAPD and ISSR data, pair-wise PhiPT values were grouped into two clusters and showed 63.34% and 67.54% of the variance in PhiPT values (Figure 3.9 & 3.10). Topography of the region mostly affects the genetic structure of plant population in Trans Himalaya, as revealed

by the BARRIER analysis (Figure 3.11). The Suru valley separated from the Indus and Nubra valley as well as the geographical location of the barrier which coincided with the Ladakh mountain range, (6500 m amsl) probably acts as a barrier to the movement of pollinators and seed dispersal agent.

Table 3.8: Overall genetic variability across the population of *Dactylorhiza*

Markers	H	I	Ht	Gst	Fst	PhiPT	Nm
RAPD	0.27 ± 0.16	0.40 ± 0.20	0.27 ± 0.02	0.24	0.13	0.57	1.47
ISSR	0.30 ± 0.15	0.46 ± 0.19	0.30 ± 0.02	0.26	0.14	0.60	1.43

H = Nei's genetic diversity, I=Shannon's information index, Ht=Total genetic diversity, Gst = Gene differentiation, Fst=Wright inbreeding coefficient, PhiPT = Total differentiation, Nm = Gene flow

Table 3.9: Inter-Population genetic identity calculated by Nei's method in RAPD data

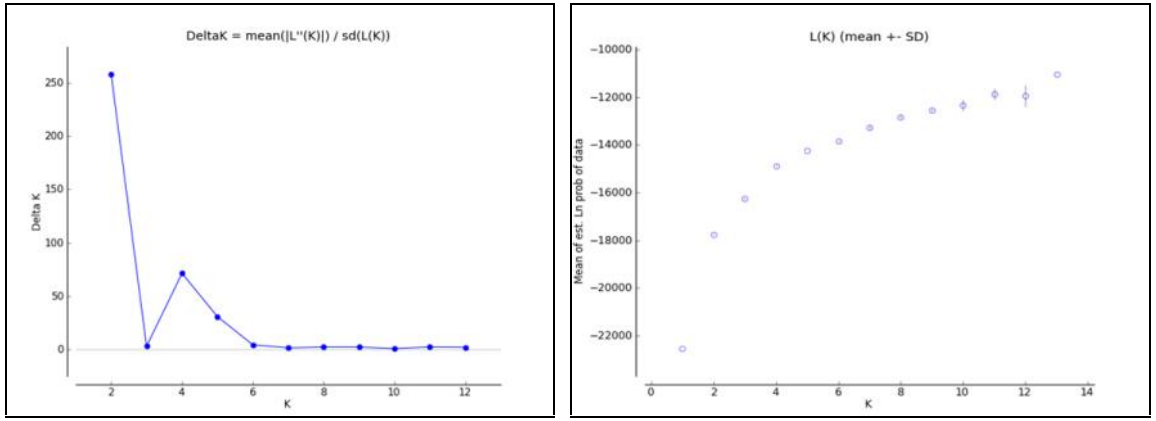
Location	Pashkum	Lochum	Mulbek	Sanjak	Skampuk	Skurru	Hunder	Bogdang	Turtuk	Staksha	Changlung	Sumur	Tirith
Pashkum	***	0.93	0.93	0.93	0.94	1.00	0.74	0.93	0.87	0.82	0.74	0.76	0.73
Lochum	0.92	***	0.91	0.95	0.96	0.97	0.74	0.90	0.87	0.87	0.73	0.75	0.73
Mulbek	0.92	0.90	***	0.95	0.95	0.93	0.76	1.01	0.88	0.81	0.75	0.78	0.75
Sanjak	0.92	0.93	0.93	***	[a]**1.02**	0.95	0.81	0.94	0.95	0.85	0.81	0.82	0.80
Skampuk	0.93	0.95	0.94	1.00	***	0.96	0.80	0.94	0.95	0.86	0.81	0.82	0.80
Skurru	0.99	0.96	0.93	0.94	0.95	***	0.75	0.93	0.89	0.84	0.75	0.76	0.75
Hunder	0.73	0.73	0.75	0.79	0.79	0.74	***	0.76	0.75	0.70	0.91	0.94	0.92
Bogdang	0.92	0.89	1.00	0.92	0.93	0.92	0.75	***	0.88	0.80	0.76	0.79	0.75
Turtuk	0.86	0.85	0.87	0.93	0.93	0.88	0.75	0.87	***	0.85	0.77	0.79	0.75
Staksha	0.81	0.86	0.80	0.84	0.85	0.83	0.69	0.79	0.84	***	0.63	0.67	0.65
Changlung	0.73	0.72	0.74	0.80	0.80	0.74	0.90	0.75	0.76	[a]**0.62**	***	0.95	0.96
Sumur	0.75	0.74	0.77	0.81	0.81	0.76	0.93	0.78	0.77	0.68	0.94	***	0.96
Tirith	0.72	0.72	0.74	0.79	0.79	0.74	0.91	0.74	0.76	0.64	0.95	0.94	***

Above diagonal values are Nei's unbiased genetic identities, those below the diagonal are Nei's genetic identities. [a]values in bold are maximum or minimum genetic identities.

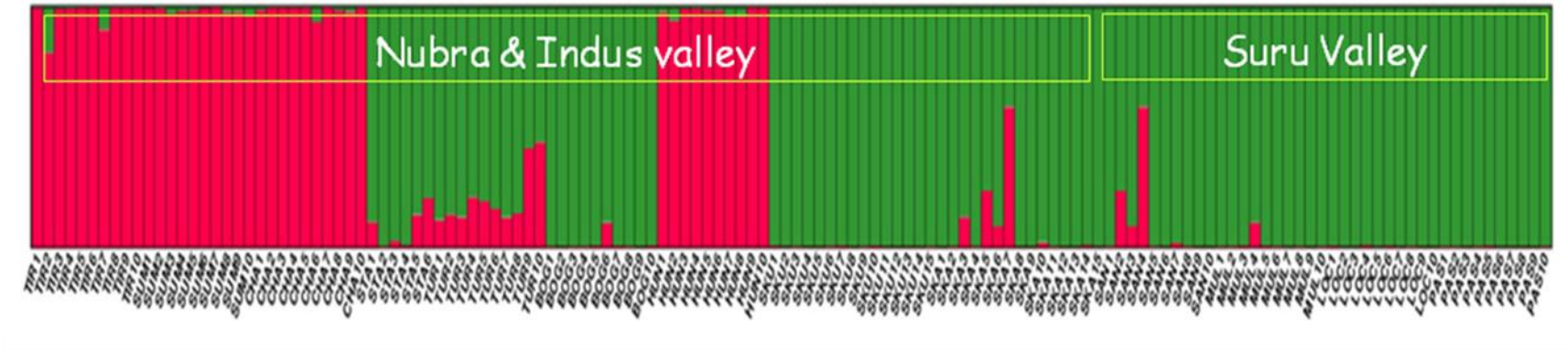
Table 3.10: Inter-Population genetic identity calculated by Nei's method in ISSR data

Location	Pashkum	Lochum	Mulbek	Sanjak	Skampuk	Skurru	Hunder	Bogdang	Turtuk	Staksha	Changlung	Sumur	Tirith
Pashkum	***	[a]**1.02**	0.99	1.00	1.00	1.01	1.00	1.00	1.01	0.94	1.00	1.01	0.97
Lochum	0.98	***	0.97	0.99	1.00	1.01	1.00	0.99	1.01	0.91	1.01	1.00	0.96
Mulbek	0.95	0.93	***	0.96	0.92	0.94	0.95	0.98	0.97	0.96	0.99	0.98	0.98
Sanjak	0.96	0.96	0.93	***	0.98	0.98	0.97	0.98	1.00	0.87	0.99	0.98	0.90
Skampuk	0.96	0.97	0.89	0.95	***	0.99	0.97	0.95	0.98	0.85	0.99	0.97	0.89
Skurru	0.98	0.98	0.91	0.95	0.97	***	1.00	0.98	1.00	0.89	1.00	1.00	0.94
Hunder	0.96	0.97	0.91	0.94	0.95	0.97	***	0.98	0.99	0.91	0.99	0.99	0.95
Bogdang	0.96	0.95	0.94	0.95	0.93	0.95	0.95	***	0.99	0.92	0.99	1.00	0.96
Turtuk	0.97	0.98	0.93	0.97	0.95	0.97	0.96	0.95	***	0.92	1.01	1.00	0.95
Staksha	0.89	0.86	0.90	0.83	[a]**0.81**	0.85	0.86	0.87	0.87	***	0.96	0.93	1.00
Changlung	0.98	0.97	0.95	0.95	0.96	0.97	0.95	0.95	0.97	0.90	***	1.01	0.98
Sumur	0.97	0.97	0.94	0.95	0.94	0.97	0.96	0.96	0.96	0.88	0.97	***	0.98
Tirith	0.93	0.91	0.93	0.87	0.86	0.90	0.91	0.92	0.91	0.96	0.94	0.94	***

Above diagonal values are Nei's unbiased genetic identities, those below the diagonal are Nei's genetic identities. [a]values in bold are maximum or minimum genetic identities

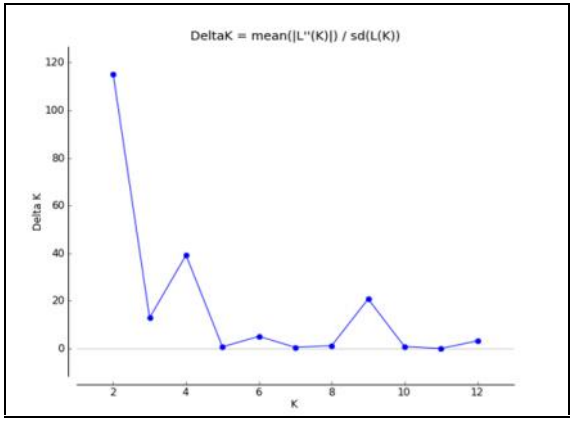


a. **b.**

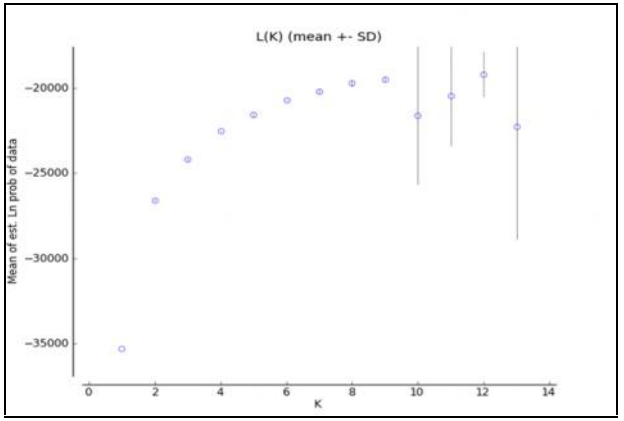


c.

Figure 3.5: STRUCTURE analysis of *D. hatagirea* population. Based on RAPD data (a: the relationship between K and Ln P (D); b: the relationship between K and ΔK; c: the grouping when K= 2)



a.



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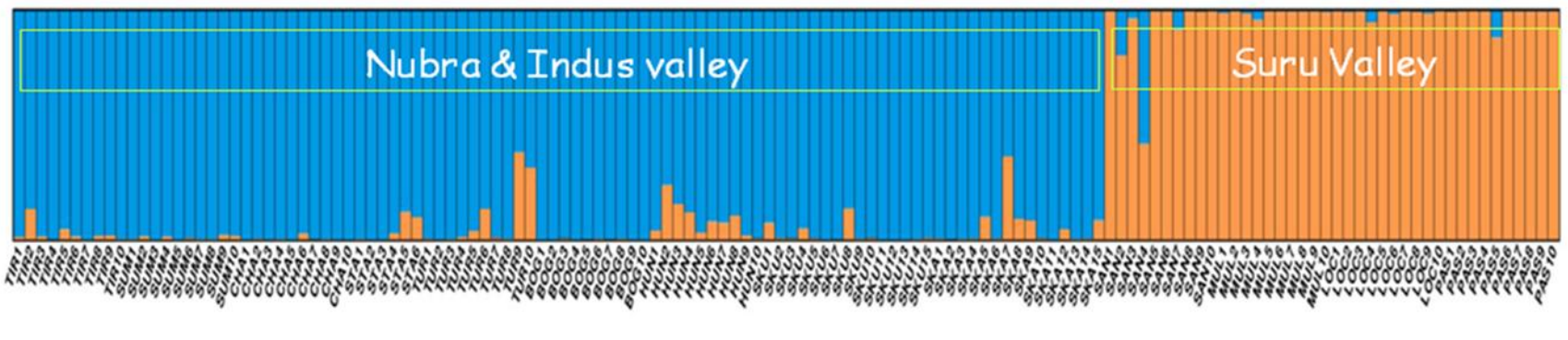


c.

Figure 3.6: STRUCTURE analysis of *D. hatagirea* population. Based on ISSR data (a: the relationship between K and Ln P (D); b: the relationship between K and ΔK; c: the grouping when K= 2)

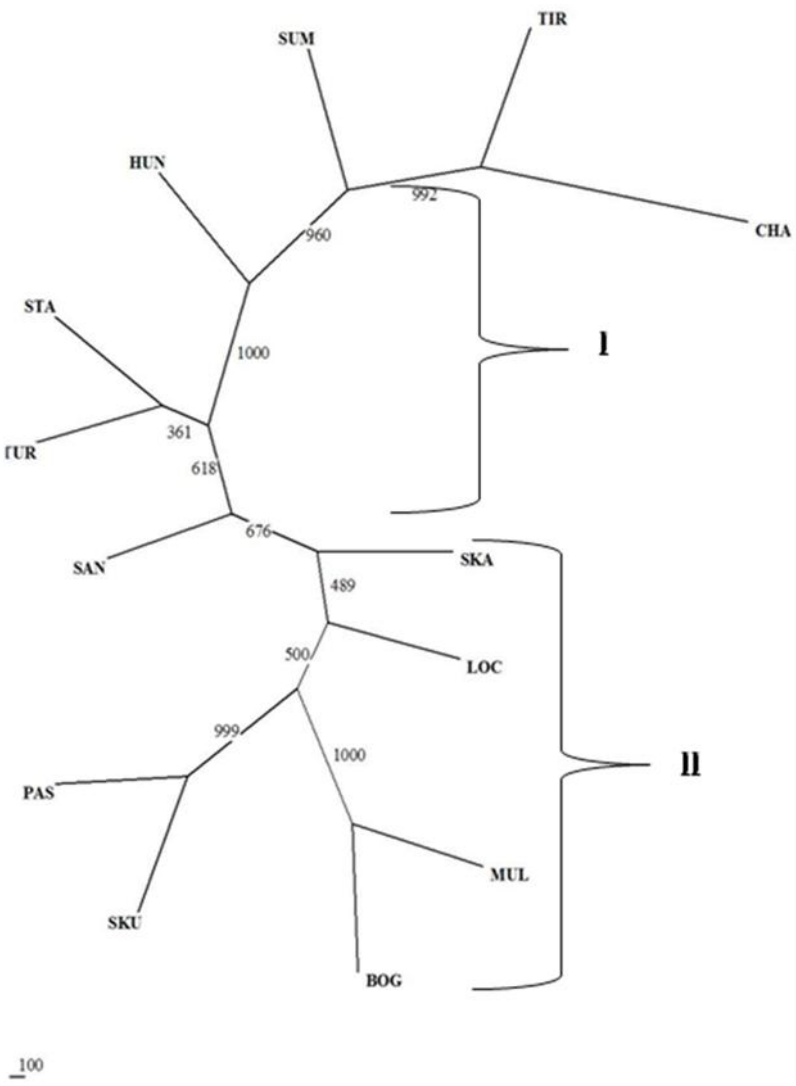


Figure 3.7: NJ tree of 13 locations of *D. hatagirea* using PHYLIP Package (Based on RAPD data)

Cluster I: Sanjak, Turtuk, Staksha, Hunder, Sumur, Tirith and Changlung

Cluster II: Skampuk, Lochum, Mulbek, Bogdang, Pashkum and Skurru

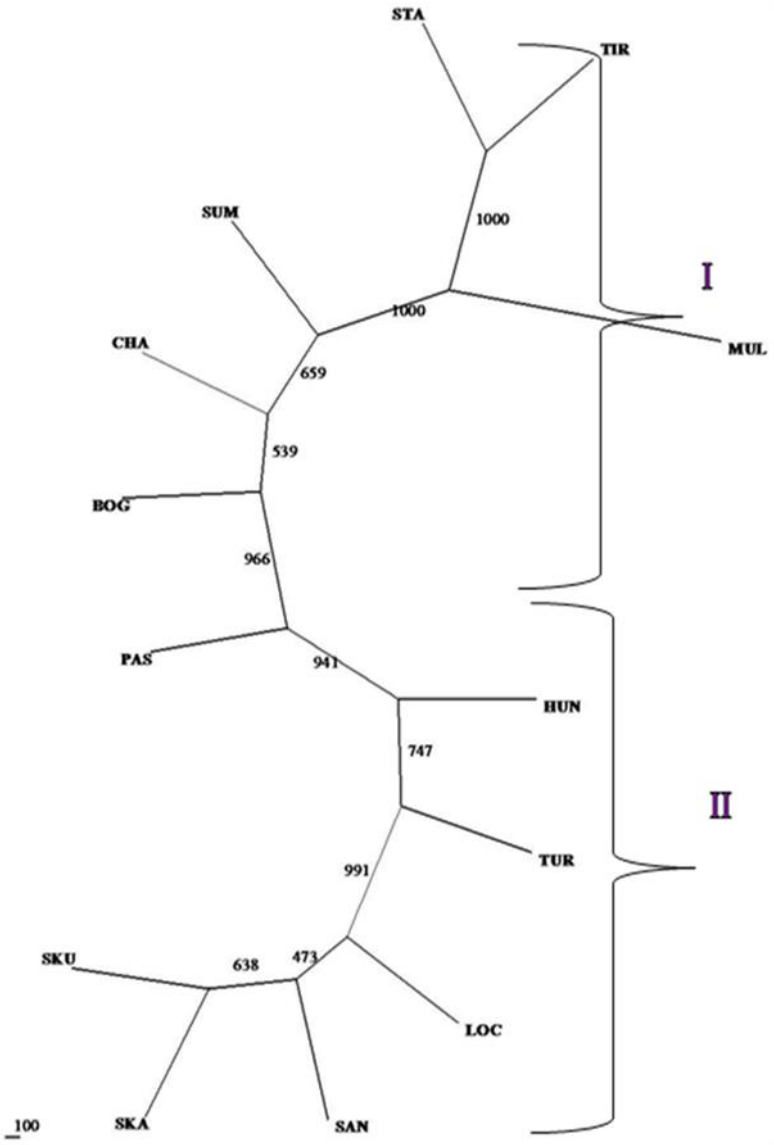


Figure 3.8: NJ tree of 13 locations of *D. hatagirea* using PHYLIP Package (Based on ISSR data)

Cluster I: Pashkum, Bogdang, Changlung, Sumur, Mulbek, Staksha and Tirith

Cluster II: Hunder, Turtuk, Lochum, Skurru, Sanjak and Skampuk

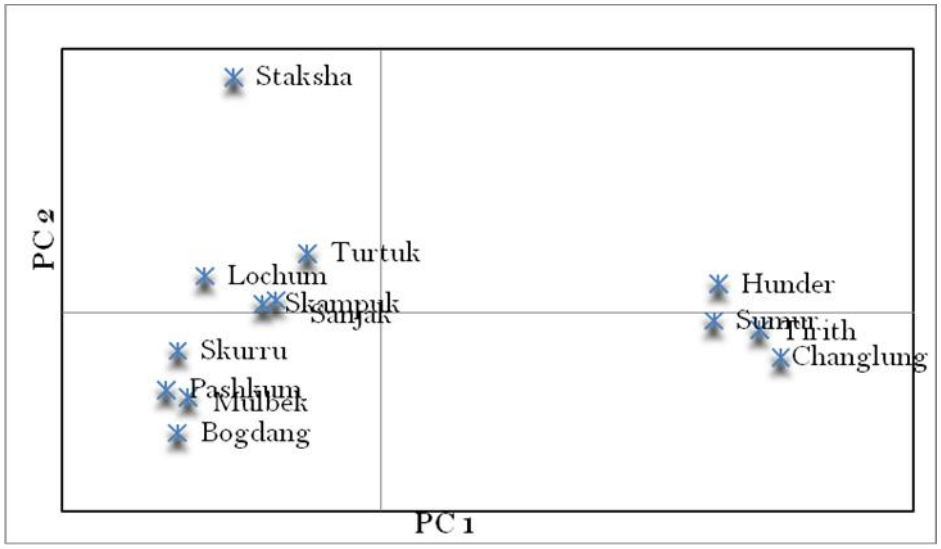


Figure 3.9: PCA analysis of PhiPT values based on RAPD data

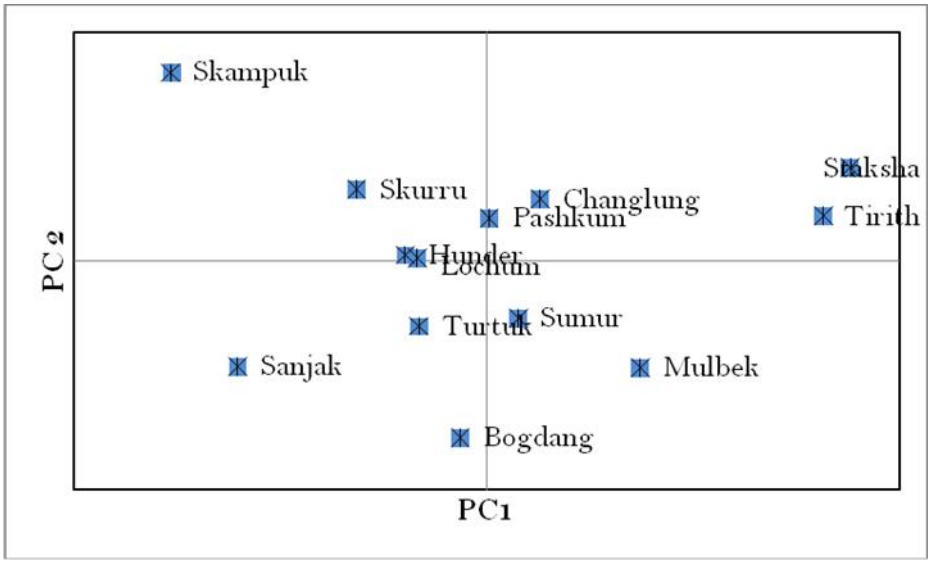


Figure 3.10: PCA analysis of PhiPT values based on ISSR data

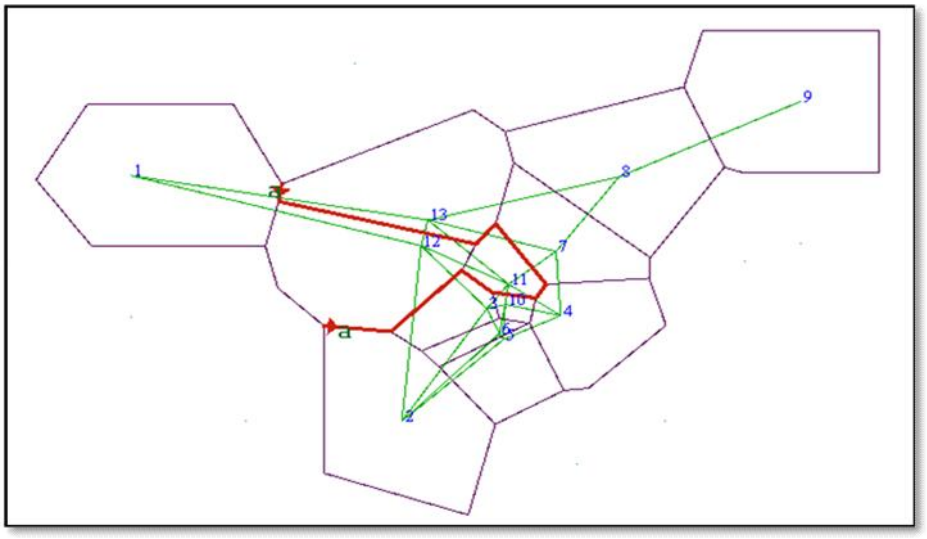


Figure 3.11: A barrier map showing the geographical barrier

1-Tirith, 2-Sumur, 3-Changlung, 4-Staksha, 5-Turtuk, 6-Bogdang, 7-Hunder, 8 Skurru, 9 Skampuk, 10-Sanjak, 11-Mulbek, 12-Lochum, 13-Pashkum

3.4 Discussions:

3.4.1 Genetic diversity

Many studies have demonstrated that endangered species tend to possess low level of genetic diversity [Xiao et al., 2004] whereas others have shown the opposite (Luan et al., 2006). Our analysis of RAPD and ISSR molecular markers indicated that *D. hatagirea* has maintained a low genetic diversity (57% and 60%), Similar results were found in closely related *Gymnadenia* species [Chung MY, 2009]. These results reinforced the population genetic hypothesis that endangered species with narrow distribution are generally depauperate and affected by a number of evolutionary factors including mating system, gene flow, founder effect, random genetic drift and seed dispersal [Hamrick and Godt, 1989]. Considering that a decline in genetic variation may represent reduced ability to survive environmental changes, resulting in an increased risk of extinction [Tansley and brown, 2000; Frankham et al., 2002] and predicting to increase inbreeding [Frankham et al., 2010], *D. hatagirea* is probably under severe threat. So the low rate of natural recruitment observed today, together with increased habitat fragmentation, isolation of population, threatened by its own demographic status and reduced distribution to a very restricted area, is seriously contributing to the low level of genetic diversity. Many terrestrial orchids are known for their ability to propagate vegetatively [Batygina et al., 2003] and typically produce numerous dust-like seeds that

contain very small nutrient reserves and are structurally adapted for wind dispersal [Whigham et al., 2006]. So, these populations probably have suffered from frequent long-term and disturbances faced by local villages. During our field investigation, we have observed that *D. hatagirea* has been characterized as perennial orchid with pollination by insects as well as high seed production which are dispersed by wind. It is very easy for the insects to facilitate pollen movement between locations and for the seeds dispersed by wind that colonize new sites, enhancing gene flow between populations [Arditti and Ghani, 2000]. Although mating system of *D. hatagirea* is not known, we believe that inbreeding through selfing or mating among closely related individuals is currently ongoing. The small fragmented population that are considerable apart from each other and the finding of viable seeds produced by several individuals in the isolated population are concordant with this inference. The reproductive biology of a given species may have important implications for its conservation and management and therefore, this is a subject that needs to be investigated.

3.4.2 Population structure

A model-based approach implemented in the software STRUCTURE for shaping the pattern of population. These methods graphically showed two well distinct clusters for the entire location which are high related to the known germplasm type information as well as the STRUCTURE subgroups. Similar population structure observations were found in *Oryza sativa* [Zhang et al., 2011] and endangered high altitude medicinal plant *Rhodiola dumulosa* [Hou et al., 2011]. The separation in the two main genetic groups of *D. hatagirea* population might be explained by several not mutually exclusive factors including stochastic events (i.e. founder effect, genetic drift, etc.) or introgression events with related co-occurring taxa in the part of its distribution range. Further investigations are necessary to test these hypotheses.

Gene differentiation and gene flow are important indices to estimate the population genetic structure of species. Significant heterogeneity in the genetic structure of orchid populations was recently documented in a comprehensive review by Forrest et al. [2004] with Gst estimates ranging from 0.012 to 0.924. Forrest et al. [2004] reported a mean Gst estimate of 0.187 for all studies. The values of Gst in this species were 0.235 and 0.253, indicating that our results are well above the overall mean Gst of 0.187. However, other studies have reported similar estimates of genetic structuring to [Li et al., 2008]. Furthermore, Mantel tests conducted on these populations revealed no significant correlation between genetic and geographical distances. Thus, RAPD and ISSR data suggested that isolation by distance

pattern may be detected across the whole range of *D. hatagirea*, the gene flow and the relationships between geographical and genetic distances have different patterns at different spatial scales.

Small fragmented plant populations are generally more prone to extinction due to the loss of genetic diversity throughout genetic drift, increased selfing, and mating among related individuals [Honnay and Jacquemyn, 2007]. On the contrary, even though the population size reduction seems to be the main cause of the genetic impoverishment of the species. *D. hatagirea* is characterized by moderate gene flow and genetic differentiation. The breeding system probably plays an important role in preventing the loss of population by sustaining gene flow in *D. hatagirea*. Nevertheless, habitat fragmentation from human pressure (earning and cultivation reduction) that reduces population size reduction persists as the main threat for the species.

3.5 Conservation consideration:

D. hatagirea is an important ornamental and medicinal plant, which is getting reduced rapidly because of low germination rate, habitat fragmentation and human overexploitation [Forest Department of Uttar Pradesh, 1998]. Lacking endosperm, the seeds of *D. hatagirea* can germinate only with symbiotic fungi in natural conditions [Aggarwal and Zettler, 2010]. The Bulk demands of *D. hatagirea* for its medicinal value causes overexploitation of wild resources. Habitat fragmentation and population deterioration will increase mating opportunities between closely related individuals and finally result in loss of genetic diversity [Warghat et al., 2012a].

Due to low genetic diversity, *D. hatagirea* may have reduced ability for evolution, unless opportunities arise for immigration of new allelic diversity into future populations. On the other hand, large percentage of aborted seeds and little seedling recruitment observed raise great concern for its long-term survival. Conflicts between conservation and local interests are a difficult issue. There is evidence that tourism has severe impact on threatened plants [Kelly et al., 2003]. Recently, we have observed rapidly increasing number of visitors in Ladakh region, due to promotion of ‘ecotourism’ by local (Jammu and Kashmir) government, the effects of which need to be investigated and monitored since locations such as Hunder and Tirith grow on the sides of camping sites are vulnerable to destruction by the infrastructure construction associated with tourism. On the other hand, the genetic threat should not be neglected. Population fragmentation will result in loss of rare alleles. Therefore, there is a

need for further study on the mechanism of abortion of seed and reproductive biology of *D. hatagirea* for survival of its population.

One primary objective of conservation management is to maintain genetic diversity of *D. hatagirea*. The strategy of conservation for this species should include both *in situ* and *ex situ* methods. Taking into account the special habitat of this species, *in situ* conservation should be given top priority. Small populations are more prone to be extinct due to environmental fluctuations and habitat destruction therefore it is necessary to protect all the existing populations and individuals *in situ* in order to preserve as much genetic variation as possible. The habitat protection will ensure species coexistence with other organisms like fungi and pollinators on which orchids depend for their cycles [Li and Ge, 2006]. For *ex situ* conservation, we need to seriously design and establish a germplasm bank and conserve germplasm through Plant tissue culture techniques.

CHAPTER 4

Population inventory and vegetation mapping of *D. hatagirea* through Ecological Niche Modelling (ENM)

4.1 Introduction:

Himalayas are one of the largest and youngest mountain chains in the world and cover roughly 10% of total land surface of India. Variations in terms of its size, climate and altitudinal ranges have created environments those are unique characteristics to this region only. Diverse climate and varied environmental conditions prevailing in Himalayas support diverse habitat and ecosystems with equally diverse life forms. It provides an important habitat to the flora and fauna including 9,000 species of angiosperms and hence, is considered as the hot spot of biodiversity. There are about 3,470 species considered exclusively endemic to the Himalayas.

The cold desert in India is occurred in Ladakh region of Jammu and Kashmir. The total area under cold deserts in Ladakh is about 45,110 km^2. Human pressure, climate change, habitat fragmentation and over-exploitation on natural ecosystems are continuously increasing, some being incompatible with survival of certain species of plants. In cold desert areas, continuous removal of plant species for various uses and overgrazing by domestic animals has resulted in desertification and loss of biodiversity. If these naturally occurring plant resources are not timely conserved they may soon become extinct. The range of many species have expanded at high latitudes and elevations but contracted at their warm margins in response to recent climate changes [Walther et al., 2002; Thomas et al. 2006]. Changes to the distribution of 'habitable climate space' may lead to extinctions if future ranges are too small or isolated from current ranges, and consequently the impact of climate change on biodiversity is of increasing concern [Thomas et al., 2004]. *Dactylorhiza hatagirea* (Family: Orchidaceae) is one of the plant species which suffered from such activities. The local people in Ladakh rely on natural resources to sustain their subsistence's and to ensure contributions to their economies/incomes. The dependency of the local people over the natural resources is coupled with a variety of factors such as population pressure, needless disturbances to ecosystems, and unsustainable harvesting/overexploitation by various pharmaceutical and aroma-chemical industries [Maikhuri et al., 1998]. Consequently, there is little room left to

naturally regenerate the endangered flora and fauna [Rao et al., 2000]. The assessment of vegetation analysis may provide key informations related to its status and its conservation.

On the other hand, species (re)introduction is one of the successful ecological engineering techniques for restoration of the depleted species populations, and degraded habitats and ecosystems [Leaper et al., 1999; Martinez-Meyer et al., 2006; Kuzovkina and Volk, 2009; Ren et al., 2009; Zai et al., 2009; Rodríguez-Salinas et al., 2010; Nazeri et al., 2010; Polak and Saltz, 2011]. In order to reintroduce and rehabilitate the threatened species in terrestrial ecosystems, a detailed knowledge on the distribution of their potential habitats is essential. Habitat distribution modelling therefore helps to identify the areas for species reserves, reintroduction, and in developing effective species conservation measures. It has been successfully used in restoring critical habitats and predicting the impact of environmental and climate changes on species and ecosystems [Brooks et al., 2004; Samways, 2005; Giriraj et al., 2008; Franklin, 2009; Gogol-Prokurat, 2011; Barik and Adhikari, 2011]. New insights into the factors governing the distribution of species have been developed using habitat distribution modelling or ecological niche modelling (ENM) [Guisan and Zimmermann, 2000; Elith et al., 2006; Kozak et al., 2008]. ENM considers environmental factors as ecological conditions e.g., temperature, precipitation, soil, vegetation and land cover, and uses the dataset from Geographic Information System (GIS) databases such as www.worldclim.org and www.diva-gis.org. Availability of high resolution satellite imageries, downscaling tools for environmental variables, and interpolated spatial datasets on climate and vegetation have enhanced the accuracy of prediction of the models manifold. ENM facilitates interpolation as well as extrapolation of species distributions in geographic space across different time periods. Therefore in this study, we have modelled the potential habitat distribution of *D. hatagirea*.

Keeping these aspects in view, a study was undertaken with the following specific objectives: (1) to investigate the population status of *D. hatagirea* through quadrat method. (2) to model the potential habitat distribution of *D. hatagirea* in its native range, (3) to identify the major factors determining the distribution of potential habitats. (4) to identify conservation baseline area for reintroduction.

4.2 Materials and Methods:

4.2.1 Study species

D. hatagirea (Orchidaceae) is a terrestrial medicinal orchid species with a height 30-76 cm and usually found in temperate region. The species starts flowering during the months of June-July, fruiting in August-September and harvesting during October-November. It bears purple flower with green capsule fruit and 2-5 lobe of tubers (Warghat et al., 2012b).

4.2.2 Associated Vegetation

As an initial step in this study, we performed a systematic review of published sources regarding vegetation analysis of *D. hatagirea*. Each sites were chosen selectively to include only natural and semi-natural vegetation where there was no earlier evidence (observational or management records). Data collection was conducted within the largest vegetation sites in the study area covering a wide geographic range belonging to three valleys of Ladakh region (Figure 4.1). For the size, number of quadrats and collection, methods of Curtis and Intosh, 1950; Smith, 1957; Misra, 1968; Kersaw, 1973; Dombois and Ellenberge, 1974; Dhar et al., 1997; Joshi and Samant, 2004; Samant and Joshi, 2005 were followed. Within each location, a plot of 50 × 50 m was laid and from that, randomly homogenous 20 quadrat of 1 × 1 m were sampled in four different habitats of each location (Figure 4.2). For vegetation study, qualitative and quantitative methods were employed. The qualitative method included collection, identification and verification of plant samples. Firstly, plant samples were shown to the local Amchis to get information on local names and availability of the species in the study area. After that, plant samples were finally identified by using standard keys from flora of Ladakh and herbarium from Medicinal and Aromatic plant division, DIHAR, Leh-Ladakh [Chaurasia et al., 2007] and consulting relevant literature [Kachroo et al.1977; Polunin and Stainton 1990; Aswal and Mehrotra 1994], expert judgment and following the rules given in ICBN (International Code of Botanical Nomenclature). While, quantitative method included species composition, density, frequency and abundance. Within each plot, species composition, density and frequency was assessed. Their abundance was scored using Braun-Blanquet scale [Westhoff and van der Maarel 1978]. Slope and aspect of each plot was measured. Density, frequency and abundance, % cover values, prominence values and dominance values of each species in each plot were calculated. We categorised habitat of *D. hatagirea* in four vegetation types such as herbaceous meadow, sedge meadow, riverine scrub and open slopes. Shannon index, Simpson index, Maturity index, Evenness, Menhinick richness index, Mergalef diversity index, Similarity index, Sorensen's coefficient and alpha

diversity were calculated for each vegetation type by PAST 2.17 software. Multivariate techniques such as principle component analysis (PCA) and Correspondence analysis (CA) were used for associated vegetation data analysis by SPSS 19 software.

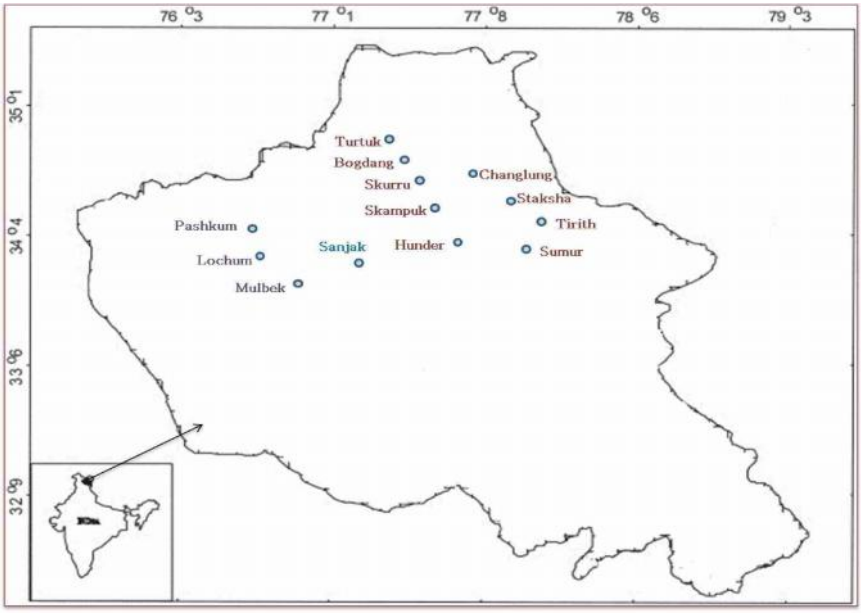


Figure 4.1: Map of Ladakh region of India

Figure 4.2: Associated vegetation of *D. hatagirea*

4.2.3 Soil parameter

In this study, we have collected a total of 52 soil samples of *D. hatagirea* from four habitats of each location. Soil samples were air-dried, sieved through a 2 mm sieves and were grind prior to analysis. The pH of soil was measured by pHEP pocket sized meter. Total Nitrogen was determined by using Kjeldahl Methods [Bremmer and Mulvancy 1982] and following formula was used for calculating Total Nitrogen.

$$\%N = \frac{14.1\times (\text{ml titrant-ml blank}) \times N \times 100}{\text{Sample Weight (gms)} \times 1000}$$

Available phosphorus was determined by spectrophotometric method [Bray and Kurtz 1945] and calculation was done by using following formula,

ppm P in soil = ppm P in solution × 15 ml/1.5 g = ppm P in solution × 10.

Available potassium was determined by flame photometer (Systronic 128 model) [Hanway and Heidel 1952], soil organic carbon and matter was determined by Walkley-Black method [1934] and following formula was used for calculation

$$\%OC = \frac{(B-S) \times M \text{ of } Fe^{2+} \times 12 \times 100}{\text{gm of soil} \times 4000}$$

$$\%\textit{Organic Matter} = \%\ OC \times 1.724$$

Where:

B = ml of Fe^{+2} solution used to titrate blank

S = ml of Fe^{+2} solution used to titrate sample

12/4000 = milliequivalent weight of C in gm

PCA was used for analysis of soil data by SPSS 19 software.

4.2.4 Habitat distribution modelling:

Thirteen primary distributional records of the species were collected through field surveys. The coordinate of all the occurrence points were recorded to an accuracy of 10-40 m using a Global positioning system (Garmin). The coordinates were then converted to decimal degrees for use in modelling the distribution of potential habitats of the species in its native range. Over the years, a variety of environmental datasets have been accumulating in the public domain websites which can be used in distributional modelling of species. Use of different formulations of environmental datasets however, yields different results for the same

set of species [Peterson and Nakazawa, 2008]. Hence, selection of appropriate data type and pixel resolution is a prerequisite prior to predictive modelling [Parra et al., 2004]. In the present study, remotely sensed data on elevation and enhanced vegetation index (EVI) were used to summarize the habitat boundaries for the species in the native range of Ladakh region of India. Digital elevation data (250 m resolution) was obtained from CGIAR-CSI [http://srtm.csi.cgiar.org, Jarvis et al., 2008]. In other words, the topographic effect is indirectly represented in the EVI dataset. Twenty three layers of MODIS images (MOD13Q1) with a spatial resolution of 250 m were obtained from Oak Ridge National Laboratory Distributed Active Archive Centre [http://daac.ornl.gov/MODIS/modis.html, santhana et al., 2009]. These layers corresponded to the year 2010-2012 during which the field survey was undertaken and the spatial aggregates of EVI at 16 days interval were characterized. Enhanced Vegetation Index (EVI) has been preferred over Normalized Difference Vegetation Index (NDVI) because of its improved sensitivity to saturation in the degree of greenness in the forested areas and higher capability to discriminate changes in vegetation across spatial and temporal scale [Huete and Justice, 1999]. The images were downloaded in geotif format and converted to ASCII raster grids in ArcGIS 9.3. In order to match with the MODIS EVI layers, 90 m elevation layer of CGIAR-CSI dataset was resampled to 250 m pixel resolution using nearest neighbourhood method of ArcGIS 9.3. Subsequently, all the analyses were conducted at the spatial resolution of 250 m.

4.2.5 Validation of model robustness

For habitat modelling, the pixel dimension was 250 × 250 m grid cell and the model was developed using maximum entropy modelling (MaxEnt version 3.3.3e) [Phillips et al., 2006]. MaxEnt estimates the maximum entropy probability distribution function to predict the geographic location of a species based on environmental variables and reconstructs the boundaries of the ecological niche by placing constraints on the probability distribution based on the environmental parameters of the grid-cell presence record [Phillips et al., 2006]. It is one amongst the 'presence-only' group of species distribution modelling methods which has been widely used. The strong attributes of MaxEnt are: (i) it holds a strict mathematical definition, (ii) gives a continuous probabilistic output, (iii) can simultaneously handle both continuous and categorical environmental data, (iv) can investigate variable importance through jackknife procedure, (v) has the capacity to handle low sample sizes, and (vi) provides simplicity for model interpretation [Phillips et al., 2006; Pearson et al., 2007; Elith et

al., 2011]. It also facilitates replicated runs to allow cross-validation, bootstrapping and repeated subsampling in order to test model robustness. Of the 16 records, seventy five percent were used for model training and twenty five percent for testing. To validate the model robustness, we executed 10 replicated model runs for the species with a threshold rule of 10 percentile training presence. In the replicated runs, we employed cross validation technique where samples were divided into replicate folds and each fold was used for test data. Other parameters were set to default as the program is already calibrated on a wide range of species datasets [Phillips and Dudík, 2008]. From the replicated runs, average, maximum, minimum, median and standard deviation were generated. Model quality was evaluated based on Area Under Curve (AUC) value and the model was graded following Thuiller et al. [2005] as: poor (AUC < 0.8), fair (0.8 < AUC < 0.9), good (0.9 < AUC < 0.95) and very good (0.95 < AUC < 1.0). Further, potential area of distribution and/or reintroduction were categorized into five classes based on logistic threshold of 10 percentile training presence i.e. very-high (0.762–1), high (0.572–0.761), medium (0.381–0.571), low (0.325–0.570) and very low (0–0.324).

4.2.6 Population status vis-à-vis model thresholds

Total population of the species was ascertained through direct count of all the individuals in each 250 × 250 m grid of occurrence within the predicted localities. The population data of *D. hatagirea* in each valley was then correlated with the corresponding threshold level of the distribution models to assess whether regions covered in the higher thresholds or they maintain higher populations thus approving better habitat conditions for the species establishment and vice versa.

4.2.7 Assessment of habitat status and identification of areas for reintroduction

Assessment of the actual habitat type of the species in the localities of occurrence as well as in the entire predicted potential area was done through repeated field surveys. We also super-imposed the predicted potential areas on Google Earth Ver. 6 [www.google.com/earth] imageries for habitat quality assessment. The predicted suitability maps were exported in KMZ format using Diva GIS ver. 7.3 [www.diva-gis.org]. KMZs are zipped Keyhole Markup Language (KML) files which specifies a set of features such as place marks, images, polygons, 3D models or textual descriptions for display in Google Earth. The exported KMZ files were overlaid on satellite imageries in Google Earth to ascertain the actual habitat condition prevailing in the areas of occurrence.

4.3 Results:

4.3.1 Vegetation composition

Extensive collection of plants in 2010-2012 and systematic enumeration during intensive sampling of various quadrat yielded species of vascular plants (angiosperms) in the study area that belonged to 16 families and 22 genera. The number of species in dicots, monocots and pteridophytes were 12, 3 and 1 respectively. The dominant vegetation of this area is herb-rich *Plantago sp.* The grasslands communities of *D. hatagirea* predominantly occur as natural grassland and pastures with shrub vegetation, sometimes with the presence of scattered tall trees of *Salix and Populas sp.* The families of flowering plants in the study area included Fabaceae (3), Asteraceae (3), Salicaceae (2), Rosaceae (2), Plantaginaceae (1), Ericaceae (1), Poaceae (1), Equisetaceae (1), Geraniaceae (1), Polygonaceae (1), Lamiaceae (1), Amarylidaceae (1), Gentianaceae (1), Ranunculaceae (1) and Orchidaceae (1). The highest density was found of *Plantago sp.* with 7186 individuals (27.63%) with respect to quadrat and followed by *Trifolium sp.* with 3626 individuals (13.94%), *G. elata* with 3306 individuals (12.71%), *Equisetum sp.* with 2793 individuals (10.74%), *O. lappanica* with 2395 individuals (9.21%), *Polygonum sp.* with 2323 individuals (8.93%), *D. hatagirea* with 1812 individuals (6.97%), *P. alpinum* with 1729 individuals (6.65%), *Gaultheria sp.* with 1653 individuals (6.36%), *H. rhamnoides* with 983 individuals (3.78%), *Allium sp.* with 899 individuals (3.45%), *Salix sp.* with 857 individuals (3.29%), *A. apthera* with 699 individuals (2.68%), *M. longifolia* with 658 individuals (2.53%), *Geranium sp.* with 542 individuals (2.08%), *P. depressa* with 419 individuals (1.61%), *Populus sp.* with 295 individuals (1.13%), *Gentiana sp.* with 254 individuals (0.97%), *M. lupulina* with 222 individuals (0.85%), *T. longifolium* with 215 individuals (0.82%), *Ranunculus hirtellus* with 209 individuals (0.80%) and *Senecio chrysanthemoides* with 152 individuals (0.58%). The highest and lowest values of frequency found in *Plantago Sp.* (98.46%) and *M. lupulina* (1.53%) as compared to other associated species. The highest and lowest values of abundance was found in *M. lupulina* (55.50) and *Populus sp.* (4.46) as compared to other associated species. The most dominant species were *Plantago sp.* with a dominance value of 10.91%, *G. elatum* (9.29%), *Trifolium sp.* (8.09%) and *Equisetum sp.* (7.16%) as compared to associated species. High importance values index were manifested among *Plantago sp.* (40.98%), *Trifolium sp.* (24.75%), *G. elatum* (23.8%) and *Equisetum sp.* (20.57%) (Table 4.1). Habitats of *D. hatagirea* were categorized into four vegetation type. Diversity indices (Shannon-Weiner index, Simpson's

index and Margalef index), richness (Menhinick index), maturity (measure index), evenness (Pielou index) and Similarity (Similarity and Sorensen's Coefficient index) for four vegetation types were calculated. Shannon-Weiner, Simpson's index and Mergalef diversity index were higher in sedge meadow and lower in herbaceous meadow. Evenness and Menhinick richness index were also higher in sedge meadow and lower in herbaceous meadow. Value of maturity index showed opposite trend; it was higher in herbaceous meadow and lower in sedge meadow. Similarity index were higher in between herbaceous meadow and sedge meadow; while lower in between riverine scrub and open slopes (Table 4.2). Alpha diversity explained more variation (i.e. 75%) and showed complexity of associated species in four different habitats of *D. hatagirea* (Figure 4.3). Correspondence analysis (CA) clearly illustrated the change of community structure along with longitudinal gradient. Samples were clustered with the relay index from sedge meadow to riverine scrub and there was a gradual shift from herbaceous meadow to open slopes. This resulted gradual shift in terrestrial ecosystem might be due to anthropogenic impact in habitat. CA of habitat data extracted two components along the axis and showed distribution of associated species according to habitat speciality. CA analysis explained 85.61% variation among the habitat of *D. hatagirea* (Figure 4.4a). The associated species distribution in four different habitats showed a good spread along both axes. The distributions of the most frequent species and the quadrats suggested a productivity gradient on both axes. The first component covered highly frequent species such as *G. elatum, A. apthera, M. longifolia, Allium sp., P. depressa, Gaultheria sp., Polygonum sp., P. alpinum, Trifolium sp., Salix sp., Populus sp., Equisetum sp., O. lappanica, H. rhamnoides, D. hatagirea, Plantago sp., Geranium sp., T. longifoium* and in second component *R. hirtellus* and *M. lupulina* (Figure 4.4b).

Table 4.1: Associated vegetation in *D. hatagirea* habitats

Scientific name	Family	Nubra	Indus	Suru	Total	Density	Frequency	Abundance	R .D.	R.F.	R.A.	IVI	% Cover values	Prominence values	Dominance values
Plantago sp.	Plantaginaceae	4870	410	1906	7186	27.63	98.46	28.07	21.53	10.89	8.56	40.98	0.1	0.99	10.91
Gaultheria sp.	Ericaceae	1196	153	304	1653	6.36	34.23	18.57	4.95	3.78	5.66	14.39	0.2	1.70	3.79
O. lappanica	Fabaceae	1635	183	577	2395	9.21	56.15	16.40	7.17	6.21	5.00	18.38	0.3	2.25	6.22
P. alpinum	Poaceae	1219	136	374	1729	6.65	45.00	14.77	5.18	4.98	4.50	14.66	0.5	3.35	4.99
G. elatum	Rosaceae	2642	271	393	3306	12.71	83.84	15.16	9.90	9.28	4.62	23.8	0.6	5.49	9.29
A. apthera	Asteraceae	557	38	104	699	2.68	22.30	12.05	2.08	2.46	3.67	8.21	0.8	3.77	0.25
Equisetum sp.	Equisetaceae	1909	219	665	2793	10.74	64.61	16.62	8.36	7.15	5.06	20.57	0.4	3.22	7.16
Trifolium sp.	Fabaceae	2536	273	817	3626	13.94	73.07	19.08	10.86	8.08	5.81	24.75	0.3	2.56	8.09
Geranium sp.	Geraniaceae	356	42	144	542	2.08	21.15	9.85	1.62	2.34	3.00	6.96	0.7	3.22	2.34
Polygonum sp.	Polygonaceae	1653	175	495	2323	8.93	57.69	15.48	6.95	6.38	4.72	18.05	0.5	3.80	6.39
M. longifolia	Lamiaceae	508	45	105	658	2.53	21.92	11.54	1.97	2.42	3.51	7.9	0.7	3.28	2.42
P. peduncularis	Rosaceae	315	26	78	419	1.61	17.30	9.31	1.25	1.91	2.83	5.99	0.6	2.50	1.92
T. longifolium	Asteraceae	135	30	50	215	0.82	3.84	21.5	0.63	0.42	6.55	7.6	1.0	1.95	0.43
H. rhamnoides	Elaeagnaceae	687	220	76	983	3.78	50.00	7.56	2.94	5.53	2.30	10.77	1.2	8.49	5.54
Allium sp.	Amarylidaceae	790	28	81	899	3.45	49.23	7.02	2.68	5.44	2.14	10.26	6.8	47.11	5.45
Salix sp.	Salicaceae	597	65	195	857	3.29	49.38	7.26	2.56	5.46	2.21	10.23	8.8	61.84	5.47
Populus sp.	Salicaceae	203	23	69	295	1.13	25.38	4.46	0.88	2.80	1.36	5.04	7.4	37.28	2.81
Gentiana sp.	Gentianaceae	108	20	126	254	0.97	9.61	10.16	0.75	1.06	3.09	4.9	0.1	0.31	1.06
S.chrysanthemoides	Asteraceae	26	25	101	152	0.58	12.30	4.75	0.45	1.36	1.44	3.25	0.5	1.75	1.36
R. hirtellus	Ranunculaceae	0	17	192	209	0.80	5.38	14.92	0.62	0.59	4.55	5.76	0.8	1.86	0.59
M. lupulina	Fabaceae	0	25	197	222	0.85	1.53	55.50	0.66	0.16	16.9	17.72	1.7	2.10	0.17
Total		**23152**	**2552**	**7523**	**33227**	**127.7**	**902.4**	**326.99**	**99.45**	**99.78**	**99.61**	**298.8**			

Table 4.2: Vegetation type and their indices

Vegetation Type	Shannon Index	Simpson Index	Maturity Index	Evenness	Menhinick richness Index	Margalef diversity index	Similarity Index	Sorensen's Coefficient
Herbaceous meadows	2.396	0.8928	0.11	0.646	0.1719	1.741	91.89	0.48
Sedge meadow	2.723	0.9256	0.07	0.6924	0.2076	2.252		
Riverine scrub	2.423	0.8957	0.10	0.6633	0.1824	1.764	81.25	0.45
Open slopes	2.543	0.9083	0.09	0.6693	0.2247	2.028		

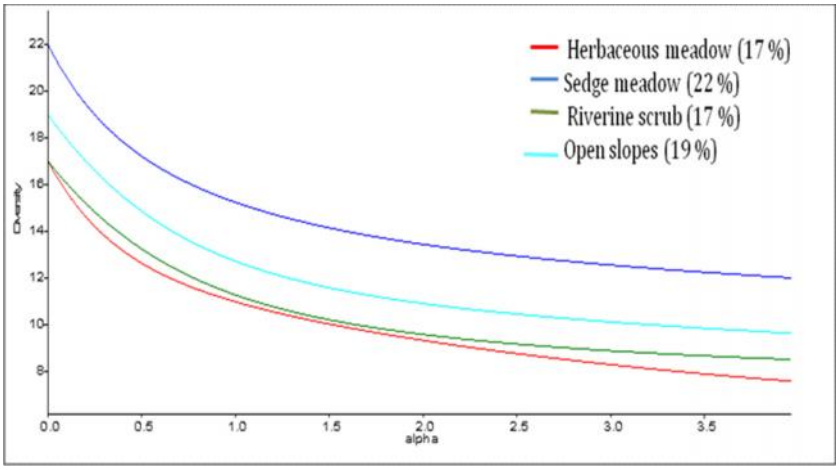
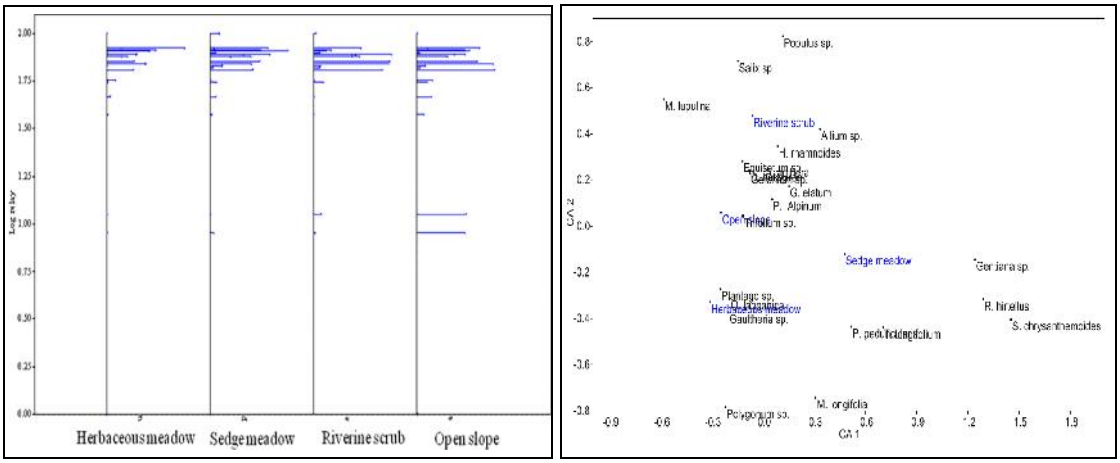


Figure 4.3: Alpha diversity in habitat of *D. hatagirea*



a. **b.**

Figure 4.4: Canonical correspondence analyses of Habitat data; a. CA axis, b. relay plot

4.3.2 Soil data

At 5 % level, Soil organic carbon with pH of soil (r= 0.414), Available Phosphorous with soil organic carbon (r= 0.483) and Available Phosphorous with total Nitrogen (r= 0.483) were positively correlated. While, Carbon/Nitrogen with pH of soil (r= -0.466), were negatively correlated. On the other hand, at 1 % level total nitrogen with pH of soil (r= 0.550), soil organic matter with soil organic carbon (r= 0.956), Total nitrogen with soil organic carbon (r= 0.953), available potassium with soil organic carbon (r= 0.535), available phosphorous with soil organic carbon (r= 0.483), Total nitrogen with soil organic matter (r= 0.871), available potassium with soil organic matter (r= 0.578), available phosphorous with soil organic matter (r= 0.566), available potassium with total nitrogen (r= 0.508), available phosphorous with total nitrogen (r= 0.483) and available phosphorous with available potassium (r= 0.761) were positively correlated. While, Carbon/Nitrogen with pH of soil (r= -0.466), Carbon/Nitrogen with soil organic carbon (r= -0.606), Carbon/Nitrogen with soil organic matter (r= -0.562), Carbon/Nitrogen with total nitrogen (r= -0.729), available potassium with Carbon/Nitrogen (r= -0.543) and available phosphorous with Carbon/Nitrogen (r= -0.743) were negatively correlated (Table 4.3). PCA components explained 81.4% of the variation in the soil parameters (Table 4.4). The soil parameter distribution according to population was observed along both axes. The first component included highly correlated characters such as Potassium, Phosphorous, Carbon/Nitrogen and Total Nitrogen. While, second component contained soil organic matter and soil organic Carbon (Figure 4.5). However, PCA of combined vegetation and soil data explained separate group of population. It can be concluded that each valley had its own vegetation flora and separate characteristics (Figure 4.6).

Table 4.3: Pearson correlation between soil parameters

	pH of soil	Soil organic carbon	Soil organic matter (%)	TN (%)	C/N	Available K (PPM)	Available K (PPM)
pH of soil	1	0.414*	0.304	0.550**	-0.466*	0.044	0.129
Soil organic carbon		1	0.956**	0.953**	-0.606**	0.535**	0.483*
Soil organic matter (%)			1	0.871**	-0.562**	0.578**	0.566**
TN (%)				1	-0.729**	0.508**	0.483*
C/N					1	-0.543**	-0.743**
Available K (PPM)						1	0.761**
Available K (PPM)							1

** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

Table 4.4: PCA component matrix of soil data

	Component	
	PC1	PC2
K	**0.920**	0.257
P	**0.876**	0.157
CN	**-0.850**	-0.292
TN	**0.690**	0.645
M	0.206	**0.934**
C	0.255	**0.932**
pH	0.198	0.636

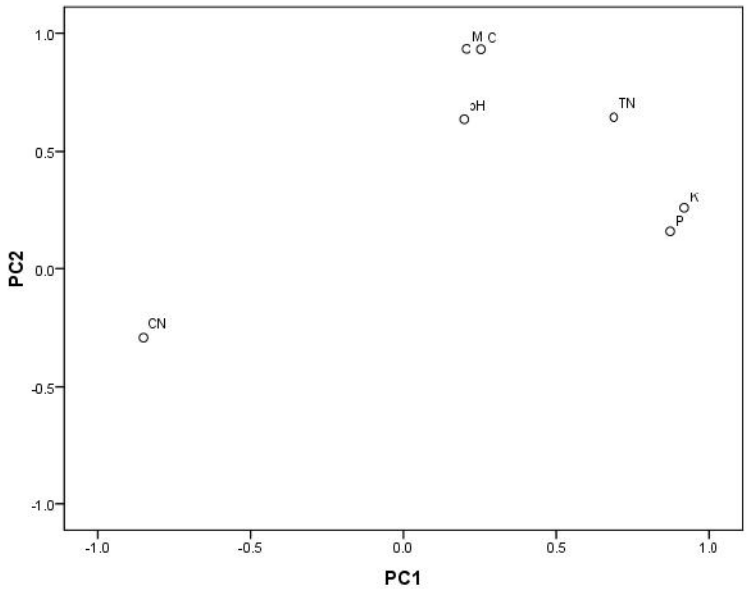


Figure 4.5: PCA plot of soil data

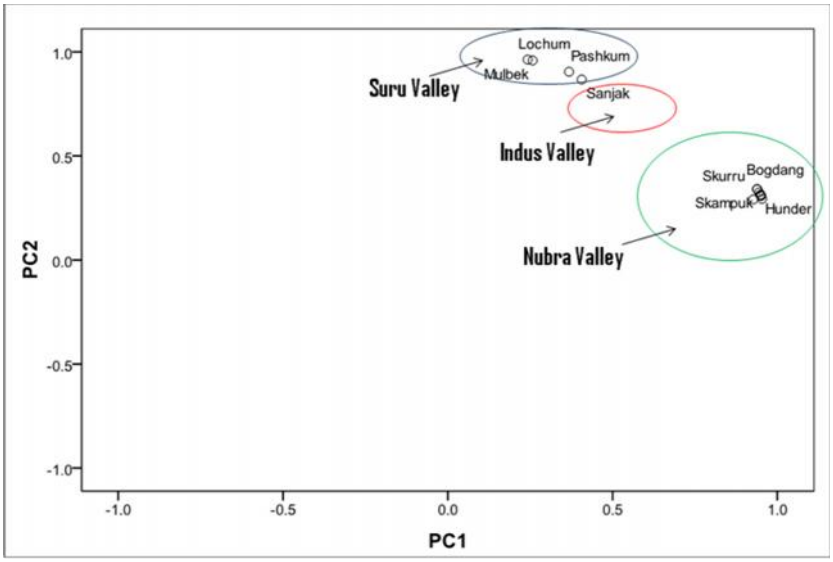
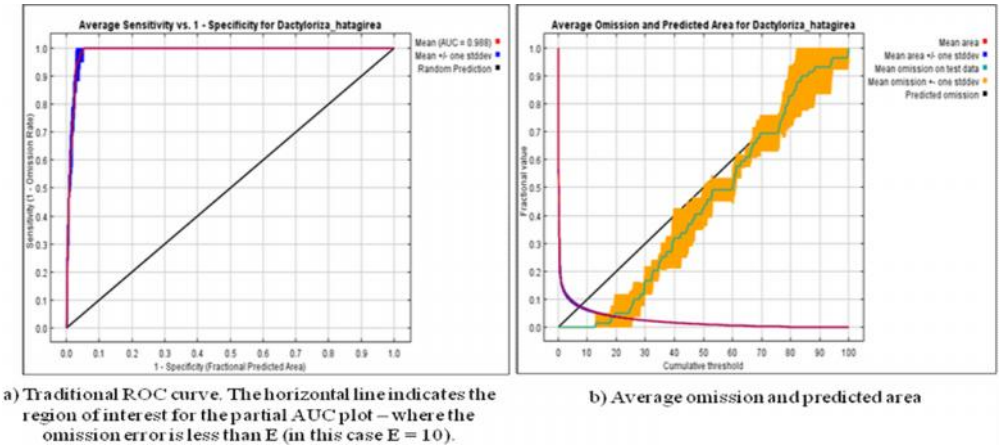


Figure 4.6: Combined PCA analysis of vegetation and soil data with respect to population

4.3.3 Calibration of models

The model calibration test for *D. hatagirea* yielded satisfactory results (AUCtrain = 0.98 ± 0.004 and AUCtest = 0.96 ± 0.020) (Figure 4.7a and 4.7b). Thirteen layers of EVI collectively contributed 99.2% in the habitat model of the species of which EVI 11 had maximum contribution (17.4%), while remaining collectively contributed to 81.8%. Considering the permutation importance, EVI 11 also had the maximum influence on the habitat model and contributed to 16.8%, while remaining together contributed to 83.2% (Figure 4.8 and Table 4.5).



a) Traditional ROC curve. The horizontal line indicates the region of interest for the partial AUC plot – where the omission error is less than E (in this case E = 10).

b) Average omission and predicted area

Figure 4.7 a & b: Comparison of traditional versus partial receiver operating characteristic (ROC) curves for the MAxEnt model

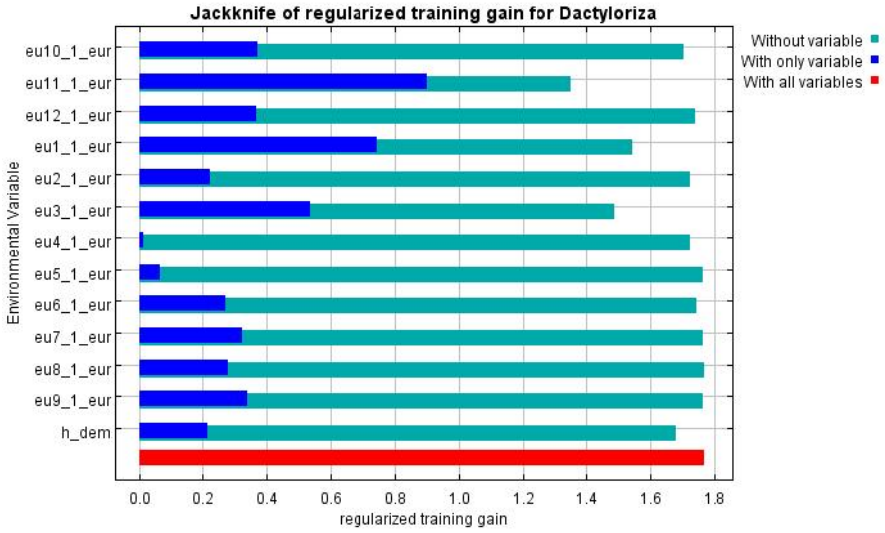


Figure 4.8: Evaluating the relative contribution of the predictor environmental variables to the habitat model

Table 4.5: Estimates of relative contribution and permutation importance of the predictor environmental variables to the MaxEnt model

Predictor variables	Percent contribution	Permutation importance
EVI 1 (1 January - 17 January)	14.5	27.8
EVI 2 (2 February- 18 February)	3.9	12.2
EVI 3 (6 March - 22 March)	12.1	22.8
EVI 4 (7 April - 23 April)	1.8	3.8
EVI 5 (9 May – 25 May)	2.6	5.4
EVI 6 (10 June – 26 June)	5.8	11.8
EVI 7 (12 July- 28 July)	7.8	16.3
EVI 8 (13 August - 29 August)	5.8	11.6
EVI 9 (14 September - 30 September)	7.9	16.5
EVI 10 (16 October- 1 November)	8.9	15.7
EVI 11 (17 November-3 December)	17.4	32.8
EVI 12 (19 December- 1 January)	8.1	15.8
h_Digital elevation model	3.4	6.8

Enhanced vegetation index (EVI) in the table represents the spatial aggregates of the degree of greenness of the same at 16 days interval

4.3.4 Potential habitat distribution area

Potential habitats with high suitability thresholds have been found in the elevations of the Indus and Suru valley in Ladakh region of India (Figure 4.9). Primary field surveys revealed that predicted potential habitats were mostly located in the willow-poplar forests and near Seabuckthorn forests. Areas with medium to very low habitat suitability were those with grasslands, cultivation lands, settlements, roads, camping sites and river banks. A total potential area of 485 km in the Indus and Suru valley was predicted to be suitable for *D. hatagirea* reintroduction. Most of the areas were fallen under medium suitability class and covered an area of 171.56 km. Area of high suitability was restricted only to about 70.26 km and 56.12 km area was very highly suitable. Area of low and very low suitability was 106.72 and 80.54 km (Figure 4.10).

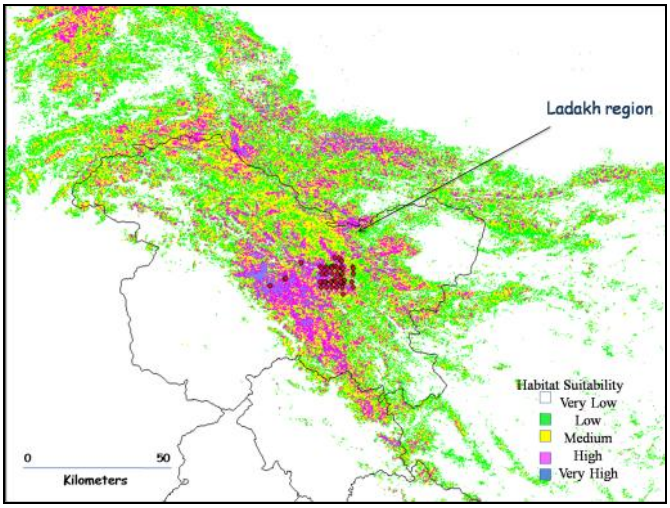


Figure 4.9: Potential habitat distribution of *D. hatagirea*

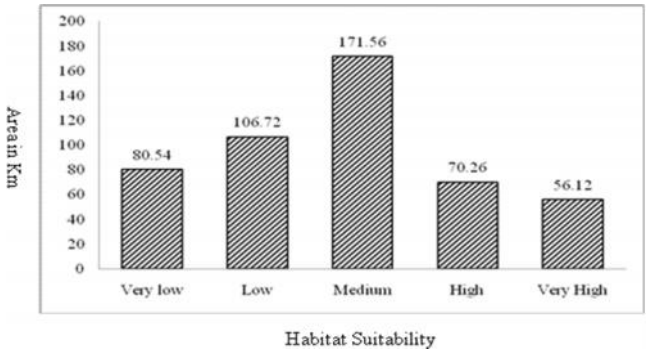


Figure 4.10: Area under different suitability grades for the optimal average model. The figures at the top of each bar represent the area.

4.3.5 Model thresholds

A total of 1812 individuals were inventoried within the surveyed area during 2010-2012. The analysis of individuals at each population were estimated highest in Nubra (1180) followed by Indus (158) and Suru (474). Areas predicted as medium to very low suitable classes represented 65.12% of the total population, while, 34.88% in high and very high thresholds. This confirms the moderate correlation between population size and level of model threshold. Of the 13 locations, 4 locations (Sanjak, Lochum, Pashkum and Mulbek) under very high, 2 locations (Turtuk and Bogdang) under high, 4 locations (Skurru, Sumur, Staksha and Skampuk) under medium, 1 location (Changlung) under low and 2 locations (Tirith and Hunder) under very low were categorised according to habitat suitability. The numbers of individuals were highest in Pashkum with 178, followed by Lochum with 174, Mulbek with 167 and Sanjak with 165 as compared to other location. The abundance structure based on individuals revealed relatively good area of regeneration in Indus and Suru valley whereas in other areas it depicted poor regeneration (Table 4.6).

4.3.6 Species recovery area

Field surveys for assessing the habitat types of *D. hatagirea* in the predicted potential areas revealed that the species occurred both in disturbed and undisturbed areas of willow-poplar forests and near Seabuckthorn forests. The species was also present in grasslands, cultivation land, along the roads and river banks. Superimposing the predicted potential habitat map of the species on Google Earth satellite imageries revealed a mosaic of habitats to be suitable for the species persistence. The areas with high to very high habitat suitability for the species were continuous patches of willow-poplar forest, Seabuckthorn forest and settled cultivation areas. The areas with medium to low habitat suitability were degraded open forest areas and scrublands. The areas with very low habitat suitability were grasslands, degraded open forests, scrublands and herbaceous meadows (Table 4.7). The superimposition of predicted potential habitat distribution map on Google Earth imageries identified four forest areas such as forest protection force in Kargil, regional wildlife in Kargil and environment and forestry centre in Jammu and Kashmir and several communities by owned forest lands which would serve as highly suitable habitats for persistence of the species. These forest areas would act as *in situ* conservation area for the species and could also be used for reintroduction/recovery of the species in the wild (Table 4.8).

Table 4.6: Population status of *D. hatagirea* through field survey (2010-2012)

Sr. No.	Population	Location	Population size	Habitat suitability class
1	Nubra	Tirith	72	Very low
2		Sumur	132	Medium
3		Changlung	116	Low
4		Staksha	129	Medium
5		Turtuk	155	High
6		Bogdang	158	High
7		Hunder	78	Very low
8		Skurru	136	Medium
9		Skampuk	152	Medium
10	Indus	Sanjak	165	Very high
11	Suru	Mulbek	167	Very high
12		Lochum	174	Very high
13		Pashkum	178	Very high
	Total		**1812**	

Table 4.7: Habitat types of *Dactylorhiza hatagirea* identified through field surveys and high resolution Google Earth satellite imageries.

Sr. No.	Habitat suitability thresholds	Habitat types identified using high resolution Google Earth satellite imageries
1	Very high	Patches of willow-poplar forest, Seabuckthorn forest and settled cultivation areas.
2	High	Patches of willow-poplar forest, Seabuckthorn forest and settled cultivation areas.
3	Medium	Degraded open forest areas and scrublands
4	Low	Degraded open forest areas and scrublands
5	Very low	Grasslands, degraded open forests, scrublands and herbaceous meadows

Table 4.8: Current protected area setting for conservation of *D. hatagirea* in the wild Indus-Suru valley of Ladakh region

Valley	Name of forest	Total area
Indus-Suru Habitat	Forest protection force Regional wildlife Environment and forestry Wildlife	485 km

4.4 Discussions:

4.4.1 Vegetation Habitat

On the basis of our field visit, we have found that 1812 individuals of *D. hatagirea* were present in our surveyed area. When we compared it with past records and observable grazing pressure, our study sites have fallen within the category of unprotected area. In our study, the density of *D. hatagirea* was 6.9 ind/m^2 which is more or less similar to density of Suru Valley of Jammu and Kashmir i.e. 8 and 6.1 ind/m^2 [Rinchen et al., 2012] and higher as compared to Garhwal Himalaya i.e. 1.8 and 0.7 ind/m^2 [Giri et al., 2008]. These data showed that there was a decrease in number of plants of this species with time. Because, in the Trans Himalayan Ladakh region of India, distinguished as global biodiversity area, ecological, vegetation and evolutionary factors favoured development of a huge diversity of species and remoteness of area prevented many large scale exploitations on natural ecosystems [Myers, 1990]. The inaccessibility of the region promoted local inhabitants to depend on local available resources for survival [Nautiyal et al., 2001]. The studies on flora of medicinal plants of this region were undertaken on contract basis from time to time. Trading of Trans-Himalayan vegetation flora has been observed very frequently across the region because of their high medicinal and aromatic values [Olsen and Larsen, 2003]**.** Many studies have documented the mode of collection of medicinal endangered plants and their trade in Himalayan region [Dobriyal et al., 1997; Farooquee and saxena, 1996; Olsen, 1998; Nautiyal et al., 2001; Olsen and Larsen, 2003; Maikhuri et al., 2005]. Based on field studies and explorations, it was noticed that in the areas where people have profitable earning sources such as tourism, the density of economically and socially important vegetation of plant species was higher as compared to other areas. Ladakh region appears very green in summer where animal reach every year during May-October for summer grazing [Nautiyal et al. 2002]. Therefore the low density in unprotected areas may be due to heavy grazing pressure. Very few studies have been conducted in Garhwal Himalaya, Uttarakhand [Nautiyal and Nautiyal, 2004; Bhatt et al., 2005] and also in Kargil District of Suru valley [Rinchen et al., 2012] but part of Nubra, Indus and Suru valley (Kargil and Leh district) have remained unexplored areas due to remoteness and this is the first information regarding vegetation along with soil data in this area. Local people destroy *D. hatagirea* underground parts i.e. tubers for Salep [Chaurasia et al., 2007]. Overexploitation due to its high medicinal value, frequently visited to potential sites (almost thrice in a year for research purposes) and

unawareness of the proper procedure of collection and propagation etc. were the major factors found for decline of this species from its natural habitats along with other level of disturbances like grazing pressure because of its edible nature. Further, some local inhabitants collect this high value medicinal plant for illegal trading. The local inhabitants could earn Rs.100 to 200 for per kg of dried roots of *D. hatagirea.* For 1 kg of dried roots, 90 to 100 mature plants are exploited [Rinchen et al., 2012]. As a result, only 1812 individuals were present in this area. This indicated that if such activity is going on, this species may become extinct within a few years.

4.4.2 Soil

Among the soil parameters, the effect of pH, C, N, P and K content have been the most important predictor variables. The pH was found to co-vary with temperature and humidity, resulting in distinct vegetation in basic soils. Our results indicated that soil K content was also significantly associated with differences in vegetation. According to Holzner [1971], indicator species of low K content usually preferred soil acidity and Andreasen et al [1991] found that increasing K content also had a negative effect on some species. But in our study, soil samples of Ladakh region contained low value of K, P, total nitrogen, total organic matter and total organic carbon as compared to high value of C/N ratio of soil samples collected from Uttarakhand [Giri et al., 2010].

4.4.3 Habitat modelling

Enhance vegetation index has played a key role in determining the distribution of potential habitats of *D. hatagirea* in its native range. Model output and field surveys revealed that suitable natural habitats of the species concurred with the distribution of willow-poplar forests in the elevations (≈ 2800-3000 m asl) of Indus and Suru valley. The restricted distribution of highly suitable habitats of *D. hatagirea* to the moderate elevations indicated that the species is absolutely near to endemic. EVI layers offered reasonable explanation on the underlying role of other environmental factors which determined the habitat suitability of the species. Various environmental factors such as geology and climate have probable influence on vegetation indices of a given place at a given time [Soleimani et al., 2008]. The effects of such underlying environmental factors were reflected through the spatial and temporal variation in the vegetation indices such as NDVI and EVI. The greater contribution of some of the EVI layers such as EVI 1, EVI 3, EVI 7, EVI 9, EVI 10, EVI 11 and EVI 12 to the overall habitat model restates the subtle role played by these factors in defining habitat

suitability. Interestingly, the layers EVI 7, EVI 9 and EVI 10 which contributed to the habitat model correspond to the period of flowering, fruiting and harvesting of the species. Hence, EVIs can also act as powerful and informative variables representing complex formulations of the underlying environmental factors which determine the boundaries of the potential habitat of the species. Better population status of the species in areas of higher model thresholds such as Indus and Suru valley indicated that these areas have ideal habitat conditions for persistence of the species. However, locations such as Hunder and Tirith had lower population size inspite of being predicted as highly suitable. The reason for this as revealed from the direct field observation was concurrent anthropogenic disturbances in these localities being hot spot for camping sites and overexploitation of *D. hatagirea* for earning purposes [Warghat et al., 2012b]. From the above observations, we can subtly assume that population status of a species in undisturbed habitats in the native range could be ascertained with reasonable level of confidence from the model output, i.e. areas with greater population size are predicted as models with higher threshold level and vice versa. Such hypothesis however, may not hold good if the habitats are modified through human pressure. Considering the numbers of individuals at different locations, the sub-populations at Sanjak, Lochum, Mulbek and Pashkum may be considered as growing while Turtuk, Bogdang, Tirith and Hunder are declining.

Overall, the results of actual habitat assessment through Google Earth superimposition and field surveys were identical. Through both the methods, the prevalence of *D. hatagirea* was in similar land use and land cover types. This analysis confirms the application of Google Earth superimposition along with field survey as a powerful tool for habitat assessment of the species and could be a substitute for extensive field survey [Benham et al., 2011]. Conservation of a species and its habitat cannot be done in isolation outside the sphere of the anthropobiome [Ellis and Ramankutty, 2008]. Habitat status assessment through primary field survey and secondary survey using Google Earth satellite imageries revealed that the predicted potential areas of the species under all suitability threshold levels i.e. Very low to very high suitability, encompass a mosaic of disturbed/undisturbed forest patches, scrubs, grasslands, settled cultivation areas, etc. which essentially are components of the anthropobiome. Species reintroduction plan should therefore carefully select appropriate areas under such a setting. In the present study, some areas consisting of continuous and intact patches of willow-poplar forest and degraded forest patches offer as potential habitats at

higher levels of probability. Hence, such forest areas could serve as habitats for *in situ* conservation and reintroduction. However, predicted less suitable areas such as grasslands and cultivation land could also be used for reintroduction of the species provided that adequate measures are taken for the habitat protection. To achieve this, awareness and active participation of local people, Non Government Organizations (NGOs), forest department and Community Based Organizations is necessary.

CHAPTER 5

Optimization of tissue culture techniques for *in vitro* multiplication of endangered orchid *D. hatagirea*

5.1 Introduction:

The ongoing loss of habitat in Ladakh region has prompted interest in preservation and restoration of these critical habitats. These habitats are considered highly productive for both plant and animal species. This loss, mostly due to habitat conversion for camping sites, road construction and habitat mis-management has greatly impacted population of rare and endangered plants. Native terrestrial orchids inhabit this threatened habitat and are at risk of population decline unless an effective method of propagation can be developed to provide plants for restoration purposes.

Seed germination represents the most efficient method of native terrestrial orchid propagation for conservation purposes. However, orchid seed germination studies are often viewed as unreliable or unrealistic since little is known concerning the germination and *in vitro* seedling developmental requirements of many terrestrial orchids (Arditti et al. 1981). Orchid seeds are microscopic and non endospermous with undiffertiated embryos. They are produced in large numbers and their germination in nature depends upon a suitable association with mycorrhizal fungus, thus making it difficult to observe their further development after they are released from the seed capsule into soil [Hardley, 1959; Temjensangba and Deb 2006]. Compounding this difficultly, Stoutamire [1974, 1989] found that many terrestrial orchids require up to eight years of *ex vitro* growth before reaching reproductive maturity. To overcome these problems some have suggested the development of optimized asymbiotic seed germination methods for entire genera or individual species [Kauth et al. 2006]. This approach shows great promise and is employed here.

The orchid, *D. hatagirea* is indigenous to Himalayas and exclusively found in the Ladakh region of Jammu and Kashmir, India. It is critically endangered and high value medicinal and ornamental orchid used in the Indian system of medicine particularly in Ayureveda, Siddha, Unani medicine and floriculture purpose [Chaurasia et al. 2007 and Pant et al. 2012]. The tubers of this species yield a high quality ‘Salep’ which is used as a tonic. The annual consumption of ‘Salep’ obtained from the species in India is about 7.38 tonnes

(valued at about Rs. 50 lakhs) and has great demand in national and international markets [Badola and Pal 2002; Olsen and Helles 1997]. The orchids are propagated through vegetative means as well as seeds. However, the rate of vegetative propagation in *D. hatagirea* is very slow and seed germination in nature is very poor, i.e. 0.2-0.3% [Vij 2002]. Both *in situ* and *ex-situ* approaches are important for the protection of rare and endangered orchid species. Tissue culture is one of the most important measures in *ex-situ* conservation of terrestrial orchids [Jakobsone et al. 2007]. Knowledge on physiological and morphological aspects of germination and development of particular orchid species is of critical importance for the establishment of tissue culture.

The current study reports for the first time optimization of increased *in vitro* immature seed germination, seedling development, plantlets formation as well as multiplication of *D. hatagirea* plants grown under *in vitro* and *in vivo* conditions.

5.2 Materials and methods:

5.2.1 Seed source

Seeds of *D. hatagirea* were obtained from immature capsules collected from naturally pollinated plants growing in a farm at Tirith village (Altitude 2817.20 m, longitude E 77°38'.481, latitude N 34°32'.378), Nubra valley of Ladakh region of India. The seeds were air-dried for two weeks at room temperature and stored in small bottles at 4°C until use.

5.2.2 Seed viability test

TTC staining

Immature seeds were soaked in filtered triphenol tetrazolium chloride (TTC) solution (1 g in 100 ml phosphate buffer, pH 6.5-7.0) for 48 hours in darkness at 20 ± 2°C and rinsed five times in sterile distilled water. Seeds were agitated between cover slides to remove testa and viewed using a Nikon microscope. The embryos retained as pink to red were considered viable, whilst seeds with embryos partially coloured, white or brown were assumed non-viable [Van and Deberg 1986].

FDA staining

The immature seeds were soaked in FDA solution composed of equal volume of distilled water and FDA stain (0.5 g in 100 ml absolute acetone) for 15 min and viewed in a Olympus BH-2 (UV light) fluorescence microscope. Seeds with embryos completely stained (fluorescent) were considered viable [Rasmussen 1995]. The same test was repeated for

viability of seeds collected from stored seed samples at 4°C at the interval of 1 week for one month.

5.2.3 Nutrient media

Ten different basal media were tested for immature seed germination. Five of the media were commercial preparations with modifications by Himedia Laboratories Pvt. Limited, Mumbai, India: Terrestrial Orchid Medium BM-1 [Van Waes and Debergh 1986]; Vacin and Went modified medium VW [Vacin and Went 1949]; Malmgreen Modified Terrestrial Orchid Medium MM [Malmgren 1996]; Mitra orchid medium PT139 [Mitra et al. 1976] and Murashige and Skoog medium MS [Murashige and Skoog 1962]. Three of the media were modified by Titan Biotech limited, Rajasthan, India: Knudson C Orchid medium KC [Knudson 1946]; Heller medium TP039 [Heller 1953] and Lindemann orchid medium TP041 [Lindemann et al. 1970]. Two of the media were procured from *Phyto* Technology Laboratories, LCC (Shawnee Mission, KS): Fast terrestrial orchid medium F522 [Fast 1976] and Orchid Seed Sowing Medium (P723) (Table 5.1). The pH of media was adjusted to 5.7 with 0.1 N HCl and 0.1 N NaOH, after the addition of sucrose and agar; 15 ml of medium was dispensed in each test tube (25 x 150 mm), plugged and autoclaved for 20 min. at 121°C and 1.05 kg cm^{-2} pressure.

5.2.4 Seed surface sterilization and inoculation

Immature seeds were surface sterilized for 2 min. in 5 ml ethanol (100%); 15-20 min. in 5 ml 0.1% NaOCl followed by rinsing in 90 ml sterile distilled deionized water for 15-20 min. Solutions were removed from the surface sterilization vials using disposable 1000 µl sterile pipette tips that were replaced after each use. Seeds were then suspended in sterile-deionized distilled water and a sterile loop was used for inoculation. The inoculating loop was immersed once into the seed suspension and seeds were placed in the culture tubes. The number of seeds used for the germination tests were 3 replications × 100-150 seeds/replication for each treatment.

5.2.5 Culture conditions

Cultures were incubated in a growth chamber maintained at 25 ± 1°C under a 16/8 hour photoperiod with illumination of 3000 lux intensity of white light.

5.2.6 *In vitro* seed germination and development of Protocorms

The seeds were germinated and differentiated into protocorm like bodies (PLBs) on the same germination medium. The cultures were monitored regularly and the data were scored at weekly intervals. After 2 and 3 weeks of germination, the protocorm development was assessed by using a dissection stereoscope. Germination and protocorm development were scored on a scale of 0-5 [Zettler and McInnis 1994] i.e. stage 0 No germination stage, stage 1 rupture of testa due to swelling of embryo (i.e. germination), stage 2 protocorm formation and emergence of rhizoids, stage 3 emergence of leaf primordium (shoot), stage 4 appearance of first leaf, stage 5 elongation of leaf and root differentiation. The percentage germination was calculated by dividing the number of seeds in each individual germination and development stage by the total number of viable seeds in the sample. The remaining seeds were dried in Petri dishes with anhydrous $CaCl_2$ for 2 weeks at 5°C. Dried seeds were transferred into Eppendorf microtubes and stored in hermetically closed containers at -20°C. All experiments were repeated three times. The experimental design was completely randomized.

5.2.7 Plantlet regeneration and mass multiplication

The PLBs developed from cultured immature embryos were maintained on optimum basal germination medium for further differentiation to form leaf primordia. The PLBs with leaf primordium were separated from the germination medium and cultured on regeneration media. For regeneration of plantlets and their multiplication, BM-2 and MS media supplemented with combinations of growth regulators (0-3 mg/L IBA and 0-3 mg/L Kin) were tested. The resulting micro shoots were separated from the regeneration medium for further mass multiplication. During plantlets regeneration, data were recorded for shoot number, shoot length, root number, root length and no. of days required for shoot/root formation. The well developed plantlets were maintained on the same regeneration medium before transferring to hardening. Three replications × 10 pots/replications for each potting mixture medium were used for hardening and transferring.

Table 5.1 Composition of different nutrient media

Component (mg/L)	BM-1	VW	MM	P723	F522	Kn C	PT139	TP041	TP039	0.5 MS
Ammonium Nitrate	-	-	-	412.5	160	500.00	-	-	-	1650
Ammonium Sulphate	-	500.00	-	-	-	500.00	-	1000.00	-	-
Aluminium chloride. $6H_2O$	-	-	-	-	-	-	-	0.560	0.054	-
Boric Acid	10	-	-	1.65	1.0	-	0.60	1.100	6.200	6.2
Calcium Chloride	-	-	-	83	-	-	-	-	56.620	-
Calcium Chloride. $2H_2O$	-	-	-	-	-	-	-	-	-	440
Calcium Nitrate	-	-	-	-	-	-	-	347.20	-	-
Calcium Nitrate. 4 H_2O	-	-	-	-	80	-	200.00	-	-	-
Calcium Phosphate, tribasic	-	200.00	75.00	75	-	-	-	-	-	-
Cobalt Chloride. $6H_2O$	0.025	-	-	-	-	-	0.04	-	-	0.025
Cobalt nitrate	-	-	-	-	-	241.30	-	-	-	-
Cupric Sulfate $\cdot 5H_2O$	0.025	-	-	0.0063	0.03	-	0.05	0.020	0.030	0.025
Sodium phosphate monobasic	-	-	-	-	-	-	150.00	-	-	-
Sodium dihydrogen phosphate	-	-	-	-	-	-	-	-	108.700	-
Sodium nitrate	-	-	-	-	-	-	-	-	600.000	-
Na2 EDTA	37.25	37.30	37.30	0.0063	-	-	22.30	-	-	-
Na2 EDTA. $2H_2O$	-	-	-	-	-	-	-	-	-	37.3

Disodium molybdate. $2H_2O$	-	-	-	-	-	-	-	-	-	-
Sodium –Fe EDTA	-	-	-	-	16	-	-	-	-	-
Ferric chloride. $6H_2O$	-	-	-	-	-	-	-	-	1.000	-
Ferrous sulphate (Anhydrous)	-	-	-	-	-	25.00	-	-	-	-
Ferrous Sulphate. $7H_2O$	27.85	27.80	27.80	13.93	-	-	16.70	-	-	27.8
Ferric citrate	-	-	-	-	-	-	-	4.400	-	-
Magnesium Sulphate	100.00	122.09	97.69	75.18	-	122.15	250.00	58.980	121.560	-
Magnesium Sulphate.7 H_2O	-	-	-	-	80	-	-	-	-	370
Manganese Sulphate	-	-	-	-	-	-	-	0.05	0.080	-
Manganese Sulphate. H_2O	25.00	5.68	1.54	4.23	-	5.680	0.42	-	-	-
Manganese Sulphate. $4H_2O$	-	-	-	-	0.1	-	-	-	-	22.3
Molybdic Acid. $2H_2O$	0.25	-	-	0.0625	-	-	0.05	-	-	-
Nickel chloride	-	-	-	-	-	-	-	0.030	-	-
Nickel chloride. $6H_2O$	-	-	-	-	0.03	-	-	-	0.025	-
Potassium Chloride	-	-	-	-	160	250.00	-	1050.00	750.000	-
Potassium Iodide	-	-	-	0.2075	0.01	-	0.03	0.100	0.015	0.83
Potassium Nitrate	-	525.00	-	475	-	-	180.00	-	-	1900
Potassium Phosphate, monobasic	300	250.00	75.00	42.5	-	-	-	135.000	-	-

Potassium dihydrogen phosphate	-	-	-	-	80	250.00	-	-	-	170
Zinc Sulfate. H_2O	-	-	-	-	-	-	-	-	1.000	-
Zinc Sulfate $\cdot 4H_2O$	-	-	-	-	-	-	-	-	-	8.6
Zinc Sulfate $\cdot 7H_2O$	10.00	-	-	2.65	1.0	-	0.05	0.570	-	-
Casein hydrolysate	500.00	-	400.00	-	-	-	-	-	-	-
D-Biotin	0.05	-	0.05	-	-	-	0.05	-	-	-
Folic acid	0.50	-	0.50	-	-	-	0.30	-	-	-
Riboflavin	-	-	-	-	-	-	0.05	-	-	-
Glycine	2.00	-	2.00	-	2.00	-	-	-	-	2.0
L-Glutamine	100.00	-	-	-	-	-	-	-	-	-
MES (Free Acid)	-	-	-	500	-	-	-	-	-	-
myo-inositol	100.00	-	100.00	100	-	100.00	-	-	-	100
Ascorbic acid	-	-	-	-	50	-	-	-	-	-
Nicotinic acid	5.00	-	5.00	1	10	0.50	1.25	-	-	0.5
Peptone	-	-	-	2000	2000	-	-	-	-	-
Pyridoxine HCl	0.50	-	5.00	1	0.5	0.50	0.30	-	-	0.5
Thiamine HCl	0.50	-	10.00	10	5	1.00	0.30	-	-	0.5
Pineapple powder	-	-	20000.0	-	-	-	-	-	-	-
Charcoal	-	-	1000.00	1000	-	-	2000.00	-	-	-
Agar	-	8000	-	8000	8000	8000	8000	8000	8000	8000
Sucrose	20000.00	20000.00	20000.00	20000	12000	20000	20000	20000	20000	30000
Fructose	-	-	-	-	5000	-	-	-	-	-

ClariGel™ (Gelrite)	3000.00	-	3000.00	-	-	-	-	-	-	-

BM-1 Terrestrial Orchid Medium (BM-1), Vacin and Went modified medium (VW), Malmgreen Modified Terrestrial Orchid Medium (MM), PhytoTechnology Orchid Seed Sowing Medium (P723), Fast terrestrial orchid medium (F522), Knudson C Orchid medium (KC), Mitra orchid medium (PT139), Lindemann orchid medium (TP041), Heller medium (TP039), Murashige and Skoog medium (MS)

5.2.8 Hardening of plantlets and *in vivo* multiplication

Fully grown plantlets were taken out from the regeneration medium and traces of agar were removed with a soft brush. Plantlets of 5-10 cm shoot length, with 5-7 roots were successfully transplanted to the controlled green house conditions into eight combinations of potting mixtures namely, C-1 (sand + soil, 1:1), C-2 (sand + soil + FYM, 1:1:1), C-3 (sand + soil + Cocopeat, 1:1:1), C-4 (sand + soil + Vermiculite, 1:1:1), C-5 (sand + soil + Perlite, 1:1:1), C-6 (cocopeat + vermiculite, 1:1), C-7 (vermiculite + Perlite, 1:1) and C-8 (cocopeat + vermiculite + Perlite, 1:1:1). Initially, for 10 - 15 days the plantlets were covered with glass jars to provide sufficient humidity and avoid desiccation till the plantlets showed new growth. During the hardening process, glass jars were taken off every day for 1 - 2 h so as to acclimatize the plantlets to the external environment and data were recorded for percent survival, number of shoots and roots, length of shoots and roots.

5.2.9 Data analysis

Univariate statistical procedures were used to analyze the data. Descriptive analysis of the data was performed using SPSS 19 software. Analysis of variance (ANOVA) with comparative Duncan's multiple range tests at 5% was used to determine significance of differences between treatments.

5.3 Results:

5.3.1 Seed viability

The interpretation of seed viability using TTC was very difficult. Viewing the immature embryos was only possible after removing the testa, a tedious and delicate process since terrestrial orchid seeds are very minute (200 to 1700 µm). TTC staining did prove effective for selecting viable seeds of *D. hatagirea* but tried FDA stain which was repeated for better staining. FDA was an excellent stain for observing viable seeds. Fluorescence was easily recognised and stained seeds were easily counted without removing the testa which was transparent. Stained viable embryos were seen in Figure 5.1. Through FDA method the highest percentage (24%) of viable embryos was observed in first week of sampling followed by significant reduction (13%) in the fourth week of sampling, whereas TTC method showed high viability (22%) in the first week and 11% in the fourth week of sampling, respectively (Table 5.2).

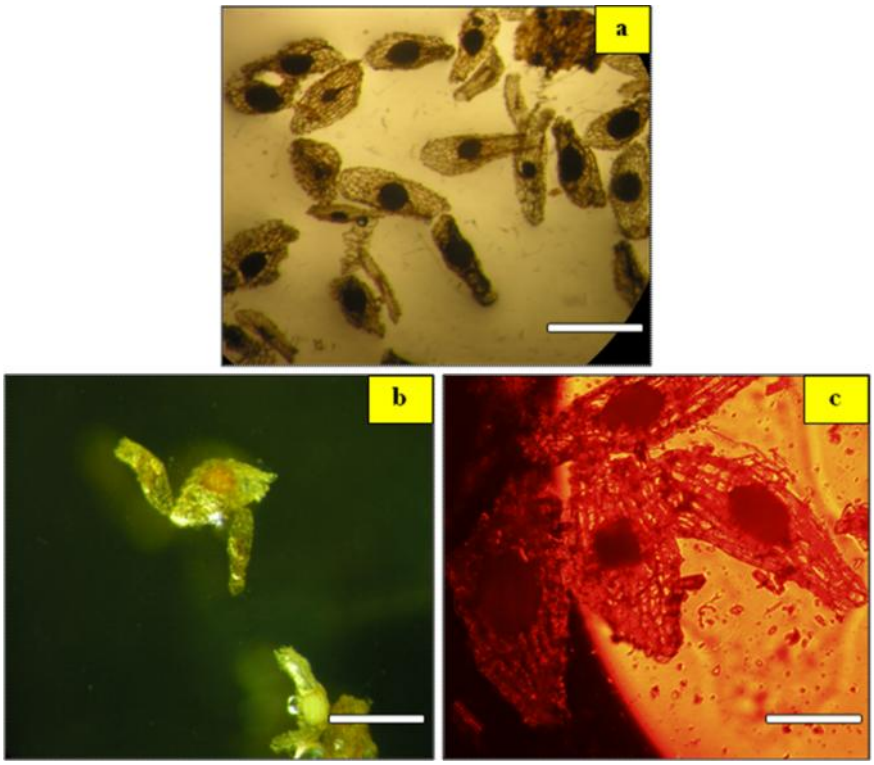


Figure 5.1 Immature seeds (3 weeks after pollination) of *D. hatagirea*; a) 10× b) Viable seeds stained with TTC (60×); c) Viable seeds stained with FDA (200×); Scale bar = 1 mm

Table 5.2 Viability of *D. hatagirea* stored seeds (4°C) at 1 week interval

Sr. No.	Stored seed (days)	Percent seed viability in TTC stain[#]	Percent seed viability in FDA stain[#]
1	7	22 ± 5	24 ± 2
2	14	14 ± 2	18 ± 7
3	21	11 ± 8	15 ± 3
4	28	11 ± 1	13 ± 9

Values are mean ± SE

5.3.2 Asymbiotic seed germination

The stages of seed germination of *D. hatagirea* were presented in figure 5.2. Seeds began swelling within two weeks after inoculation and germinated to form protocorms within third week of inoculation. Of the ten media tested, seed germination was highest (37.12%) in Lindemann orchid medium (LD), followed by 35.48%, 34.48%, 31.68% in Heller medium (TP039), Terrestrial Orchid medium (BM-1) and 0.5 Murashige and Skoog medium respectively. Maximum Protocorm formation (23.40%) occurred in Lindemann orchid medium (LD) followed by 21.40%, 20.4% and 18.6% on Heller medium (TP039), Terrestrial Orchid Medium (BM-1) and Murashige and Skoog medium (0.5 MS), respectively (Table 5.3). Seed germination was minimum in MM medium, Kn C medium, VW medium, Fast medium and Orchid seed medium even after 4 weeks of inoculation. Protocorm formation occurred on rest of the media after 7 weeks of incubation. The germinating seeds converting into PLBs were maintained on the same germination medium for further differentiation into leaf primordia. Formation of first leaf i.e. stage 3 from PLBs were developed in all media except, Mitra medium (PT139).

5.3.3 Regeneration and mass multiplication

The protocorms with first leaf primordia were cultured on BM-2 and seven different MS media supplemented with different concentrations and combinations of IBA and Kin for shoot formation. The protocorm with first leaf primordia developed into multiple shoots within 15 to 20 days of incubation in shoot regeneration media and further developed into plantlets with shoots and roots within 28 to 42 days of incubation. Growth and development of plantlets with maximum number of shoots/protocorm (18.12 ± 2.3), shoot height (17.80 cm ± 2.16), Number of roots (8.25 ± 0.69) and maximum root length (8.02 cm ± 1.45) occurred on MS medium supplemented with IBA (3 mg/L) + Kin (1 mg/L) within 28 to 30 days of incubation. Whereas MS medium containing Kin 3 mg/L showed relatively less number of shoots (16.00 ± 1.26) and other respective parameters of growth and development until 34 to 40 days of culturing (Table 5.4 and Figure 5.3 a, b and c).

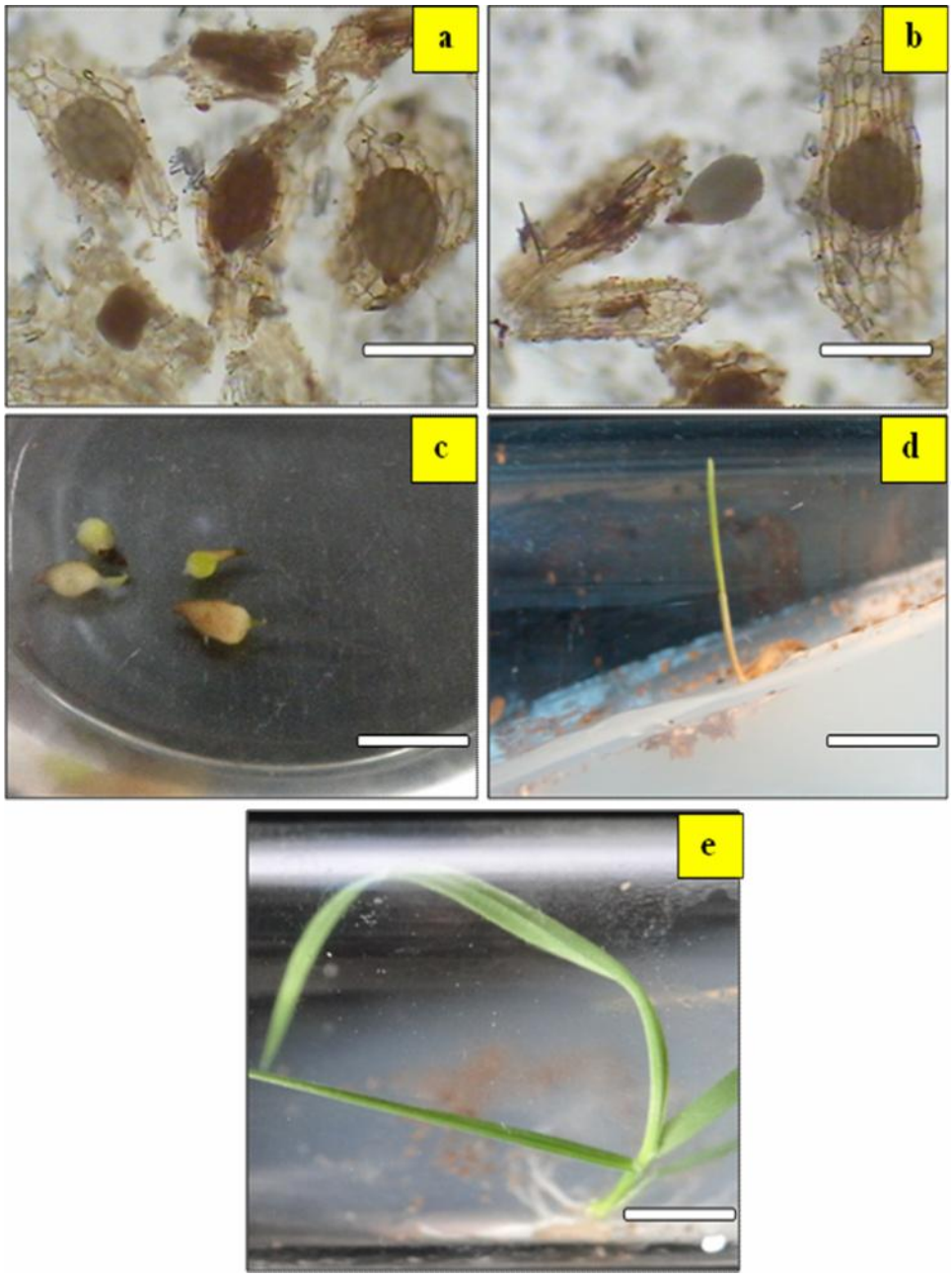


Figure 5.2 Classification of *in vitro* seed germination and seedling development into different stages in *D. hatagirea;* a) Stage 0, no germination. b) Stage 1, swelling of embryos and bursting of its testa. c) Stage 2, Protocorm formation and emergence of rhizoids d) Stage 3, emergence of leaf primordia e) Stage 4 and 5, appearance of first leaf and root development (Scale bar = 1mm for a, b & c; Scale bar = 1cm for d & e)

Table 5.3 Immature seed germination and protocorm formation in *D. hatagirea* on different nutrient media

Sr. No.	Nutrient medium	Immature seed germination[#] (%)	Immature seeds converting to PLBs[#] (%)	No. of days required for protocorm formation
1	BM1	34.48 ± 0.69^{cd}	20.4 ± 0.30^{d}	17
2	VW	1.52 ± 0.36^{ab}	3.92 ± 0.86^{bc}	25
3	MM	2.48 ± 0.35^{b}	6.64 ± 0.82^{cd}	22
4	Orchid seed	0.60 ± 0.26^{ab}	1.68 ± 0.70^{ab}	23
5	Fast	0.92 ± 0.31^{ab}	2.46 ± 0.77^{ab}	21
6	Kn C	1.58 ± 0.37^{ab}	4.08 ± 0.88^{bc}	22
7	Mitra	0	0	0
8	Lindemann	37.12 ± 0.91^{d}	23.40 ± 0.30^{d}	17
9	Heller	35.48 ± 0.70^{d}	21.40 ± 0.31^{d}	23
10	0.5 MS	31.68 ± 0.72^{c}	18.6 ± 0.43^{cd}	20

PLBs: Protocorm like bodies,

ANOVA test shows highly significant with Duncan multiple range test at 5 % ($p = 0.05$)

#Data shown are the mean of three replicates × 100-150 seeds/replication for each treatment ± standard error (SE). Data recorded within one week interval

Table 5.4 *In vitro* shoot multiplication in *D. hatagirea* on different media

Sr. No.	Medium	Shoots/protocorm[#]	Shoot length[#] (cm)	Root number[#]	Root length[#] (cm)	No. of days required for shoot/root formation
1	BM-2	7.32 ± 1.3^{d}	7.80 ± 1.16^{cd}	6.01 ± 0.15^{abc}	6.73 ± 0.68^{bc}	37 – 42
2	MS + 3 mg/L Kin	16.00 ± 1.26^{a}	17.31 ± 0.51^{a}	8.00 ± 2.04^{bc}	7.04 ± 0.16^{ab}	34 – 40
3	MS + 1 mg/L IBA + 2 mg/L Kin	14.02 ± 0.32^{abc}	16.00 ± 1.26^{a}	7.00 ± 0.51^{bcd}	6.73 ± 0.23^{abc}	31 – 35
4	MS + 1 mg/L IBA + 3 mg/L Kin	13.34 ± 0.28^{bc}	13.96 ± 1.27^{a}	7.11 ± 1.67^{bcd}	6.70 ± 0.27^{bcd}	30 – 35
5	MS + 2 mg/L IBA + 1 mg/L Kin	12.42 ± 0.43^{abc}	16.92 ± 3.78^{a}	6.11 ± 2.04^{cd}	5.94 ± 0.28^{ab}	29 – 35
6	MS + 2 mg/L IBA + 2 mg/L Kin	10.67 ± 0.67^{b}	16.67 ± 1.67^{a}	6.22 ± 2.40^{cd}	5.38 ± 0.39^{ab}	30 – 31
7	MS + 3 mg/L IBA	9.11 ± 0.44^{b}	15.00 ± 1.15^{ab}	6.11 ± 2.04^{cd}	5.34 ± 0.23^{ab}	35 – 40
8	MS + 3 mg/L IBA + 1 mg/L Kin	18.12 ± 2.3^{bc}	17.80 ± 2.16^{bc}	8.25 ± 0.69^{bc}	8.02 ± 1.45^{d}	28 – 30

BM-2: Basal medium-2, MS- Murashige and Skoog, IBA-Indole butyric acid, Kin-Kinetin

ANOVA test shows highly significant with Duncan multiple range test at 5 % ($p = 0.05$)

Data shown are the mean of three replicates ± standard error (SE)

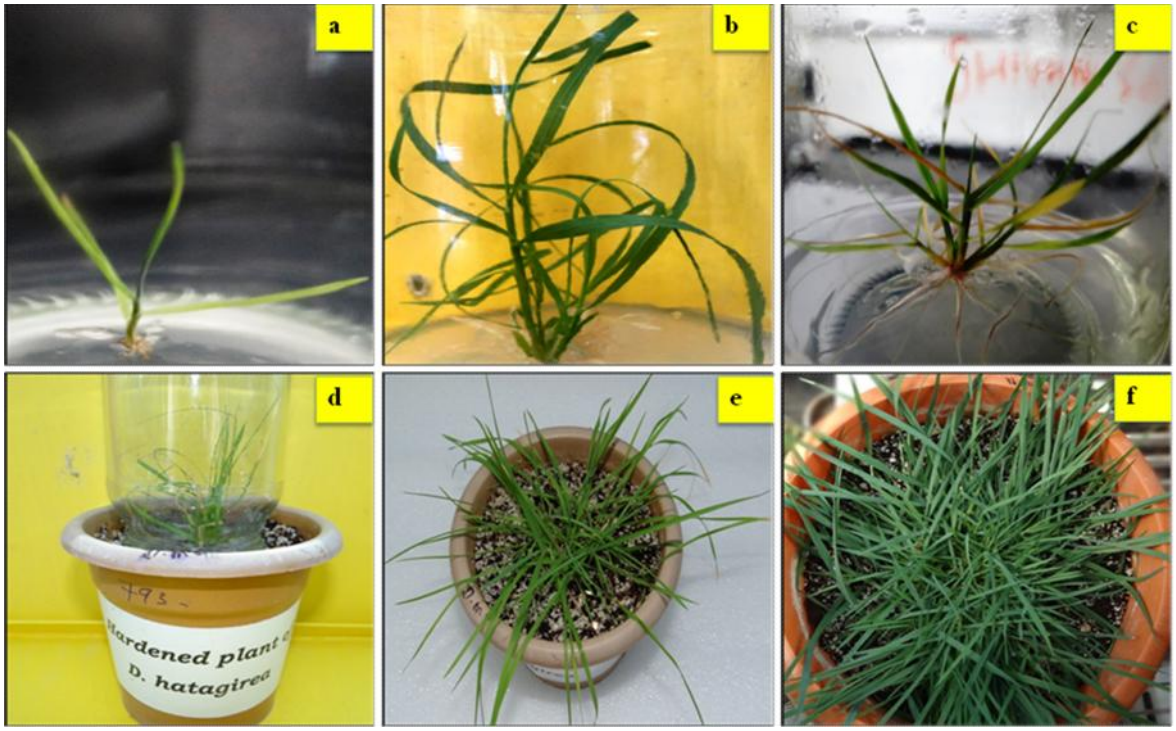


Figure 5.3 *In vitro* shoot multiplication, hardening and *in vivo* mass multiplication of *D. hatagirea* a) Initiation of shoot formation and growth b) *in vitro* shoot proliferation c) Plantlets with fully developed shoots and roots d) Hardening of plantlets e) *In vivo* growth, development and mass multiplication of plants (15 days) f) After 30 days

5.3.4 Hardening of plantlets and *in vivo* multiplication

The *in vitro* grown plantlets with 2-3 shoots were transferred to different potting mixtures for their acclimatization to field conditions and for further growth and multiplication. The data collected for each transferred plant in C-8 potting mixture (cocopeat + vermiculite + Perlite (1:1:1) which produced all 30 transferred plantlets were survived with significantly higher no. of shoots (75), maximum no. of plantlets (25), highest shoot length (18.8 cm), maximum number of roots (23) and highest root length (44.7 cm). After one month of transplantation in the glass house, satisfactory percentage survival of plantlets was observed on the potting mixtures whereas plantlets multiplication parameters were least in C-1 potting mixture with survival percentage 52.5, 29 shoots, 9.66 plantlets, 13.2 cm shoot length, 15 roots and 33.2 cm root length (Table 5.5, Figure 5.3 d, e, f and Figure 5.4).

Table 5.5 Survival and mass multiplication of *D. hatagirea* plants in different potting mixtures.

Sr. No.	Potting mixture	Survival (%)	Shoots[#] (no.) (1 month)	Plantlets[#] (no.)	Average shoot length[#] (cm)	Roots [#] (no.) (1 month)	Root length[#] (cm) [#]
1	C-1	52.5	29 ± 0.2^{bc}	9.66 ± 1.0^{cd}	13.2 ± 0.5^{a}	15 ± 0.1^{ab}	33.2 ± 0.2^{ab}
2	C-2	60.3	34 ± 0.1^{b}	11.33 ± 0.3^{cd}	13.5 ± 0.5^{bc}	16 ± 0.4^{bc}	34.7 ± 0.3^{b}
3	C-3	75.2	55 ± 0.2^{ab}	18.33 ± 0.4^{ab}	13.0 ± 0.1^{bc}	19 ± 0.6^{ab}	36.8 ± 0.2^{bc}
4	C-4	70.9	52 ± 0.3^{b}	17.33 ± 0.2^{a}	15.0 ± 0.3^{bc}	18 ± 0.5^{bc}	40.5 ± 0.4^{a}
5	C-5	70.2	51 ± 0.9^{d}	17 ± 0.0^{ab}	15.8 ± 0.7^{a}	16 ± 0.2^{a}	41.0 ± 0.2^{a}
6	C-6	90.7	63 ± 0.8^{bc}	21 ± 0.0^{ab}	16.2 ± 0.2^{b}	21 ± 0.1^{b}	42.9 ± 0.0^{ab}
7	C-7	84.9	59 ± 0.9^{ab}	19.66 ± 0.2^{bc}	17.5 ± 0.2^{bc}	19 ± 0.2^{ab}	43.5 ± 0.1^{ab}
8	C-8	100	75 ± 1.0^{ab}	25 ± 0.1^{a}	18.8 ± 0.8^{ab}	23 ± 0.9^{bc}	44.7 ± 0.9^{bc}

C-1 Sand: soil (1:1), C-2 sand: soil: FYM (1:1:1), C-3 sand: soil: Cocopeat (1:1:1), C-4 sand: soil: Vermiculite (1:1:1), C-5 sand: soil: Perlite (1:1:1), C-6 cocopeat: vermiculite (1:1), C-7 vermiculite: Perlite (1:1), C-8 cocopeat: vermiculite: Perlite (1:1:1)

ANOVA test shows highly significant with Duncan multiple range test at 5 % (p = 0.05)

Data shown are the mean of three replicates of 10 pot cultures in each treatment ± standard error (SE).

Figure 5.4 Mass multiplication of *D. hatagirea* ready for transfer to the field conditions

5.4 Discussions:

Habitat destruction, fragmentation, overexploitation and the effects of global climate changes are the main threats to the survival of orchids [Bubb et al. 2004]. *D. hatagirea* is indigenous to temperate regions of Sikkim, Uttaranchal and parts of Jammu and Kashmir. Very little information is published on growth and seedling development of *D. hatagirea* in natural habitat and no information is available as of today on *in vitro* seed germination and mass multiplication of plantlets. Low or even no germination occurred when mature seeds of *C. calceolus* and *E. palustris* were used for asymbiotic germination [Arczewska 1993]. Ramsay and Stewart [1998] suggested that the period of time when immature seeds germinate efficiently in axenic condition is short, approximately 7-10 days and should be experimentally estimated for each species. Previous studies revealed that no single nutrient medium is universally suitable for asymbiotic seed germination for most of the orchid taxa such as Mitra medium was found suitable for *Cymbidium macrorhizon* [Vij et al. 1998] and *Goodyera biflora* [Pathak et al. 1992]; Knudson C medium for *Cymbidium elegans* and *Cioelogyne punctulata* [Sharma and Tandon 1990]; VW medium for *Vanda coerulea* [Devi et al. 1998]*;* Nitsch medium for *Cymbidium iridioides* [Jamir et al. 2002] and Knudson C, VW and MS media for *Aerides rosea* [Sinha et al. 1998].

In vitro germination of seeds of terrestrial orchid species is often a long and slow process due to requirement of considerably longer periods for germination reported by de pauw et al. [1993], as in *D. majalis* seeds started to germinate after 6 weeks of culture on Fast medium [Znaniecka and lojkowska, 2004]. *D.ruthei* and *D. prastermissa* seeds started to germinate after four months of culture on Norstog medium [Vaasa and Rosenberg, 2004]. Germination of *D. ruthei* and *D. praetermissa* seeds depended on the concentration of $MnSO_4$ in the media [Vaasa and Rosenberg, 2004]. Seeds of *D. maculata* started to germinate after 3.5 months of incubation in soil [Kinderen 1995]. Vassa and Rosenberg [2004] found that a high percentage of seeds of *D. baltica, D. praetermissa* and *D. ruthei* germinated within three months of culture, and seed germination decreased afterwards. However, in our study, seeds of *D. hatagirea* germinated within a week in Lindemann and Heller orchid medium which has $MnSO_4$ as an important component. Percentage of *in vitro* protocorm formation varied in different media along with differences in the duration such as MS medium and Kn C medium, protocorm formation was 30-40% in 49 days of culture in *C. racemiferum* [Deb and

Temjensangba, 2006] While in *M. khasiana* 20-30% of protocorm formation occurred in MS medium with 500 mg/L Casein hydrolysate + 1 µM BA in 107 days [Deb and Temjensangba, 2006] and similarly in *Ophrys* species 23% of protocorms were formed in 107 days of culture on Malmgreen medium [Kitsaki et al. 2004]. We observed 22-23% of protocorm formation within 17 days of culture on Lindemann orchid medium. There are reports on multiple shoot formation under *in vitro* conditions such as in epiphytic orchid *C. racemiferum* within 30 days in MS medium supplemented with 10.0 µm NAA + 8.0 µm BAP [Deb and Temjensangba, 2006], *C. eburneum* (MS + 15 µm NAA+ 15 µm BAP) in 28 days [Gogoi et al. 2012] and *C. nervosa* (MS+ 3 mg/L BAP + 1 mg/L NAA) in 90 days [Abraham et al. 2012]. While, multiple shoots were formed in MS+ 6 µm IAA+ 18 µm BAP+ 18 µm Kin in 49-56 days in *M. khasiana* [Deb and Temjensangba, 2006]. Significant differences in multiple shoot formation on MS medium supplemented with 3 mg/L IBA and 1 mg/L Kin in a shorter duration of 28 to 30 days of incubation occurred in our study. Differences in survival of plantlets in potting mixtures have been reported such as 65% survival of transplants of *M. khasiana* in charcoal pieces + coconut husk + sterilized forest litter (1:1:1) [Deb and Temjensangba, 2006], 90% survival of transplants of *P. pallida* in charcoal chips, coconut husk and broken tiles (2:2:1) [Mulgund et al. 2012]. We observed 100% survival in C-8 potting mixture and the other potting mixtures (C-3 and C-6) also gave good shoot and root growth because of the presence of cocopeat and vermiculite as part of their potting mixtures which provided enough moisture, aeration and micronutrients for the profuse growth of plants. This observation is supported by Hartmann et al. [2007] where they found that vermiculite, when combined with perlite and cocopeat promotes faster shoot/root growth and gives quick anchorage to young plants.

In the present study, a successful attempt has been made to culture immature seed embryo of *D. hatagirea* for developing protocorms, shoot regeneration and its mass multiplication. The growth and multiplication of plants further continued after their transplantation in the glass house in potting mixtures, thereby resulting in the development of a micropropagation technology to meet the growing demand of *D. hatagirea*. This technology will help not only in multiplying the plantlets but can also play a major role in the conservation of this endangered orchid species.

CONCLUSIONS

Morphological discrimination in geographical populations of *D. hatagirea*

Plant descriptor was developed for exact identification of *D. hatagirea* and separation from *D. kafiriana*. Morphometric diversity of *D. hatagirea* was high among the populations. Tirith location showed great morphometric variation as compared to other locations. It implied that Nubra valley population showed great morphological dissimilarity with population of Suru and Indus valley. This may be due to wide geographical range, species richness and environmental factor. Being endangered *D. hatagirea*, Nubra valley showed high variation as compared to Suru and Indus. Therefore, *Dactylorhiza* populations deserve specific conservation attention as regards its habitat fragmentation. Conservation of its population's *ex-situ* and *in-situ* will have greater effects on population richness and status of such an endangered orchid.

Studies on population genetic structure and differentiation analyses of *D. hatagirea*

Low genetic diversity of *D. hatagirea* was observed due to endangered class of the plant. Significantly moderate level gene flow and genetic differentiation was observed in studied populations. Geographical barrier play a major role in shaping genetic structure of population. We found that Ladakh mountain range (6500 m amsl) act as geographical barrier in the studied population, which separated the two major gene pools. Conservation strategies involving genetic diversity studies are limited for Trans-Himalayan endangered plants and exploration of genetic diversity of this region is required. One primary objective of conservation management is to maintain genetic diversity of *D. hatagirea*. The strategy of conservation for this species should include both *in situ* and *ex situ* methods. Taking into account the special habitat of this species, *in situ* conservation should be given firstly recommended. Small populations are more prone to be extinct for environmental fluctuation and habitat destruction, it is necessary to protect all the existing populations and individuals *in situ* in order to preserve as much genetic variation as possible. Therefore, habitat protection will ensure the species coexistence with other organisms like fungi and pollinators on which orchids depend for their cycles. For *ex situ* conservation, we need to seriously design and establish a germplasm bank and conserve germplasm through Plant tissue culture technique.

Population inventory and vegetation mapping of *D. hatagirea* through Ecological Niche Modelling (ENM)

On the basis of vegetation analysis and Habitat distribution modelling, it can be concluded that low density of *D. hatagirea* found along with other medicinal plants because of famous tourist place and unexplored research area for researcher. The ecosystems of this area have been subjected to particularly intense pressure for the upcoming tourism activities, so current extinction rates of *D. hatagirea* species are expected to remain high or even. The areas identified in the present study for reintroduction of *D. hatagirea* would not only help in eco-restoration of degraded forests and habitats where the species had existed before but also in rehabilitating the species population and improving its conservation status. Therefore, there is a need to overcome with this problem with the help of research communications, policy implementation in some areas and also needs to promote cultivation, awareness of this species through participation and conserving overall biological diversity in the region by *in-situ and ex-situ* method.

Optimization of tissue culture techniques for *in vitro* multiplication of endangered orchid *D. hatagirea*

In the present study a successful attempt has been made to culture immature seed embryo of *D. hatagirea* for developing protocorms, shoot regeneration and its mass multiplication. The growth and multiplication of plants further continued after their transplantation in the glass house in potting mixtures, thereby resulting in the development of a micropropagation technology to meet the demand of *D. hatagirea*. This technology will help not only in multiplying the plantlets but can also play a major role in its conservation.

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LIST OF PUBLICATIONS

Publication in Peer-reviewed Journal

1. **Ashish R. Warghat**, Prabodh K. Bajpai, Ashutosh A. Murkute, Hemant Sood, Om P. Chaurasia and Ravi B. Srivastava, **"**Genetic diversity and population structure of *Dactylorhiza hatagirea* (Orchidaceae) in cold desert Ladakh region of India**.** Journal of Medicinal Plants Research 30 March 2012; Vol. 6(12), pp. 2388-2395 impact factor- 0 .879.

2. **Ashish R. Warghat**, Prabodh K. Bajpai, Hemant Sood, Om P. Chaurasia and Ravi B. Srivastava, "Morphometric analysis of *Dactylorhiza hatagirea* (D. Don) a critically endangered orchid in cold desert Ladakh region of India.". African journal of Biotechnology, 12 July, 2012; Vol. 11(56), pp. 11943-11951 impact factor-0 .573.

3. **Ashish R. Warghat,** Prabodh K. Bajpai, Ravi B. Srivastava, Hemant Sood and Om P. Chaurasia, "Genetic structure and conservation of small fragmented locations of *Dactylorhiza hatagirea* in Ladakh region of India". Scientia Horticulturae, 17 December; 2013, Vol. 164, pp. 448-454, impact factor- 1.39.

4. **Ashish R. Warghat**, Prabodh K. Bajpai, Ravi B. Srivastava, Om P. Chaurasia, Rajinder S. Chauhan and Hemant Sood "In vitro protocorm development and mass multiplication of an endangered orchid *D. hatagirea*." Turkish Journal of Botany, 2014, 38:737-746, impact factor-1.60.

5. **Ashish Warghat,** Prabodh Bajpai; Sahil Kapoor, Ashwani Bhardwaj, Hemant Sood, Tsering Stobdan, Om Chaurasia and Ravi Srivastava "Mapping the potential distribution of *Dactylorhiza hatagirea* (D. Don), a critically endangered medicinal orchid in cold desert Ladakh using maximum entropy model." Ecological engineering. (Accepted) impact factor-1.54.

6. **Ashish R. Warghat,** Prabodh K. Bajpai, Ravi B. Srivastava, Hemant Sood and Om P. Chaurasia "Analysis of associated vegetation and Climatic Characteristics of the endangered terrestrial orchid, *Dactylorhiza hatagirea* (D.Don) in the Ladakh Region of India" Phyton- International Journal of Experimental Botany (Accepted) impact factor-0.17.

7. **Ashish R. Warghat**, Prabodh K. Bajpai, Ravi B. Srivastava, Hemant Sood and Om P. Chaurasia **"**Multivariate analysis of the effects of soil parameters and environmental factors on the vegetation status of *D. hatagirea*.**"**. Journal of Vegetation Science (Under review) impact factor-3.7.

8. **Ashish R. Warghat,** Prabodh K. Bajpai, Ravi B. Srivastava, Hemant Sood and Om P. Chaurasia, "The influence of Natural barriers in shaping the genetic structure of *Dactylorhiza hatagirea* populations". Journal of Nature Conservation, (Communicated) impact factor-0.84.

Chapters in Book:

1. Ashutosh A. Murkute, **Ashish R. Warghat**, G. phani Kumar, G.P. Mishra and Sunil E. Jadhav, "*Dactylorhiza hatagirea* (D.don) a Critically endangered medicinal plant of Trans-Himalaya". pp. 37-46, Herbal perspectives: present and Future, ISBN 81-89304-91-7.
2. Janifer Raj X, Jitendra Kumar, Manish Bhoyar, Prabodh Kumar Bajpai, **Ashish Warghat** and R. B. Srivastava, "Biotechnological approaches for molecular characterization and Conservation of Trans-Himalayan flora". pp. 171-184, High Altitude cold Arid Agro animal Technology, ISBN 978-93-81226-02-5.

Conference Presentations and Abstracts:

(1) **Ashish R. Warghat,** Prabodh K. Bajpai, Hemant Sood, A.A. Murkute and S.B. Singh, "Exploring genetic diversity in *Dactylorhiza hatagirea* (D. Don) a Critically endangered orchid in Trans-Himalayan region". 4th Indian Horticulture Congress, held at IARI, New Delhi. 18 to 21 Nov. 2010 (Poster).

(2) **Ashish R. Warghat,** Prabodh K. Bajpai, Ashutosh A. Murkute, Hemant Sood and R. B. Srivastava, "Morphogical and Molecular differentiation among populations of *Dactylorhiza hatagirea* (Orchidaceae), a critically endangered orchid in Trans-Himalaya". 15th ADNAT Convention, a three day International Symposium on "Genomics and Biodiversity" held CCMB, Hyderabad. 23rd to 25th Feb. 2011 (Oral).

(3) **Ashish R. Warghat**, Prabodh Kumar Bajpai, Ashutosh A. Murkute, Hemant Sood and R. B. Srivastava "Morphometric and Genetic divergence among populations of *Dactylorhiza hatagirea* (Orchidaceae), an endangered terrestrial orchid in Trans-

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(4) **Ashish R Warghat**, Prabodh K Bajpai, Sahil Kapoor, Ashwani Bhardwaj, Tsering Stobdan, Bhuvnesh Kumar and Om P Chaurasia "Vegetation and environmental data sets matter in ecological niche modelling: a case study on reintroduction of *Dactylorhiza hatagirea* in Ladakh region of India" 58th Annual symposium of the International Association for vegetation science, Brno, Czech Republic. 19th -24th July, 2015. (Poster)

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