Journal of Medicinal Plants Research Vol. 6(12), pp. 2388-2395, 30 March, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR11.1007
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Genetic diversity and population structure of *Dactylorhiza hatagirea* (Orchidaceae) in cold desert Ladakh region of India.

Ashish R. Warghat[1]*, Prabodh K. Bajpai[1], Ashutosh A. Murkute[2], Hemant Sood[3], Om P. Chaurasia[1] and Ravi B. Srivastava[1]

[1]Defence Institute of High Altitude Research, DRDO, Leh-Ladakh, India, 194101.
[2]Directorate of Onion and Garlic Research, ICAR, Pune, India, 410505.
[3]Jaypee University of Information Technology, Waknaghat, Solan, India, 173215.

Accepted 24 February, 2012

Random amplified polymorphic DNA (RAPD) analysis was used to characterize the genetic diversity and population genetic structure within and among nine natural populations of *Dactylorhiza hatagirea,* a critically endangered or rare terrestrial medicinal orchid in cold desert of Ladakh region. Out of the 177 bands generated from twenty random primers, 174 were polymorphic. The genetic diversity of *D. hatagirea* that was revealed by observed number of alleles (N*a*), expected number of alleles (Ne), Nei's diversity index (H), Shannon's diversity index (I), amplificated loci, polymorphic loci and the percentage of polymorphic loci (PPL). Pair-wise population genetic distances ranged from 0.05 to 0.23. Analysis of molecular variance (AMOVA) revealed that 57% RAPD of variability was partitioned among population. The Principal coordinate's analysis (PCoA) and UPGMA supported the grouping of all 96 accessions of nine populations into four cluster groups. However, a moderate genetic differentiation among population was detected based on different measures (Nei's genetic diversity analysis: Gst = 0.2538; AMOVA analysis: Fst = 0.254)

Key words: Conservation biology,endangered orchid, genetic diversity, genetic structure.

INTRODUCTION

Dactylorhiza hatagirea (D.don) (Family: Orchidaceae) is a terrestrial ground dwelling perennial herb, 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 to 1.9 cm long with curved spur. The inflorescence consists of a compact raceme with 25 to 50 flowers developed from axillary buds. The dark purple spotted lip of the flower is rounded and lobed (1 to 5). The plants store a large amount of water in their tuberous roots to survive in arid conditions (Chaurasia et al., 2007).

Nowadays, more and more species have become threatened or extinct in the wild or in natural habitat, and plant conservations concerned with rare and endangered species are faced with an intimidating task (Holsinger and Gottlieb, 1991). The maintenance of genetic variation is one of the major objectives for conserving endangered and threatened species (Avise and Hamrick, 1996). For the management strategies, Markers are now routinely used to characterize genetic variation among and within populations which contribute towards conservation programme concerned with the critically endangered species (Milligan et al., 1994). According to our survey on this species, it is estimated that not more than 1000 individuals survived in habitat. At present, nine populations were studied but some of the populations rise towards declined stage. The threats to the species are related to certain anthropogenic activities as well as habitat destruction. In addition, population number and size are declining at an alarming rate in the last decade and genetic diversity is likely to be reduced. Considering the populations number, size and particular distribution of this critically endangered species, *D. hatagirea* should be

*Corresponding author. ashishwarghat@hotmail.com.

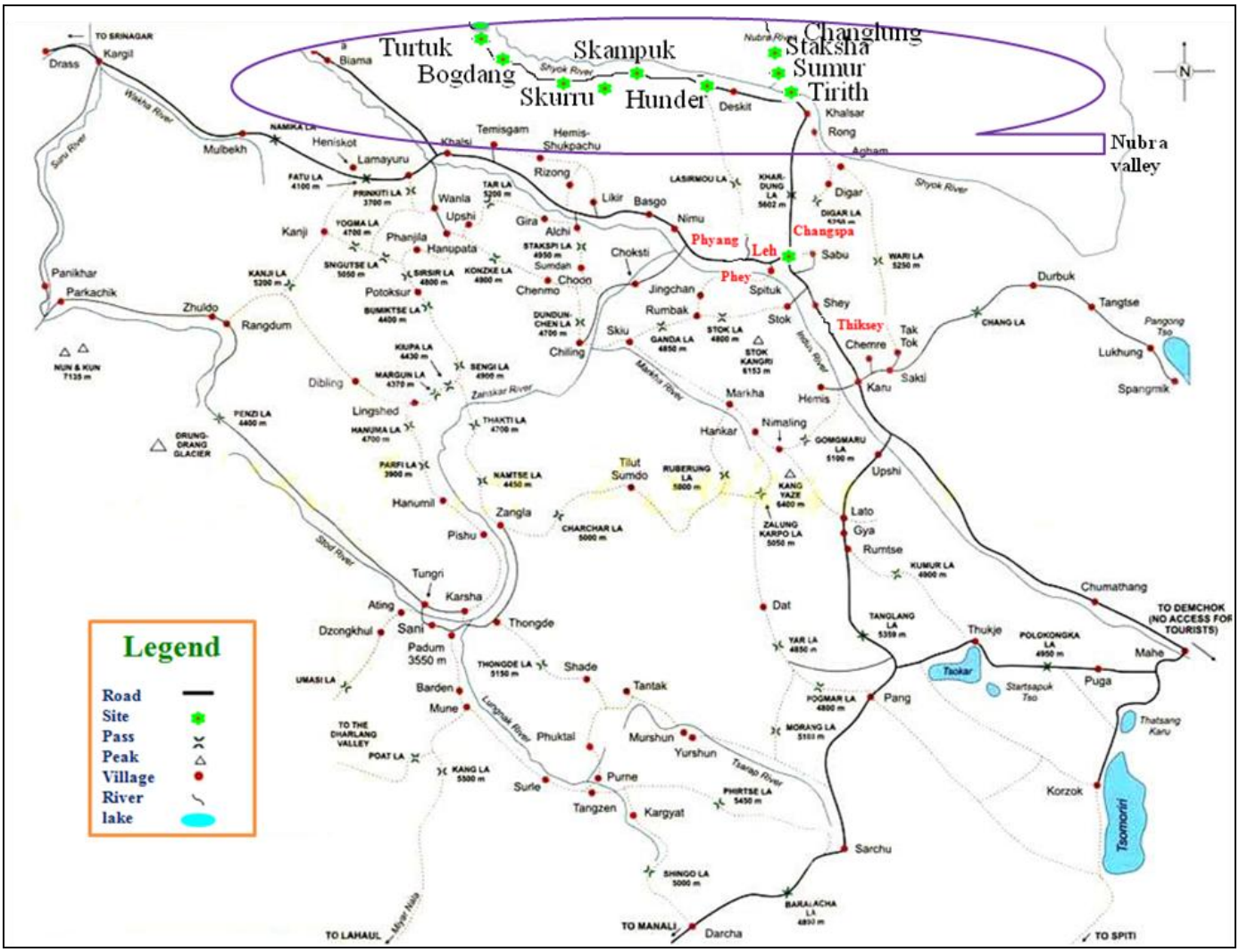

Figure 1. Map of Ladakh region of India.

assigned in a high priority for their preservation and protection. In this study, random amplified polymorphic DNA (RAPD) markers were used to detect variation at the population level in samples collected from Ladakh regions of India. The purpose of this study is to investigate the levels of variation among populations of *Dactylorhiza hatagirea*.

MATERIALS AND METHODS

Study sites and sampling

Nine populations from Ladakh regions were surveyed (Figure 1). Young leaf tissues of 96 individuals of leaves were selected randomly for molecular analysis. The number of samples taken in each population was depending on geographic distribution (Table 1). The sampling stratagem was to trace several sites in different parts of the investigated area in order to cover diverse growing habitats which represent the richly capable yet relatively stable primitive environment in the sampled regions.

RAPD analysis

Total genomic DNA was extracted from *Dactylorhiza* leaves using a cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987) with minor modifications. The quantity and quality of isolated total genomic DNA was determined using 0.8% agarose gel electrophoresis in 0.5 × TAE buffer for mobility related to known concentrations of lambda DNA. Twenty random decamer primers from integrated device technology (IDT) Tech. USA (Table 2) were used for RAPD amplification 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 $MgCl_2$, 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 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 to

Table 1. Geographic localities and sample sizes of naturally distributed *D. hatagirea.*

S/N	Population name	Population code	Longitude	Latitude	Altitude (ft)	Sample sizes
1	Bogdang	P	N34°48'.198	E77°02'.453	9240 ± 25.8	10
2	Skampuk	Q	N34°35'.238	E77°34'.481	10490 ± 17.4	15
3	Skurru	R	N34°40'.229	E77°18'.031	10295 ± 20.8	15
4	Hunder	S	N34°35'.043	E 77°28'.592	10357 ± 18.0	10
5	Turtuk	T	N34°50'.849	E76°49'.720	9240 ± 25.8	10
6	Tirith	U	N34°32'.378	E77°38'.481	10443 ± 26.9	10
7	Sumur	V	N34°31'.128	E77°34'.481	10120 ± 12.7	10
8	Changlung	W	N34°55'.884	E77°28'.276	10982 ± 39.7	10
9	Staksha	X	N34°55'.885	E77°28'.276	11081 ± 49.2	6

Table 2. List of primers used for RAPD amplification.

Primer	Primer sequence (5'→3')	Total number of fragments amplified	Percentage of polymorphic loci	Resolving power
S21	CAGGCCCTT C	597	100	12.44
S22	TGCCGAGCT G	545	100	11.35
S23	AGTCAGCCA C	341	100	7.10
S24	AATCAGCCA C	566	88.9	11.79
S25	AGGGGTCTT G	508	100	10.58
S26	GGTCCCTGA C	530	100	11.04
S27	GAAACGGGTG	423	100	8.81
S28	GTGACGTAG G	512	80	10.67
S29	GGGTAACGC C	362	100	7.54
S30	GTGATCGCA G	287	100	5.98
S31	CAATCGCCG T	485	100	10.10
S32	TCGGCGATA G	410	100	8.54
S33	CAGCACCCA C	558	100	11.63
S34	TCTGTGCTG G	455	100	9.48
S35	TTCCGAACC C	476	100	9.92
S36	AGCCAGCGA A	310	100	6.46
S37	GACCGCTTG T	459	100	9.56
S38	AGGTGACCG T	511	100	10.65
S39	CAAACGTCG G	380	100	7.91
S40	GTTGCGATC C	430	100	8.96
	Total	**9145**	**98.30**	**-**

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').

Data analysis

The resulting presence/absence binary data matrix was analyzed using POPGENE version 1.31 (Yeh et al., 1999). Jaccard's similarity coefficient (J) was used to calculate genetic similarity between pair of individuals. The similarity matrix was subjected to cluster analysis by unweighted pair group method with arithmetic mean (UPGMA) using the SAHN clustering module and dendrogram was generated using the program NTSYS-pc software ver. 2.02 (Rohlf, 1992). POPGENE software version 1.31 was used to describe the structure of studied populations with their geographic location. Observed number of alleles (Na), effective number of alleles (Ne), Nei's genetic diversity (H), Shannon's information index (I), number of polymorphic loci (NPL) and percentage polymorphic loci (PPL) across were analyzed (Zhao et al., 2006).

The non-parametric analysis of molecular variation (AMOVA) (Excoffier et al., 1992) was performed using squared Euclidean distances among all samples to partition the variation into two hierarchical levels; individual and population. 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). 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 96 genotypes

Table 3. Summary of genetic variation statistics for all loci of RAPD among the *Dactylorhiza* populations with respect to their distribution.

Markers	Sampling sites	Sample size	H (mean + SD)	I (mean + SD)	PPL
	Bogdang	10	0.1184 ± 0.1706	0.1820 ± 0.2528	36.72
	Skampuk	15	0.2346 ± 0.1647	0.3673 ± 0.2284	81.36
	Skurru	15	0.1633 ± 0.1869	0.2499 ± 0.2704	51.41
	Hunder	10	0.1925 ± 0.1791	0.2977 ± 0.2601	61.58
RAPD	Turtuk	10	0.2216 ± 0.1909	0.3629 ± 0.2716	68.36
	Tirith	10	0.2036 ± 0.1873	0.3109 ± 0.2689	62.15
	Sumur	10	0.2068 ± 0.1884	0.3151 ± 0.2700	62.71
	Changlung	10	0.1736 ± 0.1854	0.2666 ± 0.2679	54.80
	Staksha	6	0.1375 ± 0.1881	0.2055 ± 0.2749	37.29
Mean			**0.185767**	**0.284211**	**-**

H= Nei's genetic diversity, I=Shannon's information index, PPL= percentage of polymorphic loci.

Table 4. Inter-population genetic distances calculated by Nei's method.

Population	Staksha	Changlung	Sumur	Tirith	Turtuk	Hunder	Skurru	Skampuk	Bogdang
Staksha	****	0.103	0.079	0.089	0.160	0.102	0.174	0.150	0.224
Changlung	0.118	****	0.053	0.036	0.065	0.092	0.126	0.077	0.137
Sumur	0.095	0.068	****	0.044	0.081	0.058	0.086	0.061	0.108
Tirith	0.105	[a]**0.051**	0.060	****	0.062	0.079	0.101	0.066	0.112
Turtuk	0.177	0.081	0.098	0.079	****	0.090	0.118	[a]**0.051**	0.129
Hunder	0.119	0.107	0.076	0.096	0.108	****	0.084	0.067	0.135
Skurru	0.186	0.137	0.099	0.113	0.131	0.096	****	0.042	0.072
Skampuk	0.166	0.092	0.077	0.082	0.068	0.084	0.053	****	0.063
Bogdang	[a]**0.236**	0.148	0.121	0.124	0.142	0.148	0.080	0.075	****

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.

containing the band. Nei's analysis of gene diversity among population (Nei, 1978) was carried out with counting total genetic diversity (Ht), within species diversity (Hs), genetic diversity between populations (Gst) and estimation of gene flow (Nm) from parameters. Fst index (Wright, 1951) was measured via this formula (Lynch and Milligan, 1994).

RESULTS

A total of 177 reproducible bands were produced using the 20 RAPD primers (8.8 Bands per primer) of which 174 were polymorphic (PPL= 98.30%). 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 population and lowest values of Nei's genetic diversity (0.11), Shannon information index (0.18) and polymorphic loci (36.72 %) among accession from Bogdang population (Table 3). 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. Thus, the Bogdang accession varied in narrow range while, the Skampuk accessions were more diverse. Pair-wise Nei's distances (Nei's, 1973) were calculated for all populations. The greatest inter-population average distance (0.23) was between Bogdang and Staksha. While, the corresponding least distance (0.05) was between Skampuk and Turtuk (Table 4). The dendrogram was obtained from UPGMA cluster analysis based on RAPD data was presented in (Figure 2). The dendrogram showed four main clusters (I, II, III and IV). The cluster I consisted of samples from populations Bogdang, Skampuk and Skurru. Cluster II consisted of samples of population Tirith, Sumur and Changlung. Cluster III consisted of Hunder and some populations of Tirith, Sumur, Skampuk, Skurru and Turtuk. cluster IV and consisted of Staksha and some population of Skampuk, Skurru, and Turtuk.

Population genetic structure

Genetic analysis of RAPD marker showed that the highest genetic identity (0.964) existed between

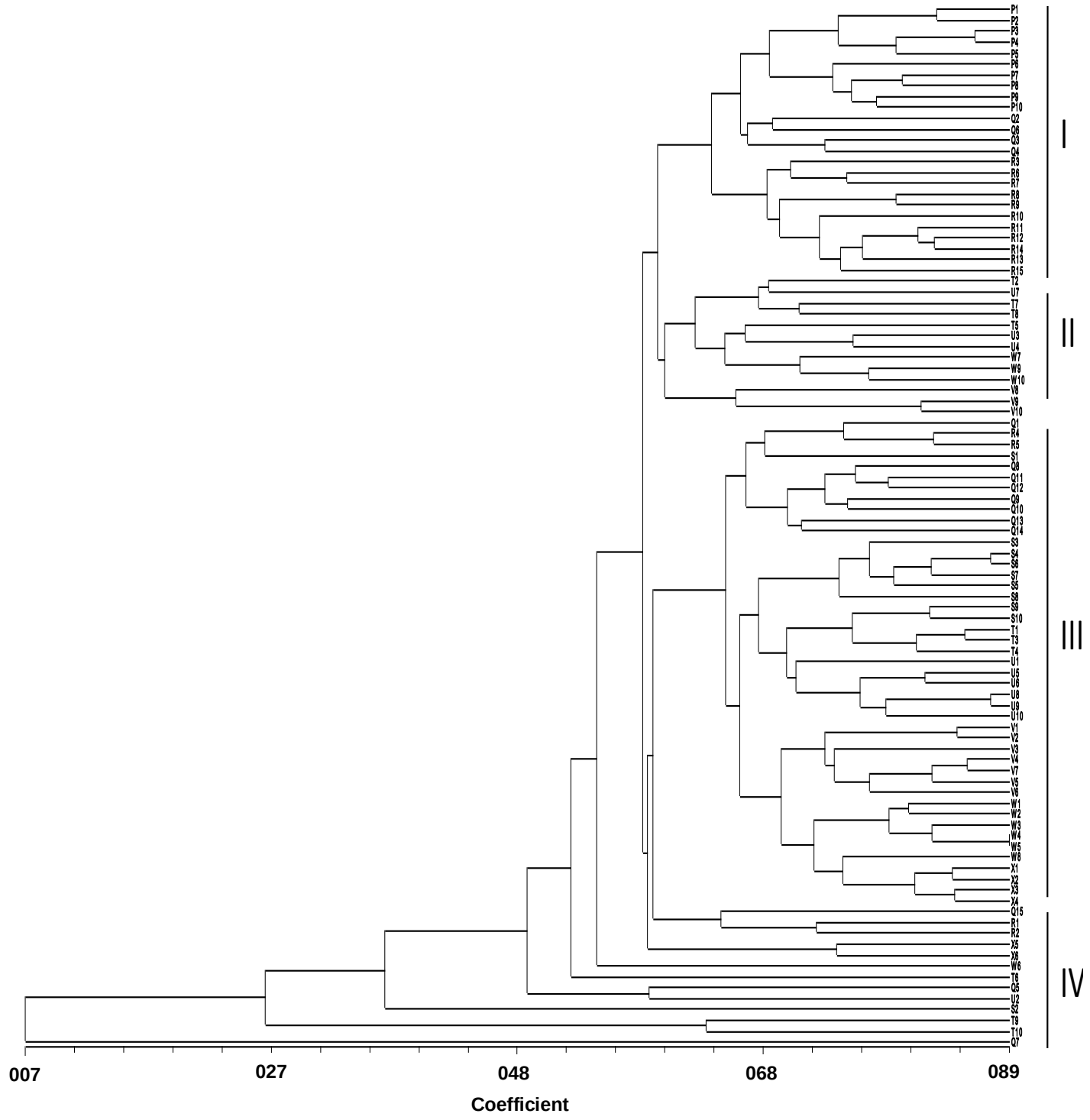

Figure 2. Dendrogram of 96 individuals of nine population of *D. hatagirea* based on UPGMA analysis of RAPD polymorphisms.

populations Changlung and Tirith. While, the lowest (0.790) occurred between the population of Bogdang and that of Staksha (Table 5). The Genetic differentiation among populations (Gst) was, estimated to be 0.2538, which indicated that 25.38% of the genetic variability was distributed among populations (Table 6). The level of gene flow (Nm) was estimated to be 1.4324 individual per generation between populations. According to results of AMOVA analysis, there were highly significant ($P < 0.001$) genetic differences within and among population of

Table 5. Inter-Population genetic identity calculated by Nei's method.

Population	Staksha	Changlung	Sumur	Tirith	Turtuk	Hunder	Skurru	Skampuk	Bogdang
Staksha	****	0.902	0.924	0.915	0.852	0.903	0.840	0.861	0.799
Changlung	0.889	****	0.948	[a]**0.964**	0.937	0.913	0.881	0.925	0.872
Sumur	0.909	0.934	****	0.957	0.923	0.943	0.917	0.941	0.897
Tirith	0.900	0.950	0.941	****	0.940	0.924	0.904	0.936	0.894
Turtuk	0.838	0.923	0.906	0.924	****	0.914	0.889	0.951	0.879
Hunder	0.888	0.899	0.927	0.909	0.898	****	0.920	0.935	0.874
Skurru	0.830	0.872	0.906	0.893	0.877	0.908	****	0.959	0.931
Skampuk	0.847	0.912	0.925	0.921	0.935	0.920	0.948	****	0.939
Bogdang	[a]**0.790**	0.862	0.886	0.883	0.867	0.863	0.923	0.928	****

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 6. Overall genetic variability across all the 96 accessions of *Dactylorhiza* based on RAPD analysis.

Marker	H	I	Ht	Hs	Gst	NPL	PPL	Fst	Nm
RAPD	0.2535 ± 0.1649	0.3984 ± 0.2106	0.2535 ± 0.027	0.1892 ± 0.015	0.2538	174	98.30	0.254	1.4324

H = Nei's genetic diversity, I = Shannon's information index, PPL= percentage of polymorphic loci, Ht = total genetic diversity, Hs = population diversity; Gst = gene differentiation, Fst = Wright inbreeding coefficient, Nm = gene flow.

Table 7. AMOVA analysis based on RAPD among the populations of *Dactylorhiza.*

Source of Variation	Among population	Within population	Total
d.f.	8	87	95
Marker		RAPD	
S.S.D.	46.917	34.000	80.917
Variance component	0.517	0.391	0.908
Percentage	57%	43%	100 %
P-value	< 0.001	< 0.001	< 0.001

Where d.f.= degree of freedom, S.S.D.= sum of square deviation, P-value = probability of null distribution.

D. hatagirea. Of the total genetic diversity, 57% was attributed to be among populations while the rest 43% was attributed to the differences within populations (Table 7). The positioning of 96 samples based on principal coordinates (PCO) analysis is shown in Figure 3. The scatter plot showed mixed population of *D. hatagirea*, which was consisted with the results of dendrogram analysis.

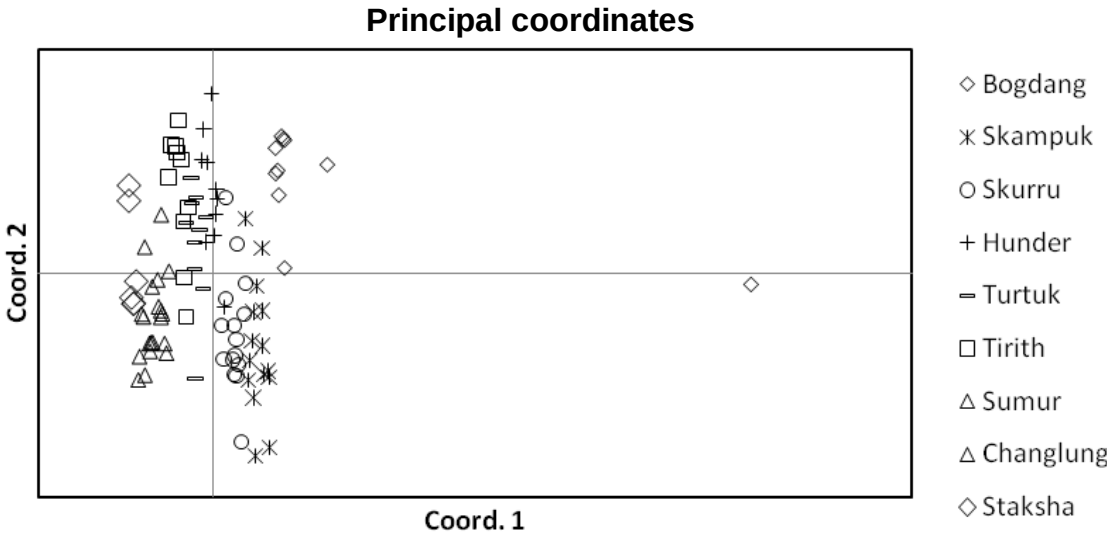

Figure 3. Two dimensional plot of Principal component analysis of nine population of *Dactylorhiza* using RAPD analysis.

DISCUSSION

Genetic diversity

According to Hamrick and Godt (1989), there is strong relationship between geographic range and genetic diversity. There is evidence that a low level of genetic diversity is a common feature of endangered endemic plant species (Gitzendanner and Soltis, 2000). *D. hatagirea* is a critically endangered, self compatible and terrestrial orchid. Being terrestrial orchid, populations are small and spatially isolated. According to population genetics predict that population of such species, affected by a number of evolutionary factors including mating system, gene flow, founder effect, random genetic drift and seed dispersal (Hamrick and Godt, 1989). We compared *D. hatagirea* and this hypothesis with related genus *Gymnadenia* which is also an endangered and endemic species (Mi Yoon Chung, 2009) and our result of RAPD analysis shows that low level of genetic diversity within populations and remarkable genetic differentiation among populations in *D. hatagirea*. In our field survey, every year large amount of population of *D. hatagirea* was destroyed because of increasing mating opportunity and habitat fragmentation. Thus, habitat fragmentation under human disturbance is an important factor leading to low genetic diversity within population of *D. hatagirea*. So the low rate of natural recruitment observed today, together with increased habitat fragmentation and isolation of population, is seriously contributing to the low level of genetic diversity.

Genetic structure

The overall degree of genetic differentiation of *D. hatagirea*, as estimated by Gst (0.2587) is slightly higher than out crossing plants (0.23). According to an AMOVA analysis, a significantly genetically differentiation among populations was estimated (Fst = 0.259), where most genetic variation existed among populations. The genetic structure of plant populations reflects the interaction of various factors, including long–term evolutionary history of the species, genetic drift, mating system and gene flow (Hogbin and Peakall, 1999). If populations are small and isolated from one another, the genetic drift could be capable of influencing the genetic structure and increasing differentiation among populations (Ellstrand and Elam, 1993). The gene flow (Nm, 1.4703) of *D. hatagirea* per generation was also slightly higher than outcrossed animal pollinated species (Nm = 1.154) could be due two geographic barrier (Nubra and Shyok river) and different environmental conditions.

In this study, we investigated variability among populations. Based on RAPD analysis, we revealed low genetic variation within the population and moderate genetic differentiation among the population. Therefore, every population deserves specific conservation attention, as habitat destruction and management for conservation will have greater effects on population richness.

REFERENCES

Avise JC, Hamrick JL (1996). Conservation genetics: Case histories from nature. New York: Chapman and Hall.

Chaurasia OP, Ahmed Z, Ballabh B (2007). In: Ethnobotany and Plants of Trans-Himalaya. Satish Serial Publishing House Delhi, p. 544.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull., 19: 11-15.

Ellstrand NC, Elam DR (1993). Population genetic consequences of small population size: implications for plant conservation. Ann. Rev. Ecol. Syst., 24: 217e-242.

Excoffier L, Smouse PE, Quattro JM (1992). Analyses of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics, 131: 479-491.

Gitzendanner MA, Soltis PS (2000). Patterns of genetic variation in rare and endangered widespread plant congeners. Am. J. Bot., 87: 783-792.

Hamrick JL, Godt MJW (1989). Allozyme diversity in plants. In: Brown, A.H.D., Clegg, M.T., Kahler, A.L., Weir, B.S. (Eds.), Plant Population.

Hogbin PM, Peakall R (1999). Evaluation of the contribution of genetic research to the management of the endangered plant *Zieria prostrata*. Con. Biol., 13: 514-522.

Holsinger KE, Gottlieb LD (1991). Conservation of rare and endangered plants: principles and prospects. In: Falk DA, Holsinger RKE eds. Genetic and conservation of rare plants. New York: Oxford University Press, pp. 195-208.

Lynch M, Milligan BG (1994). Analysis of population genetic structure within RAPD markers. Mol. Ecol., 3: 91-99.

Mi YC (2009). Low level of genetic variation within populations of the four rare orchids *Gymnadenia cucullata, Gymnadenia camptschatica, Amitostigma gracile, and Pogonia minor* in South Korea: indication of genetic drift and implications for conservation. Plant Syst. Evol., 281: 65-76.

Milligan BG, Leebens-Mack J, Strand AE (1994). Conservation genetics: beyond the maintenance of marker diversity. Mol. Ecol., 12: 844–855.

Nei M (1973). Analysis of gene diversity in subdivided populations. PNAS, 70: 3321-3323.

Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individual. Genetis, 89: 583-590.

Peakall R, Smouse PE (2001). GenAlEx V5 Genetic Analysis in Excel. Population genetic software for teaching and research. Australian National University, Canberra, Australia, (http://www.anu.edu.au/BoZo/ GenAlEx/).

Prevost A, Wilkinson MJ (1999). A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theor. Appl. Genet., 98: 107-112.

Rohlf FJ (1992). NTSYS-PC: Numerical taxonomy and multivariate analysis system version 2.02. State University of New York (Stony Brook, New York).

Williams JGK, Kubelik AR, Livak KL, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res., 18: 6531-6535.

Wright S (1951). The genetical structure of populations. Ann. Eugen., 15: 323-354.

Yeh FC, Yang RC, Boyle T (1999). POPGENE. Microsoft Windows-based freeware for population genetic analysis. Release 1.31. Edmonton: University of Alberta.

Zhao WG, Zhang JQ, Wangi YH, Chen TT, Yin Y, Huang YP, Pan Y, Yang Y (2006). Analysis of genetic diversity in wild populations of mulberry from western part of Northeast China determined by ISSR markers. J. Genet. Mol. Biol., 7: 196-203.