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Assessment of genetic diversity among *Podophyllum hexandrum* genotypes of the North-western Himalayan region for podophyllotoxin production

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Podophyllum hexandrum (Indian Mayapple) is an important medicinal plant valued all over the world. Genetic diversity among the 28 genotypes of *P. hexandrum* distributed in 11 geographical regions from Himachal Pradesh (a part of the North-western Himalaya) was analyzed using RAPD markers. The genetic diversity was high among the genotypes as measured by percentage of polymorphic bands (PPB = 92.37%) and Shanon information index (I = 0.50). The mean coefficient of gene differentiation (Gst) was 0.69, indicating that 33.77% of the genetic diversity resided within the genotypes. Analysis of molecular variance indicated that the source of variation among the groups was 53% and among the genotypes of groups was 47%. An overall value of mean estimated number of gene flow (Nm = 0.22) indicated that there was limited gene flow among the genotypes. The existence of variation among the 28 genotypes as observed through podophyllotoxin content proved to be coupled with geographical altitude (r = 0.92) and local ecological conditions (temperature, rainfall, humidity, soil *p*H, etc.) but not on genetic basis (r = -0.55). Based on the observed genetic variations among the genotypes of *Podophyllum*, we recommend for their *in situ* conservation and germplasm collection expeditions in future conservation plans.

Keywords*: Podophyllum hexandrum,* podophyllotoxin, genetic diversity, RAPD

Introduction

The quality and quantity of medicinal plants are serious issues for the pharmaceutical and dietary supplement industries. Traditionally this material has been harvested from the wild. Increasing public demand for these products is creating a serious environmental problem as demand is outpacing the supply and endangering the survival of many of these species in the world including *Podophyllum hexandrum*.

The Himalayan region is the home of numerous highly valued medicinal plants including *P. hexandrum* Royle (Berberidaceae) which is a herbaceous, rhizomatous species of great medicinal importance, now endangered in India. It has long been used by the Himalayan natives and the American Indians[1]. The rhizome of several *Podophyllum* species has been found to be the source of podophyllotoxin lignan, which has an important biological activity of blocking mitosis[2] and it is used as the precursor of semi synthetic chemotherapeutic drugs such as etoposide, teniposide and etophos[3,4]. *P. hexandrum* of Indian origin contains three times more podophyllotoxin than its American counterpart, *P. peltatum*[5]. The rhizomes are being indiscriminately harvested in large quantities from the wild to meet the ever increasing demand for the crude drug. As a result of this and lack of organized cultivation, *P. hexandrum* has been reported as a threatened species from the Himalayan region. The population size of *P. hexandrum* is very low (40-700 plants per location) and is declining every year. Some of the populations in certain pockets have virtually disappeared owing to anthropogenic activities and overexploitation[6,7]. It is distributed in restricted pockets throughout the alpine Himalayan region[8]. In the natural habitat, seed germination and seedling establishment are very poor and propagation is mostly through rhizomes. Since the species is already endangered and exploitation of its underground parts continues to exceed the rate of natural regeneration, it needs immediate attention for conservation. Population biology and genetic diversity studies are important for successful development of conservation strategies.

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Considerable variations in morphological characters such as plant height, leaf characteristics, fruit weight, seed weight and colour have been reported in *P. hexandrum* from the interior Himalayas[6-9]. At least four distinct morphological variants, with 1, 2, 3 and 4 leaves have been reported[10]. Podophyllotoxin content varies greatly between the populations of *P. hexandrum* from the northwestern Himalayas[11]. Therefore, it would be inefficient, environmentally destructive and economically unsound to randomly harvest the Indian Mayapple. For the rapid and economic development of *P. hexandrum* as an alternative crop, it would be essential to evaluate the natural populations of *P. hexandrum* for podophyllotoxin content and agronomic traits. For evaluation purposes, determination of relationship between and within populations cannot be made by just chemotyping the populations as the production of podophyllotoxin can be influenced by environmental factors like altitude, temperature, rainfall, relative humidity and soil *p*H[12.] Polypeptide profiles and esterase isozyme analyses have indicated the existence of high inter- and intra-population variations in *P. hexandrum*[6]. However, the protein markers are influenced by the stages of plant growth as well as environmental factors and hence may give erroneous results. DNA markers such as RFLP[13] and RAPD[14,15], on the other hand, are quite stable and highly polymorphic in nature. RFLP markers are less polymorphic, more expensive and laborious, compared with RAPD. Therefore, genetic diversity in *P. hexandrum* has been studied from specimens collected from two forest divisions of Himachal Pradesh (Chamba and Kullu) using RAPD markers[16]. However, for the successful establishment of *P. hexandrum* as an alternative crop for the production of podophyllotoxin, its genetic diversity needs to be understood for better identification and commercial level propagation of useful secondary compounds from the Himalayan region.

The aim of the present study was to analyse of genetic diversity and genotype structure as well as their relationship with podophyllotoxin content in the diminishing wild populations of *P. hexandrum* in the northwestern Himalayas, and to ensure appropriate methods of conservation management for the remaining populations with great genetic variations.

Materials and Methods

Plant Material

Twenty-eight accessions of *P. hexandrum* were collected for RAPD studies and podophyllotoxin estimation from 28 sites covering 11 forest divisions in the interior range of the northwestern Himalayan region, Himachal Pradesh, India. The genotypes exhibited variation in the number of leaves per plant and leaf shape, etc. These results support the findings of morphological variants on the basis of the presence of 1, 2, 3 and 4 leaves from the Garhwal region of western Himalayas[6]. Morphological variations in 280 plants were recorded in the present study (Table 1). The metrological data for all the sites have been represented in Table 2. One representative plant from each site (considered as genotype) was used for molecular characterization. Young leaves were frozen in liquid nitrogen and stored at -80°C prior to DNA isolation. The roots were trimmed and washed with running tap water to remove dirt/soil followed by washing with double distilled water. The washed roots were then dried separately at 60°C for 24 h in an oven and used for podophyllotoxin estimations.

Isolation of DNA

Total genomic DNA was extracted from frozen leaves by the CTAB method[17]. Samples of 500 mg were ground to powder using a mortar and pestle. The powder was transferred to a 30 mL sterile Falcon tube with 12.5 mL of CTAB buffer. The extraction buffer consisted of 2% (w/v) CTAB (Cetyl trimethyl ammonium bromide, Sigma), 1.4 *M* NaCl, 20 m*M* EDTA, 100 m*M* Tris-HCl, *p*H 9.5 and 0.2% (V/V) β-mercaptoethanol. After incubating the homogenate for 1 h at 65°C an equal volume of chloroform was added and centrifuged at 10,000 rpm for 20 min. DNA was precipitated with 1/10 volume (mL) of 3 *M* sodium acetate and an equal volume of isopropanol followed by centrifugation at 10,000 rpm for 10 min. RNA was removed by RNase treatment. DNA was quantified by comparing with uncut λ DNA on the agarose gel diluted to 12.5 ng.μ L^{-1} and used in PCR.

RAPD Analysis

Out of the 20, 19 random decamer primers (Operon Technologies, USA) of A, B, C and D series were used individually as primers for RAPD analysis. DNA was amplified following the protocol of Kazan *et al*[15] All the 19 primers were used previously in the study of genetic diversity among the 16 promising cultivars of ginger[18]. Amplification reactions were performed

Table 1 — 28 genotypes of *Podophyllum hexandrum* collected from different forest divisions, their podophyllotoxin content and polymorphic features

Name of forest division	Sampling site	No. of leaves	Age (yr)	Altitude (m)	Podophyllotoxin content (% dry wt) (mean±S.E.)	Total no. of bands	No. of polymorphic bands	% of polymorphic bands (PPB)
Parvati	R/4,Kasol (C-II-a-Nry)	1-2	1	1570	3.57 ± 0.75	56	46	82.14
	Twin Multivora	3-4	3	1300	4.75 ± 0.80	57	47	82.45
	Anganoala (R/9) Rajgiri	1-3	2	1300	3.02 ± 0.52	56	46	82.14
Kullu	Brundhar	1-3	2	1916	4.08 ± 0.27	79	69	87.34
	Gulaba	1-3	2	2895	5.94 ± 0.59	77	66	85.71
	ChanderKhani	1-3	2	3352	8.03 ± 0.45	83	73	87.95
	Kaned Nry	1-2	1	2150	4.66 ± 0.85	80	70	87.50
	Sanghar Nry	1-3	2	2100	4.17 ± 0.28	80	70	87.50
Dodrakwar	Madhvi Thach	1-2	1	3048	6.21 ± 0.74	90	80	88.88
	Kala Pani	2-3	2	2743	5.80 ± 0.21	85	75	88.23
Seraj	Jalora Pass (Sojha Nry)	2-3	2	2667	6.61 ± 0.35	97	87	89.69
	Jalora c-30(b)	2-3	2	2473	6.79 ± 0.86	90	80	95.55
Churah	DPF(D-1892-C1)Choundi)	1-3	2	3750	8.49 ± 0.57	67	57	85.07
	DPF (D-791-C1)	3-1	2	2700	5.75 ± 0.41	68	58	85.29
Lahaul	Myar Valley	2-3	2	4300	9.53 ± 0.48	96	86	89.58
	Nayan ghar	2-4	3	4300	8.86 ± 0.43	88	78	88.63
Palampur	Bada Bangal	1-3	2	2895	7.10 ± 0.80	90	80	88.88
	Chota Bangal	2-3	2	2700	6.57 ± 0.83	83	73	87.95
	IHBT	2-3	2	2800	5.18 ± 0.78	81	71	87.65
Rampur	Bander Thach	1-2	1	2895	6.77 ± 0.64	88	78	88.63
	Saropa Nry	2-3	2	2499	6.10 ± 0.94	83	73	87.45
Kinnaur	Nichar Nry	1-3	2	2190	4.76 ± 0.29	91	81	89.01
	Rango (N-C-8)	1-3	2	2710	5.80 ± 0.55	83	73	87.95
Pangi	Sach Range	3-4	3	2712	6.13 ± 0.22	80	71	87.65
	Killer Range	2-4	3	2850	5.97 ± 0.69	78	68	87.17
	Purthi Range	2-3	2	2900	6.23 ± 0.79	83	73	87.95
Bharmaur	Ghoei DPF	1-3	2	2080	5.70 ± 0.69	83	73	87.95
	Samara RF	2-4	3	2590	6.03 ± 0.83	85	75	88.23
			Total			2257	1977	

in volumes of 25 µL containing 10 m*M* Tris HCl, *p*H 9.0, 1.5 m*M* $MgCl_2$, 50 m*M* Tris HCl, *p*H 9.0, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 200 µ*M* of each dNTPs, 0.4 µ*M* primer, 25 ng template DNA and 0.5 unit of Taq polymerase (Sigma). DNA amplification was performed using a Gene Cycler (BioRAD, USA). The first cycle consisted of denaturation of template DNA at 94°C for 5 min, primer annealing at 37°C for 1 min and primer extension at 72°C for 2 min. In the next 42 cycles, the period of denaturation was reduced to 1 min at 92°C while the primer annealing and primer extension time remained the same as in the first cycle. The last cycle consisted of only primer extension (72°C) for 7 min.

The amplification products were resolved in 1.5% agarose gel (1x Tris-acetate-buffer) containing 40 m*M* Tris base, 20 m*M* sodium acetate, 20 m*M* EDTA and glacial acetic acid to *p*H 7.2 followed by ethidium bromide staining and visualization in UV light for photography, which have been performed three times and were reproducible[19].

Data Collection and Analysis

The relationship among the genomic DNA was assessed by comparing RAPD fragments separated according to their size and the presence/absence of shared fragments. The banding patterns obtained from RAPD were scored as present (1) or absent (0). Pairwise distance matrix was calculated using the Jaccard similarity coefficient[20]. The similarity values were used to generate a dendrogram via the unweighted pair group method with arithmetic average (UPGMA). POPGENE32 software was used to calculate Nei's unbiased genetic distance between the different populations using all RAPD markers inclusive of monomorphic markers. Nei's unbiased genetic distance is an accurate estimate of the number

Table 2—19 primers used to amplify DNA samples collected from 28 genotypes of *Podophyllum hexandrum* with number of bands generated by each primer

Primer	Nucleotide sequence	G+C content (%)	Total no. of bands	Polymorphic locii	Monomorphic loci	Percentage of polymorphic loci (PPB)	Total no. of bands amplified	Resolving power
OPA01	5'CAGGCCCTTC3'	70	3	3	0	100	52	3.71
OPA02	5'TGCCGACCTG3'	70	10	10	0	100	200	14.29
OPA04	5'AATCGGGCTG3'	60	6	5	1	83.3	112	8.0
OPA08	5'GTGACGTAGG3'	60	7	7	0	100	112	8.0
OPA11	5'CAATCGCCGT3'	60	7	7	0	100	132	9.43
OPA13	5'CAGCACCCAC3'	70	3	3	0	100	34	2.43
OPA18	5'AGGTGACCTG3'	60	11	9	2	90	232	16.57
OPB11	5'GTAGACCCGT3'	60	9	7	2	77.8	148	10.57
OPB15	5'GGAGGGTGTT3'	60	6	3	3	50	138	9.86
OPB18	5'CCACAGCAGT3'	60	9	9	0	100	148	10.57
OPB19	5'ACCCCCGAAG3'	70	10	10	0	100	134	9.57
OPC08	5'TGGACCGGTG3'	70	5	5	0	100	97	6.93
OPC12	5'TGTCATCCCC3'	60	6	6	0	100	83	5.93
OPC15	5'GACGGATCAG3'	60	4	4	0	100	28	2.0
OPC16	5'CACACTCCAG3'	60	7	7	0	100	88	6.29
OPD05	5'TGAGCGGACA3'	60	4	3	1	75	71	5.07
OPD08	5'GTGTGCCCCA3'	70	8	7	1	87.5	132	9.43
OPD11	5'AGCGCCATTG3'	60	7	7	0	100	123	8.79
OPD13	5'GGGGTGACGA3'	70	9	9	0	100	193	13.79
Total			131	121	10		2257	

of gene differences per locus when populations are small. Population diversity (Hs) and total gene diversity (Ht)[21] were calculated within 28 genotypes and within 11 major groups (as per their collection site) by POGENE software. Genetic diversity within and among populations was measured by the percentage of polymorphic bands (PPB). Estimate of gene flow (Nm) was calculated by the gene differentiation (Gst) using [0.5(1-Gst)/Gst]. In order to describe genetic variability among the genotypes, the non-parametric analysis of molecular variance (AMOVA)[22] program version 1.5 was used where the variation component was partitioned among the genotypes, among the genotypes within regions andamong regions. The input files for AMOVA were prepared by using AMOVA-PREP version-1.01[23].

Resolving Power

The resolving power (Rp)[24] of a primer was calculated using Rp = $\sum$ *IB*, where *IB* (band informativeness) takes the value of: 1-[2x (0.5 – *P*)], *P* being the proportion of the 28 genotypes of *P. hexandrum* analyzed) that contains the band. The resolving power (Rp) of the 19 RAPD primers ranged from 2.0 for primer OPC15 to 16.571 for OPA18. Three RAPD primers (OPA02, OPA18, OPD13) possess the highest Rp values (14.286, 16.571, 13.786, respectively) and each one can be able to distinguish all 28 *Podophyllum* cultivars.

Extraction and Determination of Podophyllotoxin

Dried roots were ground to powder in a pestle and mortar. Podophyllotoxin was extracted following the procedure of Broomhead *et al*[25]. HPLC analysis was carried out using a Nova Pack C18 cartridge column (250 ×4.6 mm) in Water's HPLC system. Acetonitrile :water:methanol (37:58:5) was used as a mobile phase with a flow rate of 1.0 mL min-1. Crude extract (20 µL) was used for injection into the HPLC system. Podophyllotoxin was detected at 230 nm (490 E multi wavelength Detector, Waters). Podophyllotoxin (0.1 g.L-1; Sigma, P-4405) was used as a standard for calculating podophyllotoxin content in the samples on the basis of peak heights. All the experiments on extraction of podophyllotoxin and HPLC analyses were repeated three times.

Results and Discussion

Morphological Markers

P. hexandrum genotypes collected from different forest divisions of Himachal Pradesh exhibited variation in number of leaves per plant, leaf shape, etc. The number of leaves observed were 1, 2, 3 and 4 and varied among the plant samples of different age

groups (Table 1). The results supported the findings of morphological variants on the basis of the presence of 1, 2, 3 and 4 leaves per plant from the Garhwal western Himalayas[6]. The morphological variations in 280 plants from 28 sites covering 11 forest divisions were recorded. In general, 39.5% plants had single leaves, 30% had two leaves, 20% had three leaves and 10.5% had four leaves. The single-leaf plants did not bear fruit.

Molecular Analysis using RAPD Markers

The RAPD technique had been successfully used in a variety of taxonomic and genetic diversity studies[26] and it was found suitable for use with *Podophyllum* genotypes because of its ability to generate reproducible polymorphic markers. A total of 28 plant samples were fingerprinted using 19 RAPD makers. A total of 131 (an average of 6.89 bands per primer) RAPD loci were scored out of which 121 (92.37%) were polymorphic and only 10 (7.63%) were monomorphic (Table 2). The number of amplification fragments produced per primer as well as their size ranged from 250 bp to 2,100 bp which is analytically appropriate and in conformity with those recorded with certain other plants examined analogously[27]. The observed high proportion of polymorphic loci suggests that there is a high degree of genetic variation in the *Podophyllum* genotypes. Three to ten types of amplification fragments (monomorphic + polymorphic) were produced by each primer in different genotypes. The resolving power of the 19 RAPD primers ranged from 2.0 for primer OPC15 to a maximum of 16.57 for primer OPA18. In addition to its high resolving power, RAPD primer OPA18 has the ability to distinguish all 28 *Podophyllum* genotypes.

Podophyllotoxin Content

Podophyllotoxin content was extracted and analyzed in triplicate from 28 genotypes of *P. hexandrum* distributed into 11 forest divisions at different altitudes. It was found that the podophyllotoxin content in the roots of the plants obtained from the Lahaul forest division (altitude 4300 m) was high (8.86 to 9.53% on dry wt basis) compared to the root samples collected from other forest divisions. The lowest values obtained were samples from Parvati forest division (altitude 1300 m) 3.02 to 4.75% on dry wt basis (Table 1). For genotypes in the same as well as other forest divisions, the podophyllotoxin content increased with the increase in altitude (Table 1 and Fig. 1). Podophyllotoxin content was correlated with altitude (Fig. 1a) and environmental variables like altitude, temperature, rainfall, relative humidity and soil *p*H12, but not with genetic diversity (Fig. 1b). The results have been supported by the study done by Sharma *et al*[11,16]. They reported considerable reduction in the podophyllotoxin content in the roots of plants collected from higher altitudes after growing them at lower altitudes.

Phylogenetic Analysis

Based on RAPD markers, the similarity index values ranged from 0.61 to 0.96 (Figure not shown). These values were used to construct a dendrogram using UPGMA. Genotypes from 11 forest divisions were clustered into region-specific groups with exception of Kullu forest division (Fig. 2). All the 28 genotypess were distributed into 12 main clusters (Ia-Im). Cluster Ia represented Parvati forest division with 3 different genotypes namely R/4 Kasol, TwinMultivora and Anganola. Genotypes from Kullu forest division were distributed among two clusters-Ib & Ic. The cluster Ib has genotypes from Brundhar, Gulaba and Sanghar nursery. The cluster Ic has genotypes from Kaned nursery and Chanderkhani. The cluster Id (Dodrakwar forest division) has 2 genotypes-MadhviThach and Kalapani. Similarly cluster Ie and cluster If comprise 2 genotypes each from forest division Seraj (Sojha nursery, Jellora Pass) and Lahaul (Nayanghar, Myarvelley), respectively. The Churah division (cluster Ig) has 2 genotypes-DPF-D-1892-C1 and DPF-D-791-C1. The cluster Ih (Palampur forest division) has 3 genotypes-IHBT, Bada Bangal and Chota Bangal. The cluster Ii (Rampur division) has 2 genotypes-BanderThach and Saropa nursery. The cluster Ij representing Kinnur forest division has 2 genotypes-Nichar nursery and Rango-NC-8. The cluster Im (Pangi forest division) has 3 genotypes-Sach Range, Killer Range and Purthi Range. The cluster Ik (Bharmaur forest division) has 2 genotypes-Ghoei DPF and Samara RF. The results indicate high genetic diversity among the *P. hexandrum* genotypes from Himachal Pradesh.

Genetic Diversity Analysis

A relatively high genetic variation was detected among the *Podophyllum* genotypes. The minimum value of genetic similarity is 0.47 (genetic distance 0.75) between Sach Range and Rajgiri Range and the maximum value of genetic similarity is 0.96 (genetic

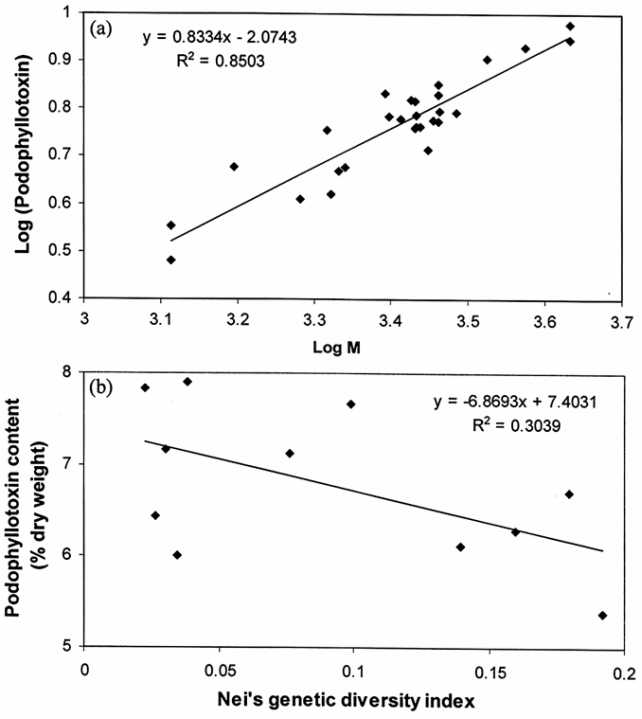

Fig. 1—Regression analysis based on (a) Log_{10} Podophyllotoxin content and Log_{10} M (altitude) between 28 populations of *P. hexandrum* and (b) Podophyllotoxin content and Nei's genetic diversity index of the *P. hexandrum* populations among 11 regions suggests that altitude affect podophyllotoxin content but not the genetics.

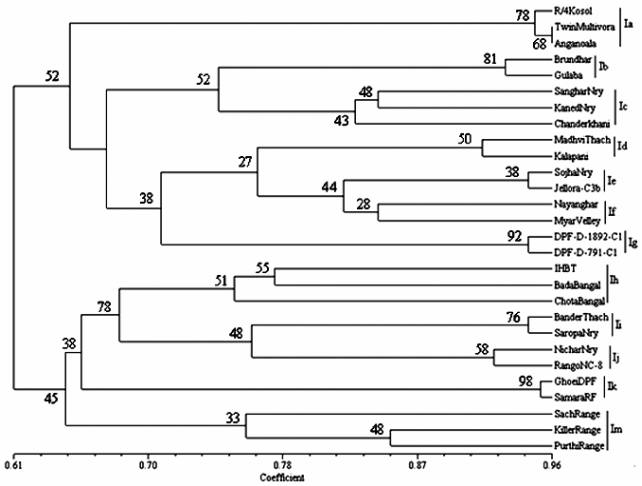

Fig. 2—Dendrogram illustrating genetic relationships among 28 populations generated by UPGMA cluster analysis calculated from 2257 RAPD bands produced by 19 primers. The values in the node indicate the bootstrap values after 1000 permutations.

distance 0.04) between R/4 Kasol and Rajgiri. Wide genetic diversity between genotypes was evident from the high number of polymorphic markers. The minimum number of polymorphic markers varied between 46 (in R/4 Kasol and Rajgiri) and a maximum of 87 (in Nayanghar) (Table 1). The total number of polymorphic loci was 121, thereby, giving an estimate of profound (>92.37%) polymorphism. Polymorphisms also varied substantially within the discrete groups of plants and the minimum was found to be 20% (Kullu) whereas the maximum was 50% (Dodrakwar, Churah, Lahaul, Rampur, Kinnaur and Bharmaur).

AMOVA analysis enabled a partitioning of the overall RAPD variations among groups (with respect to their regions) and among genotypes within groups (Table 3). More than half of the total variations (53 & 47%,) in the studied genotypes could be accounted for by difference among the forest divisions and between genotypes within a forest division, respectively. All the components of molecular variations were significant ($P < 0.001$). The gene diversity computed among different groups of genotypes was recorded in between 0.01-0.19. The effective number of alleles was the minimum for Bharmaur (1.05) and the maximum for Seraj (1.36) across the genotypes collected from different forest divisions. The same order of genetic heterogeneity was discerned through Shannon's information index, which varied from 0.03 (Bharmaur) to 0.28 (Kullu). Based on all genotype basis, the observed number of alleles was 1.92 and the effective number of alleles was found to be 1.58 per locus. Similarly, the total gene diversity (Ht) among the genotypes was 0.34 and within genotypes (Hs) was 0.10. Shannon's information index was 0.50 and estimated gene flow was found to be 0.22 among the 28 *P. hexandrum* genotypes (Tables 4 & 5).

The present study and similar studies on ginger[18], lotus[28], sweet potato[29] and *Andrographic paniculata*[30] suggest that RAPD is more appropriate for analysis of genetic variability in loosely related genotypes. Moreover, the RAPD markers used here were able to differentiate *P. hexandrum* genotypes collected from 11 forest divisions into 12 distinct region specific clusters. The study also indicates that *P. hexandrum* populations in the northwestern Himalayan region are genetically highly diverse. The high genetic variations in *P. hexandrum* may be attributed partially to the cross pollinated nature of *P. hexandrum*. AMOVA revealed that there was significant variation arising from habitat-correlated genetic difference (47%). It suggests that the effects of gene flow, genetic drift and local ecological conditions (altitude, temperature, rainfall, humidity, soil, *p*H, etc.) also play an

Table 3—Summary of analysis of molecular variance (AMOVA) based on RAPD genotypes of *Podophyllum hexandrum*

Source of variance	d.f	S.S.D.	Variance component	Percentage	P-value
Among forest division	10	429.43	12.64	53	< 0.001
Among populations within forest division	17	189.97	11.18	47	< 0.001
Total	27	619.39	23.81		

Levels of significance are based on 1000 iteration steps, d.f.degree of freedom; S.S.D.: sum of square deviation; P-value: probability of null distribution

Table 4—Summary of genetic variation statistics for all loci of RAPD among the *Podophyllum hexandrum* genotypes with respect to their distributions among eleven forest divisions

Genotypes sampled from	Sample size	Observed no. of alleles (mean±SD)	Effective no. of alleles (mean±SD)	Nei's gene diversity (mean±SD)	Shannon's information index (mean±SD)	Ht (mean±SD)	Average no. of polymorphic bands	Percentage of polymorphic bands (PPB)
Parvati	3	1.07 (0.25)	1.06 (0.20)	0.03 (0.11)	0.04 (0.16)	0.03 (0.01)	46.33	33.33
Kullu	5	1.46 (0.50)	1.34 (0.40)	0.19 (0.22)	0.28 (0.31)	0.19 (0.05)	71.80	20.00
Dodrakwar	2	1.07 (0.25)	1.07 (0.25)	0.03 (0.13)	0.05 (0.18)	0.03 (0.02)	67.50	50.00
Seraj	2	1.36 (0.48)	1.36 (0.48)	0.18 (0.24)	0.25 (0.33)	0.18 (0.06)	76.50	49.67
Churah	2	1.15 (0.36)	1.15 (0.36)	0.08 (0.18)	0.11 (0.25)	0.08 (0.03)	70.00	50.00
Lahual	2	1.20 (0.40)	1.20 (0.40)	0.10 (0.20)	0.14 (0.28)	0.102 (0.04)	81.00	50.00
Palampur	3	1.36 (0.48)	1.29 (0.39)	0.16 (0.21)	0.23 (0.31)	0.16 (0.05)	74.66	33.33
Rampur	2	1.05 (0.23)	1.05 (0.23)	0.03 (0.11)	0.04 (0.16)	0.03 (0.01)	75.50	50.00
Kinnaur	2	1.08 (0.27)	1.08 (0.27)	0.04 (0.13)	0.05 (0.18)	0.04 (0.02)	77.00	50.00
Pangi	3	1.31 (0.47)	1.25 (0.37)	0.14 (0.21)	0.20 (0.30)	0.14 (0.04)	70.66	33.33
Bharmaur	2	1.05 (0.21)	1.05 (0.21)	0.02 (0.11)	0.03 (0.15)	0.02 (0.01)	74.00	50.00

Table 5—Genetic variability across all the genotypes of *Podophyllum hexandrum*

Observed no. of alleles	Effective no. of alleles	Nei's gene diversity	Shannon's Information index	Ht	Hs	Gst	Estimate of gene flow 0.5(1Gst)/Gst	No. of polymorphic alleles	Per cent of polymorphic alleles
1.94 (0.27)	1.58 (0.32)	0.34 (0.15)	0.50 (0.20)	0.34 (0.02)	0.10 (0.01)	0.69	0.22	121	92.37

important role in the variation of the genetic structure of *P. hexandrum* genotypes (Table 6). Considering the high genetic differentiation among the wild populations of *P. hexandrum*, preservation of only a few populations may not adequately protect the genetic variation within the species in the Himalayan region. At present, the rate of propagation of *P. hexandrum* is far less than the rate of its exploitation. This species or at least a large part of its genetic diversity may be lost in the near future owing to its importance and over exploitation as a medicinal plant if appropriate conservation measures are not adopted. Since a single or even a few plants will not represent the whole genetic variability in *P. hexandrum,* there appears to be a need for maintenance of sufficiently large populations in natural habitats for conservation of its genetic diversity and avoidance of genetic erosion.

Table 6—Environmental factors with respect to the site of collection of *Podophyllum hexandrum* genotypes

Name of forest division	Sampling site	Altitude	Temeprature		Rainfall (mm)	Rainfall (mm)	Humidity (%)	Humidity (%)	Soil *p*H
			Min	Max	Max	Min	Noon	Morning	Top layer
Parvati	R/4,Kasol(C-II-a-Nry)	1570	2	18	100	30	30	80	4.74
	Twin Multivora	1300	0	35	80	60	60	85	4.5
	Agan nala (R/9) Rajgiri	1300	-1.5	15	150	30	40	75	5.2
Kullu	Brundhar	1916	-1.5	28	134.86	18.01	50	70	7.46
	Gulaba	2895.6	-10	15	134.86	18.01	30	50	5.67
	ChanderKhani	3352.8	-10	15	134.86	18.01	30	50	5.8
	Kaned Nry	2150	0	28	164	50	20	40	6.86
	Sanghar Nry	2100	0.4	35	164	0	20	40	6.72
Dodrakwar	Madhvi Thach	3048	-3	24	70	65	0	75	4.6
	Kala Pani	2743.2	-5	34	75	40	0	78	5.01
Seraj	Jalora Pass (Sojha Nry)	2667	-10	26	300	100	20	30	5.88
	Jalora c-30(b)	2473.2	-5	33	125	70	50	80	5.77
Churah	DPF(D-1892-CI) (Choundi)	3750	-4	16	32	20	40	75	5.55
	DPF(D-791-C1)	2700	-4	15	35	15	40	75	5.09
Lahaul	Myar Valley	4300	-2	22	15	10	20	40	4.78
	Nayan ghar	4300	-5	12	15	10	20	50	7.35
Palampur	Bada Bangal	2895	-10	13	400	147	30	50	6.15
	Chota Bangal	2700	-10	18	350	150	30	60	5.31
	IHBT	2800	-2	25	84	25	30	90	4.73
Rampur	Bander Thach	2895.6	0	25	15	10	25	85	4.5
	Saropa Nry	2499.4	0	30	170	20	20	50	5.51
Kinnaur	Nichar Nry	2190	-2	28.3	170	20	50	80	5.78
	Rango (N-C-8)	2710	-4	24	90	20	50	60	5.19
Pangi	Sach Range	2712.7	-5	27	100	20	50	60	4.58
	Killer Range	2850	-2.5	30	70	30	50	60	7.34
	Purthi Range	2900	-10	30	70	40	50	60	4.88
Bharmaur	Ghoei DPF	2680	-10	15	145.5	12.75	25	60	5.4
	Samara RF	2590.8	-10	15	134.86	18.01	25	60	6.02

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