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Congruence of RAPD and ISSR Markers for Evaluation of Genomic Relationship Among 28 Populations of *Podophyllum hexandrum* Royle from Himachal Pradesh, India

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Abstract: Twenty eight populations of *Podophyllum hexandrum* Royle were selected to study genetic relationship using RAPD and ISSR markers from north-western Himalayas, Himachal Pradesh, India. Nineteen RAPD primers and 11 ISSR primers amplified a total of 131 and 68 scorable bands, of which 92.37% and 83.82%, respectively, were polymorphic. The mean coefficient of gene differentiation (Gst) was 0.6933 and 0.6296, indicating that Nei's gene diversity of 33.77% and 29.44% reside in all the populations. Estimated value of gene flow for RAPD (Nm = 0.11059), for ISSR (Nm = 0.1470) individually, and the combination of RAPD+ISSR (Nm = 0.1211) markers indicated that there was limited gene flow among the populations. The dendrogram obtained from UPGMA analysis revealed grouping of populations with respect to their forest division, except with Kullu forest division. The existence variation among 28 populations based on percentage of polymorphic bands (PPB) was proved to be coupled with geographical altitude (r = 0.474). The genetic similarity matrices generated by ISSR and RAPD markers were highly correlated (r = 0.721 at P = 0.001), showed similar estimation between the 2 systems. Both markers were equally useful in providing some understanding about the genetic relationship of different populations in *Podophyllum* L.

Key Words: *Podophyllum hexandrum*, RAPD, ISSR, RAPD+ISSR, Genetic Diversity

Introduction

The Himalayan region is home to numerous highly valued medicinal plants including *Podophyllum hexandrum* Royle (Berberidaceae), also known as Indian May Apple. It is distributed in very restricted pockets in the Himalayan zone at the altitudes ranging from 1300 to 4300 m above sea level (Alam et al., 2008). *P. hexandrum* is an herbaceous plant and has an extensive rhizogenous system that allows it to spread and survive as established colonies. It is recognized for its anti-cancer properties. The rhizomes and roots of *P. hexandrum* contain anti-tumor lignans such as podophyllotoxin, 4'-dimethyl podophyllotoxin, and podophyllotoxin 4-o-glucoside (Imbert, 1998). Recently, podophyllotoxin has been reported to be produced by endophytes from *P. hexandrum* (Puri et al., 2005) and *P. peltatum* L. (Eyberger et al., 2006). Among the plethora of physiological activities and potential medicinal and agricultural applications, the antineoplastic and antiviral properties of podophyllotoxin congeners and their derivatives are arguably the most eminent from a pharmacological perspective. Semisynthetic derivatives of epipodophyllotoxin, e.g. etoposide (VP-16) (Allevi et al., 1993), etopophos (Schacter, 1996), and teniposide (VM-26), are effective agents in the treatment of lung cancer, a variety of leukemias, and other solid tumours (Pandey et al., 2007). Growing demand in the world of anti-cancer

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drugs adds much to the importance of podophyllotoxin and thus the *Podophyllum* L. species. Presently, *P. hexandrum* (an Indian species) and *P. peltatum* L. (an American species) are the commercial source of podophyllotoxin for the pharmaceutical industry. However, the Indian species *P. hexandrum* contain 3 times more podophyllotoxin than the American species *P. peltatum* (Fay & Ziegler, 1985; Pandey et al., 2007) adding more to the importance of *P. hexandrum.* However, the annual supply is at present estimated 50 – 80 tonnes, while the demand is more than 100 tonnes. To meet the ever increasing demand for the crude drug, the rhizomes of *P. hexandrum* are being indiscriminately collected in large quantities. As a result of this and the 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 each year. Some of the population in certain pockets has virtually disappeared owing to anthropogenic activities and overexploitation (Bhadula et al., 1996). In the natural habitat, seed germination and seedling establishment are very poor and propagation is mostly through rhizomes. Because 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. In this regard, studies of its population biology and genetic diversity are important for successful development of conservation strategies.

Considerable variation in morphological characters, such as plant height, leaf characteristics, fruits weight, seed weight, and colour, has been reported in *P. hexandrum* from interior Himalayas (Bhadula et al., 1996; Airi et al., 1997; Purohit et al., 1999). Therefore, it would be inefficient, environmentally destructive, and economically unsound to randomly harvest *P. hexandrum* from the field. Polypeptide profiles and esterase isozyme analysis have indicated the existence of high inter- and intra-population variation in *P. hexandrum* (Bhadula et al., 1996). 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 ISSR (Salimath et al., 1995) and RAPD (Williams et al., 1990; Kazan et al., 1993), on the other hand, are quite stable and highly polymorphic in nature. To our knowledge, no report has been published on the genetic diversity, population structure, and gene flow among the populations of *P. hexandrum* in Himalayan region with high resolution molecular markers like ISSR and RAPD for combined analysis.

Inter-Simple Sequence Repeats (ISSR) amplifies inter-microsatellite sequences at multiple loci throughout the genome (Salimath et al., 1995; Li & Xia, 2005). An ISSR molecular marker technique permits the detection of polymorphism in microsatellites and inter-microsatellite loci without previous knowledge of DNA sequences (Zietkiewicz et al., 1994). Furthermore, they are highly reproducible due to their primer length and to the high stringency achieved by the annealing temperature. This technique has been widely used to investigate genetic diversity and population genetic structure (Reddy & Nagaraju, 1999; Li & Xia, 2005; Chen et al., 2005) because of its advantages in overcoming limitations of allozyme and RAPD techniques (Wolfe et al., 1998; Ratnaparkhe et al., 1998; Esselman et al., 1999).

In this study, we investigated the genomic relationship among the wild populations of *P. hexandrum* and their relationship with geographical altitude in north-western Himalayas, Himachal Pradesh, India, with the aim to provide insight to facilitate conservation management of the remaining populations. Appropriate conservation management should be adopted including in situ conservation and germplasm collection from those of the remaining populations with the greatest genetic variation.

Materials and Methods

Plant Material

Twenty-eight populations of *P. hexandrum* were collected from 28 sites covering 11 forest divisions with an altitude ranging from 1300 m to 4300m from the interior range of the north-western Himalayan region, Himachal Pradesh, India (Table 1). One forest division has 2-5 selected sampling sites. From each site, in total 7-8 plants, representative plant samples, for all age groups (1st, 2nd, 3rd, and 4th year) combined, were collected (one population). Fresh leaves (about 5 g) from these plants were harvested, mixed together, and placed in a zip-lock plastic bag containing silica gel which speeded up the drying process. The pair-wise distance between populations within a forest division was 0.5 – 32 km, whereas, the pair-wise distance between forest divisions was 10 – 400 km (Figure 1). The samples were brought to the laboratory and stored at -80 °C prior to DNA isolation.

Table 1. Twenty eight populations of *Podophyllum hexandrum* collected from different sites, at different altitudes covering 11 forest divisions and their polymorphic features using RAPD, ISSR, and RAPD+ISSR markers.

Forest Division	Sampling site	Sample size	Altitude (m)	Total Number of bands			% of Polymorphic Band (PPB)		
				RAPD	ISSR	RAPD +ISSR	RAPD	ISSR	RAPD +ISSR
Parvati	R/4 Kasol	7	1570	56	38	94	82.14	71.05	77.65
	Twin Multivora	7	1300	57	36	93	82.45	69.44	77.41
	Anganoala	8	1300	56	37	93	82.14	70.27	77.41
Kullu	Brundhar	7	1916	79	58	137	88.88	81.03	91.97
	Gulaba	8	2895	76	48	124	88.23	77.08	83.06
	Sanghar Nry	8	2100	80	51	131	85.07	78.43	83.96
	Kaned Nry	7	2150	80	53	133	85.29	79.24	84.21
	ChanderKhani	7	3352	83	52	135	89.69	78.84	84.44
Dodrakwar	Madhvi Thach	8	3048	90	50	140	95.55	78.00	85.00
	Kala Pani	8	2743	85	51	136	89.58	78.43	84.55
Seraj	Sojha Nry	7	2667	97	42	139	88.63	73.80	84.89
	Jalora C-3b	7	2473	90	41	131	87.34	73.17	83.96
Churah	DPF-D-1892-C1	7	3750	67	45	112	85.71	75.55	81.25
	DPF-D-791-C1	8	2700	68	47	115	87.95	76.59	81.73
Lahaul	Nayanghar	8	4300	88	48	136	87.50	77.08	84.55
	Myar Valley	7	4300	96	50	146	87.50	78.00	85.61
Palampur	IHBT	7	2800	81	42	123	88.88	73.80	82.92
	Bada Bangal	7	2895	90	45	135	87.95	75.55	84.44
	Chota Bangal	8	2700	83	43	126	87.65	74.41	83.33
Rampur	Bander Thach	7	2895	88	50	138	88.63	78.00	84.78
	Saropa Nry	7	2499	83	49	132	87.45	77.55	84.09
Kinnaur	Nichar Nry	7	2190	91	48	139	89.01	77.08	84.89
	Rango (N-C-8)	7	2710	83	50	133	87.95	78.00	84.21
Pangi	Sach Range	7	2712	81	48	129	87.65	77.08	83.72
	Killer Range	8	2850	78	45	123	87.17	75.55	82.92
	Purthi Range	7	2900	83	46	129	87.95	76.08	83.72
Bharmaur	Ghoei DPF	7	2680	83	45	128	87.95	75.55	83.59
	Samara RF	8	2590	85	45	130	88.23	75.55	83.84
Total				2257	1303	3560	87.50	76.07	83.50

Isolation of DNA

Total genomic DNA from each population was isolated using CTAB method (Saghai-Maroof et al., 1984). Samples of 500 mg were ground to powder in liquid nitrogen, using a mortar and pestle. The powders were 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 mM

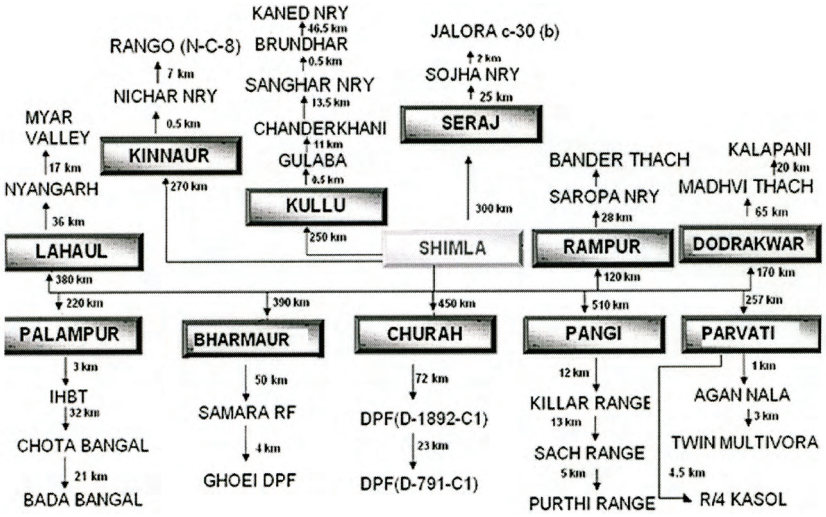

Figure 1. The difference in distance (Km) between sampling sites of respective forest divisions, which is measured from a centre point Shimla.

EDTA, 100 mM Tris-HCl, pH 9.5, and 0.2% (v/v) β-mercaptoehanol. After incubating the homogenate for 1 h at 65 °C, an equal volume of chloroform was added and centrifuged at 15.000 rpm at 10 °C for 20 min. DNA was precipitated with 1/10 volume (ml) of 3M 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 known quantity of uncut DNA on the agarose gel, diluted to 12.5 ng.μl^{-1} and used in PCR.

RAPD Amplification

A total of 20 RAPD primers (Operon Tech, USA) were initially screened and, out of them, 19 random decamer primers, A, B, C, and D series (Table 2), which produced clear reproducible fragments, were selected for further analysis. DNA was amplified following the protocol of Williams et al. (1990). Amplification reaction was performed in volumes of 25 µl containing 10 mM Tris HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM Tris HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTPs, 0.4 µM 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 the 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 the primer extension (72 °C) for 7 min. The PCR products were stored at 4 °C before analysis.

ISSR Amplification

A total of 30 ISSR primers (commercially synthesized from Sigma Incorporation) were screened with 10 plant samples. After assessing the effects of Mg^{+2}concentration, template DNA concentration, and temperature during the annealing stage of the amplification, 11 primers, which produced clear and reproducible fragments, were selected for further analysis. The sequences of these ISSR primers are listed in (Table 2). The PCR amplification was performed in a 25 µl reaction volume containing 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 10 mM each of DNTP, 0.4 µM of primer, 0.01% gelatin, 1 unit of Taq polymerase, and 25 ng of genomic DNA. Initial denaturation for 5 min at 94 °C was followed by 40 cycles of 1 min at 94 °C, 1 min at specific annealing temperature, 2 min at 72 °C, and a 10 min final extension step at 72 °C. The annealing temperature in this study ranged from 45 °C to 55 °C.

Agarose Gel Electrophoresis

Amplification products, electrophoresed on 1.5% agarose gels, were run at a constant voltage (50 V) in 1 × TBE for approximately 2 h, visualized by staining with

Table 2. RAPD and ISSR primers used, total number of recorded markers for each primer, and their percentage of polymorphic band along with resolving power of DNA samples collected from 28 populations of *Podophyllum hexandrum*.

Primer type	Nucleotide sequence	No. of recorded bands	Percentage of polymorphic band (PPB)	Resolving power
RAPD				
OPA01.	5'CAGGCCCTTC3'	3	100	3.714
OPA02.	5'TGCCGACCTG3'	10	100	14.286
OPA04.	5'AATCGGGCTG3'	6	83.3	8.0
OPA08.	5'GTGACGTAGG3'	7	100	8.0
OPA11.	5'CAATCGCCGT3'	7	100	9.429
OPA13.	5'CAGCACCCAC3'	3	100	2.429
OPA18.	5'AGGTGACCTG3'	11	90	16.571
OPB11.	5'GTAGACCCGT3'	9	77.8	10.571
OPB15.	5'GGAGGGTGTT3'	6	50	9.857
OPB18.	5'CCACAGCAGT3'	9	100	10.571
OPB19.	5'ACCCCCGAAG3'	10	100	9.571
OPC08.	5'TGGACCGGTG3'	5	100	6.929
OPC12.	5'TGTCATCCCC3'	6	100	5.929
OPC15.	5'GACGGATCAG3'	4	100	2.0
OPC16.	5'CACACTCCAG3'	7	100	6.286
OPD05.	5'TGAGCGGACA3'	4	75	5.071
OPD08.	5'GTGTGCCCCA3'	8	87.5	9.429
OPD11.	5'AGCGCCATTG3'	7	100	8.786
OPD13.	5'GGGGTGACGA3'	9	100	13.786
ISSR				
P02	5'AGAGAGAGAGAGAGAGT3'	5	20	9.358
P08	5'TGTGTGTGTGTGTGTGA3'	3	66.7	4.071
P10	5'AGAGAGAGAGAGAGAGYT3'	3	66.7	4.500
P 13	5'CTCTCTCTCTCTCTCTRA3'	5	80	5.714
P 16	5'CCGCCGCCGCCGCCGCCG3'	5	80	4.642
P 17	5'GGCGGCGGCGGCGGCGGC3'	8	100	10.428
P 21	5'CTTCACTTCACTTCA3'	7	85.7	11.071
P 22	5'TAGATCTGATATCTGAATTCCC3'	12	100	12.642
P 23	5'AGAGTTGGTAGCTCTTGATC 3'	6	100	9.500
P 24	5'CATGGTGTTGGTCATTGTTCCA3'	5	60	7.000
P 25	5'ACTTCCCCACAGGTTAACACA3'	9	100	14.142

ethidium bromide (@0.5 µg ml^{-1}) and a total of 2.5 µl loading buffer (1.0 × TBE, 50% glycerol, 0.25% xylene cyanol) was added to each reaction before electrophoresis. After electrophoresis, the gels were observed under an UV-transilluminator, documented in a Gel-Doc 2000 (Bio-Rad). Gel-Pro analyzer version 3-1 software was used to score ISSR profile. The reproducibility of the amplification was confirmed by repeating each experiment 3 times.

Data Analysis

RAPD and ISSR amplified fragments were scored for band presence (1) or absence (0) and a binary qualitative data matrix was constructed. Data analyses were performed using the NTSYS PC version 2.0 computer package program (Rohlf, 1992). Pair-wise distance matrix was calculated using the Jaccard similarity coefficient (Sneath & Sokal, 1973). The similarity values were used to generate a dendrogram via the un-weighted pair group method with arithmetic average (UPGMA). The data matrix of RAPD and ISSR were also used for assessment of genetic structure, genetic differentiation, gene flow, and diversity. Measurement of diversity including gene diversity (H), gene flow, and Shannon's information index (I) were estimated by POPGEN 1.32 software (Table 3).

Resolving Power

According to Prevost & Wilkinson (1999) the resolving power (Rp) of a primer is: $Rp = \sum IB$, where *IB* (band informativeness) takes the value of: 1-[2× (0.5 – *P*)] *P* being the proportion of the 28 genotypes (*P. hexandrum* populations analyzed) containing the band.

Table 3. Summary of genetic variation and polymorphic features estimated using RAPD, ISSR, and RAPD+ISSR markers among the *Podophyllum hexandrum* populations with respect to their distributions among 11 forest divisions.

Forest Division	Nei's Genetic Diversity (H) (mean ± SD)			Shannon's information index (I) (mean ± SD)			Percentage of polymorphic loci		
	RAPD	ISSR	RAPD+ISSR	RAPD	ISSR	RAPD+ISSR	RAPD	ISSR	RAPD+ISSR
Parvati	0.0305	0.0784	0.0469	0.0437	0.1123	0.0672	33.33	17.65	10.55
	-0.1129	-0.1707	-0.1369	-0.1616	-0.2445	-0.1961			
Dodrakwar	0.0344	0.1029	0.0578	0.0476	0.1427	0.0801	50	20.59	11.56
	-0.127	-0.2037	-0.1603	-0.176	-0.2824	-0.2222			
Churah	0.0763	0.0588	0.0704	0.1058	0.0815	0.0957	50	11.76	14.07
	-0.1805	-0.1623	-0.1743	-0.2503	-0.225	-0.2416			
Seraj	0.1794	0.1176	0.1583	0.2487	0.1631	0.2194	49.67	23.53	31.66
	-0.2407	-0.2137	-0.2332	-0.3337	-0.2962	-0.3232			
Lahual	0.0992	0.1985	0.1332	0.1376	0.2752	0.1846	50	39.71	26.63
	-0.2002	-0.2465	-0.2216	-0.2775	-0.3417	-0.3072			
Kullu	0.1918	0.2282	0.2042	0.2779	0.3375	0.2983	20	58.82	50.25
	-0.216	-0.2018	-0.2114	-0.3088	-0.292	-0.3038			
Palampur	0.1595	0.085	0.134	0.2284	0.1217	0.1919	33.33	19.12	30.15
	-0.214	-0.1761	-0.2045	-0.3065	-0.2522	-0.2928			
Rampur	0.0267	0.0662	0.0402	0.037	0.0917	0.0557	50	13.24	8.04
	-0.1129	-0.1707	-0.1363	-0.1565	-0.2366	-0.189			
Kinnaur	0.0382	0.0294	0.0352	0.0529	0.0408	0.0488	50	5.88	7.04
	-0.1333	-0.1185	-0.1282	-0.1848	-0.1643	-0.1777			
Pangi	0.1391	0.0523	0.1094	0.1992	0.0749	0.1567	33.33	11.76	24.62
	-0.2069	-0.1443	-0.192	-0.2963	-0.2066	-0.2749			
Bharmaur	0.0229	0.0588	0.0352	0.0317	0.0815	0.0488	50	11.76	7.04
	-0.1049	-0.1623	-0.1282	-0.1455	-0.225	-0.1777			
Mean	0.090727	0.128227	0.093164	0.128227	0.138445	0.131564	42.69	21.25	40.09

Results

RAPD Band Patterns

The 19 RAPD makers used in the study generated a total of 131 bands (an average of 6.89 bands per primer) out of which 121 (92.37%) were polymorphic and only 10 (7.63%) were monomorphic bands. The size of amplified fragments produced ranged from 250 bp to 2100 bp. The numbers of polymorphic bands varied in between 46 (in R/4 Kasol) and a maximum of 87 (in Sojha Nry) (Table 1). The total number of polymorphic alleles were 121, thereby giving an estimate of profound (>92%) polymorphism. Also polymorphism differed substantially within the discrete groups of plants with an average 42.69% and was found to be in between 20.0% (Kullu) and a maximum of 50.0% (Dodrakwar, Churah, Lahaul, Rampur, Kinnaur, and Bahrmaur). The mean coefficient of gene differentiation (Gst) was 0.6933 (Table 4), indicating 33.77% of the total genetic diversity within the populations. The gene flow (Nm) calculated based on the Gst value (Slatkin & Barton, 1989) between populations was found to be 0.11059 (Table 4).

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, and OPD13) possess the highest Rp values (14.286, 16.571, and 13.786, respectively) and are each able to distinguish between all the 28 populations of *P. hexandrum* collected from the field (Table 2).

ISSR Band Patterns

Eleven ISSR primers chosen generated a total of 68 ISSR bands (an average of 6.18 bands per primer), out of

Table 4. Genetic variability estimated among 28 populations of *Podophyllum hexandrum.*

Nei's gene diversity	Shannon's Information index	Hs	Gst	Estimate of gene flow (Nm) (0.25(1-Gst)/Gst	Number of polymorphic alleles	% of polymorphic alleles
RAPD						
0.3377	0.5014	0.1036	0.6933	0.11059	121	92.37
-0.1545	-0.2036	-0.0056				
ISSR						
0.2944	0.4413	0.109	0.6296	0.147	57	83.82
-0.1731	-0.2391	-0.0076				
RAPD+ISSR						
0.3229	0.4809	0.1054	0.6735	0.1211	178	89.45
-0.162	-0.2176	-0.0062				

Hs = Population diversity; Gst = Gene differentiation.

which 57 were polymorphic (83.82%). The amplified PCR fragment size ranged from 250 bp to 1350 bp. Out of these 11 primers, P02, P08, P13, P16, P21, and P24 revealed 11 monomorphic bands existed in all of the 28 populations. The high reproducibility of ISSR markers may be due to the use of longer primers and higher annealing temperature than those used for RAPD. The annealing temperature in this study ranged from 45 °C to 55 °C. The numbers of polymorphic bands varied in between 25 (in Twin Multivora) and a maximum of 47 (in Brundhar) (Table 1). The total number of polymorphic alleles is 57, thereby giving an estimate of >83% polymorphism. It differed substantially within the discrete groups of plants with an average of 21.25% and was found to be in between 5.88% (Kinnaur forest division) and a maximum of 58.82% (Bahrmaur forest division). The mean coefficient of gene differentiation (Gst) was 0.6296, indicating 29.44% of the total genetic diversity within the populations. The total gene flow (Nm) between populations was found to be 0.1470 (Table 4).

The resolving power (Rp) of the 11 ISSR primers ranged from 4.071 for primer P08 to 12.642 for P22. Three ISSR primers (P21, P22, and P25) possess the highest Rp values (11.071, 12.642, and 14.142, respectively) and are each able to distinguish all the 28 populations of *Podophyllum* collected from the field (Table 2).

Dendrogram Analysis

Genetic similarity was calculated from the Jaccard similarity index value for all the 28 accessions of *P. hexandrum* considering ISSR and RAPD approaches individually, as well as together. Based on RAPD marker alone, the similarity index values ranged from 0.61 to 0.96 (figure not shown). These values were used to construct a dendrogram using the UPGMA method. Populations from 11 Forest Divisions were clustered into region-specific groups with the only exception of Kullu Forest Division.

Similarly, based on ISSR markers alone, the similarity index values ranged from 0.57 to 0.96. All the populations clustered among 12 main clusters (figure not shown). The populations collected from Kullu Forest Division were distributed in 2 clusters similar to RAPD markers. The similarity index values did not differ significantly for ISSR and RAPD systems.

Based on the both marker systems together, the similarity index values ranged from 0.59 to 0.94 and both systems have been used to construct the dendrogram (Figure 2). All the 28 *P. hexandrum* populations were distributed into 12 main clusters (Ia-Im), similarly, for both RAPD and ISSR, when used separately. Cluster Ia represented Parvati forest division with 3 different populations, namely, R/4, Kasol, TwinMultivora, and Anganola. Population from Kullu Forest Division distributed between 2 clusters; Ib & Ic. Ib has Brundhar and Gulaba populations and Ic has SangharNry, KanedNry, and Chanderkhani populations. Cluster Id (Palampur Forest Division) had 3 populations: IHBT, BadaBangal, and ChotaBangal. Cluster Ie (Rampur Division) has 2 populations: BanderThach and SaropaNry. Cluster If represents Kinnaur Forest Division with 2 populations:

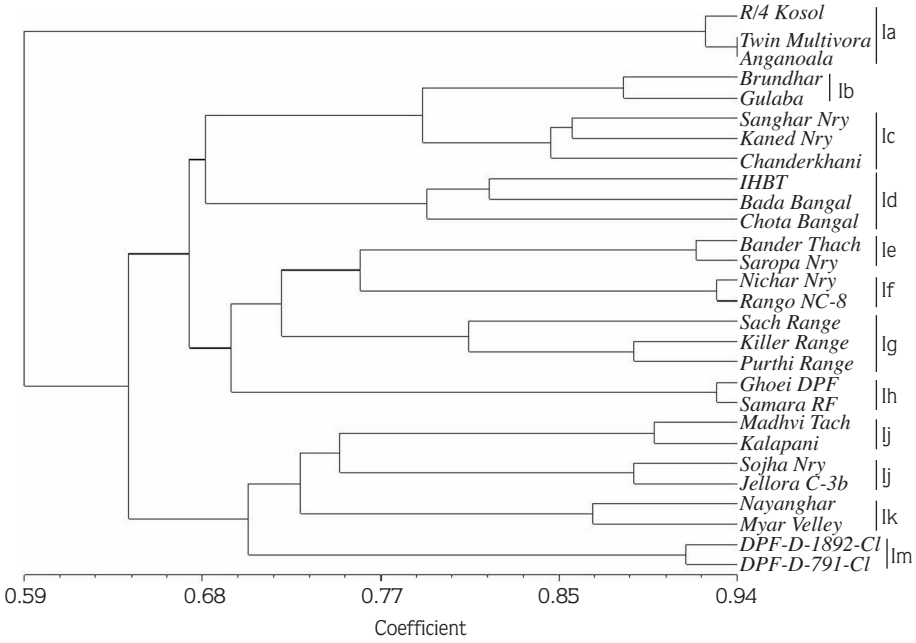

Figure 2. Dendrogram illustrating genetic relationships among 28 populations generated by UPGMA cluster analysis calculated from 3560 RAPD+ISSR bands produced by 30 primers.

NicharNry and RangoNC-8. Cluster Ig (Pangi Forest Division) has 3 populations: SachRange, KillerRange, and PurthiRange. Cluster Ih (Bharmaur Forest Division) has 2 populations: GhoeiDPF and SamaraRF. Cluster Ii (Dodrakwar Forest Division) has 2 populations: MadhviThach and Kalapani. Similarly, the forest division Seraj (Cluster Ij) and Lahaul (cluster Ik) have SojhaNry and JelloraC-3b, and Nayanghar and Myarvelley, respectively. Churah division (cluster Im) has 2 populations: DPF-D-1892-C1 and DPF-D-791-C1. The results indicate high genetic diversity in *P. hexandrum* from Himachal Pradesh.

Comparison of Genetic Relationship Estimates in *P. hexandrum*

Data collected with ISSR and RAPD markers were used to compare genetic similarities between various populations of *P. hexandrum.* In *P. hexandrum*, when the similarity matrices were generated using RAPD and ISSR markers and compared, a value of r = 0.721, at P = 0.001 indicated a good correlation between the data generated by both systems (Figure 3). Further, the existence variation among 28 populations, as observed through percentage of polymorphic band (PPB), was proved to be coupled with geographical altitude (r = 0.474) (Figure 4).

Heterozygosity and Molecular Variance

Heterozygosity and molecular variance were calculated using RAPD and ISSR marker systems individually as well as together. Nei's gene diversity (H) values calculated for RAPD, ISSR, and RAPD+ISSR were 0.3377 ± 0.1545, 0.2944 ± 0.1731, and 0.3229 ± 0.1620, respectively, which showed overall 9.07% to 12.82% heterozygosity among the population of *P. hexandrum*. Similarly, the Shannon's information indices (I) were 0.5014 ± 0.2036, 0.4413 ± 0.2391, and 0.4809 ± 0.2176 for RAPD, ISSR, and RAPD+ISSR markers, respectively, show a gene diversity measurement with an average of 0.4745 among the forest divisions (Table 4).

Discussion

Considering the high genetic differentiation among the wild populations of *P. hexandrum*, conservation of only a few populations may not adequately protect the genetic variations 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 near future, owing to its importance and consequent

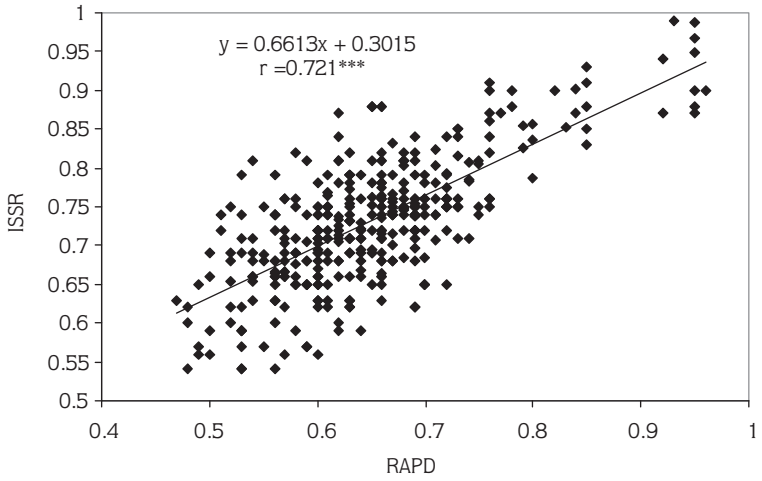

Figure 3. Regression analysis of similarity matrices obtained by RAPD and ISSR markers in *Podophyllum hexandrum* populations. The symbol *** indicates that the value is significant at P = 0.001 level of significance.

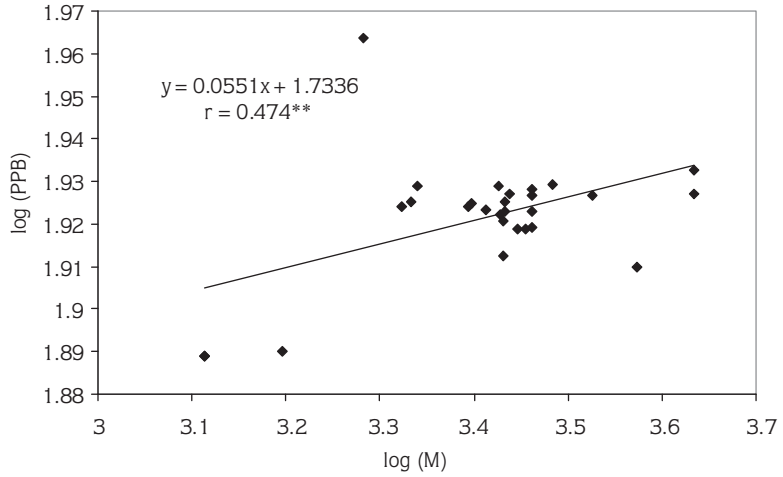

Figure 4. Regression analysis based on $\log_{10}$ M (geographical altitude) and Log PPB (percentage of polymorphic band) between 28 populations. The symbol ** indicates that the value is significant at P = 0.01 level of significance.

exploitations as a medicinal plant, if appropriate conservation measures are not adopted. Since no single, or even a few plants, will represent the whole genetic variability in *P. hexandrum*, there appears to be a need to maintain sufficiently large populations in natural habitats to conserve genetic diversity in *P. hexandrum* and avoid genetic erosion.

Analysis of Polymorphic Feature

Twenty eight accessions of *P. hexandrum* were fingerprinted using 19 RAPD and 11 ISSR markers. RAPD markers produced 2257 bands on an average 118.78 bands per primer and ISSR markers generated 1303 bands on an average of 118.45 bands per primer. A high percentage of polymorphism was detected in all the populations of *P. hexandrum*, which were examined. The high proportion of polymorphic loci suggests that there is a high degree of genetic variation existing among the *Podophyllum* population. The genetic variation estimated by RAPD markers is PPB = 92.37% and I = 0.5014, whereas for ISSR markers it is 83.82% and 0.4413 at the

population level of *P. hexandrum*. RAPD markers were more efficient than the ISSR assay, as they detected 92.37% polymorphic DNA markers in *P. hexandrum* compared to 83.82% for ISSR markers. This is in contrast to the result obtained for several other plant species like wheat (Nagaoka et al., 1997) and vigna (Ajibade et al., 2000). This may be due to less number of ISSR markers used in the study in comparison to RAPD. It may be also due to the higher annealing temperature used for ISSR markers in comparison to RAPD, and thus the chances of PPB are higher in RAPD. The 19 RAPD and 11 ISSR primers in the present study yielded 178 polymorphic markers that unambiguously discriminated 28 genotypes into 12 clusters. Geographically isolated population accumulates genetic differences as they adapt to a different environment. Genetic variation among elite genotypes of *P. hexandrum* based on RAPD and ISSR analysis could be useful in selecting parents to be crossed for generating appropriate populations intended for both genome mapping and breeding purposes. In general, dispersal resulting in colonization and gene flow into existing populations is very important for both the persistence and genetic success of a species (Hamrick & Godt, 1996). In population genetics, a value of a gene flow (Nm) <1.0 (less than one migrant per generation into a population) or, equivalently, a value of gene differentiation (Gst) >0.25 is generally regarded as the threshold quantity beyond which significant population differentiation occurs (Slatin, 1987).

The average Resolving Power (Rp) of ISSR primers (8.48) was higher than RAPD primers (6.89). There is seemingly a linear relationship between the Rp of each primer and the number of recorded marker. This relationship was stronger for RAPDs (r = 0.81) than for ISSRs (r = 0.42). Prevost and Wilkinson (1999) have studied the nature of this relationship using a total of 371 hypothetical primers producing 8, 10, or 12 band positions (r = 0.98). However, they found a seemingly linear relationship between the Rp of real ISSR primers and the number of genotype of potato cultivars identified (r = 0.80). In our case, we have observed a greater correlation using RAPDs, compared to ISSRs primers, probably due to the low number of cultivars analyzed.

Estimation of genetic relatedness

Cluster analysis using RAPD, ISSR, and the combination of RAPD+ISSR revealed the distribution of different populations with respect to their forest divisions. This indicated that gene flow in the study populations of *P. hexandrum* occurred mainly within the same Forest Division rather than between divisions. Moreover, RAPD and ISSR markers used here were able to differentiate *P. hexandrum* populations collected from 11 forest divisions into 12 distinct region specific clusters except the Kullu forest division (Figure 2). The study also indicates that *P. hexandrum* populations in the north-western 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*. Partitioning of diversity is mainly influenced by the system of reproduction. Instead, the resultant genetic diversity may also be due to clonal propagation of *P. hexandrum.* Although clonal propagation contributes towards genetic uniforming within each population, Hangelbroek et al. (2002) reported that clonal plant species can have high levels of genetic variation in some cases. The low gene flow among populations detected in this study points towards the possibility of instances of single isolated populations possessing unique genotypes not found in other populations. It is, therefore, imperative for conservation planners in designing conservation strategies for wild populations of *P. hexandrum* to ensure that as many as possible separate populations are targeted for conservation rather than conserving a few selected populations.

The correlation between RAPD and ISSR, Jaccard's similarity coefficient value (r = 0.721), indicated a good correlation between the data generated by both systems (Figure 3). The observed increase in the genetic variation among the populations was coupled with an increase in altitude (r = 0.474) (Figure 4). A similar finding was observed by Fahima et al. (2002) who reported that microsatellite polymorphisms in natural populations of wild emmer wheat were best explained by the variation of altitude and temperature in August. The primers with poly (GC)n and poly (GA)n motifs produced more polymorphism than any other motif. Somewhat similar result was also reported by Ajibade et al. (2000) where they found that the primer containing the CT repeats was one of those, which did not give interpretable phenotype when analyzed, while primers with GA and CA repeats revealed polymorphism in the genus Vigna.

Based on polymorphic feature, genetic diversity, genetic similarity, and gene flow among the populations of *Podophyllum* based on RAPD and ISSR study, we recommend that any future conservation plans for this

species should be specifically designed to include representative populations with the highest genetic variation for both in situ conservation and germplasm collection expeditions.

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