Research Article

Assessment of genetic diversity through RAPD, ISSR and AFLP markers in *Podophyllum hexandrum*: a medicinal herb from the Northwestern Himalayan region

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ABSTRACT

Total synthesis of podophyllotoxin is an expensive process and availability of the compound from the natural resources is an important issue for pharmaceutical companies that manufacture anticancer drugs. In order to facilitate reasoned scientific decisions on its management and conservation for selective breeding programme, genetic analysis of 28 populations was done with 19 random primers, 11 ISSR primers and 13 AFLP primer pairs. A total of 92.37 %, 83.82 % and 84.40 % genetic polymorphism among the populations of *Podophyllum* were detected using RAPD, ISSR and AFLP makers, respectively. Similarly the mean coefficient of gene differentiation (Gst) were 0.69, 0.63 and 0.51, indicating that 33.77 %, 29.44 % and 26 % of the genetic diversity resided within the population. Analysis of molecular variance (AMOVA) indicated that 53 %, 62 % and 64 % of the genetic diversity among the studied populations was attributed to geographical location while 47 %, 38 % and 36 % was attributed to differences in their habitats using RAPD, ISSR and AFLP markers. An overall value of mean estimated number of gene flow (Nm) were 0.110, 0.147 and 0.24 from RAPD, ISSR and AFLP markers indicating that there was limited gene flow among the sampled populations. **[Physiol. Mol. Biol. Plants 2010; 16(2) : 135-148]** *E-mail : pknaik73@rediffmail.com*

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INTRODUCTION

Podophyllum hexandrum Royle commonly known as the Himalayan Mayapple, is an endangered perennial herb belonging to the family Berberidaceae. It is distributed in restricted pockets form the Northwestern Himalayan region: India, China, Nepal, Pakistan, Afghanistan and other areas (Chatterjee, 1952; Fu, 1992; Ying, 1979). It has long been used by the Himalayan natives and the American Indian (Anon, 1970) for the treatment of certain types of cancers. The roots and rhizomes of several *Podophyllum* species have been found to be the source of podophyllotoxin lignan which is a precursor to synthesis of anticancer drugs such as etoposide, teniposide and etophos (Stahelin and Wartburg, 1991; Imbert, 1998). Hence, *P. hexandrum* and *P. peltatum* (an American species) are presently the commercial source of podophyllotoxin for the pharmaceutical industry. However, the yield of podophyllotoxin content in *P. hexandrum* is 3 times larger than its counterpart *P. peltatum* (Fay and Ziegler, 1985). Also the quality of podophyllotoxin in *P. hexandrum* is better than *P. peltatum.* The rhizomes of this important medicinal plant 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 a lack of organized cultivation, *P. hexandrum* has been reported as a threatened species from the Himalayan region. The demand for the compound continues to increase and thus encourages domestication and conservation of *P. hexandrum* in the Himalayan region.

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A thorough understanding of the level and distribution of genetic variation in *P. hexandrum* populations is essential for the conservation and management policies (Fritsch and Rieseberg, 1996). PCR-based DNA fingerprinting techniques such as random amplified polymorphic DNA analysis (RAPD), inter simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) represents a very informative and cost-effective approach for assessing genetic diversity of a wide range of organisms (Williams *et al*., 1990; Zietkiewicz *et al*., 1994; Vos *et al*., 1995). The usefulness of RAPD and ISSR for genetic characterization of *P. hexandrum* populations has been confirmed in several studies (Alam *et al*., 2008; Sharma *et al*., 2000). AFLP fingerprinting combine universal applicability with high power of discrimination and reproducibility in detecting polymorphism in species (Janssen *et al*., 1996). All these markers do not require any prior knowledge of a species genetics (Ajmone-Marsan *et al*., 1998; De Knijff *et al*., 2001; Han *et al*., 2000). The utility, repeatability and efficiency of the AFLP lead to the broader application of this technique.

To our knowledge no work has been done to analyze the genetic variation in *Podophyllum hexandrum* population for comparisons of different DNA markers for diversity studies as well as their relatedness with the yield of podophyllotoxin. The objectives of the study were to examine the levels of genetic diversity of populations of *P. hexandrum* based on DNA profiling using DNA markers like RAPD, ISSR and AFLP in order to optimize sampling strategies for the efficient maintenance of variability in the species.

MATERIALS AND METHODS

Sampling of plants

Eleven forest divisions covering 28 genotypes of *P. hexandrum* were collected with an altitude ranging from 1300 m to 4300 m from the interior range of the northwestern Himalayan region, Himachal Pradesh, India (Table 1). One forest division has 2-5 selected sampling sites. From each site representative plant samples (7-8 plants) of different age groups (1st, 2nd, 3rd and 4th year) were collected. Fresh leaves (about 5 g) from these plants were harvested, mixed together and placed in a zip-lock plastic bag containing silica gel to speed up the drying process. The samples were brought to laboratory and stored at -80 ^{o}C prior to DNA isolation.

Isolation of DNA

Total genomic DNA was extracted from frozen leaves by the CTAB method (Saghai-Maroof *et al*., 1984). Samples of 500 mg were ground to powder in liquid nitrogen using a mortar and a 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 EDTA, 100 mM Tris-HCl pH 9.5 and 0.2 % (v/v) β-mercaptoethanol. After incubating the homogenate for 1 hour at 65 ^{o}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 minutes. RNA was removed by RNase treatment. DNA was quantified by Nano Drops and comparing with known quantity of uncut λ DNA on the 1 % agarose gel, diluted as per requirement of PCR analysis.

Extraction and determination of podophyllotoxin

Dried roots/rhizomes were ground to powder in a mortar with a pestle. Podophyllotoxin was extracted following the procedure of Broomhead *et al*., (1990) and estimated using HPLC. All the experiments on extraction of podophyllotoxin and HPLC analysis were repeated three times. Fifty milligrams of root powder was suspended in 20 ml of ethanol and continuously stirred at 60 ^{o}C for 20 min using a magnetic stirrer. The extract was filtered through Whatman filter paper No. 1. The second, third and fourth extractions of the same samples were done with 10 ml ethanol for 10 min under the same conditions. All the extracts were pooled and ethanol was evaporated to dryness in a water bath shaker at 60 ^{o}C. The resultant residue was dissolved in 10 ml acetonitrile (HPLC grade) and filtered with 0.22 µm durapore membrane filter (Millipore) for HPLC analysis.

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 ml/min. 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; 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.

Molecular analysis

Nineteen random decamer primers (Operon Tech USA) A, B, C and D series were used for RAPD analysis. DNA was amplified following the protocol of Kazan *et al*., (1993). DNA amplification was performed using a Gene Cycler (BioRAD, USA). The first cycle: Denaturation 94 ^{0}C for 5 min, primer annealing at 37 ^{o}C for 1 min and primer extension at 72 ^{o}C for 2 min. Next 42 cycles: Denaturation time was reduced to 1 min at 92 ^{o}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 ^{o}C) for 7 min. The amplification products were resolved in 1.5 % agarose gel (0.5 TBE buffer).

Eleven primers (Sigma Inc) (after being screened out of 30 primers) were selected and were used for ISSR study. PCR amplification was performed as per the method of Zietkiewicz *et al*. (1994). Initial denaturation for 5 min at 94 ^{o}C was followed by 40 cycles of 1 minute at 94 ^{o}C, 1 min at specific annealing temperature ±50 ^{o}C, 2 min at 72 ^{o}C and a 10 min final extension step at 72 ^{o}C. Amplification products were electrophoresed on 1.5 % agarose gels.

AFLP was performed as described by Vos *et al*. (1995) and was conducted using the Small Genome Primer Kit AFLP System II (Invitrogen life technology) and visualized with the Polyacrylamide Gel Electrophoresis (PAGE) system. DNA fragments were amplified using the procedure by Vos *et al*. (1995) modified as follows. Template DNA (150 ng) was digested by 1 µl mixture of *Eco*R I/*MSe* I (1.25 units/µl) at 37 ^{o}C for 2 h and at 70 ^{o}C for 15 min to inactivate the restriction endonuclease and ligated to commercial *EcoR* I and *Mse* I oligonucleotide adapters using 1 µl (1 units/µl) of T4 DNA ligase at 22 ^{o}C for 2 hrs. The adapter-ligated DNA is diluted 1:10 ratio and was amplified using a mixture of 5 µl of DNA from the ligation reaction (diluted), 40 µl of preamp primer mix II, 5 µl of 10 X PCR buffer, and 1 µl of *Taq* polymerase (3 units/µl). The pre-amplification reactions were performed on an Eppendorf Gradient Thermal cycler, using the following cycling parameters: 20 cycles at 94 ^{o}C for 30 s, 56 ^{o}C for 60 s and 72 ^{o}C for 60 s. Preamplification products were diluted in the ratio 1:50. Primer labeling is performed by phosphorylating the 5' end of the *EcoR* I primers with γ^{32}P dATP (5000 Ci/mmol) and T4 kinase at 37 ^{o}C for 1h and heat inactivate the enzyme at 70 ^{o}C for 10 min. Selective amplification was performed using reaction mix composed of 2.5 µl of diluted DNA from preamp, 4.5 µl of *MSe* I primer (6.7 ng/ µl), 0.5 µl of labeled *EcoR* I primer (27.8 ng/ µl), 2.5 µl of 10X PCR buffer, 1.5 µl of distilled water, and 1 µl of *Taq* polymerase (3 units/µl). The selective amplification PCRs were performed by another touchdown program as follows: One cycle at 94 ^{o}C for 30 s, 65 ^{o}C for 30 s, and 72 ^{o}C for 60 s lower the annealing temperature each cycle 0.7 ^{o}C during 12 cycles. This gives the touchdown phase of 13 cycles. After completing the touchdown phase of 13 cycles, continued with 23 more cycles at 94 ^{o}C for 30 s, 56 ^{o}C for 30 s and 72 ^{o}C for 60 s. Both pre- and selective amplification conditions were modified according to Myburg *et al*. (2000). An aliquot of 2 µl of selective amplification product was mixed with 2 µl of formamide dye, denatured for 3 min at 95 ^{o}C, and chilled on ice immediately. The reaction products were then size-fractioned on 6 % polyacrylamide gel on a DNA sequencing apparatus. Electrophoresis was carried out for 3.5 hr in 1X TBE buffer at 1000 V. Gel photograph has been developed by X-ray film and also seen by using phosphor measure (BioRad system). The resulting banding patterns were analyzed manually from all different kind of DNA markers.

Resolving power

According to Prevost & Wilkinson (1999) the resolving power (Rp) of a primer is: Rp = Σ *IB*, where *IB* (band informativeness) takes the value of: 1-[2x (0.5 – *P*)], *P* being the proportion of the 28 sample containing the band.

Data analysis

A total of 19 RAPD primes, 11 ISSR primer and 13 *EcoR* I + 2 bases/*Mse*I +3 bases AFLP has been used for in this study. Each sample was scored as '1' if a fragment was present and '0' for absence. The numbers of polymorphic loci produced by each primer set are listed in Table 2. Data were imported into the multivariate data analysis program, NTSYSpc, (version 2.1, ExeterSoftware, Setauket, NY). The module SimQual was used to generate a similarity matrix based on simple matching coefficients and the module SAHN performed sequential, agglomerative, hierarchical and non-overlapping clustering by unweighted pair-group method, arithmetic average (UPGMA). The module Tree Plot was used to convert the data generated by SAHN into a dendrogram. Pairwise distance matrix was calculated using the Jaccard similarity coefficient (Sneath & Sokal, 1973). Support for clusters was evaluated by bootstrapping analysis (Felsenstein, 1995). One thousand permutation data sets were generated by

resampling with replacement of characters within the combined 1/0 data matrix. POPGENE32 software was used to calculate Nei's unbiased genetic distance between the different populations using all markers. Nei's unbiased genetic distance is an accurate estimate of the number of gene differences per locus when populations are small. Population diversity (Hs) and total gene diversity (Ht) were calculated within 28 populations and within 11 forest division (as per their collection site) by POPGENE 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 (0.25(1-Gst)/Gst). The non-parametric analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) was done using GenAlex. The correlation and regression analysis were examined by using MINITAB statistical package.

RESULTS

Podophyllotoxin estimation

Podophyllotoxin from roots/rhizomes were estimated in triplicate from all 28 populations of *P. hexandrum* covering 11 forest divisions at different altitudes. It has been seen that the podophyllotoxin content in the root of the plants estimated from the Lahaul forest division was comparatively more (8.857 to 9.533 % on dry weight basis) than that of the root samples collected from other forest divisions with the lowest from Parvati (3.020 to 4.753 % on dry weight basis) (Table 1). For populations in the same forest division as well as among the forest divisions, the podophyllotoxin content increased with the increase in altitude. For populations in the same as well as other forest divisions, the podophyllotoxin content increased with the increase in altitude (Table 1).

Level of polymorphism

The 19 RAPD primers generated a total of 131 loci (an average of 6.89 loci per primer) out of which 121 (92.37 %) were polymorphic and only 10 (7.63 %) were monomorphic loci, the total number of bands generated were 2257 in 28 individual with an average of 118.78 bands per primer. The size of amplified fragments produced ranged from 250 bp to 3100 bp. The number of polymorphic loci were observed to be in the range of 46 (in R/4 Kasol) to a maximum of 87 (in Sojha nursery) (Table 2). The polymorphism differed substantially within the discrete groups of plants with an average of 42.69 % and was found to be between 20.0 % (Kullu) and 50.0 % (Dodrakwar, Churah, Lahaul, Rampur, Kinnaur and Bharmaur) (Table 3). The mean coefficient of gene differentiation (Gst) was 0.69 (Table 4) revealed that 33.77 % of the total genetic diversity lies within the populations. The gene flow (Nm) calculation based on the Gst value (Slatkin & Barton, 1989) between populations was found to be 0.11 (Table 4). The resolving power (Rp) of the 19 RAPD primers ranged from 2.0 (for primer OPC15) to 16.57 (for OPA18) which differentiated all the 28 populations of *P. hexandrum* collected from the wild (Table 2). Further, the existing variations among 28 populations as observed through percentage of polymorphic band (PPB) were proved to be coupled with geographical altitude (r = 0.606) (Figure 1).

Eleven ISSR primers were used in this study which generated a total of 68 ISSR loci (an average of 6.18 loci per primer) out of which 57 were polymorphic (83.82 %) and also generated a total of 1303 bands with an average of 118.45 bands per primer. The amplified PCR fragments ranged from 250 bp to 1350 bp. 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 is ±50 °C. The number of polymorphic loci ranged between 25 (in Twin Multivora) and 47 (in Brundhar) (Table 1). The mean coefficient of gene differentiation (Gst) was 0.63, indicating 29.44 % of the total genetic diversity within the populations. The total gene flow (Nm) between populations was

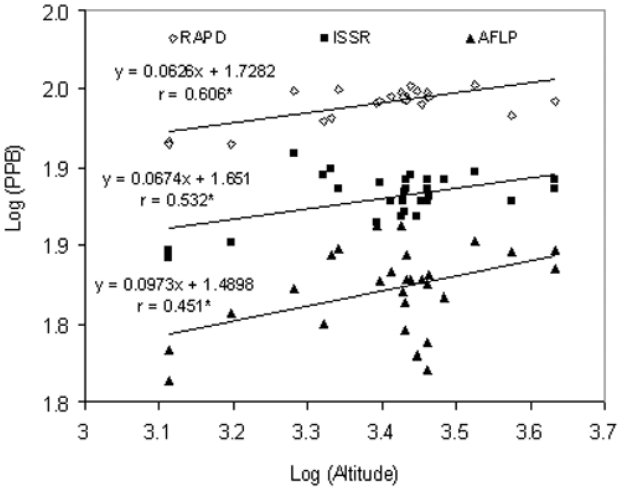

Fig. 1. Regression analysis based on log10 M (geographical altitude) and Log PPB (percentage of polymorphic band) between 28 populations. The symbol *, indicates the value is significant at p = 0.01 level of significance.

Table 1. Twenty eight populations of *Podophyllum hexandrum* collected from different sites, at different altitude covering eleven forest divisions: Polymorphic features using RAPD, ISSR, RAPD+ISSR markers and their podophyllotoxin content.

Forest Division	Sampling site	Altitude (m)	Podophyllotoxin content [% dry weight] (mean ± S.E.)	Total Number of bands			% of Polymorphic Band (PPB)		
				RAPD	ISSR	AFLP	RAPD	ISSR	AFLP
Parvati	R/4 Kasol	1570	3.567 ± 0.747	56	38	237	82.14	71.05	64.14
	Twin Multivora	1300	4.753 ± 0.796	57	36	216	82.45	69.44	60.65
	Anganoala	1300	3.020 ± 0.524	56	37	203	82.14	70.27	58.13
Kullu	Brundhar	1916	4.077 ± 0.270	79	58	254	88.88	81.03	66.54
	Gulaba	2895	5.943 ± 0.591	76	48	207	88.23	77.08	58.94
	Sanghar Nry	2100	8.033 ± 0.454	80	51	230	85.07	78.43	63.04
	Kaned Nry	2150	4.657 ± 0.850	80	53	282	85.29	79.24	69.86
	ChanderKhani	3352	4.173 ± 0.276	83	52	295	89.69	78.84	71.19
Dodrakwar	Madhvi Thach	3048	6.207 ± 0.743	90	50	247	95.55	78.00	65.59
	Kala Pani	2743	5.800 ± 0.212	85	51	261	89.58	78.43	67.43
Seraj	Sojha Nry	2667	6.607 ± 0.348	97	42	314	88.63	73.80	72.93
	Jalora C-3b	2473	6.790 ± 0.855	90	41	313	87.34	73.17	72.84
Churah	DPF-D-1892	3750	8.487 ± 0.565	67	45	285	85.71	75.55	70.18
	DPF-D-791	2700	5.753 ± 0.411	68	47	227	87.95	76.59	62.56
Lahaul	Nayanghar	4300	9.533 ± 0.484	88	48	286	87.50	77.08	70.28
	Myar Valley	4300	8.857 ± 0.427	96	50	269	87.50	78.00	68.40
Palampur	IHBT	2800	7.097 ± 0.797	81	42	214	88.88	73.80	60.28
	Bada Bangal	2895	6.573 ± 0.827	90	45	220	87.95	75.55	61.36
	Chota Bangal	2700	5.183 ± 0.780	83	43	244	87.65	74.41	65.16
Rampur	Bander Thach	2895	6.773 ± 0.640	88	50	257	88.63	78.00	66.93
	Saropa Nry	2499	6.097 ± 0.942	83	49	259	87.45	77.55	67.18
Kinnaur	Nichar Nry	2190	4.760 ± 0.291	91	48	287	89.01	77.08	70.38
	Rango (N-C-8)	2710	5.797 ± 0.552	83	50	282	87.95	78.00	69.86
Pangi	Sach Range	2712	6.133 ± 0.216	81	48	261	87.65	77.08	67.43
	Killer Range	2850	5.967 ± 0.692	78	45	261	87.17	75.55	67.43
	Purthi Range	2900	6.233 ± 0.790	83	46	264	87.95	76.08	67.80
Bharmaur	Ghoei DPF	2680	5.700 ± 0.692	83	45	251	87.95	75.55	66.14
	Samara RF	2590	6.030 ± 0.825	85	45	266	88.23	75.55	68.05
Total				**2257**	**1303**	**7192**	**87.50**	**76.07**	**66.45**

Table 2. RAPD, ISSR and AFLP 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 loci	Percentage of polymorphic loci	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
AFLP				
P01	E+AG/M+CAG	52	96.15	51.929
P02	E+TC/M+CTG	78	94.87	83.929
P03	E+TC/M+CAT	32	90.63	31.571
P 04	E+TC/M+ATG	26	100	20.500
P 05	E+AT/M+CAA	40	90	38.286
P 06	E+AA/M+CTC	24	95.83	19.929
P 07	E+TC/M+CTC	28	100	17.071
P 08	E+TG/M+CTG	53	84.91	43.214
P 09	E+AT/M+CAT	40	67.5	35.714
P 10	E+TA/M+CTC	27	88.89	31.571
P11	E+TG/M+CAT	37	89.19	26.857
P12	E+TG/M+CTC	50	76	41.429
P13	E+AT/M+CTG	64	51.56	71.714

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 primer P22) which was able to differentiate all the 28 populations of *Podophyllum* collected from the wild (Table 2). Further, the existing variations among 28 populations as observed through percentage of polymorphic band (PPB) were proved to be coupled with geographical altitude (r = 0.532) (Figure 1).

The 13 AFLP primer pairs used in this study generated a total of 7192 bands (an average of 553.23 bands per primer) out of which 4616 (mean = 355.08 per pair) were polymorphic across 28 regionally adapted *P. hexandrum* genotypes, along with a total of 2576 monomorphic bands (Table 1) (Figure 2). The total number of polymorphic loci is 466 thereby, giving an estimate of profound (>84.40 %) polymorphism (Table 4). Polymorphism varied substantially within the discrete groups of plants and the minimum was found to be 10.34 % (Lahual and Bharmaur forest divisions) whereas the maximum was 68.78 % (Kullu forest division) Table 3. The fragment size ranged from 20 bp to 750 bp. The mean coefficient of gene differentiation (Gst) was 0.51, indicating 26.0 % of the total genetic diversity within the populations. The total gene flow (Nm) between populations was found to be 0.24 (Table 4). The resolving power (Rp) of the 13 AFLP primers ranged from 17.071 (for primer combination E+TC/M+CTC) to 83.929 (for primer combination E+TC/M+CTG) which differentiated all the 28 populations of *Podophyllum* collected from the wild (Table 2). Further, the existing variations among 28 populations as observed through percentage of polymorphic band (PPB) were proved to be coupled with geographical altitude (Figure 1).

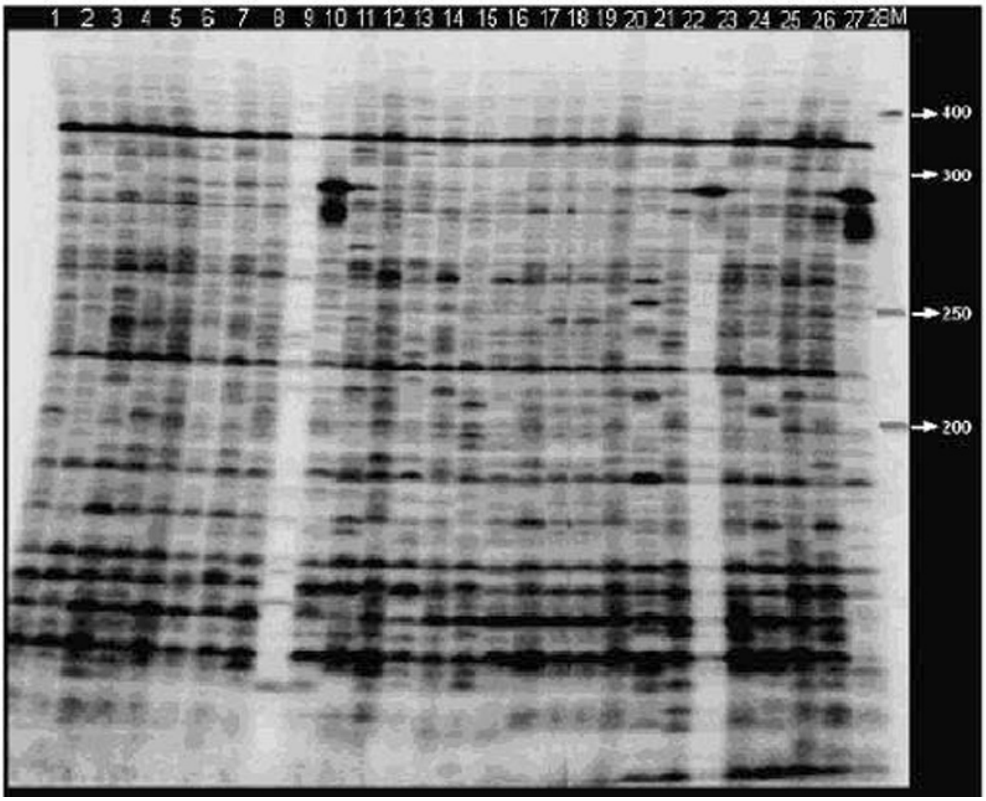

Fig. 2. Representative gel of AFLP amplification products obtained from the 28 genotypes of *P. hexandrum* studied using E+TC/M+CTG primer combination. 1. R/4 Kasol; 2. Twin Multivora; 3. Anganoala; 4. Brundhar; 5. Gulaba; 6. ChanderKhani; 7. Kaned Nursery; 8. Sanghar Nursery; 9. Madhvi Thach; 10. Kala Pani; 11. Jalora Pass (Sojha Nursery); 12. F (D-791-C1); 13. DPF (D-1892-C1); 14. DPGhar; 15. Myar Valley; 16. Nayan Jalora c-30(b); 17. Bada Bhangal; 18. Chota Bangal; 19. IHBT; 20. Bander Thach; 21. Saropa Nursery; 22. Nichar Nursery; 23. Rango (NC-8); 24. Sach Range; 25. Killer Range; 26. Purthi Range; 27. Ghoei DPF; 28. Samara RF. M = the size of molecular markers in base pairs using λ DNA.

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

Forest Division	Nei's Genetic Diversity (H) (mean ± SD)			Shannon's information index (I) (mean ± SD)			Percentage of polymorphic loci		
	RAPD	ISSR	AFLP	RAPD	ISSR	AFLP	RAPD	ISSR	AFLP
Parvati	0.03 (0.11)	0.08 (0.17)	0.15 (0.21)	0.04 (0.16)	0.11 (0.24)	0.22 (0.30)	33.33	17.65	33.94
Kullu	0.19 (0.22)	0.23 (0.20)	0.26 (0.19)	0.28 (0.31)	0.34 (0.29)	0.39 (0.27)	20.00	58.82	68.78
Dodrakwar	0.03 (0.13)	0.10 (0.20)	0.06 (0.16)	0.05 (0.18)	0.14 (0.28)	0.09 (0.22)	50.00	20.59	12.34
Seraj	0.18 (0.24)	0.12 (0.21)	0.11 (0.21)	0.25 (0.33)	0.16 (0.30)	0.15 (0.29)	49.67	23.53	22.32
Churah	0.08 (0.18)	0.06 (0.16)	0.08 (0.18)	0.11 (0.25)	0.08 (0.23)	0.11 (0.25)	50.00	11.76	15.61
Lahual	0.10 (0.20)	0.20 (0.25)	0.05 (0.15)	0.14 (0.28)	0.28 (0.34)	0.07 (0.21)	50.00	39.71	10.34
Palampur	0.16 (0.21)	0.09 (0.18)	0.13 (0.20)	0.23 (0.31)	0.12 (0.25)	0.18 (0.29)	33.33	19.12	28.31
Rampur	0.03 (0.11)	0.07 (0.17)	0.06 (0.16)	0.04 (0.16)	0.09 (0.24)	0.09 (0.23)	50.00	13.24	12.34
Kinnaur	0.04 (0.13)	0.03 (0.12)	0.11 (0.21)	0.05 (0.18)	0.04 (0.16)	0.15 (0.29)	50.00	5.88	21.60
Pangi	0.14 (0.21)	0.05 (0.14)	0.14 (0.21)	0.20 (0.30)	0.07 (0.21)	0.20 (0.30)	33.33	11.76	31.76
Bharmaur	0.02 (0.10)	0.06 (0.16)	0.05 (0.15)	0.03 (0.15)	0.08 (0.23)	0.07 (0.21)	50.00	11.76	10.34
Mean	**0.09**	**0.13**	**0.11**	**0.13**	**0.14**	**0.16**	**42.69**	**21.25**	**24.33**

Level of diversity and molecular variance

Heterozygosity and molecular variance were calculated using RAPD, ISSR and AFLP markers individually. Nei's gene diversity (H) value calculated for RAPD, ISSR and AFLP were 0.34 ± 0.15, 0.29 ± 0.17 and 0.26 ± 0.17 respectively which showed overall 9.0 % to 13.0 % heterozygosity among the populations of *P. hexandrum*. Similarly, the Shannon's information indices (I) were 0.50 ± 0.20, 0.44 ± 0.24 and 0.41 ± 0.23 for RAPD, ISSR and AFLP markers respectively showed gene diversity with an average of 0.45 among the forest divisions. The gene diversity (H) computed among different groups of populations was recorded in between 0.02 (Bharmaur forest division) – 0.19 (Kullu forest division), 0.03 (Kinnaur division) – 0.23 (Kullu forest division) and 0.05 (Bharmaur and Lahaul forest division) – 0.26 (kullu forest division) based on RAPD, ISSR and AFLP markers. The Shanon's information index (I) obtained among different groups of populations was recorded in the range of 0.03 (Bharmaur forest division) – 0.28 (Kullu forest division), 0.04 (Kinnaur division)

Table 4. Genetic variability estimated among 28 populations of *Podophyllum exandrum* using 3 different markers.

	Nei's gene diversity	Shannon's Information index	Hs	Gst	Estimate of gene flow (Nm) $\frac{0.25\ (1\text{-}Gst)}{Gst}$	Number of polymorphic loci	% of polymorphic loci
RAPD	0.34 (0.15)	0.50 (0.20)	0.10 (0.01)	0.69	0.11	121	92.37
ISSR	0.29 (0.17)	0.44 (0.24)	0.11 (0.01)	0.63	0.15	57	83.82
AFLP	0.26 (0.17)	0.41 (0.23)	0.13 (0.01)	0.51	0.24	466	84.40

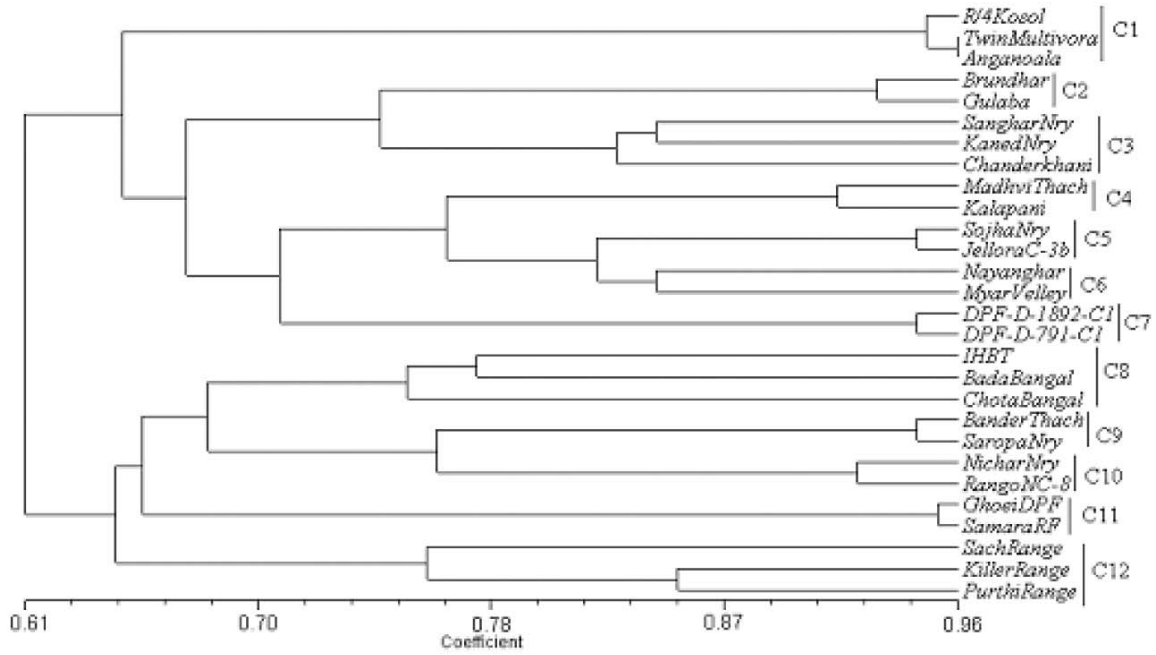

Fig. 3. Dendrogram illustrating genetic relationships among 28 populations generated by UPGMA cluster analysis calculated from 2257 RAPD bands produced by 19 primers.

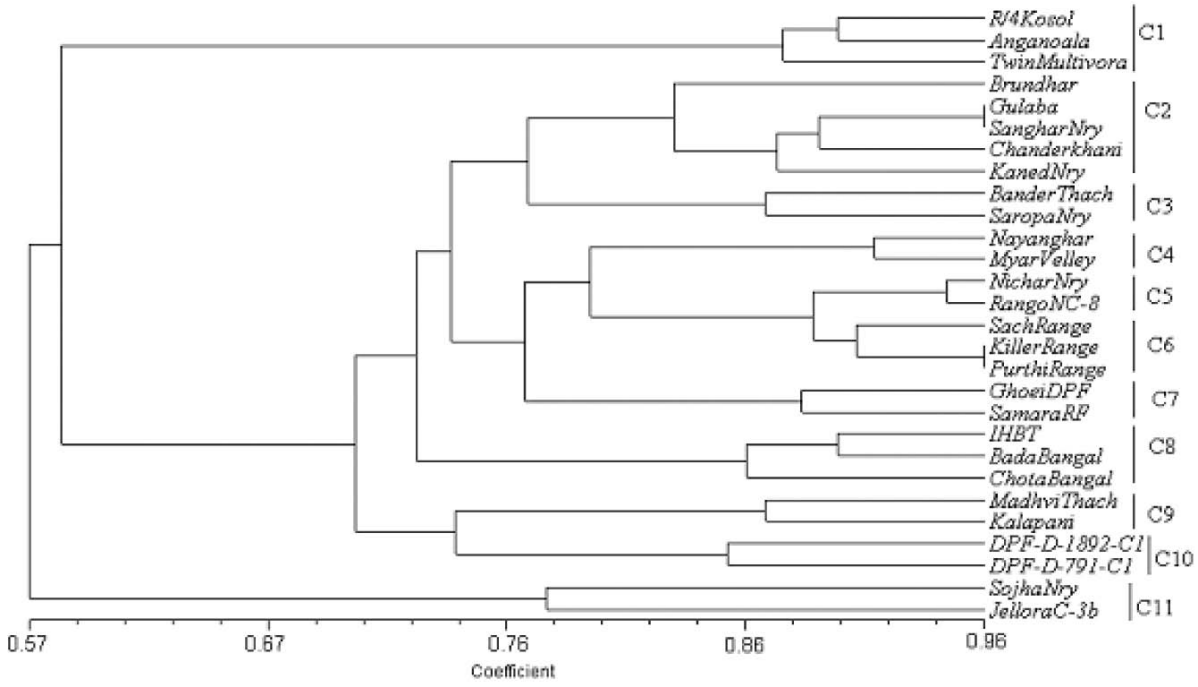

Fig. 4. Dendrogram illustrating genetic relationships among 28 populations in population diversity study generated by UPGMA cluster analysis calculated from 1303 ISSR bands produced by 11 primers.

– 0.34 (Kullu forest division) and 0.07 (Bharmaur and Lahaul forest division) – 0.39 (kullu forest division) using RAPD, ISSR and AFLP markers respectively. An average value of percentage of polymorphic loci among forest divisions were 42.69 %, 21.25 % and 24.33 % obtained from RAPD, ISSR and AFLP marker respectively (Table 3). More than half of the total variations in the studied populations: 53 % and 47 % could be accounted for by difference among the forest divisions and between populations within a forest division using RAPD marker (Table 5). The analysis of molecular variance indicated that over half of the total variation in the studied populations (62 %) could be accounted for by differences among the 11 forest divisions with a further 38 % being accounted for by the variation among populations within a forest division using ISSR marker (Table 5). Further the analysis of molecular variance based on AFLP marker revealed 64 % of variations among the 11 forest divisions and 36 % of variation among populations within a forest division (Table 5). All components of molecular variations were significant ($P < 0.001$).

Phylogenetic analysis

Genetic similarity was calculated from the Jaccard similarity index value for all the 28 accessions of *Podophyllum hexandrum* considering RAPD, ISSR, AFLP markers individually. Based on the RAPD marker, the similarity index values ranged from 0.61 to 0.96. These values were used to construct a dendrogram using UPGMA method. Populations from 11 forest divisions were clustered into region-specific groups with the exception of the Kullu forest division (Figure 3). Similarly, cluster analysis of ISSR data generated a dendrogram with 11 clusters (C1-C11). All the populations in each region clustered together having similarity co-efficient values ranging from 0.57 to 0.96 (Figure 4). Based on AFLP marker, the similarity index values ranged from 0.59 to 0.90. All the 28 *Podophyllum hexandrum* populations were distributed into 14 main clusters (C1-C14). Populations from 11 forest divisions were clustered into region-specific groups with the exception of Kullu forest division which is distributed in 4 different clusters C5, C7, C10 and C14 (Figure 5).

Based on the combined markers (RAPD + ISSR + AFLP), the similarity index value ranged from 0.62 to 0.91 and has been used to construct the dendrogram (Figure 6). All the 28 *Podophyllum hexandrum* populations were distributed into 14 main clusters (C1 – C14) and all 28 populations is clustered into region specific except Kullu forest division. Cluster C1 represents Parvati forest division with 3 different populations, R/4 Kasol, Twin Multivora and Anganola. The cluster C2 (Palampur forest division) and C3 (Churah) has 3 populations each- IHBT, Bada Bangal, Chota Bangal and (DPF-D-1892-C1 and DPF-D-791-C1 respectively. The cluster C6 (Rampur forest division) and C7 (Dodrakwar forest division) having genotypes Bander Thach, Saropa nursery and Madhvi Thach, Kala Pani respectively. Genotypes from Bharmaur and Pangi forest divisions were distributed within cluster – C8 and C9 having populations Ghoei DPF, Samara RFand Sach Range, Killer Range, Purthi Range population respectively. The clusters C11, C12, C13 comprise 6 populations each from forest division Lahaul (Myar Valley and Nayagarh), Kinnaur (Rango-NC-8 and Nichar nursery) and Seraj (Sojha nursery and Jellora C-3b), respectively. Genotypes of Kullu forest division scattered with 4 clusters C4, C5, C10, and C14 having Brundhar and Gulaba, Sanghar Nry, Kaned Nry and Chander Khani repectively. The results indicated high genetic diversity in *P. hexandrum* from Himachal Pradesh.

Table 5. Analysis of molecular variance (AMOVA) (levels of significance are based on 1000 iteration steps

	Source of variance	d.f	S.S.D.	Variance component	Percentage	P-value
RAPD	Among forest division	10	429.43	12.64	53	< 0.001
	Among populations within forest division	17	189.97	11.18	47	< 0.001
ISSR	Among regions	10	209.75	6.69	62	< 0.001
	Among populations within regions	17	70.50	4.15	38	< 0.001
AFLP	Among forest division	10	454.41	18.64	64	< 0.001
	Among genotypes within forest division	17	167.86	9.87	36	< 0.001

d.f.: degree of freedom; S.S.D.: sum of square deviation; P-value: probability of null distribution).

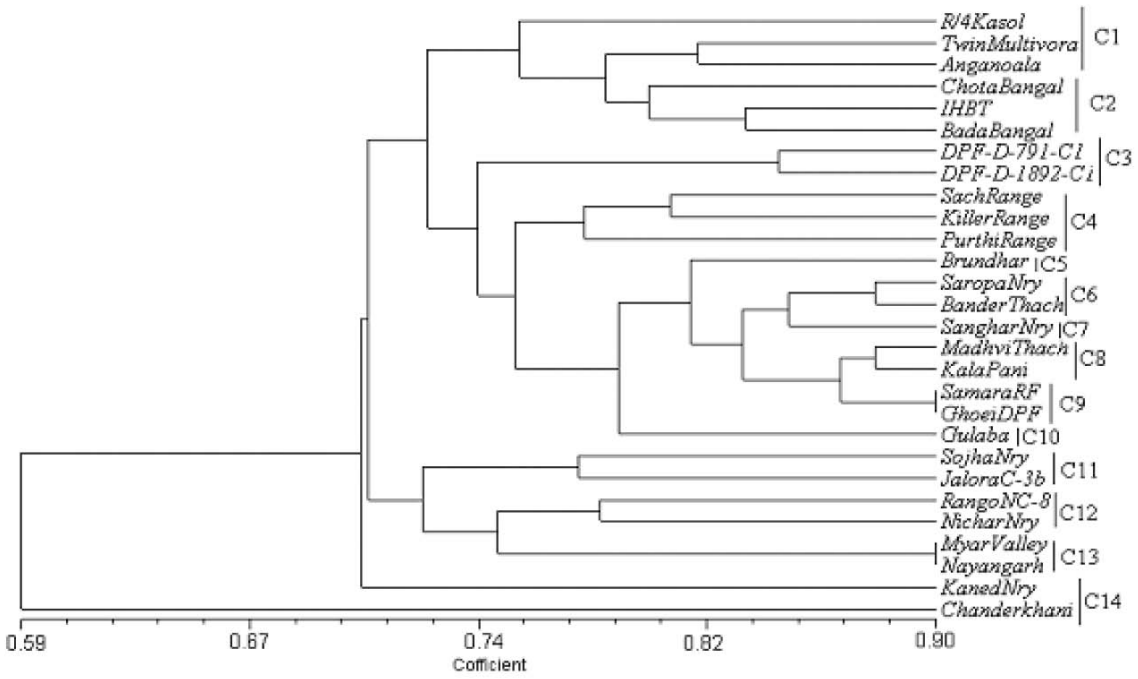

Fig. 5. Dendrogram illustrating genetic relationships among 28 populations in population diversity study generated by UPGMA cluster analysis calculated from 7192 AFLP bands produced by 13 primers.

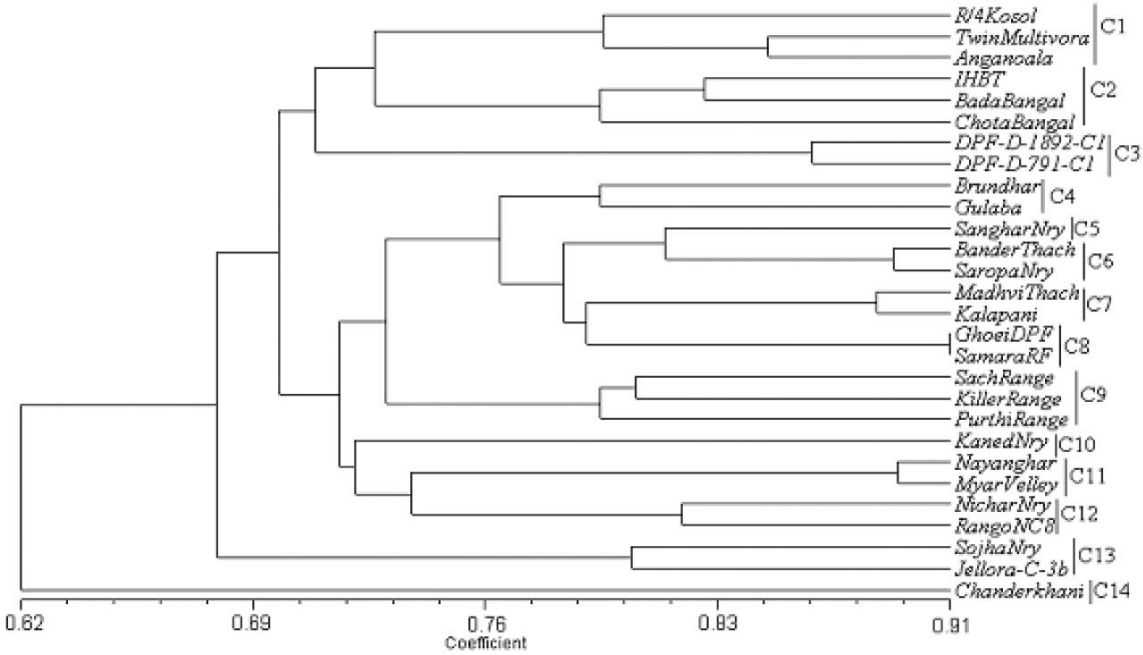

Fig. 6. Dendrogram illustrating genetic relationships among 28 populations in population diversity study generated by UPGMA cluster analysis calculated from 10752 RAPD, ISSR and AFLP bands produced by total 43 primers.

DISCUSSION

Variation in podophyllotoxin content

The variation in podophyllotoxin content was found to be related with altitude. However, it showed no significant correlation with genetic diversity index. The podophyllotoxin content was found to be maximum (8.857 to 9.533 % on dry weight basis) from the Lahaul forest division (altitude of 4300 m) in comparison to Parvati forest division (3.020 to 4.753 % on dry weight basis) at an altitude of 1300 m. The results have been supported by the study done by Sharma *et al*. (2000). They reported considerable reduction in the podophyllotoxin content in the roots of plants collected from higher altitude after growing them at lower altitude.

Genetic variations based on RAPD, ISSR and AFLP

In our studies a high percentage of polymorphic bands (PPB) were detected among the populations of *P. hexandrum.* The increase in genetic variations among the populations were correlated with log (PPB) and with log (altitude) using all the three molecular markers (r = 0.606 for RAPD; r = 0.532 for ISSR and r = 0.451 for AFLP). A similar finding was made by Fahima *et al*. (2002) who reported that microsatellite polymorphisms in natural populations of wild emmer wheat were best explained by variation of altitude and temperature in August.

In this study RAPD was found to be more efficient than the ISSR and AFLP as it detected 92.37 % polymorphic DNA markers in *P. hexandrum* as compared with 83.82 % and 84.40 % for ISSR and AFLP respectively. Such high level of percentage polymorphism >80 is also reported in ten population of two different habitat types of *R. acris* (Odat *et al*., 2004) by using all three markers RAPD, ISSR and AFLP. In our studies the low level of genetic variation within the forest division is in congruence with the study of Fu *et al*. (2004) on little blue stem (*Schizachyrium scoparium*), where AFLP revealed high (>91 %) inter-population and low (only 7.9 %) intra-population genetic diversity. The high proportion of polymorphic loci suggests that there is a high degree of genetic variation among the *Podophyllum* populations for all 3 markers. In population genetics, a value of 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 low gene flow among populations detected in this study point 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 many separate populations are targeted for conservation rather than conserving a few selected populations. AMOVA revealed that there was significant variation arising from habitat-correlated genetic difference which suggests that the effects of gene flow and genetic drift, local ecological conditions (altitude, temperature, rainfall, humidity, soil, pH etc) also play an important role in the variation of the genetic structure in the study of populations of *P. hexandrum*. In general, dispersal resulting in colonization and gene flow into existing populations is very important for both persistence and genetic success of a species (Hamrick & Godt, 1996).

The Rp value was calculated to be more than 5 times higher in AFLP than in RAPD and ISSR, which highlights the unique advantage of the high multiplex ratio provided by the AFLP technology. This confirmed the better discrimination power of AFLP. This superiority of AFLP among multilocus markers have been reported earlier in other species (Sharma *et al*., 1996; Russel *et al*., 1997; Barker *et al*., 1999; McGregor *et al*., 2000).

Cluster analysis by using RAPD, ISSR, AFLP and combination of RAPD+ISSR+AFLP revealed the same pattern of distribution of different populations with respect to their forest divisions. This indicated that gene flow in the studied populations of *P. hexandrum* occurred mainly within the same forest division rather than between divisions. Moreover, RAPD, ISSR and AFLP markers used here were able to differentiate *P. hexandrum* populations collected from 11 forest divisions into distinct region specific clusters except the Kullu forest division. The region-specific cluster in our studies can be used for the authentication of this medicinal herb as the biochemical properties of the pharmaceutical active ingredients is known to be influenced by the geographic region. Wang *et al*. (2003) have already exploited RAPD for confirming the genuineness of *Bupleurum Chinese.* Na *et al*. (2004) have detected three RAPD primers which can discriminate between Korean and Chinese *Astragali radix*. 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*.

CONCLUSION

Based on polymorphic feature, genetic diversity, genetic similarity and gene flow among the populations of *Podophyllum* based on RAPD, ISSR and AFLP study, we conclude that all markers proved to be efficient tools in assessing the genetic diversity of *Pododophyllum* genotype and 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. This species or at least a large part of its genetic diversity may be lost in the near future owing to its importance and its exploitation as a medicinal plant if appropriate measures for conservation are not adopted. At present, the rate of its exploitation of *P. hexandrum* is much more than the rate of propagation. Since no single or even a few plants represent the whole genetic variability of *P. hexandrum*, there appears to be a need to maintain sufficiently large populations in natural habitats to conserve the genetic diversity and avoid genetic erosion. For conservation aspect, only a few populations may not adequately protect the genetic variations within the species in the Himalayan region. Genetic variations among elite genotypes of *Podophyllum hexandrum* based on RAPD, ISSR and AFLP analysis could be useful in selecting parents to be crossed for generating appropriate populations intended for both genome mapping and breeding purposes.

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REFERENCE

Ajmone, Marsan P, Castiglioni P, Fusari F, Kuiper M and Motto M (1998). Genetic diversity and its relationship to hybrid performance in maize as revealed by RFLP and AFLP markers. *Theor Appl Genet*. 96: 219-227.

Alam MA, Pallavi G, Gulati AK, Mishra GP and Naik PK (2008). Characterization of genetic structure of *Podophyllum hexandrum* populations–an endangered medicinal herb of Northwestern Himalaya using ISSR-PCR markers and its relatedness with podophyllotoxin content. *African journal of Biotechnology.* 7(8): 1028-1040.

Anon (1970). *Podophyllum hexandrum*. The wealth of India: A Dictionary of Indian Raw Material and industrial products: Raw Materials. Vol. VIII: Ph.Re. Publication and Information Directorate. CSIR, New Delhi. pp. 170-174.

Barker JHA, Matthes M, Arnold GM, Edwards KJ, Ahman I, Larsson S and Karp A (1999). Characterization of genetic diversity in potential biomass willows (*Salix spp*.) by RAPD and AFLP analyses. *Genome* 42: 173-183.

Broomhead AJ and Dewick PM (1990). Tumor inhibitory aryltralin lignans in *Podophyllum versipelle, Diphyllcia cymosa* and *Diphyllicia grayi*. *Phytochemistry* 29: 3831-3837.

Chatterjee R (1952). Indian Podophyllum. *Econ. Bot.* (Indian). 6: 342-354.

De Knijff P, Denkers F, van Swelm ND, and Kuiper M (2001). Genetic affinities within the herring gull *Larus argentatus* assemblage revealed by AFLP genotyping. *J. Mol. Evol.* 52: 85–93.

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

Fahima T, Roder MS, Wendehake K, Kirzhner VM and Nevo E (2002). Microsatellite polymorphism in natural population of wild emmer wheat, *Trititum dicoccoides* in Israel. *Theor. Appl. Gent.* 104: 17-29.

Fay DA and Ziegler HW (1985). Botanical source differentiation of *Podophyllum* resin by high performance liquid chromatography. *J liq Chromatogr* 8: 1501-1506.

Felsenstein J (1995). Phylogenies from restriction sites, a maximum likelihood approach. *Evolution*. 46: 557-574.

Fritsch PW and Rieseberg LH (1996). The use of random amplified polymorphic DNA (RAPD) in conservation genetics. *In* T. Smith and B. Wayne (eds.), *Molecular Genetic Approaches in Conservation*, pp. 54–73. Oxford Univ. Press, New York.

Fu L-G (1992). *Plant Red Book of China: Rare Threatened Plants*, Science Press, Beijing, pp. 184–185.

Fu YB, Phan AT, Coulman B and Richards KW (2004). Genetic diversity in natural populations and corresponding seed collections of little bluestem as revealed by AFLP markers. *Crop Sci.* 44: 2254-2260.

Hamrick JL and Godt MJW (1996). Conservation genetics of endemic plant species. Pp. 281-304. In Avise JC and JL Hamrick (eds). *Conservation Genetics*, Chapman and Hall, New York.

Han TH, Jeu MD, Eck HV and Jacobsen E (2000). Genetic diversity of Chilean and Brazilian *Alstroemeria* species assessed by AFLP anaslysis. *Heredity*. 84: 564-569.

Imbert TF (1998). Discovery of podophyllotoxins. *Biochimie* 80: 207-22.

Janssen P, Coopman R, Huys G, Swings J, Bleeker M, Vos P, Zabeau M and Kersters K (1996). Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiol.* 142: 1881-1893.

Kazan K, Manners JM and Cameron DF (1993). Genetic variation in agronomically important species of *Stylosanthes* determine using random amplified polymorphic DNA markers. *Theor. Appl. Genet.* 85: 882-288.

McGregor CE, Lambert CA, Greyliing MM, Louw JH and Warnich L (2000). A comparative assessment of genetic diversity in dent and pocorn (*Zea mays)* inbred lines using inter-simple sequence repeat (ISSR) amplification. *Mol. Breed.* 1: 365-373.

Myburg AA, O'Malley D, Sederoff RR and Whetten R (2000). Highthroughput multiplexed AFLP analysis of interspecific hybrids of *Eucalyptus* trees species. Plant & Animal Genome VIII conference, San Diego, CA, p 544.

Na HJ, Um JY, Kim SC, Koh KH, Hwang WJ, Lee KM, Kim CH and Kim HM (2004). Molecular discrimination of medicinal Astragali radix by RAPD analysis. *Immunopharmacol Immunotoxicol* 26: 265–272

Odat N, Jetschke G and Hellwig FH (2004). Genetic diversity of *Ranunculus acris* L. (Ranunculaceae) populations in relation to species diversity and habitat type in grassland communities. *Mol. Breed*. 2: 225-238.

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

Russel JR, Fuller JD, Macaulay M, Hatz BGB, Jahoor A, Powell W and Waugh R (1997). Direct comparison of level of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. *Theor. Appl. Genet.* 95: 714-722.

Saghai-Maroof MA, Soliman KM, Jorgensen RA and Allard RW (1984). Ribosomal spacer length in barley: Mendelian inheritance, Chromosomal location and population dynamics. *Proc. Natl. Acad. Sci. (USA)* 81: 8104-8118.

Sharma SK, Knox MR and Ellis THN (1996). AFLP analysis of the diversity and phylogeny of *Lens* and its comparison with RAPDanalysis. *Theoretical and Applied Genetics* 93(5-6): 751-758.

Sharma TR, Singh BM, Sharma NR and Chauhan RS (2000). Identificatin of high podophyllotoxin producing biotypes of Podophyllum hexandrum form north-western Himalaya. *J. Plan Biochem. & Biotech*. 9: 49-51.

Slatin M (1987). Gene flow and geographic structure of natural populations. *Science (Washington, DC)* 236: 787-792.

Sneath PHA and Sokal K (1973). *Numerical Taxonomy*, pp. 100-308.

Stahelin HF and Wartburg AV (1991). The chemical and biological route form podophyllotoxin glucoside to etoposide: Ninth Cain Memorail Award Lecture. *Cancer Res.* 51: 5-15.

Vos PR, Hogers M, Bleeker M, van de Lee Reijans T, Hornes M, Fritjers A, Pot J, Peleman J, Kuiper M and Zabeau M (1995). AFLP: a new concept for DNA fingerprinting. *Nucleic Acids Res*. 23: 4407–4414.

Wang X, Li Y, Li H, Zhang Y, Zhao L and Yu Y (2003). RAPD analysis of genuineness on source of Bupleurum chinense. *Zhong Yao Cai* 26: 855–856

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

Ying TS (1979). On *Dysosma*Woodson and *Sinopodophyllum* Ying, gen. nov. of the Berberidaceae. *Acta Phytoxon. Sin.* 17: 15–23.

Zietkiewicz E, Rafalski A and Labuda D (1994).Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20: 176-183.