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Genetic Diversity of Indian Liverwort *Plagiochasma appendiculatum* Revealed by RAPD Marker

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Abstract: Genetic diversity of the Monoceious thalloid liverwort *Plagiochasma appendiculatum* was investigated by Rapid Amplified Polymorphic DNA (RAPD) analysis. The species is explained and demonstrated with its genetic diversity on the basis of morphological variations. Samples were collected from different parts of India growing on different habitat at variable altitude. After the study of its morphology, it has been observed that the population of this taxon shows significant variation in plant size, shape, colour, ventral scales, appendages of scales, rhizoids, position of male and female receptacles etc. Based on such morphological variations, we have used the RAPD marker to estimate the genetic diversity within and between the populations. Approximately 75% of the variations have been observed within and between genotypes of *P. appendiculatum* as revealed with both phenotypic and genotypic data. The RAPD markers are being used increasingly to analyze the phylogenetic relationship among the liverworts to give the exact framework of taxonomic identification of naturally occurring liverwort *P. appendiculatum.*

Key words: Genetic diversity, RAPD marker, morphological variation, *Plagiochasma appendiculatum*

INTRODUCTION

Plagiochasma appendiculatum is one of the important Indian liverwort belongs to the order Marchantiales under family Aytoniaceae. Lehmann and Lindenberg (1832) first instituted the species. *Plagiochasma* is a thalloid liverwort represented by 30 species (Bischler, 1978), but in India only 10 species have been reported, viz., *Plagiochasma appendiculatum* Lehm. et Lindb, *Plagiochasma articulatum* Kash., *Plagiochasma. bicornutum* Steph., *Plagiochasma cordatum* Lehm. and Lindb, *Plagiochasma cordotii* Steph., *Plagiochasma intermedium* L. et Gott., *Plagiochasma martensii* Steph., *Plagiochasma nepalensis* Steph., *Plagiochasma pauriana* Udar et Chandra and *Plagiochasma quadricornutum* Steph. (Parihar *et al*., 1994). Out of these taxa *P. appendiculatum* abundantly grows in India.

Plagiochasma appendiculatum is widely distributed in western, eastern Himalayas, central India and south India and generally grow to an altitude upto 8000 ft from sea level. This species in known from the east part of the central and south African continent Eritrea, Ethiopia, Kenya, Tanzania to Rhodesia, Zimbabwe and South Africa (Perold, 1999;

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Wigginton, 2002). Frey and Kürschner (1988) reported that in Asia it is widespread and ranging from the southwest of the Arabian Peninsula and Socotra Islant to the southern part of the Himalayas, Formosa, Philippines and Celebes (Bischler, 1979). The species grow in xeric and mesic conditions in all continents, they are absent in areas of equatorial and continental climate (Bischler, 1978, 1979). Mahabale and Bhate (1945) noted that it is a monoecious plant and usually grows in moist places, on rocks surface, soil covered rocks, walls of old buildings and show extra ordinary regeneration. Besides this, *P. appendiculatum* also represents the maximum xerophytic habit and can grow on comparatively naked and exposed rocks (Kachroo, 1954). Ghates and Chapekar (2000) reported that *P. appendiculatum* could be used as a biotest for water quality assessment. *Plagiochasma appendiculatum* is significant taxon which possesses antimicrobial property (Banerjee, 2000). In India, it is used by Gaddi tribes in Himachal Pradesh for the treatment of cuts, wounds and burns (Kumar *et al*., 2000; Singh *et al*., 2006).

After the study of morphological variation in *P. appendiculatum*, we have used the Random Amplified Polymorphic DNA (RAPD) markers to investigate the genetic diversity from all the different geographical regions. Adams and Demeke (1993) reported that RAPD is a powerful technique for systematic investigation and for analyzing polymorphism among and within taxa. Boisselier-Dubayle and Bischler (1994) studied the phylogeny of liverworts and solving out the taxonomical problems at the genetic level viz. in *Porella canariensis.* Freitas and Breham (2001) have been reported the existence of genetic variations and close relationship between and within the different populations of *Porella canariensis*. Similarly, in case of three subspecies of *Marchantia polymorpha,* isozymes, RFLP and RAPD markers were used to characterize the genetic variation. All these markers support the taxonomic distinctness of the subspecies from different ecological niches and subsequent reproductive isolation (Boisselier-Dubayle *et al*., 1995). As well as genetic variation in *Riccia dictyospora* (Ricciaceae) were detected to show the phylogenetic relationship within and between populations (Dewey, 1989). Similarly, Odrzykoski and Szweykowski (1991) reported that the thalloid liverwort *Conocephalum conicum*, genetic differentiations were detected to show the association between different populations etc.

Many different taxonomic treatments of the bryophyte can be found in classified systematic literature (Schuster, 1984; Frahm and Frey, 1992; Fukarke *et al*., 1992; Walther, 1983). In this preliminary survey of the Indian representatives of *P. appendiculatum*, the morphological characteristics and RAPD banding patterns were analyzed to estimate genetic variation within and among populations of different geographical regions.

MATERIALS AND METHODS

Study conduct: The study was carried out from 27th June 2003 to 30th October 2006 in plant genomic lab, National Botanical Research Institute (NBRI), Lucknow, India.

Plant Material

The samples of *P. appendiculatum* were collected from various localities of the country: Western Himalaya (Kilbury, Jeolikot, Thandi Sadak from Nainital, Kalika Forest, Chaubatia Road, Hotel Parvati Inn. from Ranikhet, Sahastradhara, Dehradun, Library road, Mussoorie), eastern Himalaya, (Bishop's house, Darjeeling) and from central India (Kewanchi, Achanakmar; – Chattisgarh). Voucher specimens have been deposited in NBRI Bryophyte Herbarium, Lucknow (LWG) India. Each population has been identified by their characteristic features through literature and authentic specimens available in the NBRI Herbarium.

Morphology Study

Thalli of *Plagiochasma appendiculatum* is characterized by large, purplish green patches, thick, 20 mm long and 5 mm wide, dichotomously branched and occasionally with adventitious shoots. Lobes oblong, dorsal surface smooth margins undulate. Midrib distinct passing into lamina, ventral surface purple. Scales in one row on each side of the midrib purple, broadly lunate, body with 1 or 2 appendages reaches half way to the margins, appendages large, usually hyaline, fan shaped, entire and occasionally purple. Male receptacle horse-shoe shaped sometimes scattered on the thallus or in acropetal order. Female receptacle sessile or stalked, usually with 2-5 lobes, situated on the thallus in row or scattered or some time at the basal part of the plant. Spores yellowish brown and elaters bispirate. This species is mainly separated from the other species on the basis of its scale structure which has typical from shaped on broad lunate appendages.

Plant DNA Preparation

Fresh and matured thalli of *P. appendiculatum* have been used to isolate the genomic DNA. All samples were carefully washed and checked under the microscope to be sure that no possible contaminations (microalgae, fungi) were left. Doyle and Doyle (1988) proposed that DNA was extracted from 1 g of plant material with some modification of several standard protocols (Williams *et al*., 1990). Fresh material was crushed in liquid nitrogen and mixed with modified CTAB extraction buffer. Add 1% β -mercaptoethanol, 2% proteinase K (20 mg mL^{-1}) and 100 mg PVP (Sigma) and then incubated in a water bath at 68°C for 5 h, add Chloroform: Isoamyl alcohol (24: 1) to this mixture, followed by centrifugation for 10 min at 12,000 rpm. The supernatant was transferred to a corex tube, add 0.7 Vol. iso-propanol, mixed well, store in -20°C for 5 h to precipitate the DNA. Centrifuge 10 min at 12,000 rpm. Supernatant was discarded and the pellet washed with a solution of 70% ethanol. Tubes were incubated at 37°C for 15 min and resuspended the pellet in TE buffer. Purify the DNA followed by phenol/chloroform method, then precipitate and washing as before. Finally the aqueous phase was discarded and the pellet was dried for 15 min in incubator at 37°C. Resuspended the pellet in 100 µL TE buffer. This procedure recovered at least 800 ng μL^{-1} genomic DNA which is good quality for RAPD analysis.

Primers and PCR

Decamer primers from OPERON Technologies (USA) were screened on individual representative of the populations under study. Many of the primer produced either complex banding pattern of non-reproducible and inconsistent amplification products. Hence only 45 primers scored good result out of 60 primers used for the subsequent analysis (Table 2). Reproducibility of bands was assessed by replicating extraction of DNA and amplifications of selected samples. Polymorase Chain Reaction (PCR) was carried out in 20 µL volumes using DNA, dNTPs (2 mM of each of four nucleotides: Fermentas), 10X Taq Buffer, 5 pmol primer, 1 unit Taq DNA polymerase (Bangalore Genei). The PCR conditions were initiated at 92°C followed by 44 cycles of denaturation at 92°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 1:30 min, followed by the final extension of 5 min. Amplified products were separated in 1.4% agarose gel, stained with ethidium bromide and visualized under ultraviolet (UV) light. The image of gel was taken by multiimager TM 3400 (Alpha Innotech Co.).

Data Analysis

The RAPD profiles were scored for each individual as discrete characters (presence or absence of amplified products) across all individuals from all populations and for each primer

used. Sneath and Sokal (1973) proposed that relationships among and between the populations were evaluated via the unweighted pair-group method UPGMA and all analysis was performed by using NTSYS (Rohlf, 1993). Researchers studied the genetic diversity in blackgram by Jaccard's coefficient calculated by using SIMQUAL program, a common estimator of genetic identity and was calculated as follows:

$$\text{Jaccard's coefficient} = N_{AB} / (N_{AB} + N_A + N_B)$$

where, N_{AB} is the number of bands shared by samples, N_A represents fragments in sample B. Similarity matrices based on these indices were calculated. Similarity matrices were utilized to construct the UPGMA (unweighted pair group method to construct arithmetic average) dendrogram. Yap and Nelson (1996) establish a statistical stability of the branches in the cluster was estimated by bootstrap analysis with 1,000 replicates, using the winboot software program.

RESULTS AND DISCUSSION

Out of 60 primers used, 45 decamer primers amplified 623 RAPD bands in the 22 populations of *P. appendiculatum* collected from different geographical sites (Fig. 1). The number of polymorphic sites varied considerably among the population studied (Table 1). Total genetic diversity estimated as the ratio of polymorphic sites within a population to the numbers of individuals. A substantial amount of variation in RAPD profiles of *P. appendiculatum* was found within and among populations (Fig. 2a, b). Similar results have been found using its morphological studies. The RAPD analysis revealed two identical accessions (Plant No. 14-15) found within colony of Deharadun indicated that very less differences observed might correspond to different individuals sampled from the same patch. There are some minor variation may be due to environmental factor through they belong to same habitat, locality etc. and show identical RAPD banding pattern.

Cluster Analysis Based on RAPD

Total genetic diversity estimated as the ratio of polymorphic sites within a population to the numbers of individuals. A dendrogram based on UPGMA (Fig. 3) analysis for 22 genotypes of *P. appendiculatum* were clustered according to the geographical regions with Jaccard's similarity coefficient ranged from 0.099 to 0.741 (Table 2).

Cluster I contain 14 genotypes that were further clustered based on their collection sites (Fig. 3). Genotype 11 and 12 formed a separate OUTs with similarity coefficient of 0.332. Genotype 22 and 17 also formed a separate out-group but close to each other with 0.308 similarity coefficient. Population of Ranikhet (R_1) comprised four genotypes (6, 7, 9 and 10). Genotype 6 and 7 and 9 and 10 appeared to be close to each other with similarity coefficient 0.47 and 0.492, respectively. Similarly, group of Deharadun (D_1) comprised four genotypes (13, 14, 15 and 16). Genotype 14 and 15 appeared to be identical with similarity coefficient of 0.741 indicates that these individuals sampled from the same patch. Genotype 13 and 16 formed a separate OUTs but appeared to be close to genotype 14 and 15 with similarity coefficient of 0.729 and 0.652, respectively. Genotype 4 (N_2) appeared to close to genotype 8 (R_3) with similarity coefficient of 0.55 and genotype 5 (N_3) formed a separate OUTs but close to R_3 with 0.531 similarity coefficient (Fig. 3).

Genotype 18 collected from Mussoriee that formed a distinct group in dendrogram according to their different morphological and geographical features (Table 1). Genotypes

Table 1: Morphological data of Plagiochasma appendiculatum showing variations from different geographical regions of India

LWG No.	Group code	Locality	Habitat	Altitude	Characteristic features	Position of ♂ receptacles	Position of ♀ receptacles	Spores and elaters
Nainital 225562	N_1	Kilburry	On stony wall	Ca 8100 ft	25-32 mm long and 5-10 mm Dark green, margin slightly wavy, rhizoids simple and tuberculate	Horse-shoe shaped, below to female receptacles 2-3 together found	Stalked, 1-3 mm long stalk, 3 lobed female receptacles.	Spores- light yellow Elaters- bispirate
225596	N_2	Jeolikot	On soil	Ca 6000 ft	18-20 mm long and 9-12 mm broad Thallus dark green, margin highly wavy, rhizoids simple and tuberculate	Not present	Immatured in developing stage, situated on apex of thallus	Not present
226869		Thandi Sadak	On soil	Ca 6000 ft	15-20 mm long and 8-11 mm broad Thallus light green, margin highly wavy, rhizoids simple and tuberculate	Absent	Both stalked and sessile but dehished	Absent
226877		Jeolikot	On soil	Ca 6000 ft	15-19 mm long and 7-10 mm broad Thallus light green, margin highly wavy, rhizoids simple and tuberculate	1-3 on one thallus	Sessile bi-trilobed	Spores-yellow elaters-bispirate
226880	N_3	Jeolikot	On stony wall	Ca 6000 ft	10-25 mm long and 5-10 mm broad Thallus dark green, margin highly wavy, rhizoids simple and tuberculate	Absent	Sessile, 3-4 presenton the thallus bi-penta lobed	Spores- dark yellowish brown and elaters bispirate.
Ranikhet 226358	R_1	Hotel Parvati Inm	On soil covered rock	Ca 5200 ft	15-22 mm long and 8-11 mm broad Thallus dark green, margin slightly wavy, rhizoids simple and tuberculate	Absent	Sessile, tri lobed	Spores-dark yellow bispirate elaters
226848		Hotel Parvati Inm	On soil covered rock	Ca 5200 ft	15-22 mm long and 8-11 mm broad Thallus dark green, margin slightly wavy, rhizoids simple and tuberculate	Absent	Stalked, bilobed, dark brown	Dark brown spores bispirate
226850	R_2	Chaubatia road	On soil covered rock	Ca 5500 ft	30-35mm long and 12-17 mm broad Thallus dark green, margin highly wavy, rhizoids tuberculate are more in no.	Absent	Not fertile	Absent
226853	R_1	Chaubatia road	On soil covered rock	Ca 5500 ft	15-25 mm long and 8-15 mm broad Thallus dark green, margin slightly wavy, rhizoid simple and tuberculate	Absent	Trilobed with 2-3 mm long stalk, dishes	Absent
226854		Chaubatia road	Soil covered rock	Ca 5500 ft	16-23 mm long and 8-14 mm broad Thallus dark green, margin slightly wavy, rhizoid simple and tuberculate	Arranged in acropetal order	Trilobed, sessile and green	Yellow sores, bispirate elaters few are branched

Table 1 : Continued

LWG No.	Group code	Locality	Habitat	Altitude	Characteristic features	Position of ♂ receptacles	Position of ♀ receptacles	Spores and elaters
226852	R_2	Chaubatia road	Soil covered rock	Ca 5000 ft	12-20 mm long and 8-12 mm broad Thallus light green, margin highly wavy, rhizoids simple and tuberculate	Absent	1 cm long stalk, plenty of female receptacles on single thallus, bi-penta lobed	Deep brown spores, bispirate elaters
226868		Chaubatia road	On rock	Ca 5500 ft	10-18 mm long and 5-10 mm broad Thallus light green, margin highly wavy, rhizoids simple and tuberculate	Absent	Stalked and sessile both dehised	Absent
Dehradun 226334	D_1	Sahastradhara	On soil covered rocks	Ca 1000 ft	19-22 mm long and 8-10 mm broad Thallus dark green, margin slightly wavy, rhizoids only tuberculate	Absent	Not present	Absent
226335		Sahastradhara	On soil covered rocks	Ca 1000 ft	18-23 mm long and 8-11 mm broad Thallus dark green, margin slightly wavy, rhizoids only tuberculate.	Absent	Not present	Absent
226336		Sahastradhara	On soil covered rocks	Ca 1000 ft	15-28 mm long and 5-12 mm broad Thallus dark green, margin slightly wavy, rhizoids only tuberculate	Absent	Not present	Absent
226337		Sahastradhara	On soil covered rocks	Ca 1000 ft	14-28 mm long and 4-13 mm broad Thallus dark green, margin highly wavy, rhizoids simple and tuberculate	Absent	Not present	Absent
226338	D_2	Sahastradhara	On rocks	Ca 1000 ft	19-32 mm long and 7-15 mm broad Thallus dark green, margin highly wavy, rhizoids simple and tuberculate	Absent	Not present	Absent
Mussoorie 226344	-	Library Road	On soil	Ca7000 ft covered rocks	34-36 mm long and 16-18 mm broad Thallus Comparatively very large and light green, margin highly wavy, rhizoids simple and tuberculate	Absent	Not present	Absent
Darjeeling 225531	J_1	Bishop's house	On rocks	Ca 7042 ft	32-36 mm long and 16-18 mm broad Thallus large, light green margin slightly wavy, rhizoids simple and tuberculate	Absent	Situated on the center and lower portion thallus, sessile, 3-4 lobed	Spores light yellow

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Table 1: Continued

LWG No.	Group code	locality	Habitat	Altitude	Characteristic features	Position of ♂ receptacles	Position of ♀ receptacles	Spores and elaters
225532	J_2	Bishop's house	On stony wall	Ca 7042 ft	24-32 mm long and 5-10 mm broad Thallus small, dark green, Margin, highly wavy, rhizoids simple and tuberculate	Absent	Found on the midrib of the thallus with a very small stalk 5-lobed	Spores dark yellow elaters bispirate
Amarkantak 226211	-	Chowatiya road	On soil	Ca 1600 ft	20-22 mm long and 8-10 mm broad Thallus dark green, margin highly wavy, rhizoids simple and tuberculate	Absent	A small stalk, trilobed	Dark yellow bispirate
Ranikhet 226849	R_2	Hotel Parvati inn.	On soil covered rocks	Ca 5200 ft	15-28 mm long and 3-10 mm broad Thallus light green, margin highly wavy, rhizoids simple and tuberculate	Absent	Absent	Absent

Table 2: Jaccard's similarity coefficient between 22 accessions of *P. appendiculatum* collected from various geographical regions of India

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	1																					
2	0.163	1																				
3	0.200	0.192	1																			
4	0.124	0.250	0.340	1																		
5	0.165	0.260	0.396	0.561	1																	
6	0.185	0.250	0.323	0.455	0.464	1																
7	0.189	0.232	0.346	0.465	0.461	0.470	1															
8	0.168	0.269	0.347	0.550	0.531	0.509	0.511	1														
9	0.179	0.277	0.407	0.452	0.484	0.450	0.454	0.522	1													
10	0.174	0.280	0.353	0.417	0.474	0.432	0.449	0.493	0.492	1												
11	0.196	0.225	0.319	0.268	0.325	0.293	0.297	0.306	0.366	0.349	1											
12	0.181	0.255	0.302	0.344	0.390	0.388	0.356	0.397	0.413	0.404	0.393	1										
13	0.202	0.280	0.326	0.436	0.481	0.427	0.456	0.507	0.456	0.396	0.283	0.356	1									
14	0.163	0.255	0.336	0.491	0.506	0.452	0.468	0.556	0.480	0.402	0.307	0.351	0.729	1								
15	0.150	0.235	0.380	0.504	0.525	0.441	0.475	0.555	0.474	0.409	0.290	0.348	0.628	0.741	1							
16	0.147	0.238	0.365	0.461	0.470	0.414	0.476	0.509	0.415	0.412	0.277	0.342	0.591	0.623	0.652	1						
17	0.191	0.243	0.296	0.324	0.339	0.328	0.358	0.365	0.345	0.355	0.292	0.289	0.376	0.376	0.389	0.416	1					
18	0.189	0.204	0.262	0.213	0.242	0.207	0.261	0.236	0.262	0.219	0.233	0.203	0.204	0.200	0.224	0.222	0.254	1				
19	0.169	0.294	0.280	0.389	0.358	0.320	0.337	0.362	0.368	0.348	0.264	0.333	0.337	0.323	0.316	0.333	0.310	0.236	1			
20	0.269	0.135	0.117	0.115	0.132	0.134	0.145	0.132	0.101	0.118	0.127	0.121	0.143	0.113	0.105	0.105	0.123	0.099	0.133	1		
21	0.195	0.186	0.223	0.211	0.253	0.209	0.226	0.241	0.206	0.262	0.242	0.253	0.248	0.229	0.228	0.236	0.231	0.157	0.199	0.206	1	
22	0.183	0.270	0.305	0.377	0.408	0.372	0.374	0.433	0.361	0.382	0.268	0.318	0.363	0.390	0.376	0.371	0.308	0.229	0.364	0.170	0.258	1

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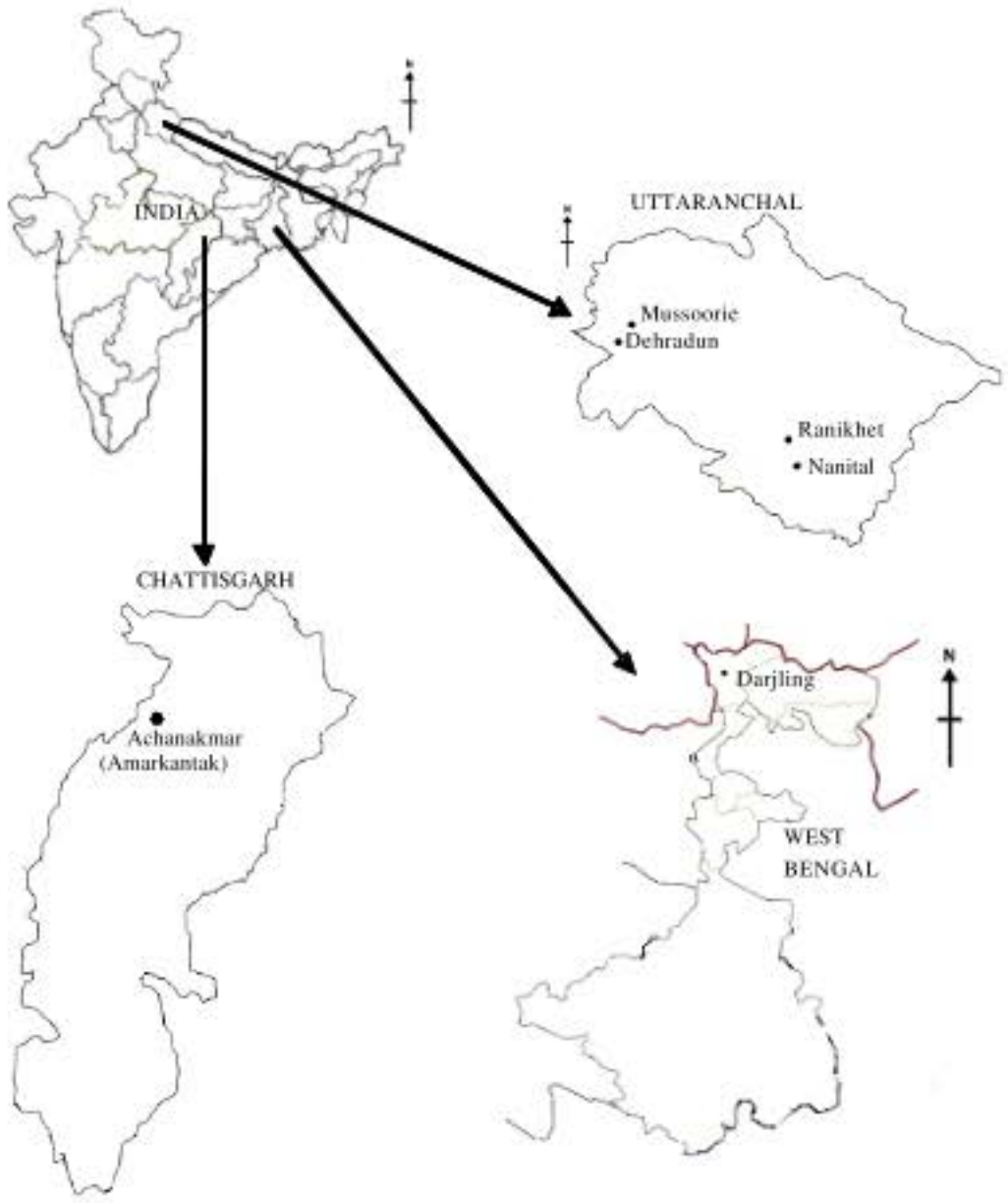

Fig. 1: Collection sites of *P. appendiculatum* from different places in India

19 (J_1) and 20 (J_2) of Darjling were separated to each other in dendrogram according to their different habitat and morphological feature. J_2 and N_1 appeared to be close to each other with 0.269 similarity coefficient (Table 2).

The RAPD profiles revealed a structuring of populations completely congruent with their geographical location as well as their morphological data. High levels of genetic variation have been found in bryophytes (Wyatt *et al*., 1989) based on their microhabitats. In fact, the variance among regions is smaller than that among populations within single regions. Geographical isolation appears to be one of the more important factors affecting genetic structure in *P. appendiculatum*. These markers, assumed selectively neutral, indicate that historical effects, drift and/or migration would cause any pattern of variation found between different populations. This way, variation within populations must be determined by the mating system of the liverwort. The genetic relationships among the population studied, partly covering the species distribution range, will be addressed which may offer insights into the spreading and immigration history of *P. appendiculatum* in India.

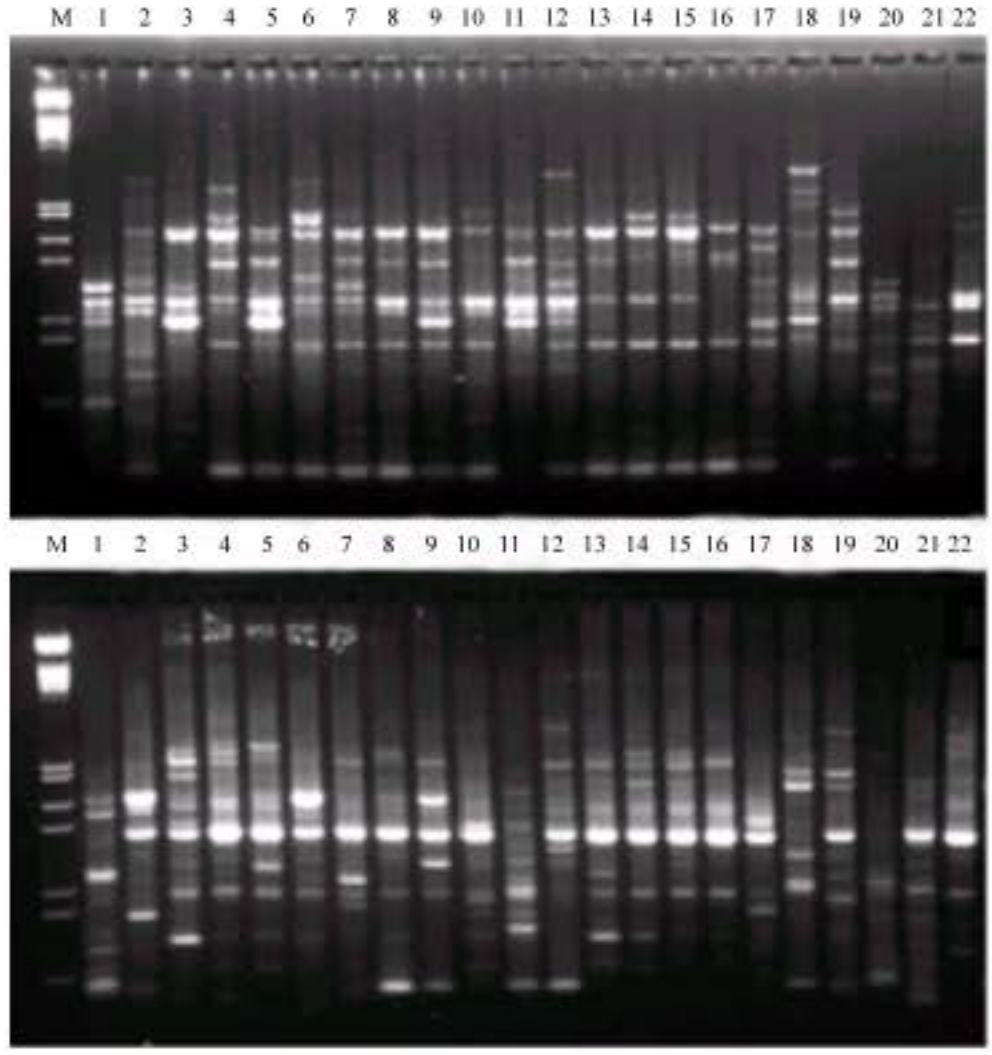

Fig. 2: The representative polymorphic profiles of all the 22 accessions of *P. appendiculatum* and with primer (A) OPB-10, (B) OPB-5. Lane M: λ DNA marker digested with Hind III and EcoRI, Lane 1-22: Accessions of *P. appendiculatum* of different places

Taking into consideration in population of *P. appendiculatum* it is clear that colonies are comprised of more than one individual. Moreover, besides vegetative reproduction, several recombinations appear to be more common than usually admitted. Researcher found high variation among and within regions confirms that spore dispersal, even over short distance, may effective within colonies. Ecological constraints may be strong factor limiting the possibility for colonies to diverge. An alternative explanation could be that liverworts which reproduces vegetatively during most of its long haploid life cycle, accumulates mutations, Soane and Watkinson (1979) producing an extremely rich clonal diversity. In a study of uniclonal populations of sea grasses, proposed that long lived individuals characterized by vegetative growth might have accumulated genotype differences via the accumulation of somatic mutation and recombinations, a fact also predicated in theoretical model. The present study showed that genotype collected from Ranikhet, Nanital and Dheradun were closely clustered each other because of their approximately similar altitude. This finding indicated that gene flow between short distances is very high. Loveless and Hamrick (1984) revealed that environmental factor i.e., habitat and altitude affect the morphological characters of the different populations. In fact, the amount of the gene flow is a major factor determining genetic variation found throughout the species

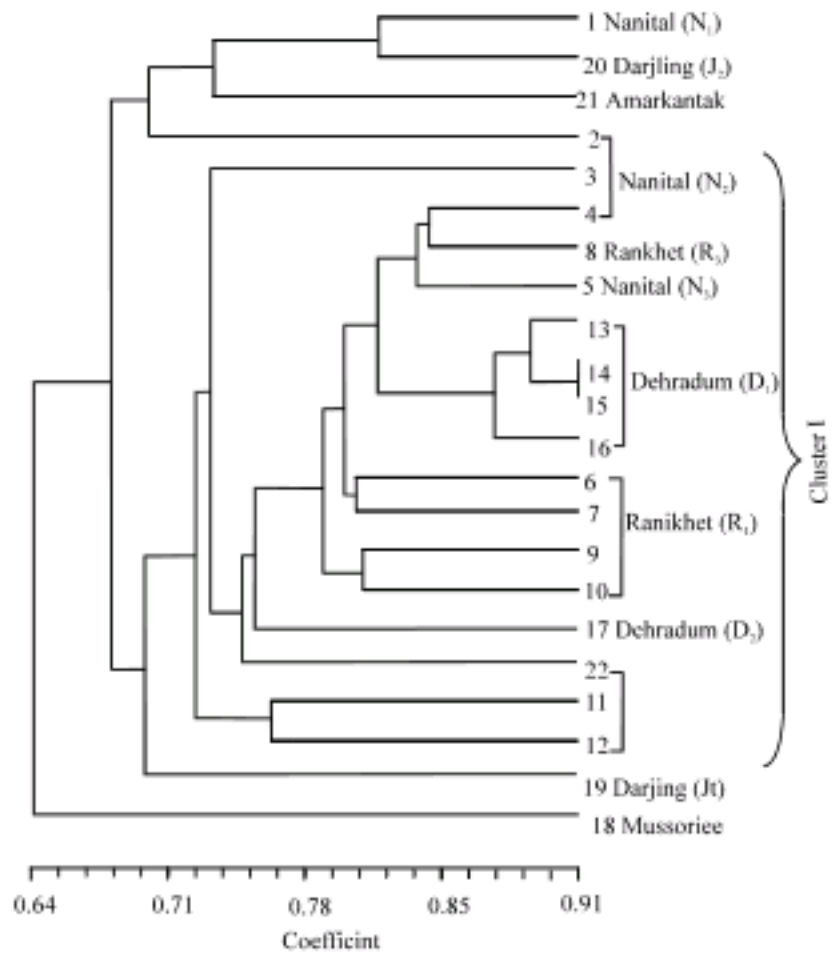

Fig. 3: Dendrogram showing the phylogenetic

distribution. Gene flow depends on reproductive mode, breeding system and selective constraints. Species with limited dispersal or that reproduced by selfing present a pattern of geographical genetic variation where drift may play the primary role. Thus, all these morphotaxonomical account and genomic profile of this potential species with critical variations in different populations has been provided with the objective to correlate these variations with environmental factors, habitat changes and genetic level. These finding clearly stated that there were high level of genetic diversity between and within population of *P. appendiculatum* which was totally differentiate the previous studies based on their morphological basis. A larger sample of this taxon and more comparative material of the *P. appendiculatum* have to be investigated to permit specification of its relationship with the latter species.

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