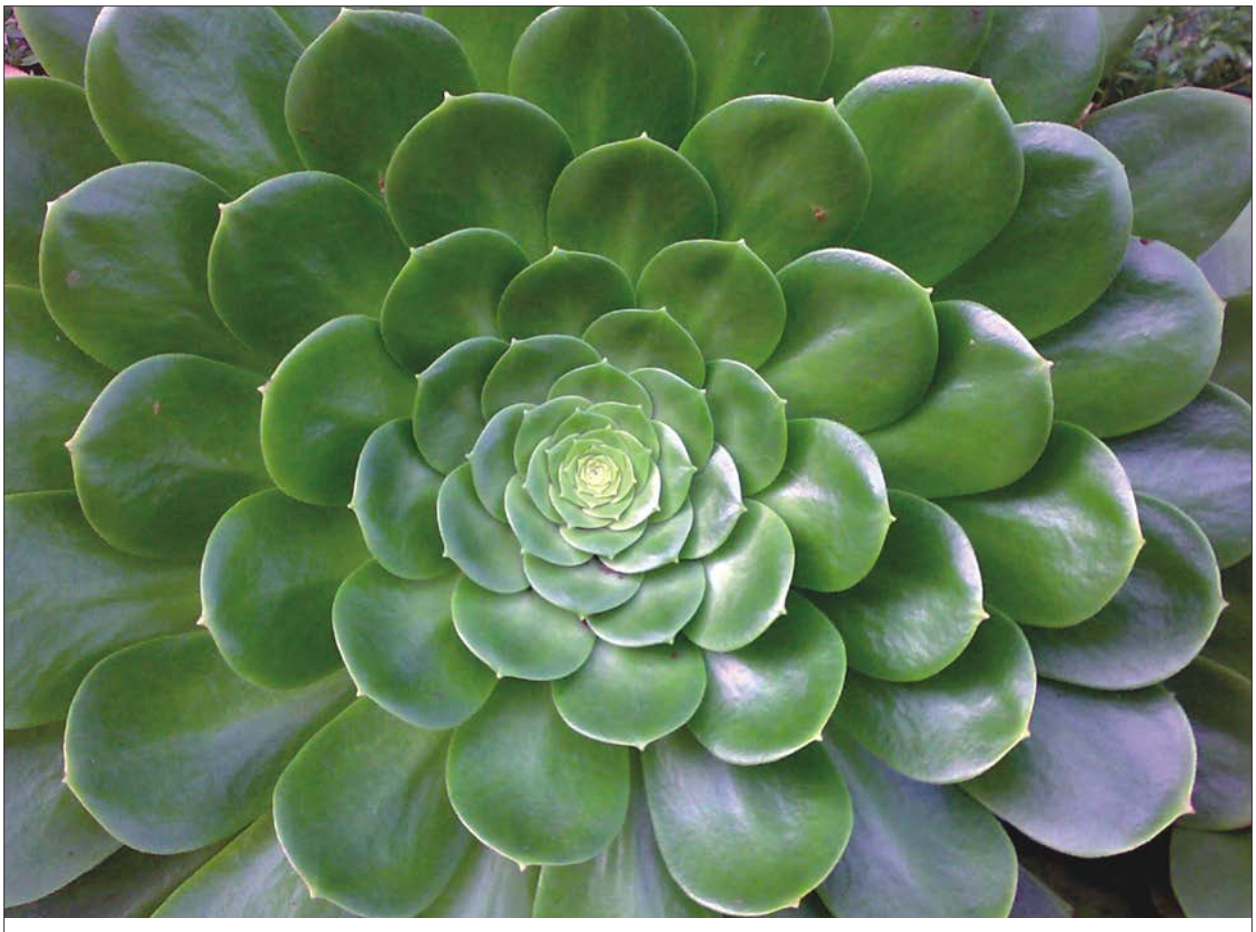

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Uses of Random Amplified Polymorphic DNA (RAPD) Markers in the Altitudinal Diversity of *Plagiochasma appendicualtum*

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ABSTRACT

Random amplified polymorphic DNA markers were used to determine the altitudinal variation within and between *Plagiochasma appendicualtum* collected from different altitude of Western Himalaya especially from Mussoorie region of India. Findings of UPGMA cluster analysis and band frequency of all the nine accessions were separated according to their altitudes supporting to their morphological differences as well. Gene flow and spore dispersal plays an important role in the polymorphism. Gene flow within *P. appendiculatum* growing on same altitude is very high as compared to accessions collected from different gradient of altitudes i.e., the genotypes collected from same altitude showing not so much polymorphism compared to different altitude. It has been concluded that the RAPD markers would be useful to characterize the altitudinal variation between different accessions of *P. appendiculatum* and may be also valuable to other bryophytes collected from various environmental condition.

Key words: RAPD, morphological and altitude variation, *Plagiochasma appendicualtum*, gene flow

INTRODUCTION

Bryophytes are the diverse group of land plants after the flowering plants (Mishler, 2001) but due to complexity in their identification and lack of literature from tropical areas, they have rarely been included in biodiversity analysis (Pharo *et al*., 1999). They are found from the tropics to the polar regions, from sea level to mountain summits and are principle candidates for latitudinal and altitudinal revision (Andrew *et al*., 2003). Several evocative studies of bryophytes and their altitudinal zonation have been reported in South America, in Puerto Rico along a transect of 200-1075 m by Fulford *et al*. (1971), in the Sierra Nevada de Santa Marta in Colombia (Van Reenen and Gradstein, 1983, 1984). Also in Northeastern Peru (Gradstein and Frahm, 1987), Bolivia, Peru and Columbia (Kessler, 2000), on Mt. Kinabulu, in the eastern part of Borneo (Frahm, 1990) and in New Zealand (Frahm and Ohlemuller, 2001; Pfeiffer, 2003) as well as in Africa on Mount Kilimanjaro (Pocs, 1994).

Mussoorie is a city about 30 km from Dehradun located in Dehradun district, Indian state of Uttarakhand. This hill station, situated in the foothills of the western Himalaya ranges, is also known as the Queen of the Hills. Mussoorie, with its green hills and varied flora and fauna, is notable for its unique geographical location with varied topography and associated altitudinal diversity supporting unique assemblage of biodiversity. Except for the coast and backwaters, almost

all habitats in Western Himalaya, from low altitude to the high altitude grasslands and shoal forests, occur in Mussoorie, which forms the major part of the Himalaya Biosphere Reserve.

Plagiochasma appendiculatum is one of the important liverwort belongs to the order Marchantiales under family Aytoniaceae. Lehman and Lindenberg (1832) first described 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. et 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 Mussoorie and also on other parts of India as well.

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 (Mahabale and Bhate, 1945). Besides this, *P. appendiculatum* also represents the maximum xerophytic habitat and can grow on comparatively naked and exposed rocks (Kachroo, 1954). Ghate and Chaphekar (2000) proved that this taxon could be used as a biotest for water quality assessment. *P. appendiculatum* is significant taxon which possesses antimicrobial property. Banerjee (2000), Kumar *et al*. (2000) and Singh *et al*. (2006) stated that in India, it is used by Gaddi tribes in Himachal Pradesh for the treatment of cuts, wounds and burns. Genetic variation of *P. appendicualtum* collected from different geographical conditions have been reported by using RAPD markers (Soni *et al*., 2009). Under the same study, RAPD markers were also stated the genetic diversity of *P. appendicualtum* within and between populations.

Random amplified polymorphic DNA markers (RAPD) can be widely used as DNA fingerprinting techniques (Williams *et al*., 1990, 1993) that has been utilized in bryophytes to survey population genetic structural, dispersal of spores, phylogeographic patterns and species relationship (Skotnicki *et al*., 2000; Scotnicki *et al*., 2001; Freitas and Brehm, 2001; Boisselier-Dubayle and Bischler, 1994; Boisselier-Dubayle *et al*., 1995). There were several reports related to RAPD genetic variation of various species of Antarctic moss (Selkirk *et al*., 1997; Skotnicki *et al*., 1997, 1998a, b).

P. appendiculatum is widely distributed in western, eastern Himalayas, central India and south India and generally growing upto an altitude of 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; Wigginton, 2002). In Asia, it is widespread ranging from the southwest of the Arabian Peninsula and Socotra Islant (Frey and Kurschner, 1988) to the southern part of the Himalayas, Formosa, Philippines and Celebes (Bischler, 1979). Due to occurrence of such variable altitudinal range, present study was planned out to determine altitudinal variation in *P. appendiculatum* through random amplified polymorphic DNA marker.

MATERIALS AND METHODS

Field sampling: The samples of *P. appendiculatum* were collected from various localities of Mussoorie e.g., Library road, Camel's back road, Kempty fall, Wood stock collage, Lal-Tibbs and Company garden (Fig. 1). Voucher specimens have been deposited at NBRI Bryophyte Herbarium, Lucknow, India (Table 1). Each specimen has been identified by their characteristic features through literature and authentic specimens available in the NBRI Herbarium.

Plant identification: Thalli of *Plagiochasma appendiculatum* is characterized by large, purplish green patches, thick, 20 mm long and 5 mm wide, dichotomously branched and occasionally with

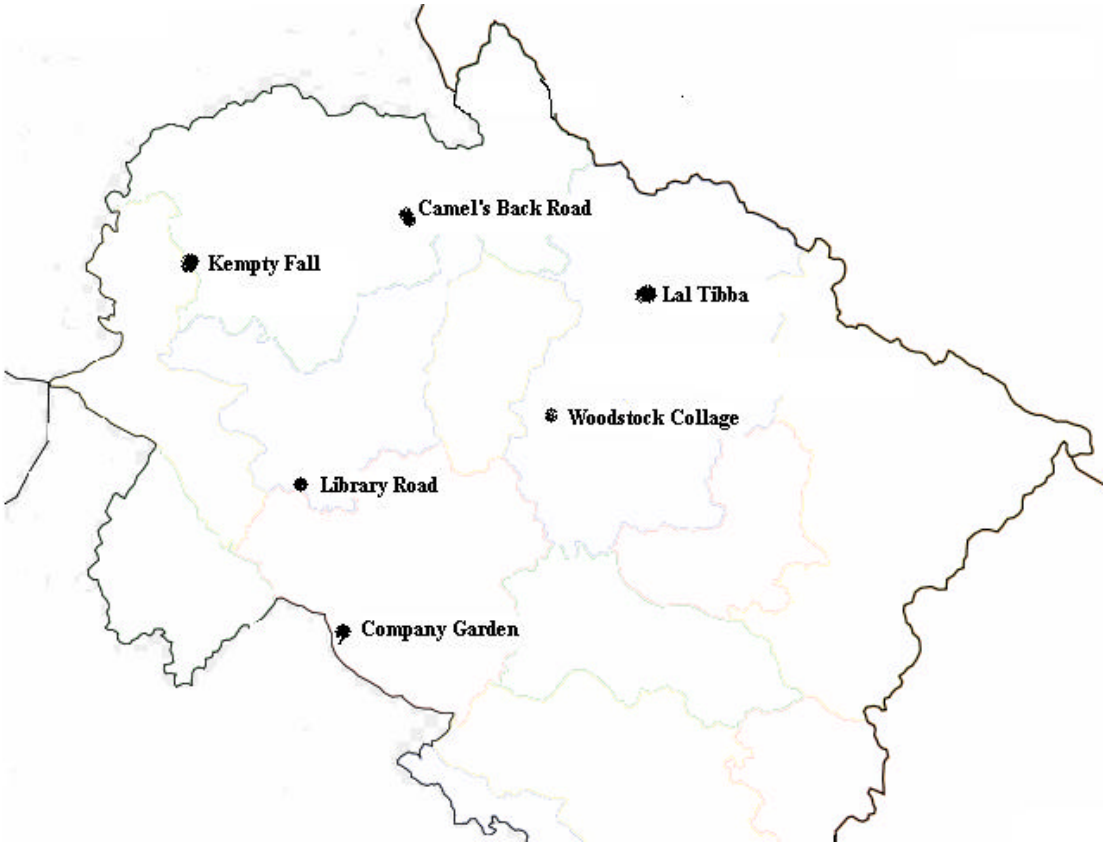

Fig. 1: Collection sites of *P. appendiculatum* from various altitude of Mussoriee (Western Himalaya) India

Table 1: Genotype collection of *P. appendiculatum* from various localities of mussoorie, growing at different altitude

LWG No.	Group code	Locality	Habitat	Altitude
226342	MS1	Library road	On soil covered rocks	Ca7000 ft
226958	MS2	Camel's bark road	On soil covered rocks	Ca 6000 ft
226932	MS3	Kempty fall (taxi stand)	On soil covered rocks	Ca 4000 ft
226925	MS4	Kempty fall	On soil covered rocks	Ca 4000 ft
226928	MS5	Kempty fall	On soil covered rocks	Ca 4000 ft
226941	MS6	Wood stock collage	On soil covered rocks	Ca 6800 ft
226942	MS7	Lal-Tibbs	On rocks	Ca 6400 ft
226943	MS8	Company garden	On soil covered rocks	Ca 6400 ft
226955	MS9	Company garden	On soil covered rocks	Ca 6400 ft

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 that has typical from shaped on broad lunate appendages (Table 2).

Table 2: Morphological data of *Plagiochasma appendiculatum* collected from geographical localities of Mussoorie (India)

LWG No.	Group code	Locality	Habitat	Altitude	Characteristic features	Position of ♂ receptacles	Position of ♀ receptacles	Spores and elaters
Mussoorie 226342	MS1	Library road	On soil covered rocks	Ca7000 ft	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
226958	MS2	Camel's bark	On soil covered rocks road	Ca 6000 ft	1-2.8 cm long and 0.8-1.2 mm broad, small delicate thallus light green, slightly wavy margin, ventral surface light purple, beak appendages	Absent	Absent	Absent
226932	MS3	Kempty fall	On soil covered rocks (taxi stand)	Ca 4000 ft	2.0-2.8 cm long, 0.4-0.9 cm broad, slightly wavy margin, dark green thallus cordate, ventral scales dark purple, fan-shaped dark purple appendages	Absent	Absent	Absent
226925	MS4	Kempty fall	On soil covered rocks	Ca 4000 ft	0.8-1.2 cm long, 0.4- 0.6 cm broad, slightly wavy margin, dark green in colour, shape-cordate, simple and tuberculate, rhizoid ventral scales light purple, small and rounded appendage (hyaline)		Trilobed stalked, stalk 1-2 mm long, growing on the midrib of the thallus in series (one by one); dehised	Absent
226928	MS5	Kempty fall	On soil covered rocks	Ca 4000 ft	1.5-2.2 cm long, 0.3-0.6 cm broad, highly wavy margin, Thallus light green in colour, strap shaped, both type of scales, ventral scales dark purple, fan dark pink appendages	Absent	Stalked, 1-3 mmm long stalk, bilobed, situated on the centre of thallus, dehised	Absent
226941	MS6	Wood stock collage	On soil covered rocks	Ca 6400 ft	2.5-3.6 cm long, 1.2-2.0 cm broad, slightly wavy margin, dark green cordate thallus which is spongy in texture, dark purple ventral scales, appendages large fan-shaped	2-3 in acropetal order	Bilobed-trolobed, sessile scattered on the thallus	Spores dark yellowish brown, elaters bispirate
226942	MS7	Lal-Tibbs	On rocks	Ca 6900 ft	0.5-1.0 cm long and 0.2- 0.6 cm broad,	Absent	Absent	Absent

Table 2: Continue

LWG No.	Group code	Locality	Habitat	Altitude	Characteristic features	Position of ♂ receptacles	Position of ♀ receptacles	Spores and elaters
226943	MS8	Company garden	On soil covered rocks	Ca 6400 ft	Thallus very delicate and light green in colour. Both type of rhizoids present, ventral surface and scales very light purple, small light hyaline, beak shaped appendages. (slightly wavy margin) 3-4.5 cm long, 1-1.8 cm broad, cordate, thick and dark green thallus, highly wavy margin, ventral surface dark purple, ventral surface is smaller than appendages, appendages light purplish, transparent	Horse-shoe shaped male receptacles 1-2 in number on every thallus	Absent	Absent
226955	MS9	Company garden	On soil covered rocks	Ca 6400 ft	2-2.8 cm long 0.5-1.2 cm broad, very brittle and thick thallus, dark green, large highly wavy margin, dark purple scales, fan-shaped dark pink appendages	Absent	Penta lobed, stalked 1-2 mm long situated on the mid rib of the thallus	Absent

Plant DNA preparation: Fresh and matured thallii of *P. appendiculatum* were 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. DNA was extracted from 1 g of plant material with some modification of several standard protocols (Doyle and Doyle, 1990; Soni and Kumar, 2009). Fresh material was crushed in liquid nitrogen and mixed with modified CTAB extraction buffer. Add 1% α-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 3). 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). 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: 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. Relationships among and between populations were evaluated via the unweighted pair-group method UPGMA (Sneath and Sokal, 1973) and all analysis was performed by using NTSYS (Rohlf *et al.*, 1990). Jaccard's coefficient was calculated by using FREETREE program, a common estimator of genetic identity and was calculated as follows:

$$\text{Jaccard's coefficient} = \frac{N_{AB}}{N_{AB} + N_A + N_B}$$

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. Statistical stability of the branches in the cluster was estimated by bootstrap analysis with 1,000 replicates, using the winboot software program (Yap and Nelson, 1996).

RESULTS

Altitudinal variation: Altitudinal variation was found in the genotypes of *P. appendiculatum*. Genotypes M_3, M_4 and M_5 and M_7, M_8 and M_9 were collected from 4000 and 6000 ft, respectively. Similarly, genotypes M_1 (7000ft), M_6 (6800 ft) and M_2 (6000 ft) showed variation related to their altitude. Thus, these genotypes clearly separated on the basis of their collection site (Fig. 2a) and by band frequency (Fig. 2b) obtained by amplified bands.

RAPD analysis: Genomic DNA amplification of 9 accessions of *P. appendiculatum* were carried out by using 30 random primers out of which 27 primers yielded 875 reproducible fragments and rest of the 3 primers were not given the amplification (Fig. 3). All the chosen primers amplified across the 9 accessions, with the number of amplified fragments ranging from eighteen (OPA-13) to fourty (OPD-08 and OPAP-03) which varied from 300 to 3500 bp. Out of the 875 amplified bands, 863 were polymorphic, with an average of 29 polymorphic fragments per primer and rest of the 12 fragments were monomorphic (Fig. 3). Percentage of polymorphism ranged from 93.5% (OPD-07) to a maximum 100% with an average of 98.5% polymorphism (Table 3).

The Polymorphism Information Content (PIC) obtained by random amplified bands were obtained with an average of 0.228, ranged from 0.000 to 0.62. Primers, OPA-04, OPA-07, OPA-15, OPA-16, OPA-17, OPA-19 and OPD-10 gave highest PIC values (Table 3).

Cluster analysis: Dendrogram based on UPGMA cluster analysis all the 9 accessions of *P. appendiculatum* were clearly separated according to their altitudinal sites. Genotypes MS1 and MS6 formed separate OUTs from other genotypes according to their high altitude (Fig. 4). However, MS1 and MS6 genotypes appeared to be closer to each other with similarity coefficient of 1.000 and 1.149, respectively (Table 4). Group I consist of genotypes MS3, MS4 and MS5, in which MS3 appeared to closer to M4 with similarity coefficient of 0.511 and genotype MS5 make separate out group with less difference similarity coefficient of 0.550. Similarly, genotype MS2 formed separate out group but appeared close to Group I. In the same manner, Group II consist MS7, MS8 and MS9 genotypes out of which MS7 and MD8 appeared close to each other with similarity coefficient of 0.419 and MS9 formed a separate out group but close to MS7 with similarity coefficient of 0.493. Therefore, all the genotypes are separated in relation to their collection site and altitude.

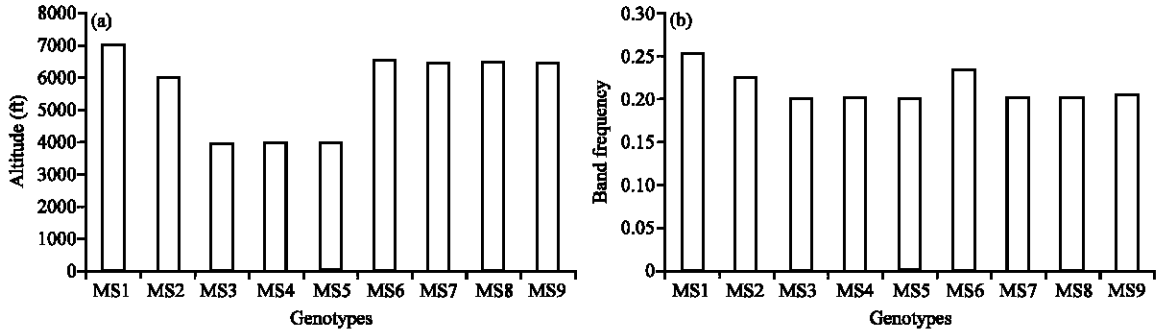

Fig. 2(a-b): Graph representing the (a) Altitudinal variation and (b) Band frequency obtained by amplified fragments

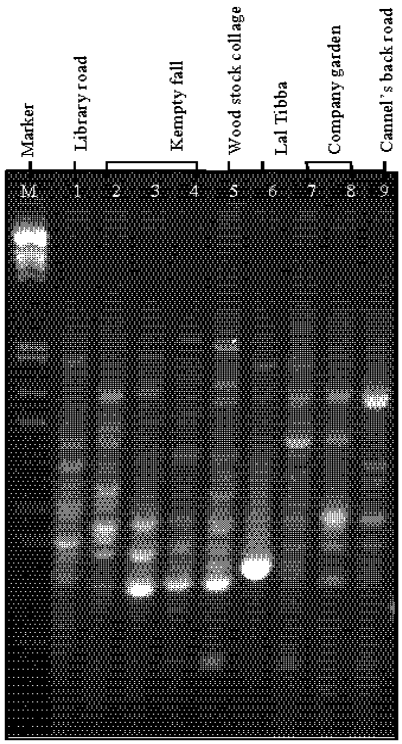

Fig. 3: RAPD profile of *P. appendiculatum* collected from different altitude of Mussoorie. Lane M: EcoRI and Hind III double digested marker. Lane 1: *P. appendicualtum* collected at 7000 ft; Lane 2: at 6000ft ; Lane 3-5: at 4000ft; Lane 6: at 6800ft; Lane 7-9: at 6400 ft

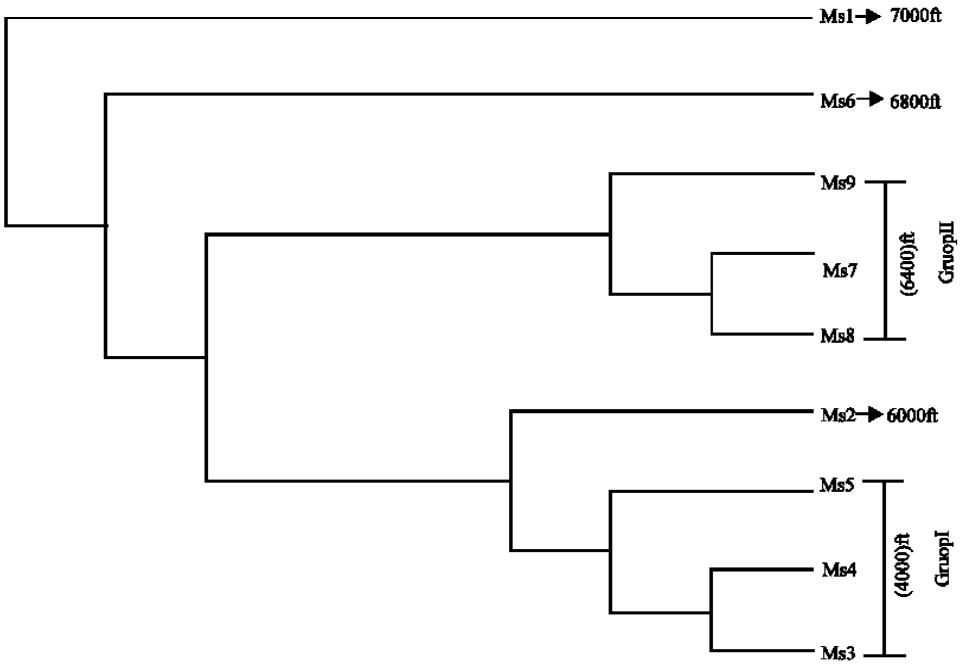

Fig. 4: UPGMA based Dendrogram showing the altitudinal variation and genetic relation ship between nine accessions of *P. appendiculatum* collected from various altitude of Mussoorie

Table 3: Sequences, bands, fingerprints and calculated parameters for the 30 RAPD primer used in *Plagiochasma appendiculatum*

						PIC		
Primer	Sequences (5'-3')	NB	NPF	NMF	PPB	Average	Range	Fragment size
OPA-01	CAGGCCCTTC	34	34	0	100	0.205	0.101-0.50	400-2500
OPA-02	TGCCGAGCTG	32	31	1	96.8	0.211	0.000-0.61	564-2100
OPA-03	AGTCAGCCAC	32	31	1	96.8	0.236	0.000-0.49	300-2100
OPA-04	AATCGGGCTG	30	29	1	96.6	0.260	0.000-0.62	300-2300
OPA-07	GAAACGGGTG	30	30	0	100	0.266	0.051-0.62	400-2100
OPA-09	GGGTAACGCC	31	31	0	100	0.211	0.051-0.47	564-2000
OPA-13	CAGCACCCAC	18	17	1	94.4	0.227	0.000-0.50	400-2100
OPA-15	TTCCGAACCC	25	24	1	96.0	0.185	0.000-062	564-2100
OPA-16	AGCCGCCGAA	32	32	0	100	0.236	0.000-0.62	400-2200
OPA-17	GACCGCTTGT	25	24	1	96.0	0.247	0.000-0.62	400-2027
OPA-18	AGGTGACCGT	43	43	0	100	0.262	0.051-0.50	400-2300
OPA-19	CAAACGTCGG	33	32	1	96.9	0.186	0.000-0.62	500-2200
OPB-11	GTAGACCCGT	34	34	0	100	0.204	0.051-0.47	300-3500
OPB-12	CCTTGACGCA	35	35	0	100	0.286	0.051-0.49	564-3000
OPB-15	GGAGGGTGTT	34	34	0	100	0.188	0.051-0.47	564-3500
OPB-17	AGGGAACGAT	30	29	1	96.6	0.223	0.000-0.50	400-3000
OPD-06	ACCTGAACGG	37	36	1	97.2	0.200	0.000-0.46	564-2200
OPD-07	TTGGCACGGG	31	29	2	93.5	0.148	0.000-0.48	300-1564
OPD-08	GTGTGCCCCA	40	40	0	100	0.221	0.051-0.48	300-2500
OPD-09	CTCTGGAGAC	22	22	0	100	0.195	0.051-0.50	300-1964
OPD-10	GGTCTACACC	31	31	0	100	0.278	0.051-0.62	400-2500
OPD-15	CATCCGTAAG	33	32	1	96.9	0.193	0.000-0.50	400-2027
OPD-17	TTTCCCACGG	29	29	0	100	0.251	0.051-0.50	564-2000
OPD-18	GAGAGCCAAC	20	20	0	100	0.298	0.101-0.50	564-2500
OPD-19	CTGGGGACTT	32	32	0	100	0.243	0.051-0.50	400-2500
OPD-20	ACCCGGTCAC	28	28	0	100	0.239	0.051-0.50	300-2027
OPAP-03	GTAAGGCGCA	40	40	0	100	0.236	0.051-0.45	300-2500
OPAP-13	TGAAGCCCCC	34	34	0	100	0.244	0.051-0.43	400-2500
		875	863	12	98.5	0.228	0.000-0.62	300-3500

Table 4: Jaccard's similarity coefficient matrix followed by UPGMA analysis of *P. appendiculatum* based on RAPD markers

Parameters	MS1	MS2	MS3	MS4	MS5	MS6	MS7	MS8	MS9
MS1	1								
MS2	0.931	1							
MS3	1.089	0.775	1						
MS4	1.170	0.748	0.511	1					
MS5	1.201	0.834	0.550	0.467	1				
MS6	1.149	0.918	0.889	0.977	0.76	1			
MS7	1.09	0.539	0.754	0.774	0.750	0.910	1		
MS8	1.157	0.650	0.795	0.836	0.768	0.873	0.419	1	
MS9	1.087	0.631	0.834	0.877	0.778	0.934	0.493	0.454	1

DISCUSSION

The RAPD marker is simple and reproducible technique that allowing comparison of genetic variation between wide range of bryophytes (Boisselier-Dubayle and Bischler, 1989; Bischler-Causse and Boisselier-Dubayle, 1991). Bopp and Capesius (1996) reported that RAPD is

used for identification and gene sequencing of bryophytes. However, other methods, such as isozymes and microsatellite have been reported to detect colonization and dispersal in many bryophytes. (Cronberg and Natcheva, 2002; Fang *et al*., 1997; Stenoien and Sastad, 1999; Rumsey *et al*., 2001).

The results obtained from the RAPD analysis indicated significant altitudinal variation among the *P. appendiculatum*, as collected from their different growing site (Fig. 3). UPGMA based dendrogram obtained by RAPD analysis indicated that the genotypes of Group I viz., MS3, MS4 and MS5 comes closer to each other because of short geographical distance and different altitudinal range. On the other hand, similar result has been found within genotypes of Group II i. e., MS7, MS8, MS9. Similarly, genotype MS1 and MS6 are separated according to their higher altitude i.e., 7000 and 6800 ft, respectively (Fig. 4). Soni *et al*. (2009) deescribed the morphological and genetic variation within and between the genotypes of *P. appendiculatum* due to growing at different altitude and habitat. These variation is due to the gene flow within closely and far located genotypes. Low gene flow may be one of the major cause of altitudinal variation in *P. appendiculatum* because high gene rate flow within closly growing individuals occurs by environmental factors indicating not so much vatiation. It means that gene flow takes place from higher to lower altitude and it has been reported that water is essential for the sexual reproduction by mean of dispersal of spores (Wyatt and Anderson, 1984). Restricted gene flow can generally promote local adaptation and genetic divergence between different microhabitats (Via and Lande, 1985). The magnitude of gene flow between the habitats in the current study is difficult to assess. Gamete dispersal distances are considered highly restricted in bryophytes (Wyatt and Anderson, 1984). Spore dispersal distances are probably orders of magnitude higher than gamete dispersal distances.

There is an important role of substrate in bryophyte species diversity and composition has been well established (Pharo and Beattie, 2002), but little is known about the effects of microhabitat and altitude in bryophytes (Romero *et al*., 2006). Andrew *et al*. (2003) did not find an overall pattern of bryophyte diversity on different mountains in Tasmania and New Zealand. They considered that altitudinal gradient may control community structure and diversity but suggested that factors operating at smaller scales (moisture, microhabitats) should be studied to understand the underlying mechanisms.

Water plays important role in the spreading of bryophyte propagules from higher to lower altitude along short drainage channels indicating water dispersal where at mixing of geographically divergent populations divided by numerous kilometers and snow confirmed that wind also is imperative for long-range dispersal (Skotnicki *et al*., 1999). Gene flow in bryophyte is caused during asexual reproduction by dispersal of sperm and spores. All the views of sperm dispersal in mosses and liverworts have reached to the same conclusion: sperm dispersal is very short (Wyatt and Anderson, 1984). Even in large species with splash cups, only rarely do sperms get dispersal more than 50 cm in species without splash cups, fertilization typically occurs within a radius of 10 cm. In the same manner *P. appendiculatum* growing on short distance having high gene flow so less amount of genetic variation that is why genotypes collected from same altitude comes closer to each other. It is therefore concluded that the RAPD markers may be beneficial for the revealing of altitudinal variation among the genotypes of *P. appendiculatum* collected from various environmental condition.

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