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MOLECULAR MARKERS (RAPD AND SSR) BASED CHARACTERISATION OF GENETIC DIVERSITY AND POPULATION STRUCTURE OF MOSS *HYOPHILA INVOLUTA*

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Genetic relationships among 24 genotypes of *Hyophila involuta* collected from five different natural populations of Mount Abu (Rajasthan) is analysed using RAPD and SSR markers. Based on efficiency parameters calculated for each marker system such as polymorphic information content (RAPD = 0.34; SSR = 0.66), marker index (RAPD = 2.78; SSR = 2.62) and resolving power (RAPD = 8.13; SSR = 2.23), the RAPD marker system shows higher values for some indices but microsatellites are more accurately reproducible than RAPD. Moreover, in case of the SSR, the average number of alleles was almost twice compared to RAPD. Mean coefficient of genetic differentiation between populations with RAPD was Gst = 0.269, while with SSR marker was Fst = 0.224. The UPGMA cluster analysis assembled genotypes into two main clusters with diverse levels of sub-clustering within the clusters. Also, the Mantel test showed no significant correlation between geographical and genetic distances. The observed moderately high genetic variability can be explained by efficient spore dispersal. Other factors such as reproductive mode, somatic mutation, continuous propagule recruitment and high degree of intermingling have great impact on the level of genetic variability in moss populations.

Key words: genetic diversity, moss, RAPD, SSR, population structure

INTRODUCTION

Mosses are the land plants which comprise about 13,000 species and exhibit pivotal role in many terrestrial ecosystems (Shaw 2009). The relationships between and within the major groups of mosses have not been explored as done in other higher land plants (Chang and Graham 2011). Since they have a characteristic life cycle with dominant haploid phase bryophytes have been increasingly used as the model plant to understand basic biology processes in plants and also in various research fields.

Several reports suggest the contradictory views regarding the evolutionary potential of mosses and the dynamics influencing genetic variability in moss populations (Shaw 1991). While several reports based on the study of anatomical traits, suggest a slight evolution in mosses since the end of the Palaeozoic era (Smoot and Taylor 1986), some suggest subsistence of intra-spe-

cific biodiversity. Thus the distinct populations with indistinguishable morphology are probably differentiated at the genetic level (Wyatt *et al.* 1989). In addition, some authors reported comparable levels of intrapopulation genetic diversity in natural populations of both mosses and vascular plants (Stoneburner *et al.* 1991), whereas other molecular studies reported lower molecular variability in mosses (Stenøien and Såstad 2001).

Hyophila involuta (Hook.) Jaeg. belonging to family Pottiaceae is an acrocarpous, dioecious moss growing predominately on rocks covered with sandy soil or on walls, moist soil. They are widely distributed species throughout the world. However, from India it is reported from Himalayas, Central India, South India, Gangetic Plain, Rajasthan and Nicobar Island (Alam 2015). Rajasthan remains one of the country's most overlooked bryological regions due to its harsh environmental condition. Bryofloristically Mount Abu is the richest place in Rajasthan, climatically suitable for the growth of mosses. Till now 63 moss species have been reported from this region. However, *H. involuta* is mostly abundant in natural population of Mount Abu (Rajasthan) reported from diverse altitudinal gradient. The plants are frequently fertile and reproduction occurs via both sexual as well as asexual mode. Asexual reproduction occurs by means of numerous multicellular polymorphous gemmae (size *ca* 100–250 μm) on branched stalks that are common in leaf axils (Britton 1904). The asexual diaspores cover short distance whereas spores cover long distances, dispersed via wind or water. Moreover, this species also exhibits high medicinal properties. Thus, this study will provide the overview of molecular diversity and factor affecting variation in this region.

Several previous studies have utilised various molecular markers such as inter simple sequence repeats (ISSR) (Spagnuolo *et al.* 2007), Random Amplified Polymorphic DNA (RAPD) (Manju *et al.* 2012) and Simple Sequence Repeats (SSRs) (Pandey *et al.* 2016) to evaluate genetic variability in different taxa of mosses. SSRs can also be present in organelle genomes such as those of mitochondria and chloroplast. The chloroplast and mitochondrial genomes usually have a uniparental mode of transmission, so they display different patterns of genetic differentiation compared to nuclear alleles (Provan *et al.* 1999). RAPD markers were utilised efficiently to study the intraspecific diversity within genus *Sphagnum* (Såstad *et al.* 1999). RAPDs also, proved efficient in distinguishing the three species of *Aerobryopsis* and closely related taxon *Aerobryidium* (Manju *et al.* 2012), analysis of source of colonising moss propagules and dispersal of moss species (Skotnicki *et al.* 2002). The SSR markers were widely used in several species of bryophytes to study reproductive biology and its effect on genetic variation and genetic structure within populations (Leonardia *et al.* 2006). The molecular markers' based analysis of several bryophytes exhibit high levels of intrapopulation genetic diversity (Hutsemékers *et al.* 2010), in contrast to earlier reports that assumed that bryophytes

being haploid, succeeded to retain a low level of genetic variations during the course of natural selection. Understanding genetic diversity and population structure is necessary for the conservation management of threatened and diminishing species, as the loss of genetic variability can reduce the adaptation potential of species to changing environment (Weeks *et al.* 2016).

The aim of the present study was to investigate the genetic diversity and structuring within natural populations of the dioecious moss *H. involuta* as well as genetic differentiation among populations by using RAPD and SSR markers. This study also made an attempt to check whether there is isolation by distance between populations and also describes the pattern of distribution of genetic clusters across the species range in Mt Abu. The efficiency of these marker systems in the diversity analysis was studied. This is the first effort to systematically understand the genetic diversity and population genetic structure of the widespread moss *H. involuta* in Mount Abu (Rajasthan), India.

MATERIAL AND METHODS

Collection of plant samples and genomic DNA isolation – Plant samples were collected from the different localities of Mount Abu (Rajasthan), *viz.* Sunset Point, Nakki Lake, Achalgadh, Way to Trevor's Tank, Oriya Road during the month of July–September 2014–2015 and site samples were coded as in Table 1, Figure 1. In each site, random samplings were done from the population. The samples were washed properly to remove mud, dirt and microscopic organisms as these microscopic organisms may cause undesirable PCR amplification. It was stored at –80 °C till further use. The clump of moss with stem, leaves and rhizoids are used for the extraction of total genomic DNA via modified CTAB method (Pandey *et al.* 2018). The concentration and purity of extracted DNA were assessed by micro-volume spectrophotometer (Thermofisher Scientific Nanodrop) and then diluted (25 ng/µl) for further use.

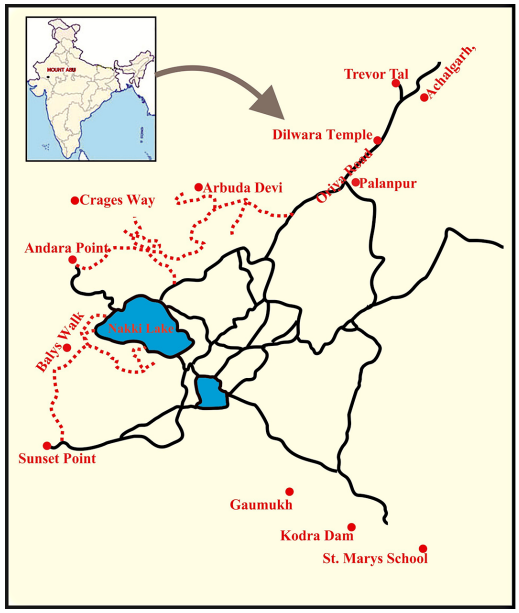

Fig. 1. Map showing different sites of sample collection in Mount Abu

In silico data mining and primer design – The chloroplast genome

Table 1
Genotypes of *H. involuta* collected from different localities of Mount Abu, Rajasthan, India

S. no	Sample name	Population location	Latitude, longitude
1	OR1	Oriya Road	24.6242° N, 72.7307° E
2	OR2	Oriya Road	24.6267° N, 72.7421° E
3	OR3	Oriya Road	24.6274° N, 72.7442° E
4	OR4	Oriya Road	24.6313° N, 72.7548° E
5	OR5	Oriya Road	24.6311° N, 72.7534° E
6	OR6	Oriya Road	24.6299° N, 72.7456° E
7	AG1	Achalgadh	24.6240° N, 72.7598° E
8	AG2	Achalgadh	24.6161° N, 72.7687° E
9	AG3	Achalgadh	24.6239° N, 72.7601° E
10	AG4	Achalgadh	24.6203° N, 72.7638° E
11	AG5	Achalgadh	24.6177° N, 72.7677° E
12	NL1	Nakki Lake	24.5819° N, 72.7192° E
13	NL2	Nakki Lake	24.5935° N, 72.7060° E
14	NL3	Nakki Lake	24.5965° N, 72.7077° E
15	NL4	Nakki Lake	24.5932° N, 72.7059° E
16	NL5	Nakki Lake	24.5833° N, 72.7193° E
17	SP1	Sunset Point	24.5995° N, 72.6979° E
18	SP2	Sunset Point	24.5967° N, 72.6893° E
19	SP3	Sunset Point	24.5992° N, 72.6990° E
20	SP4	Sunset Point	24.5999° N, 72.6975° E
21	SP5	Sunset Point	24.5975° N, 72.7020° E
22	TT1	Trevor's Tank road	24.6194° N, 72.7271° E
23	TT2	Trevor's Tank road	24.6170° N, 72.7267° E
24	TT3	Way to Trevor's Tank	24.6145° N, 72.7263° E

sequence of *Tortula ruralis* (Hedw.) P. Gaertn., B. Mey. et Scherb. (NC-012052) and mitochondrial genome sequence of *Physcomitrella patens* (Hedw.) Bruch et Schimp., (NC-005087) were downloaded from the NCBI (National Centre for Biotechnology Information) and mined by MISA software (http://pgrc.ipkgatersleben.de/misa/misa). PCR primers (forward and reverse) flanking the repeat sequence was designed using the Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) having default parameters set for GC content, melting temperature, PCR product and primer size. The primers were purchased from Xcleris, India.

DNA amplification – DNA amplification reactions were assembled in 15 µl reaction mixture containing 25 ng/µl of DNA, 1X PCR buffer, 0.2 U of Taq DNA polymerase (Genei, Banglore, India), 2.5 mM each of dNTPs (Genei, Ban-

Table 2
The sequence and parameters calculated for each RAPD primer used to study diversity in moss *H. involuta*

S. no.	Primer code	Primer sequence	T °C	TB	PB	MB	Pp (%)	PIC	MI	RP
1	RS01	GGGTAACGCC	36	17	17	0	100	0.29	2.49	10.0
2	RS02	GTCCACACGG	36	15	15	0	100	0.33	2.34	8.53
3	RS03	CCACAGCAGT	36	12	12	0	100	0.30	2.04	6.41
4	RS04	CCGGCCTTAC	36	12	12	0	100	0.33	2.83	8.25
5	RS05	TTCCCGGAGC	36	13	13	0	100	0.30	2.61	7.92
6	RS06	GATGACCGCC	36	12	12	0	100	0.34	2.82	7.66
7	RS07	GGTGCGCCTT	36	14	14	0	100	0.35	2.90	8.75
8	RS08	TTTGCCCGGA	36	11	11	0	100	0.37	3.55	7.91
9	RS18	GTTGCGATCC	36	11	11	0	100	0.40	3.60	9.25
10	RS20	TTCCGAACCC	36	10	10	0	100	0.36	2.61	6.58
Total	10			127	127	0				
		Average/Primer		12.7	12.7		100	0.34	2.78	8.13

T°C = annealing temperature; TB = number of bands; PB = number of polymorphic bands; MB = number of monomorphic bands; Pp = percentage of polymorphic bands; PIC = polymorphism information content; MI = marker index; Rp = resolving power

glore, India), 1.5 mM $MgCl_2$ and 1 μM primer concentration (Xcelris, India) was amplified in 96 wells gradient thermal cycler (Primus 96) using following program: initial denaturation at 94 °C for 5 min; 35 cycles of 1 min at 94 °C, 36 °C annealing temperature for 1 min, and 72 °C for 2 min; and a final elongation step of 72 °C for 5 min. The annealing temperature (Ta) for RAPD primers was kept 36 °C, whereas for SSR primers the annealing temperature varies from 48 to 52 °C. Primers were synthesised from Xcelris (Xcelris Lab Limited, Gujrat, India). The RAPD-PCR and SSR-PCR amplification products for all samples were resolved on agarose (Himedia) gel 1.5% and 2%, respectively. The gels were stained with 0.25 μg/ml Et-Br (ethidium bromide), then photographed and analysed using Gel doc (Alphaimager MINI ProteinSimple, Japan). From the preliminary screening of 10 RAPD and 9 SSR primers, the clear, visible and reproducible bands were preferred for subsequent analysis (Tables 2 and 3).

Band scoring and data analysis – For RAPD marker system reproducible and high intensity bands were scored 0 and 1 for the presence and absence of bands and used for construction of binary matrix. Whereas for the SSR marker the allele size of each PCR products was measured with Gel doc (Alphaimager MINI ProteinSimple, Japan) using Alpha View 3.4.0.0 analysis tool software by manual editing to increase accuracy. GenAlEx version 6.5 (Peakall and Smouse 2012) and Microsatellite software were utilised to create the input

Table 3
The sequence and parameters calculated for each SSR primer used to study diversity in moss *H. involuta*

S. no.	Primer code	Primer sequence (5′→ 3′)	T °C	No. of alleles	Size range (bp)	PIC	MI	RP
1	PpMt1	F TCTCTGGGAAAAATGCGAAC R TGCTTAAGGATTCCAGCAAAA	49	3	247–252	0.65	1.82	2.00
2	PpMt2	F TACGTGGGAGCCCTATACAA R GGCAAAAGCATCTTTGGTTC	49	4	204–212	0.71	2.56	3.50
3	PpMt3	F TGGTGTTGGATCCCAAGTTT R TCGAACGAAAGGATTGGAAC	51	4	175–184	0.53	1.27	2.51
4	SrCp1	F TGGATGAAGTAAAATACCCCGTA R AGGTTGAAGTGTGATAATCATTCG	52	4	181–188	0.76	2.12	2.07
5	SrCp2	F TGCGAGTTATCGAACGAGGT R TTTGAAAGAAATAAGGGAAATGAA	45	4	170–194	0.64	1.66	2.01
6	SrCp3	F AATTCGCCCAACATAAAACC R GCTGCAGTTTCTACTCCTGCT	51	4	193–205	0.71	1.99	2.01
7	SrCp4	F GTGTCAACAACCCCCTTACG R CTCGCGGAAGTACATCACAG	52	3	179–193	0.62	1.36	2.04
8	SrCp5	F ACCGGATACACACGGAGAAG R CACAAAGCCCATGTGTGAAA	48	3	139–167	0.64	1.41	2.05
9	SrCp6	F ACCGAGTGCAGCTAATGCTT R ATGCAATGGAAATTGTGCAG	48	3	229–238	0.66	1.32	2.00
Total	9			32				
		Average/Primer		3.6		0.66	2.62	2.23

T °C = annealing temperature; n, number of alleles; PIC = polymorphism information content; MI = marker index; Rp = resolving power

data file for POPGENE version 1.32 (Yeh *et al.* 1999).

Firstly, the primer banding characteristics, including the total number of bands (TB), number of polymorphic bands (PB) and percentage of polymorphic bands (Pp) was calculated to estimate the utility of these marker systems in the genetic variability analysis of selected moss species. The utility of the markers was calculated using three parameters: RP (resolving power), PIC (polymorphic information content) and MI (marker index). These parameters were estimated as described by Powell *et al.* (1996).

The genetic diversity indices such as observed (H_O) and expected heterozygosity (H_E) (Nei 1973), observed and expected number of alleles (Kimura and Crow 1964), Nei's gene diversity (h) (Nei 1973), Shannon's index (I) (Lewontin 1972), average heterozygosity and Nei unbiased genetic identity, estimate of gene flow (Nm) and genetic differentiation (G_{ST}/F_{ST}) for each population was calculated by POPGENE software. G_{ST} and F_{ST} is calculated for RAPD and SSR markers, respectively. As the F_{ST} is calculated for codominant markers (SSR) with dominant markers (RAPD) may give ambiguous result. The formula used for the estimation of G_{ST} and F_{ST} is based on Nei (1973) and Wright (1969), respectively.

$G_{ST} = D_{ST}/H_T = (H_T–H_S)/H_T$ (where H_S = intrapopulation genetic diversity, D_{ST} = interpopulation diversity, H_T = total genetic diversity). For biallelic marker G_{ST} is identical to Wright's F_{ST}, whereas for multiple alleles G_{ST} is equal to the median of F_{ST} for all alleles.

The molecular variance among and within the population or individual was determined by using "AMOVA" analysis of molecular variance (Excoffier *et al.* 1992) with the help of GenAlEx software. Further, principal coordinates analysis (PCoA) was carried out by GenAlEx software. The Mantel test (Mantel 1967) was performed to calculate isolation by distance effect by using GenAlEx software. A dendrogram was constructed by the UPGMA (unweighted pair group method with arithmetic average) method for studying the genetic distance among all the genotypes by POPGENE software.

To interpret the genetic structure of population the Structure version 2.3.4 (Pritchard *et al.* 2000) was used. The analysis was repeated twice and the parameters such as K (number of presumed populations) (1 to 20), burn in (50,000), MCMC (50,000) and iterations (20) were set to specific value. Evanno *et al.* (2005) method was used for determination of optimal K and ΔK which was retrieved using Structure Harvester software (Earl and Von-Holdt 2012).

RESULTS

Allele diversity in the moss H. involuta using RAPD and SSR marker system

Both the marker techniques (RAPD and SSR) proved to be extremely valuable in discriminating the 24 genotypes of *H. involuta*. The analysis of five *H. involuta* populations with the 10 RAPD primers that were polymorphic identified a total of 127 reproducible fragments (Table 2, Fig. 2A). The amplicons differ in their size ranging from 150 to 2500 bp. The number of fragments amplified per primer ranged between 11 and 17, with an average of 12.7 bands. The size and patterns of amplicons were found to be species and primer dependent as reported earlier (Sripaoraya *et al.* 2001).

For SSR, the number of alleles per locus was found to be 3 and 4, which differ in size ranging from 139 to 252 bp (Table 3, Fig. 2B). The primers yielded a total of 32 alleles of which 30 were found to be polymorphic resulting on an average of 3.6 alleles and 3.33 polymorphism per locus, respectively. More or less similar mean value of alleles has been reported in earlier studies also in *Sphagnum platyphyllum* (Ricca and Shaw 2010), in *Polytrichum juniperinum* (Vander-Velde and Bijlsma 2003) and in *Platyhypnidium riparioides* (Hutsemékers *et al.* 2008). Studies suggest that the different SSR motifs vary in their frequency and number of repeats in different species (Neelamraju and Neeraja 2005). Li *et al.* (2002) reported that dinucleotide repeats account for the majority of microsatellites for several species and relatively better source

for developing polymorphic SSR markers. Five out of 9 primers that produced polymorphism were dinucleotide repeats.

PIC is an important feature of primer which indicates its potential to differentiate various individuals. The result showed higher levels of gene diversity for SSR (0.66) based markers than RAPD (0.34). The PIC value ranged from 0.5 to 1 for co-dominant, whereas for dominant marker it has maximum value of 0.5 (Boopathi 2013). Highest PIC was observed for primers RS18 (0.40) in RAPD assay, while the highest PIC was observed for primer SrCp1 (0.76) in SSR assay. SSR allelic diversity is generated by replication slippage unlikely with single nucleotide mutations and insertions/deletions, probably responsible for the expected higher level of polymorphism (Park *et al.* 2009). Marker index is a feature of a marker, which elucidates its discriminatory power, thus determined for all the markers. Due to high multiplex ratio component (8.25)

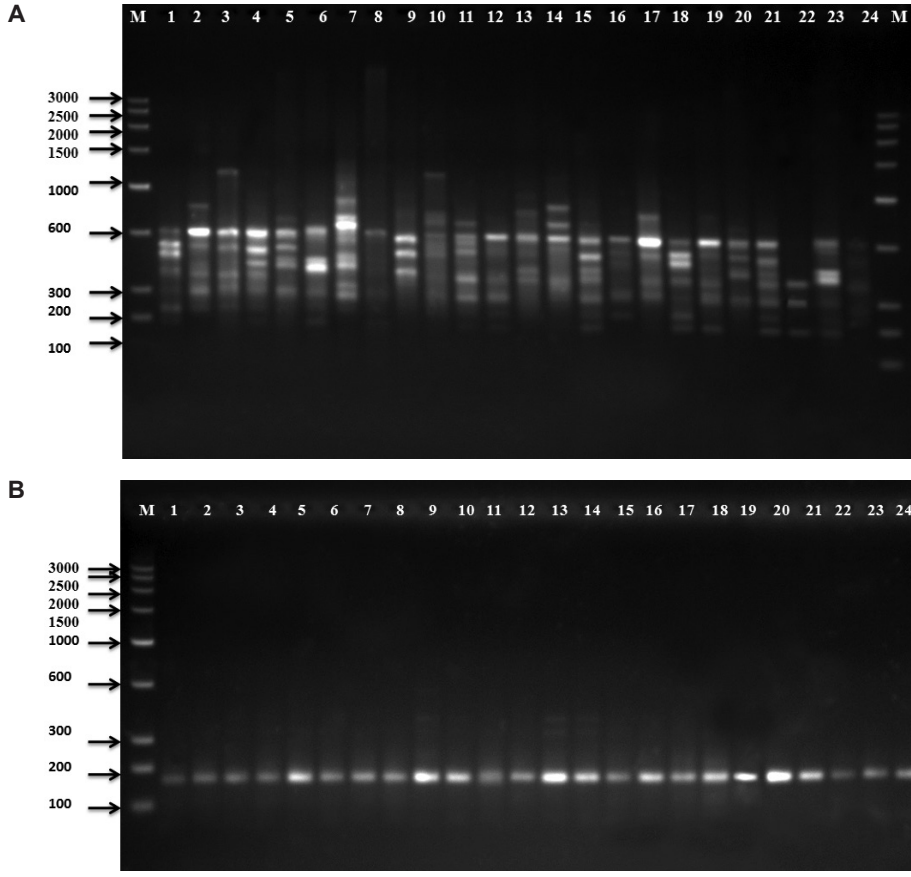

Fig. 2. Gel images showing (A) RAPD profile; Primer RS01 (B) SSR profile; Primer SrCp2 of *H. involuta.* Lane M, Low range DNA ladder; Lane 1–24, *H. involuta* genotypes as given in Table 1

Table 4
Efficacy of RAPD and SSR marker systems based on discriminatory power in five *H. involuta* populations

Indexes with their abbreviations		Marker systems	
		RAPD	SSR
Number of assay units	U	10	9
Number of loci	L	127	9
Number of loci/assay unit	n_u	12.7	1
Average number of allele/locus	n_{qv}	2	3.6
Fraction of polymorphic loci	β	1	1
Effective multiplex ratio	EPR	8.25	3.85
Polymorphic information content	PIC	0.34	0.66
Marker index	MI	2.78	2.62
Resolving power	RP	8.13	2.23

for RAPD, the marker index obtained for RAPD (2.78) was higher as compared to SSR (2.62). Zargar *et al.* (2016) observed a higher multiplex ratio for RAPD (2.69) than for SSR (1.279) in 52 common bean accessions. Further, resolving power/discriminatory power of a marker is defined by the tendency of primers to distinguish the genotypes and was estimated for each primer. An average resolving power of 8.13 was observed for RAPD whereas for SSR it was 2.23. Highest resolving power of 10 was observed for primer RS03 among RAPD markers while the highest resolving power of 3.50 was observed for primer PpMt2 among the SSR markers. The comparisons of different parameters to evaluate the efficacy of marker systems are summarised in Tables 4 and 5.

Genetic analysis of H. involuta *populations*

In RAPD markers, observed and effective number of alleles ranged between 1.370–1.850 and 1.261–1.3688, respectively. The Nei's gene diversity (h) ranged between 0.148 and 0.255 with total diversity at the species level of 0.205. The value of Shannon's index ranged between 0.218 and 0.399 with an overall diversity of 0.313. The mean expected heterozygosity (H_E) was found to be 0.205±0.007 with the Trevor's Tank (TT) and Nakki Lake (NL) populations having lowest and highest mean value of H_E 0.148±0.018 and 0.255±0.013, respectively (Table 6).

Table 5
Variation partitioning based upon RAPD and SSR data in five *H. involuta* populations

Indexes with their abbreviations		Marker systems	
		RAPD	SSR
Shannon's index	I	0.301	0.712
Expected heterozygosity	H_E	0.205	0.437
Genetic differentiation	G_{ST}/F_{ST}	0.269	0.224
Gene flow	Nm	1.361	1.529

Table 6
Genetic diversity statistics and differentiation parameters for five populations of *H. involuta* using RAPD primers

Populations	N	Na±SD	Ne±SD	h±SD	H_E±SD	I±SD	Pp (%)	G_{ST}	Nm
OR	6	1.717±0.452	1.368±0.350	0.223±0.182	0.223±0.016	0.343±0.256	71.65		
AG	5	1.661±0.475	1.366±0.366	0.218±0.190	0.218±0.017	0.332±0.268	66.14		
NL	5	1.850±0.358	1.401±0.298	0.255±0.151	0.255±0.013	0.399±0.209	85.04		
SP	5	1.559±0.498	1.291±0.338	0.178±0.183	0.178±0.016	0.274±0.266	55.91		
TT	3	1.370±0.485	1.262±0.378	0.148±0.201	0.148±0.018	0.218±0.290	37.01		
	24	1.631±0.036	1.338±0.014	0.205±0.187	0.205±0.007	0.313±0.011	63.15	0.269	1.361

N = sample size; Na = observed number of alleles; Ne = effective number of alleles; h = gene diversity; H_E = expected heterozygosity; I = Shannon's information index (Pp = percentage of polymorphic loci; SD = standard deviation; G_{ST} = diversity among populations; Nm = gene flow; F_{ST} = fixation index. Parameter calculations assume Hardy–Weinberg equilibrium

Table 7
Genetic diversity statistics and differentiation parameters for five populations of *H. involute* using SSR primers

Populations	N	Na±SD	Ne±SD	H_O±SD	H_E±SD	I±SD	Pp (%)	Nm	F_{ST}
OR	6	2.555±0.881	1.895±0.614	0.388±0.300	0.476±0.141	0.711±0.260	100		
AG	5	2.777±1.092	2.100±0.756	0.444±0.296	0.501±0.263	0.771±0.419	88.89		
NL	5	2.444±0.726	1.960±0.614	0.266±0.300	0.479±0.243	0.703±0.361	88.89		
SP	5	2.555±0.726	1.885±0.542	0.333±0.200	0.464±0.231	0.700±0.348	88.89		
TT	3	2.666±1.00	2.368±0.888	0.370±0.351	0.585±0.339	0.825±0.483	77.78		
	24			0.389±0.289	0.437±0.243	0.712±0.375	88.89	1.529	0.224

N = sample size; Na = mean number of alleles; Ne = effective number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; I = Shannon's information index (Pp = percentage of polymorphic loci; SD = standard deviation; Nm = gene flow; F_{ST} = fixation index. Parameter calculations assume Hardy–Weinberg equilibrium

Table 8
Analysis of molecular variance within and among *H. involuta* populations based on RAPD and SSR data set.

Source of variation	df		Sum of squares		Variance components		Percentage of variance	
	RAPD	SSR	RAPD	SSR	RAPD	SSR	RAPD	SSR
Among populations	4	4	138.68	87.83	3.02	3.37	13%	36%
Within populations	9	9	385.86	112.50	20.31	5.921	87%	64%

The percentage of polymorphic loci (Pp) was found to be in the range of 37.01–85.04 with an average of 63.15. The value of mean coefficient of genetic differentiation (G_{ST}) and gene flow (Nm) in the population was found to be 0.269 and 1.369, respectively, which indicates the moderately high population differentiation. The result of AMOVA showed that the genetic variation within the population (87%) is higher than among the populations (13%) for *H. involuta* (Table 8).

In SSR markers, the observed and the effective number of alleles ranged between 2.444–2.777 and 1.885–2.368, respectively. The mean expected heterozygosity (H_E) was found to 0.437±0.243 with the Sunset Point (SP) and Achalgadh (AG) populations having lowest and highest mean value of H_E 0.464±0.231 and 0.501±0.263, respectively. The value of mean observed heterozygosity was found to be lower than mean expected heterozygosity. The Shannon's index was found to 0.71 at the level of inter-population. The percentage of polymorphic loci ranged between 77.78 and 100% with a mean value of 88.89%. The value of mean coefficient of genetic differentiation and gene flow was found 0.224 and 1.102, respectively, which indicate moderate population differentiation (Table 7). AMOVA analysis showed that the individuals of *H. involuta* are more diverse at intra-population level with a high percentage of variation (64%) than at inter-population level with comparatively lower variation (36%) (Table 8).

Analysis of genetic divergence through dendrogram construction

The pair wise genetic distances based on RAPD and SSR banding pattern had been used for cluster analysis to find the genetic relationship between 24 genotypes of moss *H. involuta* in the form of dendrogram. The clusters generated by UPGMA method are represented in Figures 3 and 4. The Nei's unbiased genetic distance for RAPD based diversity analysis ranged from 0.239 to 0.701 with a mean of 0.435. The highest (0.811) and lowest (0.465) genetic identity was found between (SP1 and SP2) and (AG3 and NL3), respectively. Whereas for SSR based diversity analysis, Nei's unbiased genetic distance

vary from 0.159 to 0.968 with the mean of 0.708. Highest genetic identity (0.897) was found between OR1 and OR3, while the lowest (0.100) between three pairs (OR6 and SP3, TT2 and OR5, TT2 and OR6).

Careful observation in the UPGMA tree shows a marked tendency of different populations that remain genetically different and form separate groups (Fig. 3). The dendrogram obtained with RAPD markers divided all 24 genotypes of *H. involuta* into two main clusters. Cluster-I represented only two genotypes (NL4 and NL3). Whereas, the remaining genotypes were grouped in the cluster-II, which further divided the genotypes into three sub clusters. Cluster-II consists of 22 samples in which first sub-cluster IIa consists of all the isolates Oriya Road population (OR1–OR6), sub-cluster IIb contains samples of population way to Trevor's Tank (TT1–TT3) and Achalgadh (AG1–AG6) whereas, sub-cluster IIc contains sample of population Sunset point (SP1–SP6) and Nakki lake (NL1, NL2, NL5). Thus, basically, samples of four populations of viz. Sunset Point (SP), Achalgadh (AG), Trevor's Tank (TT) and Oriya Road (OR) form distinct clusters and show genetic differentiations while samples of Nakki Lake (NL) do not form separate cluster but distributed in different clusters.

The dendrogram obtained with SSR markers also divides all 24 isolates of *H. involuta* into two main clusters (Fig. 4). Cluster-I represented by eleven samples, divided into two sub-clusters (Ia and Ib). Sub-cluster Ia consists of all the genotype of Sunset Point population (SP1–SP6) and sub-cluster Ib contains all the genotypes of Nakki Lake (NL1–NL6) population and genotype AG5. Whereas, the remaining genotypes were grouped in the cluster-II which further divided the genotypes into three sub clusters. Cluster-II consists of 13 samples in which first sub-cluster IIa consists of all the isolates Oriya Road population (OR1–OR6), sub-cluster IIb contains samples of population way to Trevor's Tank (TT1–TT3) and sub-cluster IIc contains sample of population Achalgadh (AG1-AG4, AG6). Thus, basically, samples of two populations of *viz.*, Sunset Point (SP) and Nakki Lake (NL) form distinct clusters and show genetic differentiations while samples of Achalgadh (AG), Trevor's Tank (TT) and Oriya Road (OR) were distributed with different groups.

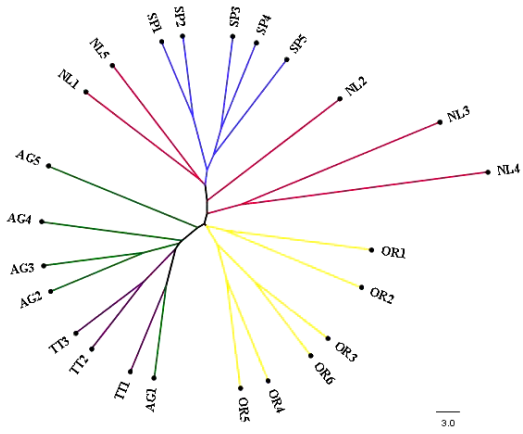

Fig. 3. UPGMA clustering based on RAPD analysis obtained through collected dataset of 24 different samples of *H. involuta*. Numbers indicated in the tree are individual's sample name as shown in Table 1

Principle Coordinate Analysis

PCoA obtained on account of RAPD data showed total 52.49% of the genetic similarity variance with first three principal coordinate components accounted for 9.51, 17.61 and 25.37% variation, respectively. PCoA based on the RAPD were comparable to the result of dendrogram which also revealed two distinct clusters with NL samples more scattered than rest of samples (Fig. 5A).

PCoA derived on the basis of SSR data illustrated that the first three principal coordinate accounted for 23.79, 15.08 and 12.91% variation, respectively, accounted for 51.78% of the genetic similarity variance (Fig. 5B). PCoA based on the SSR were comparable to the results of dendrogram, which also revealed two distinct clusters with TT samples more scattered than rest of samples.

Isolation by distance

The significance of the geographical pattern for the study of genetic relationships among these populations was estimated by comparing the F_{ST} / $(1–F_{ST})$ ratio and PHI_{PT} matrix for pairs of populations to the log-transformed geographical distance for SSR and RAPD data (Figs 4 and 5), respectively, so called isolation by distance hypothesis. The Mantel test results gave r value of −0.167, P = 0.367, for RAPD data (Fig. 4), whereas for SSR data r value of −0.462, P = 0.373 (Fig. 5), signifying that isolation-by-distance effect is not found in the populations. Engh (2012) reported similar results in boreal moss *Hylocomium splendens.*

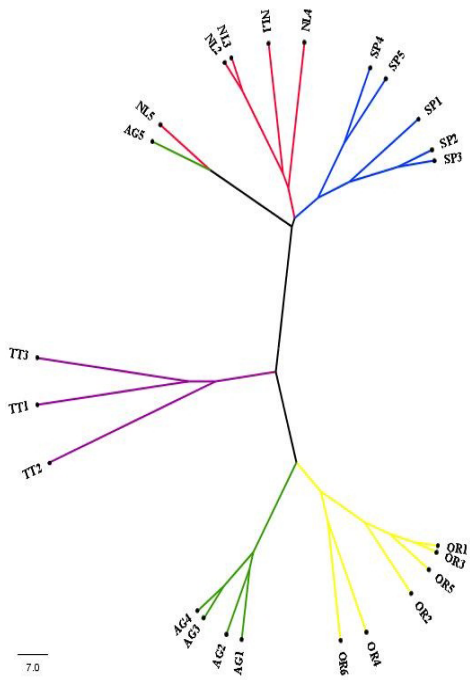

Fig. 4. UPGMA clustering based on SSR analysis obtained through collected dataset of 24 different samples of *H. involuta*. Numbers indicated in the tree are individual's sample name as shown in Table 1

Population structure of 24 genotypes of H. involuta

Further Structure analysis was conducted to examine the number of populations that may be generated from 24 genotypes using 10 RAPD and 9 SSR markers. Structure analysis identified K = 2 as the most suitable configuration consistent with

the parameters set for the software. Figures 6 and 7 show the fraction of each population that contributed to each of two clusters obtained from RAPD and SSR markers. However with RAPD data, cluster I included individuals from Nakki lake (NL) and Sunset Point (SP) populations and few individual from Oriya Road (OR) population, leaving the rest to form Cluster II. Whereas with SSR markers, cluster I included individuals from Nakki lake (NL) and Sunset Point (SP) populations and single individual from Achalgadh (AG) and Trevor's Tank (TT) population, leaving the rest to form Cluster II. Since the locations of collections of *H. involuta* genotypes are connected to each other, this may be the reason of admixing among two distinguished population. Moreo-

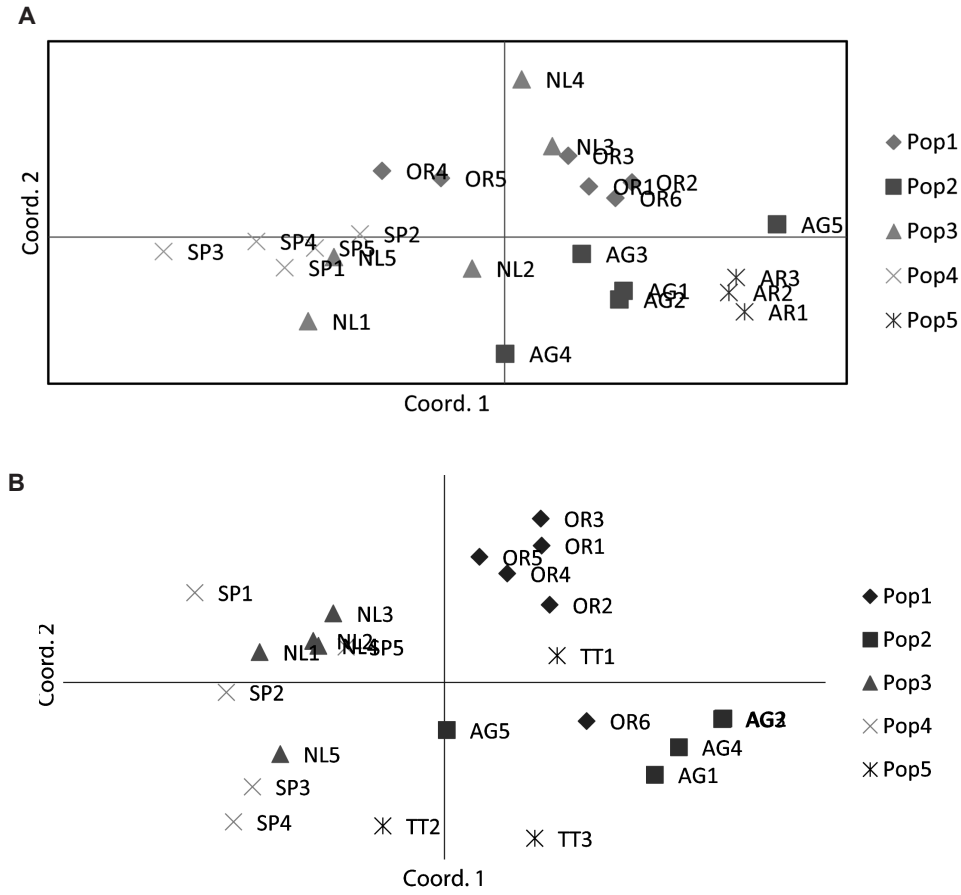

Fig. 5. Principal coordinate analysis of *H. involuta* genotypes in two-dimensional space based on (A) RAPD dataset, (B) SSR dataset (OR = Oriya Road; SP = Sunset Point; NL = Nakki Lake; AG = Achalgadh; TT = Trevor's Tank)

ver, the population structure analysis confirmed the grouping of genotypes as observed by PCoA and UPGMA.

DISCUSSION

The level of genetic variability in moss *H. involuta* is found to be moderately high across all samples in populations as reported earlier by other researchers studied in different moss species. However, no evidence of isolation by distance, but samples seems to group in two clusters that are found throughout the distribution range. This can be elucidated, as likely the ecotypic differentiations are frequently superimposed by population genetic processes, for instance the founder effects or genetic drift (Nevo *et al.* 1988).

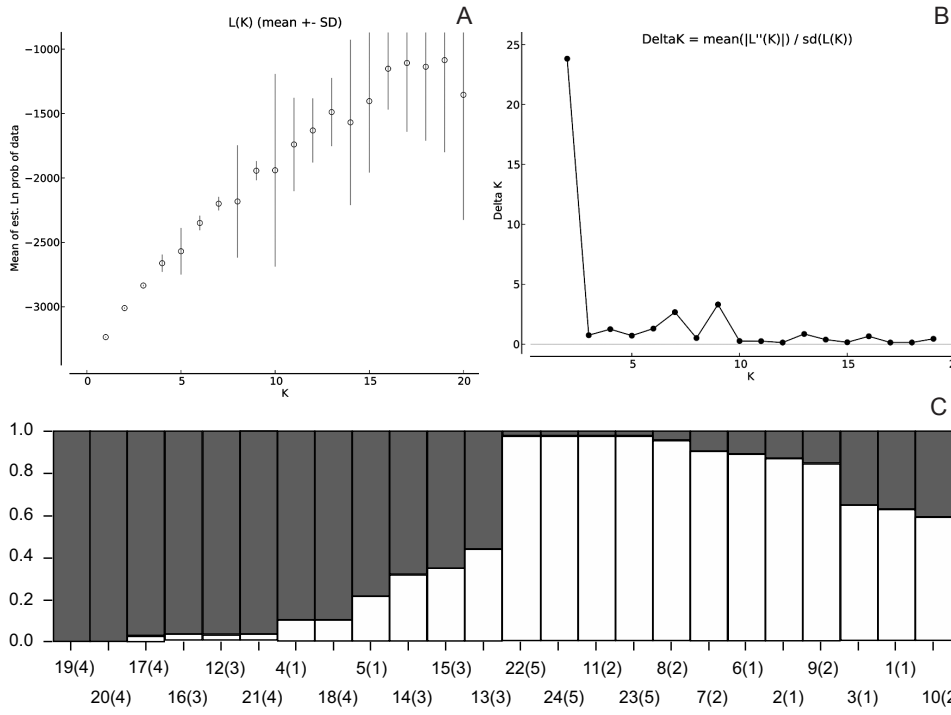

Fig. 6. Population structure analysis**.** (A) Plot of Ln P(D) ± SD using Structure Harvester software based on RAPD data. (B) Plot of delta K (ΔK) obtained using Structure Harvester software based on RAPD data. (C) Clustering of 24 genotypes of *H. involuta* based on RAPD data collected from different locations of Mount Abu, Rajasthan using Structure version 2.3.4 software. The numbers corresponds to serial number of samples as shown in Table 1. The numbers in brackets represent the 5 distinct population (1 = Oriya Road; 2 = Achalgadh; 3 = Nakki Lake; 4 = Sunset Point; 5 = Trevor's Tank)

Also the lower value of H_O than H_E in the present study is an indication of inbreeding, as inbreeding increases the frequency of homozygotes at the expense of heterozygotes. In haploid moss organism, inbreeding along with multiple-niche selection is responsible for increased rate of allelic frequency change occur due to expression of recessive alleles in inbred homozygotes (Stenøien and Såstad 2001). Due to inbreeding the increase in genetic diversity may be slow as it dominates the sexual reproduction in many moss species (Wyatt *et al.* 1989).

The level of genetic diversity in *H. involuta* is intermediate to high when compared to other mosses. For instance, genetic diversities in the genus *Sphagnum* based on microsatellite markers are reported as of 0.594 for *S. cossonii*, 0.457 for *S. revolvens* (Kophimai *et al.* 2014), 0.174 for *S. junghuhnianum*, 0.367 for *S. palustre* and 0.298 for *S. imbricatum*. Korpelainen *et al.* (2008) based on microsatellite markers reported comparatively, lower value of genetic di-

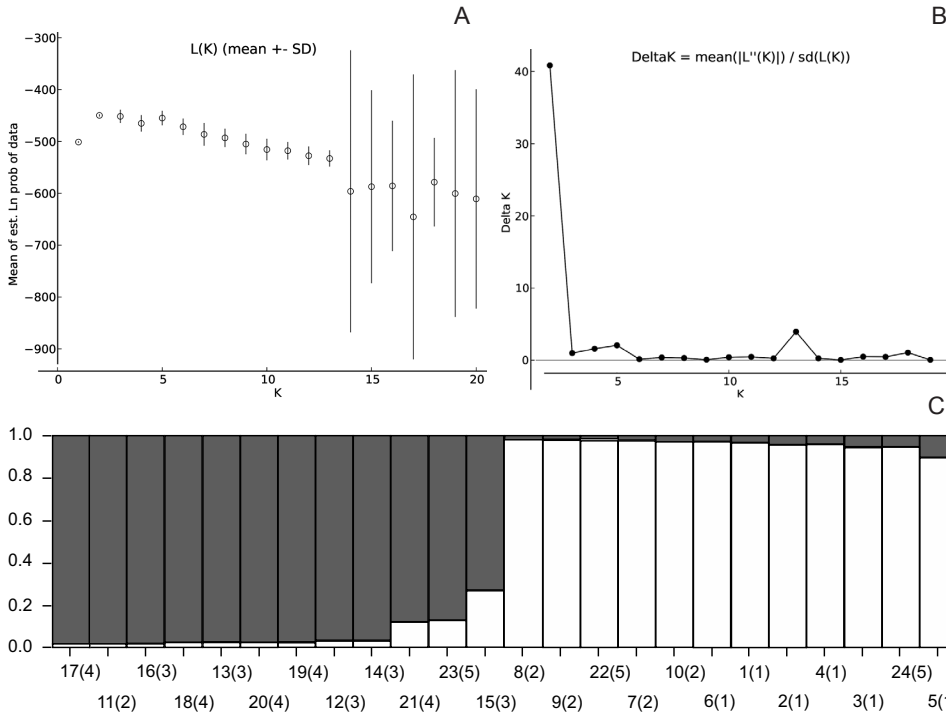

Fig. 7. Population structure analysis. **(A)** Plot of Ln P(D) ± SD using Structure Harvester software based on SSR data. **(B)** Plot of delta K (ΔK) obtained using Structure Harvester software based on SSR data. **(C)** Clustering of 24 genotypes of *H. involuta* based on SSR data collected from different locations of Mount Abu, Rajasthan using Structure version 2.3.4 software. The numbers corresponds to serial number of samples as shown in Table 1. The numbers in brackets represent the 5 distinct population (1 = Oriya Road; 2 = Achalgadh; 3 = Nakki Lake; 4 = Sunset Point; 5 = Trevor's Tank)

versity i.e. 0.28 and 0.20 in *Rhytidiadelphus subpinnatus* and *R. squarrosus* populations, respectively. The neutral somatic mutation developing in various vegetative parts of the plant can give a probable elucidation of the observed genetic diversity as has been assumed for other bryophytes (Buczkowska *et al.* 2010, Karlin *et al.* 2012) as well as seed plant (Wolf *et al.* 2000).

The factors such as reproductive mode, recruitment, somatic mutation and geographic range have great effect on the level of genetic variation in moss populations (Cronberg 2002, Vander-Velde and Bijlsma 2003). However, reproduction in many bryophytes may be both sexual (spores) and vegetative (propagules), as in the case of *H. involuta*. The mode of spore dispersal (wind or water) plays a significant role in rate of gene flow among populations. Generally, vegetative propagules are responsible for short range dispersal and spores for long distance dispersal. Thus, the contribution of long distance spore dispersal to distant patches in increasing local genetic diversity is acceptable for study, as lately suggested for *Ceratodon purpureus* and *Pyrrhobryum mnioides* (McDaniel and Shaw 2005).

Further, the timing of recruitment of diaspores also affects the level of genetic diversity. The level of genetic variability after continuous recruitment was higher, while it decreases periodically due to clone loss (Cronberg 2002). Consequently, the genetic diversity found in *H. involuta* populations implies continuous recruitment of diaspores from adjacent areas. The spores as well as asexual propagules (gemmae) dispersed from adjacent region might intermittently provide a genetic mosaic which transforms the genetic structure of pre-existing colony. According to Cronberg (2002), the recruitment and habitat disturbance measures are interrelated, for instance, fragmentation of propagules (gemmae or leaf) during the dry season in the present study. Thus, both events might produce highly dispersed propagules which result in high intra-population genetic diversity. Also, Shaw *et al.* (2008) based on microsatellite markers reported a higher (59%) variation within population than among population (51%) of moss *Sphagnum imbricatum.*

As well, widely distributed, common species show higher genetic diversity compared to rare species. This is a known phenomenon recognised in flowering plants (Cole 2003). As due to random genetic drift the rare alleles may be lost by chance and common alleles may become fixed, resulting in low diversity (Frankham *et al.* 2004). Thus, populations of *H. involuta* commonly distributed in Mount Abu show moderately high genetic variation.

The most probable explanation for genetic variation among populations is efficient spore dispersal. In *Hyophila involuta*, the long distance spore dispersal (Frahm 2012) through wind and gradual dispersal of asexual diaspores (gemmae, range *ca* 100–250 μm) (Britton 1904) through water channels may account for high genetic variability. There is sufficient evidence of long distance dispersal in bryophytes (Karlin *et al.* 2012). Vander-Velde and Bijlsma

(2003) used both allozyme and microsatellite markers to study the genetic variation in five species of the genus *Polytrichum* along a north–south gradient in Europe. Isolation by distance cannot be found in three of the *Polytrichum* species, *viz. P. commune*, *P. uliginosum* and *P. piliferum*. Thus, authors have concluded that the efficient spore dispersal might be responsible for abundant gene flow. Results similar to those found for *H. involuta* are known in other spore producing organisms.

In the present study, the variation within the populations is higher than the variation among populations. This was supported by the value of F_{ST}/G_{ST} and AMOVA analysis, which demonstrated that a higher fraction of the genetic variations reside within the populations, whereas lower proportion of genetic variation among populations. Earlier reports (Buczkowska *et al.* 2010, Shaw *et al.* 2008) employing different moss species also support the present findings. Similarly, Travadon *et al.* (2011) reported high genetic variability and haplotypic richness in fungal plant pathogen with higher differentiation within populations and lower variation among populations and there was no isolation by distance. Thus, he proposed that high dispersal rates and/or large effective populations' size may be responsible for this pattern. Furthermore the value of estimate of Nm (gene flow) in the present study is 1.361 for RAPD and 1.529 for SSR, indicating a moderately low profile (Nm>1<4) (Kumar *et al.* 2014). And also habitat fragmentation, which causes population isolation or dispersal distances for asexual propagules, which are generally short owing to their weight (Laaka-Lindberg *et al.* 2003) may delimit the rate of gene flow (Padmesh *et al.* 2012). Consequently, genetic variation within populations of *H. involuta* is moderately high.

In the present study, the low Mantel test values represent several aspects such as random distribution of the genetic variation, successful propagation of spores, etc. However, the long-distance dispersal of spores in *H. involuta* and also considerably high genetic variation over short geographic distances (average height of Mount Abu, 1400 m) and low genetic variation over significantly large geographic distances may be interpreted from the mantel test. Similar result was reported by Engh (2012) in moss *H. splendens* populations. He concluded that the high genetic variation in moss *H. splendens* across short geographical distances may be due to extreme gene flow between closely situated populations.

The comparable result obtained from both marker system in terms of moderately high level of genetic differentiation between populations of *H. involuta* occur due to moderate gene flow. Gene flow may possibly be facilitated by spore and asexual propagules dispersal in moss *H. involuta*. As the dispersal of asexual propagules in this species is generally restricted to a few meters, thus the effective spore dispersal must be responsible for the moderate levels

of gene flow. This supports the assumption of Van-Zanten and Pócs (1981), which proposed that the long distance dispersal of spores may be accountable for absence of genetic differentiation in geographically distant populations. As evident from the present study, a combination of various factors might be accountable for high level of genetic variability in *H. involuta* which exhibits a totally contrast view point compared to well established traditional view which states that the bryophyte display a low level of variation, owing to their dominating haploid phase (Wilson and Provan 2003). Thus, the intermingling, continuous and massive recruitment and mode of spore dispersal probably played remarkable roles in maintaining the high genetic variability among *H. involuta* population of Mount Abu, Rajasthan. However, further studies are needed to demonstrate the effect of the microhabitat (moisture, pH, temperature, etc.) on population structure of moss species prevailing in the study area.

The outcome from both marker systems conclude that sexual reproduction is considerably more significant component than asexual reproduction for determining the genetic structure of this species. These markers can be efficiently utilised to facilitate the understanding of population structure of other moss species and thus potentially provide a much deeper understanding of different modes of reproduction and their effects on the genetic structure of plant species.

CONCLUSIONS

In conclusion, the results demonstrated that both RAPD and SSR markers proved to be valuable and efficient tools to study genetic variation in *H. involuta*. RAPD based analysis has the limitation of reliability and transferability. But if a standard protocol is followed the reliability of RAPD data can become high. Both marker systems are equivalently accurate in clustering genotypes according to location of collection but microsatellites are more accurately reproducible than RAPD. However, the two markers system indicate different aspects of genetic variation, but both have revealed moderately high levels of polymorphism in populations of *H. involuta*.

*

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