Nucleus (2018) 61:19–27
https://doi.org/10.1007/s13237-017-0219-2

ORIGINAL ARTICLE

Molecular analysis of genetic diversity and population structure in *Everniastrum cirrhatum* (Fr.) Hale (Parmeliaceae) in India

Niraj Singh[1] · Rohit K. Verma[1] · Narender Kumar[1,2] · Rajesh Bajpai[3] · Dalip K. Upreti[3] · Tikam S. Rana[1]

Received: 16 December 2016 / Accepted: 16 August 2017 / Published online: 31 August 2017

Abstract *Everniastrum cirrhatum* is a medicinally important lichen used in Ayurvedic and Unani systems of medicine. In the present study, DAMD and ISSR methods were used to estimate the genetic variation and population structure of *E. cirrhatum* collected from different geographical regions of India. Four DAMD and ten ISSR primers detected 42 and 110 polymorphic bands and accounted for 95.65 and 94.24% polymorphisms, respectively. Cumulative band data generated for DAMD and ISSR markers resulted into 94.95% polymorphism across all the accessions of *E. cirrhatum*. The UPGMA dendrogram showed two major clusters. The clustering pattern in the UPGMA dendrogram revealed that the groupings are largely in congruence with the geographical distribution of the accessions. Clustering patterns in STRUCTURE revealed that geographical diversity is perfectly in congruence with the genetic diversity. The clustering pattern in STRUCTURE was also supported by PCoA. Mantel test for matrix correlation showed a weak but positive correlation between genetic and geographical distance. The hierarchical analysis of molecular variance revealed that maximum percentage of variation was found within a population (57%), followed by among regions (28%) and among populations (15%). The present study provides significant insight into the genetic variability and population structure of *E. cirrhatum*. Understanding population structure would provide baseline information for developing its sustainable management strategies. It would also be important to conserve populations of *E. cirrhatum* in different localities of the Himalayan regions to prevent population decline caused by anthropogenic and environmental stochastic effects.

Keywords DAMD · DNA marker · Genetic variations · ISSR · Molecular tools

Introduction

Lichens occur in very different climatic conditions ranging from the poles to the tropics. *Everniastrum* is characterized by regularly dichotomously branched lobes, apothecia with hollow stipe, relatively large asci and a thin hypothecium [28]. *Everniastrum cirrhatum* (Fr.) Hale (Parmeliaceae) is a foliose lichen and has been reported from different countries such as India, Bhutan, China, Japan, Nepal, Sri Lanka and Taiwan, central and South America and southern Asia. It is commonly known as '*Charila*' in India and found luxuriantly in the tropical Himalayas, central India and higher altitudes of southern India [17]. *E. cirrhatum* is medicinally very important and is used in Ayurvedic and Unani systems of medicine for dyspepsia, stomach disorders, excessive salivation, bronchitis, bleeding piles, scabies, leprosy, spermatorrhoea, amenorrhea, enlarged spleen, tooth ache, soreness of throat and wound healing. It is also useful as a diuretic, soporific and sedative [3, 16].

Electronic supplementary material The online version of this article (doi:10.1007/s13237-017-0219-2) contains supplementary material, which is available to authorized users.

✉ Tikam S. Rana
ranatikam@gmail.com; ranats@nbri.res.in

[1] Molecular Systematics Laboratory, CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, India

[2] Academy of Scientific and Innovative Research (AcSIR), Anusandhan Bhawan, 2 Rafi Marg, New Delhi 110 001, India

[3] Lichenology Laboratory, CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, India

Though thallus morphology has been very useful in discriminating different species of lichenized fungi, morphological attributes, however, can be variable within a species. Habitat and microclimate significantly influence the morphology of lichenized fungi [9]. Molecular markers could be helpful to explore the mode and effectiveness of dispersal, gene exchange, and intra as well as inter-specific relationships in lichens [25]. DNA based methods are simple, rapid and unaffected by the environmental conditions and have been successfully used to estimate the genetic variability in many lichenized fungi [8, 11, 14, 15, 24, 27, 31, 32]. In the present study, directed amplification of minisatellite DNA (DAMD) [10] and inter simple sequence repeats (ISSR) [23] methods were considered to estimate the genetic diversity and population structure in *E. cirrhatum*. These methods are quick, inexpensive, reveal high levels of polymorphism and only require small amounts of template DNA for PCR amplification, and they do not require any sequence information about the genome. There is no information available on genetic diversity and population structure of *E. cirhatum*. The present study, therefore, was envisaged to estimate the genetic diversity and population structure of *E. cirrhatum* occurring in India using DAMD and ISSR markers.

Materials and methods

Plant materials and genomic DNA isolation

A sampling of *E. cirrhatum* accessions was carried out in different localities of Sikkim, West Bengal, Arunachal Pradesh, Meghalaya, Himachal Pradesh and Uttarakhand states of India (Fig. 1). A total of forty-five accessions of *E. cirrhatum* and a closely related taxon-*Flavoparmelia caperata* (out-group) were analyzed (Table 1). Voucher specimens for all accessions have also been deposited in the herbarium of CSIR-National Botanical Research Institute (LWG), Lucknow. Total genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden Germany), according to the instructions of the manufacturer. The quantitative analysis of genomic DNA was carried out on 0.8% TAE agarose gel and NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

DAMD and ISSR amplification

The DAMD primers were custom synthesized from Sigma Aldrich Chemicals Pvt. Ltd. India, as per the primer sequences available in the public domain [12]. A set of 20 DAMD and 100 ISSR primers (University of British Columbia, Canada) were initially screened with two template DNAs, and only four DAMD and ten ISSR primers resulted in discrete profiles consisting of well-separated fragments (Table 2). DNA amplification with DAMD and ISSR primers were carried out according to [27].

Agarose gel electrophoresis

The PCR products were resolved on 1.5% agarose gel using 0.5× TBE buffer at a constant voltage (5 V/cm). After electrophoresis, the gel stained with ethidium bromide was visualized and documented using Gel Documentation System (UV Tech, UK).

Statistical analysis

The data for DAMD and ISSR markers were scored manually as presence (1) and absence (0) of bands. The

Fig. 1 Map showing the collection sites of *E. cirrhatum* in India. Sampling localities of the populations like Sikkim, Darjeeling, Arunachal Pradesh, Meghalaya, Uttarakhand and Himachal Pradesh along with their geographical coordinates are indicated

Table 1 Locations of *E. cirrhatum* (EC) accessions used in DAMD and ISSR analyses

S. no.	Sample code	Voucher no.	Locality	Lattitude/longitude	Altitude (m)	Substratum
1.	EC_SKM 01	14-024954	Kupup, South Sikkim, SK	27°17′26.2″N 88°50′02.4″E	3702	On Twig
2.	EC_SKM 02	14-024919	Kupup, South Sikkim, SK	27°17′45.6″N 88°49′54.8″E	3902	On Twig
3.	EC_SKM 03	14-024905	Kupup, South Sikkim, SK	27°17′26.2″N 88°50′02.4″E	3702	On Bark
4.	EC_SKM 04	14-024928	Kupup, South Sikkim, SK	27°17′04.3″N 88°48′11.2″E	3894	On Twig
5.	EC_SKM 05	14-024941	Kupup, South Sikkim, SK	27°18′27.7″N 88°49′17.5″E	3865	On Twig
6.	EC_SKM 06	14-025038	Tsongmo Lake, North Sikkim, SK	27°52′03.5″N 88°32′38.4″E	3682	On Twig
7.	EC_SKM 07	14-024901	Kupup, South Sikkim, SK	27°18′27.7″N 88°49′17.5″E	3865	On Bark
8.	EC_SKM 08	14-022286	Kupup, South Sikkim, SK	27°18′27.7″N 88°49′17.5″E	3865	On Twig
9.	EC_SKM 09	14-024930	Kupup, South Sikkim, SK	27°17′37.2″N 88°49′41.4″E	3749	On Bark
10.	EC_DRL 10	14-022281	Darjeeling, WB	26°58′12.8″N 88°21′26.1″E	1451	On Twig
11.	EC_DRL 11	14-024985	Darjeeling, WB	27°05′18.3″N 88°30′22.2″E	1662	On Bark
12.	EC_DRL 12	14-022066	Darjeeling, WB	26°58′24.9″N 88°21′01.2″E	1642	On Twig
13.	EC_DRL 13	14-022916	Darjeeling, WB	27°03′07.7″N 88°16′25.3″E	1871	On Bark
14.	EC_DRL 14	14-024993	Darjeeling, WB	26°58′24.9″N 88°21′01.2″E	1642	On Twig
15.	EC_DRL 15	14-022279	Darjeeling, WB	26°58′12.8″N 88°21′26.1″E	1451	On Twig
16.	EC_DRL 16	14-022992	Darjeeling, WB	26°58′24.9″N 88°21′01.21″E	1642	On Twig
17.	EC_DRL 17	14-022290	Darjeeling, WB	27°05′11.8″N 88°30′32.8″E	1630	On Twig
18.	EC_SML 18	02-79122	Shimla, HP	31°05′51.3″N 77°16′07.6″E	2602	On Twig
19.	EC_SML 19	02-79019	Shimla, HP	31°05′55.9″N 77°16′03.9″E	2607	On Twig
20.	EC_SML 20	02-81590	Shimla, HP	31°15′29.5″N 77°27′36.3″E	2677	On Twig
21.	EC_SML 21	02-81590	Shimla, HP	31°15′26.1″N 77°27′36.7″E	2687	On Twig
22.	EC_ARP 22	09-12346	West kameng, ARP	27°47′58.7″N 91°48′24.4″E	4009	On Twig
23.	EC_ARP 23	12-017788	Tawang, ARP	27°38′18.5″N 91°51′58.9″E	3918	On Bark
24	EC_ARP 24	12-017752	West kameng, ARP	27°31′28.1″N 92°07′42.3″E	3758	On Bark
25.	EC_ARP 25	12-017782	West kameng, ARP	27°38′18.5″N 91°51′58.9″E	3918	On Bark
26.	EC_MLH 26	08-019101	Chrrapungi, MG	25°17′37.9″N 91°42′16.1″E	1485	On Twig
27.	EC_MLH 27	20-67508	Shora, MG	25°16′25.6″N 91°43′24.”E	1335	On Twig
28.	EC_MLH 28	20-67510	Cherrapungi, MG,	25°17′57.5″N 91°41′58.1″E	1527	On Twig
29.	EC_MLH 29	20-67508	Cherrapungi, MG	25°17′13.4″N 91°42′19.9″E	1454	On Twig
30.	EC_PTH 30	05-09770	Pithoragarh, UK	29°35′02.2″N 81°14′44.5″E	1550	On Twig
31.	EC_PTH 31	09-12193	Pithoragarh, UK	29°58′31.9″N 80°39′22.6″E	2740	On Bark
32.	EC_PTH 32	09-121771	Pithoragarh, UK	29°58′31.9″N 80°39′22.6″E	2740	On Bark
33.	EC_PTH 33	09-12193	Pithoragarh, UK	29°58′32.1″N 80°39′19.3″E	2707	On Bark
34.	EC_PTH 34	09-121771	Pithoragarh, UK	29°58′22.2″N 80°39′25.6″E	2604	On Bark
35.	EC_CHT 35	10-015288	Champawat, UK	29°24′19.1″N 80°04′54.3″E	1696	On Bark
36.	EC_CHT 36	10-015286	Champawat, UK	29°24′05.3″N 80°04′57.2″E	1663	On Bark
37.	EC_CHT 37	10-015288	Champawat, UK	29°24′22.2″N 80°05′17.2″E	1694	On Bark
38.	EC_CHT 38	10-015287	Champawat, UK	29°24′20.5″N 80°04′56.9″E	1676	On Bark
39.	EC_CHT 39	10-015285	Champawat, UK	29°21′14.7″N 79°26′41.1″E	1633	On Bark
40.	EC_CHT 40	10-015286	Champawat, UK	29°21′14.7″N 79°26′41.1″E	1633	On Bark
41.	EC_UTK 41	12-018321	Uttarkashi, UK	31°04′44.9″N 78°11′03.7″E	1975	On Bark
42.	EC_UTK 42	13-021979	Uttarkashi, UK	31°02′58.3″N 78°10′59.2″E	3050	On Twig
43.	EC_UTK 43	13-020024	Uttarkashi, UK	31°03′08.2″N 78°11′05.5″E	2820	On Bark
44.	EC_UTK 44	13-021984	Uttarkashi, UK	31°05′52.3″N 78°11′72.5″E	2312	On Twig
45.	EC_UTK 45	13-021971	Uttarkashi, UK	31°05′52.3″N 78°11′72.6″E	2312	On Twig
46.	Fc_OG	12-017421	Uttarkashi, UK	31°06′50.6″N 78°20′25.5″E	2592	On Rock

SK Sikkim, *WB* West Bengal, *HP* Himachal Pradesh, *ARP* Arunachal Pradesh, *MG* Meghalaya, *UK* Uttarakhand

Table 2 Details of DAMD and ISSR primers, total band (TB), polymorphic band (PB), percentage polymorphic band (PPB), polymorphic information content (PIC), resolving power (RP), band size (BS), effective multiplex ratio (EMR), marker index (MI)

Primer name	Sequence (5′–3′)	TB	PB	PPB	PIC	RP	BS	EMR	MI
DAMD								9.83	4.20
URP-9F	GGTGTGCGATCAGTTGCTG	11	10	90.91	0.36	10.36	180–1500		
OGRB01	AAGGGCTGGAGGAGGGC	12	11	91.67	0.37	9.24	120–1600		
6.2H+	AGGAGGAGGGGAAGG	11	11	100	0.45	9.29	110–1000		
14C2	AGGCAGGATTGAAGC	8	8	100	0.38	6.89	240–1600		
Total		42	40						
Average/primer		10.5	10	95.65	0.39	8.95	110–1600		
ISSR									
UBC 808	AGAGAGAGAGAGAGAGC	10	10	100	0.43	9.42	220–1200	9.52	3.87
UBC 809	AGAGAGAGAGAGAGAGG	13	12	92.31	0.40	10.93	200–1200		
UBC 811	GAGAGAGAGAGAGAGAC	14	13	92.86	0.40	11.16	180–1100		
UBC 825	GACACACACACACACACT	11	11	100	0.43	7.38	200–1000		
UBC 836	AGAGAGAGAGAGAGAGYA	7	6	85.71	0.37	6.67	220–1000		
UBC 841	GAGAGAGAGAGAGAGAY	9	8	88.89	0.39	7.82	180–1000		
UBC 861	GCCACCACCACCACCACC	12	11	91.67	0.39	11.56	100–1000		
UBC 886	VDVCTCTCTCTCTCTCT	14	14	100	0.41	10.93	180–1400		
UBC 888	BBDBCACACACACACACA	9	9	100	0.44	7.47	200–900		
UBC 889	DBDACACACACACACAC	11	10	90.91	0.39	10.09	100–800		
Total	T	110	104						
Average/primer		11	10.4	94.24	0.41	9.34	100–1400		
Total	(DAMD + ISSR)	152	144						
Average/primer	(DAMD + ISSR)	10.7	10.2	94.95	0.40	9.14	100–1600	9.68	4.04

R = A/G, Y = C/T

polymorphic information content (PIC) was calculated according to [2]. Pair wise genetic distances were computed using Jaccard's coefficient in the Free Tree program (ver. 0.9.1.5) [20]. UPGMA tree was computed using 1000 replicate bootstrap test using the same program. All the trees were viewed annotated and printed using Tree View (ver. 1.6.5) [19]. Binary data (1/0) were further utilized to calculate different genetic diversity parameters such as observed number of alleles (Na), effective number of alleles (Ne), percentage of polymorphic loci (P), allelic diversity (A), expected heterozygosity (He) and Shannon's information index (I) using GenAlEx version 6.5 [21]. The software was also used to undertake analysis of molecular variance (AMOVA), principal coordinate analysis (PCoA) and mantel test for matrix correlation between genetic distance and geographic distance.

Bayesian clustering model implanted in STRUCTURE software version 2.3 [5, 26] was used to estimate the number of natural populations (k) and the proportion of assignment of individuals from each of the assumed and inferred populations to natural genetic clusters. An admixture model with correlated allele frequencies was used to infer the number of 'k' with prior population information. All analyses were performed with a burn in period of 20,000 and a Markov Chain Monte Carlo (MCMC) replication number set up to 50,000. The program was run 20 times for each 'k' ranging from 1 to 8. The number of 'k' was estimated as per the method described by [4]. The output from STRUCTURE analysis was the proportion of ancestry membership of each of the given population in the inferred genetic cluster calculated by averaging the membership coefficient obtained from 20 runs of the real 'k'.

Results

DAMD polymorphism and genetic diversity

In the present analysis 20, DAMD primers were screened with two template DNAs, out of which 4 DAMD primers gave distinct and reproducible profiles. These DAMD primers amplified a total number of 42 bands, out of which 40 (95.65%) bands were polymorphic. The maximum number of amplified bands (12) was obtained with primer OGRB-01, whereas the primer 14C2 resulted into the minimum

number (8) of amplified bands. The primer $6.2H^+$ and 14C2 showed 100% polymorphism, whereas primer URP-9F revealed 90.91% polymorphism. Primer $6.2H^+$ produced highest PIC value (0.45), while primer URP-9F resulted into lowest PIC value (0.36). The mean PIC value obtained for 4 DAMD primers was 0.39 (Table 2).

ISSR polymorphism and genetic diversity

A set of 100 ISSR primers were screened to examine their amplification efficiency of which ten primers were chosen for further DNA amplification of *E. cirrhatum* samples. The selected ISSR primers amplified a total of 110 bands, of which 104 (94.24%) bands were polymorphic. Primer UBC-811 and UBC-886 showed the maximum number of bands (14), while primer UBC-836 produced the minimum number of bands (7). The primers UBC-808, UBC-825, UBC-886 and UBC-888 showed 100% polymorphism, whereas primer UBC-836 showed 85.71% polymorphism. Primer UBC-888 revealed maximum PIC value (0.44), while primer 836 resulted in minimum PIC value (0.37). The average PIC value obtained for 10 ISSR primers was 0.41 (Table 2). The intra-specific polymorphism resulted significantly and suggested that the natural populations of *E. cirrhatum* possess significant genetic variability.

Cumulative data analysis

A total of 14 primers (4 DAMD and 10 ISSR) revealed 94.95% polymorphism across all the *E. cirrhatum* accessions. The mean PIC value obtained with 14 primers was 0.40. The cumulative genetic distance varied from 0.19 to 0.85 with an average value of 0.65 among all the accessions. The highest intra-specific average genetic distance was (0.85) between Ec_ARP23 (Arunachal Pradesh) and Ec_UTK33 (Uttarakhand) accessions, while the lowest genetic distance was (0.19) found in between EC_SKM 01 (Sikkim) and Ec_SKM 03 (Sikkim) accessions, respectively (Data not shown). In order to ascertain the utility of both the markers, marker index (MI), effective multiplex ratio (EMR), resolving power (RP), diversity index (DI) were also estimated. The MI value for DAMD was 4.2, whereas MI value in case of ISSR was 3.87. The EMR value for DAMD and ISSR were 9.83 and 9.52, respectively. The RP of DAMD ranged from 6.9 to 10.4 with an average value of 8.95 followed by ISSR (6.7–11.6) with an average value of 9.34 (Table 2).

The number of alleles identified in each population across all loci ranged from 81 to 180. Gene diversity, inferred from Nei's heterozygosity (He) varied from 0.148 to 0.265 in all populations. Total gene diversity within the species was He = 0.226. The percentage of polymorphic loci, when pooled together from all the populations, varied from 32.24% (SML) to 65.13% (SKM) with an average value of 52.30%. The accessions belonging to SKM populations revealed highest (1.408 ± 0.069) observed number of alleles (Na) while the corresponding lowest number of observed alleles was found in the SML populations (0.849 ± 0.072), with an average value 1.182 ± 0.026 (Table 3). The effective number of alleles (Ne) was invariably higher than Na values for each population and varied from 1.234 ± 0.031 (SML) to 1.438 ± 0.034 (CHT) with an average of 1.365 ± 0.012. Shannon information index (I) among all the populations were highest in populations collected from CHT population (0.354 ± 0.025), and the lowest was recorded for SML populations. Therefore, among all the eight populations used in the present study, the CHT population exhibited the greatest level of genetic variability (Table 3). The hierarchical analysis of molecular variance (AMOVA) revealed the maximum percentage of variation within a population (57%), followed by among region (28%) and among the population (15%) (Table 4).

The genetic distance data were used to generate the UPGMA dendrogram which showed two major clusters, Clusters I and II with 100% bootstrap support (BS). The out-group taxon (*F. caperata*) was separated out clearly from rest of the *E. cirrhatum* accessions. Cluster I was divided into four sub-clusters with 98% BS (SKM, DRL, MLH, and ARP). SKM population grouped all the accessions from Sikkim, whereas sub-cluster DRL clustered together all the accessions from Darjeeling, West Bengal (WB) in two distinct groups. Sub cluster MLH grouped all the accessions of Meghalaya except one accession Ec_ARP24 from Arunachal Pradesh. Cluster II grouped together all the accessions from Western Himalaya. Cluster II was also divided into four sub-clusters with 93% BS (CHT, UTK, SML, and PTH). Sub-cluster CHT grouped all the accessions from Champawat, Sub-cluster UTK grouped all the accession from Uttarkashi, Sub cluster SML grouped all the accessions of Himachal Pradesh, and PTH grouped all the accessions of Pithoragarh. The clustering of the *E. cirrhatum* accessions in the UPGMA dendrogram revealed that the groupings are largely in congruence with the geographical affinities of the accessions (Fig. 2).

Population genetic structure

The Bayesian model of analysis of genetic structure of the natural populations of *E. cirrhatum* in STRUCTURE revealed the number of k as 2. The STRUCTURE confirmed two genetic clusters (k = 2), and four out of eight populations showed an average ancestry membership participation ≥75% to the first inferred cluster (SML-99, PTH-75, CHT-100 and UTK-100%), while the cluster 2 showed an average inferred ancestry membership participation

Table 3 Genetic variations as revealed through combined (DAMD + ISSR) markers among eight populations of *E. cirrhatum*

Population	N	N_A	Na (SD)	Ne (SD)	I (SD)	H (SD)	%P	G_{ST}
SKM	9	115	1.408 ± 0.069	1.408 ± 0.031	0.350 ± 0.023	0.249 ± 0.017	65.13%	0.432
DRL	8	114	1.382 ± 0.070	1.415 ± 0.032	0.348 ± 0.024	0.252 ± 0.018	63.16%	
ARP	4	81	0.974 ± 0.078	1.319 ± 0.033	0.259 ± 0.025	0.204 ± 0.020	44.08%	
MLH	4	87	1.020 ± 0.076	1.324 ± 0.033	0.262 ± 0.025	0.206 ± 0.020	44.74%	
PTH	5	95	1.164 ± 0.077	1.357 ± 0.032	0.301 ± 0.024	0.227 ± 0.019	53.95%	
CHT	6	115	1.362 ± 0.069	1.438 ± 0.034	0.354 ± 0.025	0.265 ± 0.019	60.53%	
UTK	5	114	1.296 ± 0.068	1.423 ± 0.034	0.335 ± 0.026	0.258 ± 0.020	54.61%	
SML	4	180	0.849 ± 0.072	1.234 ± 0.031	0.188 ± 0.023	0.148 ± 0.018	32.24%	
Total Populations	45		1.182 ± 0.026	1.365 ± 0.012	0.300 ± 0.009	0.226 ± 0.007	52.30 ± 3.98	

N, sample size; N_A, total number of alleles across loci; Na, observed no. of alleles; Ne, effective no. of alleles; H, Nei's genetic diversity; I, Shannon's information index; %P, percentage of polymorphic loci; G_{ST}, diversity among populations; SD, standard deviation

Table 4 AMOVA analysis within and between populations of *E. cirrhatum* collected from Himalayan regions of India

Source of variations	Degree of freedom	Sum of squares	Mean of squares	Variance component	Percentage of variations
Among regions	1	286.217	286.217	10.416	28
Among pops	6	311.314	51.886	5.634	15
Within pops	37	779.536	21.069	21.069	57
Total	44	1377.067		37.118	100

(SKM-100, DRL-98, ARP-99, and MLH-99%). The assignment of the geographic populations revealed a weak population structure among them (Fig. 3). The eight populations analyzed represented two discrete genetic clusters showing a weak admixture of individuals across the natural populations from North Eastern Himalaya and Western Himalayan region of the India.

The principal coordinate analysis (PCoA) of DAMD and ISSR data showed two major clusters. Cluster I grouped together all the accessions from North eastern Himalaya (SKM, DRL, ARP and MLH) and Cluster II, grouped all the accession from Western Himalaya (PTH, CHT, UTK, and SML) (Fig. S1). Mantel test for matrix correlation showed weak but positive correlation ($r = 0.185$, $P = 0.143$) between genetic distance and geographic distance.

Discussion

There are several DNA-based molecular approaches available for diversity analysis of plants and fungal genomes. Although RAPD, DAMD, and ISSR markers have been used to assess genetic variability in a number of lichenized fungus species [11, 15, 24, 27, 29, 32], there is, however, no study on the estimation of genetic diversity and population structure of *E. cirrhatum* accessions. The present study, to the best of our knowledge, seems to be the first attempt to analyze genetic diversity and population structure in *E. cirrhatum* employing DAMD and ISSR markers. The study revealed high levels of genetic diversity within the *E. cirrhatum* accessions collected from different locales of the North Eastern and Western Himalayan regions of India. Earlier studies on the genetic diversity in lichenized fungi like *Pandora neoaphidis* [29], *Lobathallia radiosa* [32], *Zwackhia viridis* [31], *Flavoparmelia caperata* [27] using different molecular markers also revealed high levels of genetic diversity within species. The present study, therefore, is in congruence with the earlier studies and revealed that DAMD and ISSR markers are significantly efficient and reliable to estimate the genetic diversity in *E. cirrhatum.*

The population diversity indices like allelic frequency (Na, Ne), Nei's genetic diversity (H), Nei's genetic differentiation (G_{ST}), Shannon's information index (I) define the genetic composition of a population. The present study revealed reasonably high genetic differentiation ($G_{ST} = 0.432$) within the population. This was also supported by the hierarchical analysis performed by AMOVA, which showed that maximum percentage of variation occurred within a population (57%), followed by among regions (28%) and among populations (15%). Similar

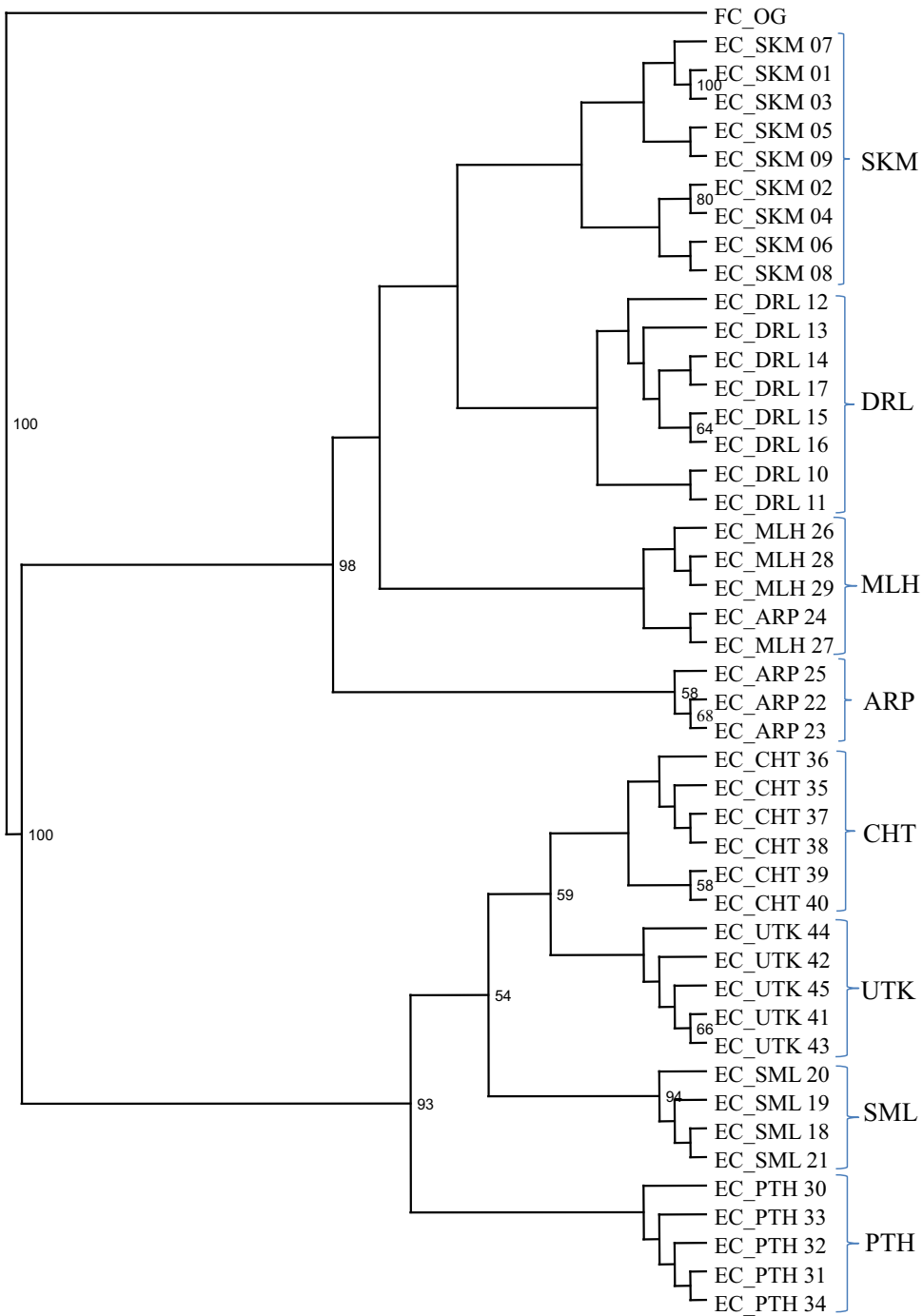
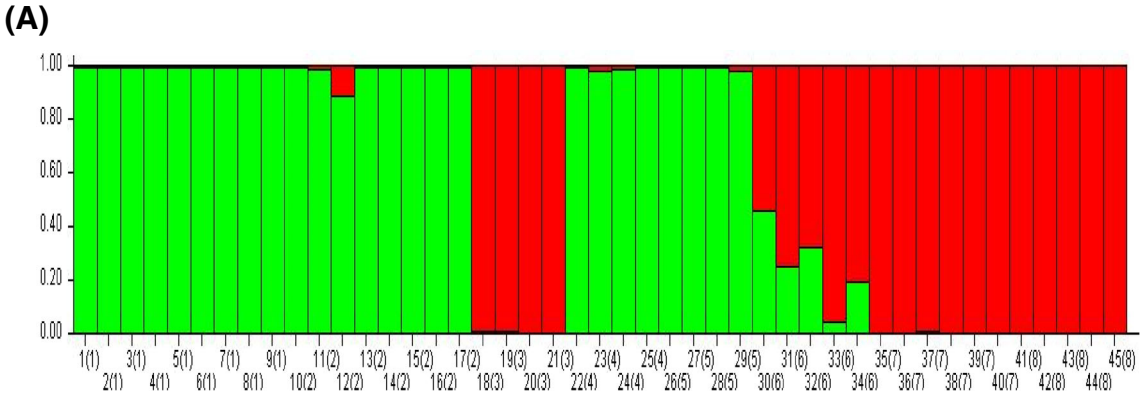


Fig. 2 UPGMA dendrogram showing the clustering patterns of 45 accessions of *E. cirrhatum* analyzed using cumulative data of DAMD and ISSR markers

levels of genetic differentiation were recorded for *Fusarium culmorum* [8], *Fusarium graminearum* [14], *Fusarium oxysporum* [1], *Lobaria pulmonaria* [18] and *Corollospora maritima* sensu lato [30]. In the present study, SML population was characterized by the minimum allelic diversity, heterozygosity, and polymorphism in comparison to its corresponding populations (Table 3). The low genetic diversity in the SML population was due to an unprecedented level of human induced habitat destruction and fragmentation in the western Himalayas in relatively recent times. The genetic diversity in *Lobaria pulmonaria* was found higher in populations where the fungal symbiont reproduced sexually [33], and this is in congruence with the level of genetic diversity obtained in the case of *E. cirrhatum.* However, several factors may affect the estimate of genetic variabilities like a number of markers used, distribution of markers in the genome, and the nature of evolutionary mechanism [22]. Genetic variability forms the



(B)

Given Pop	Inferred Clusters		Number of Individuals
	Cluster 1	Cluster 2	
EC_SKM (1)	0.002	**0.998**	9
EC_DRL (2)	0.017	**0.983**	8
EC_SML (3)	**0.994**	0.006	4
EC_ARP (4)	0.009	**0.991**	4
EC_MLH (5)	0.006	**0.994**	4
EC_PTH (6)	0.745	0.255	5
EC_CHT (7)	**0.995**	0.005	6
EC_UTK (8)	**0.999**	0.001	5

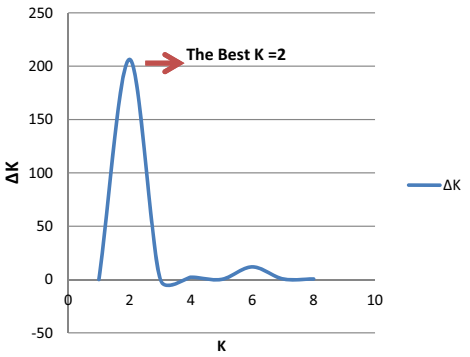


Fig. 3 **a** Proportional membership of 45 individuals from the eight studied populations of *E. cirrhatum* in two clusters identified by STRUCTURE. *Each individual* is represented by a *single vertical bar*. **b** A number of individuals, geographical populations and average genetic membership fractions in the two genetic clusters. **c** The *graph* is representing the best K

basis of the evolutionary potential of a species to respond to environmental changes. Loss of genetic variability reduces the future adaptability to environmental changes [6, 7, 13]. Therefore, maintenance of genetic variation within a species is very important for long term survival.

On the basis of STRUCTURE analysis, eight natural populations of *E. cirrhatum* differentiated into two genetic populations. Cluster-I consisted of all the populations from North Eastern Himalaya (SKM, DRL, ARP, MLH), where as Cluster-II consisted of populations from Western Himalayan region (PTH, CHT, UTK, SML). Clustering patterns in STRUCTURE revealed that geographical diversity is perfectly in congruence with the genetic diversity. The clustering pattern in STRUCTURE was also supported by PCoA. The present study provides significant insights in unraveling the genetic variability and population structure in *E. cirrhatum* using DAMD and ISSR markers. Understanding population structure of *E. cirrhatum* would provide baseline information for developing sustainable management strategies. It would also be important to conserve populations of *E. cirrhatum* in different locations of Himalayan regions to prevent population decline caused by large scale anthropogenic and environmental stochastic effects predominantly operating in the Himalayan regions.

The extent of genetic diversity and population differentiation resulted from the present study indicated that *E. cirrhatum* has no immediate threat perception operating on the different populations of the species. However, the fragmented state of its habitats requires adequate measures for maintenance of genetic diversity. The present endeavour seems to be the first report on the molecular analysis of genetic diversity and population structure of *E. cirrhatum* from its distributional range in India.

Acknowledgements The study was financially supported by the Department of Biotechnology, New Delhi (No. BT/PR1457/39/204/2011).

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

References

1. Bayraktar H, Dolar FS, Maden S. Use of RAPD and ISSR markers in detection of genetic variation and population structure among *Fusarium oxysporum* Fr. sp. *ciceris* isolates on chickpea in Turkey. J Phytopathol. 2008;156:146–54.

2. Botstein D, White RL, Skolnick M, Davis RW. Construction of a genetic linkage map in man using restriction fragment length polymorphism. Am J Hum Genet. 1980;32:314–31.
3. Chandra S, Singh A. A lichen crude drug (Chharila) from India. J Res Educ Indian Med. 1971;6:209–15.
4. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol. 2005;14:2611–20.
5. Falush D, Stephens M, Pritchard JK. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics. 2003;164:1567–87.
6. Frankham R. Quantitative genetics in conservation biology. Genet Res. 1999;74:237–44.
7. Frankham R. Relationship of genetic variation to population size in wildlife. Conserv Biol. 1996;10:1500–8.
8. Gargouri S, Bernier L, Hajlaoui MR, Marrakchi M. Genetic variability and population structure of the wheat foot rot fungus, *Fusarium culmorum*, in Tunisia. Eur J Plant Pathol. 2003;109:807–15.
9. Grube M, Hawksworth DL. Trouble with lichen: the evaluation and re-interpretation of thallus form and fruit body types in the molecular era. Mycol Res. 2007;111:1116–32.
10. Heath DD, Iwama GK, Devlin RH. PCR primed with the VNTR core sequences yields species specific patterns and hypervariable probes. Nucl Acids Res. 1993;21:5782–5.
11. Honegger R, Zippler U. Mating system in representatives of Parmeliaceae, Ramalinaceae and Physciaceae (Lecanoromycetes, lichen-forming ascomycetes). Mycol Res. 2007;111:424–32.
12. Hu J-B, Li J-W, Wang L-J, Liu L-J, Si S-W. Utilization of a set of high-polymorphism DAMD markers for genetic analysis of a cucumber germplasm collection. Acta Physiol Plant. 2011;33:227–31.
13. Lande R. Genetics and demography in biological conservation. Science. 1988;241:1455–60.
14. Mishra PK, Tewari JP, Clear RM, Turkington KT. Molecular genetic variation and geographical structuring in *Fusarium graminearum*. Ann Appl Biol. 2004;145:299–307.
15. Murtagh GJ, Dyer PS, Furneaux PA, Crittenden PD. Molecular and physiological diversity in the bipolar lichen-forming fungus *Xanthoria elegans*. Mycol Res. 2002;106:1277–86.
16. Nadkarni KM. Indian materia medica. Mumbai: Popular prakashan; 1996.
17. Nash TH III, Ryan BD, Gries C, Bungartz F. Lichen flora of the greater Sonoran desert region, vol I. Lichens unlimited. Tempe: Arizona State University; 2002.
18. Otalora MAG, Belinchon R, Prieto M, Aragon G, Izquierdo P, Martinez I. The threatened lichen *Lobaria pulmonaria* in the Iberian Peninsula: genetic diversity and structure across a latitudinal gradient. Fungal Biol. 2015;119:802–11.
19. Page RDM. TreeView (Win32), Ver. 1.6.5; 2001. Available from: http://taxonomy.zoology.gla.ac.uk/rod/treeview.html.
20. Pavlicek A, Hrda S, Flegr J. Free tree—freeware program for construction of phylogenetic trees on the basis of distance data and bootstrapping/jackknife analysis of the tree robustness. Application in the RAPD analysis of the genus *Frenkelia*. Fol Biol (Praha). 1999;45:97–9.
21. Peakall R, Smouse PE. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics. 2012;28:2537–9.
22. Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, et al. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol Breed. 1996;2:225–38.
23. Prevost A, Wilkinson MJ. A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theor Appl Genet. 1999;98:107–12.
24. Printzen C, Lumbsch HT, Schmitt I, Feige GB. A study on the genetic variability of *Biatora helvola* using RAPD markers. Lichenologist. 1999;31:491–9.
25. Printzen C. Fungal specific primers for PCR-amplification of mitochondrial LSU in lichens. Mol Ecol Res. 2002;2:130–2.
26. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000;155:945–59.
27. Singh N, Bajpai R, Mahar KS, Tiwari V, Upreti DK, Rana TS. ISSR and DAMD markers revealed high genetic variability within *Flavoparmelia caperata* in Western Himalaya (India). Physiol Mol Biol Plants. 2014;20:501–8.
28. Sipman HJM. Studies on Colombian cryptogams. X. The genus *Everniastrum* Hale and related taxa (Lichens). Proc K Ned Akad van Wet C. 1980;83:333–54.
29. Tymon MA, Pell JK. ISSR, ERIC and RAPD techniques to detect genetic diversity in the aphid pathogen *Pandora neoaphidis*. Mycol Res. 2005;109:285–93.
30. Velez P, Quintero CA, Merino G, Gasca-Pineda J, González MC. An ISSR-based approach to assess genetic diversity in the marine arenicolous fungus *Corollospora maritima* sensu lato. Mycoscience. 2016;3:187–95.
31. Wieczorek A, Achrem M, Mitka JR, Rogalski M, Werczynska K. Genetic variability of the populations of *Zwackhia viridis* (ach.) poetsch and schied (lecanographaceae, lichenized ascomycetes) in the eastern poland: geographic *versus* habitat distance. Pol J Ecol. 2014;62:253–61.
32. Yuzbasioglu E, Halici MG, Karabacak M, Aksoy A. RAPD and ISSR markers indicate high genetic variation within *Lobathallia radiosa* in Turkey. Mycol Prog. 2011;10:219–28.
33. Zoller S, Lutzoni F, Scheidegger C. Genetic variation within and among populations of the threatened lichen *Lobaria pulmonaria* in Switzerland and implications for its conservation. Mol Ecol. 1999;8:2049–59.