RESEARCH ARTICLE

Molecular analyses of genetic variability in the populations of *Bergenia ciliata* in Indian Himalayan Region (IHR)

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Abstract *Bergenia ciliata* is an important medicinal plant species of Indian Himalayan Region (IHR). Genetic variability and population genetic structure of *B. ciliata* sampled from IHR was studied using two single primer amplification reaction (SPAR) methods (DAMD: Directed Amplification of Minisatellite region DNA; ISSR: Inter Simple Sequence Repeats). To provide a reasonable scientific basis for management and conservation of *B. ciliata* populations in IHR, genetic diversity analysis of 11 populations with 24 SPAR markers (15 ISSR and 9 DAMD) revealed significantly high level of (90.03%) polymorphism at species level. However, genetic variability was low at population level and KUL and BWS populations showed maximum while SHM population revealed least genetic diversity among the 11 populations. Analysis of molecular variance revealed highest percentage of variation (73%) within populations, followed by 17% among populations and least (10%) among the Himalayan regions. Clustering pattern obtained from UPGMA dendrogram was supported by STRUCTURE and principal coordinate analysis, segregating all the 11 natural populations of *B. ciliata* into two genetic clusters: Eastern and Western Himalayan populations. The clustering patterns of all the three statistical methods indicated that populations of *B.*

ciliata have structured in response to the local micro-climates of the habitats in IHR, and therefore, it can be concluded that genetic variability is in congruence with the geographical diversity.

Keywords *Bergenia ciliata* · SPAR markers · Genetic variability · Population structure · Indian Himalayan Region

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Introduction

Bergenia ciliata (Haw.) Sternb. (family—Saxifragaceae) is a perennial rhizomatous herb, predominantly found in temperate and sub-temperate Himalayan regions at an elevation of 350–4000 m from Afghanistan to eastward in Pakistan, India, Nepal and Bhutan (Singh and Rawat 2000; Rana et al. 2003). In India, it occurs mostly in humid, damp, moist to dry and open to shady habitats; on rock surfaces, crevices, hill slopes and near water channels. It is commonly known as pashanbheda or stone breaker because of its habitat characteristics and applications in dissolving urinary calculi in Indian System of Medicine (Kirtikar and Basu 1935). The plants of *B. ciliata* are traditionally used to cure various ailments such as boils, burns, cuts and wounds, fever, liver complaints, thirst, asthma, ophthalmia, piles, kidney stones, and urinary problems and diarrhoea in cattle (Kirtikar and Basu 1935; Rana and Samant 2011). Young shoots are edible, while rhizomes, leaves and roots possess anthelmintic, antitussive, anti-inflammatory, antiulcer, anticancer, antioxidant, antidiabetic, antibacterial, antifungal, antimalarial, antineoplastic activities and cytoprotective effects (Khan and Kumar 2016; Ahmad et al. 2018). Due to the presence of various bioactive metabolites, *B. ciliata* is used as a chief botanical source

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for preparations of various Herbal and Unani medicines. It constitutes one of the major ingredients of indigenous drugs like Cystone and Calcuri used clinically to treat kidney and urinary bladder stones (Asolkar et al. 1992), and Herbenol-herbal antiseptic cream useful in treatment of burns and wounds.

Bergenia ciliata has been investigated with respect to morphology (Chowdhary and Verma 2010), cytology (Kumar et al. 2012), tissue culture and micropropagation (Rafi et al. 2016), therapeutic activities and phytochemistry (Khan and Kumar 2016; Ahmad et al. 2018). Recent studies in different parts of Indian Himalayan Region (IHR) have categorized this species as vulnerable (Khan et al. 2013; Gajurel et al. 2015), and near threatened (Kumari et al. 2012). The increased demand of *B. ciliata* in medicine and healthcare has adversely affected the natural habitats of this species, leading to population size depletion, fragmentation and loss of genetic diversity. Therefore, the conservation of available genetic diversity of this high value plant resource is of utmost importance. *B. ciliata* is also esteemed in ornamental horticulture trade as several hybrids have been produced with desired traits from *B. ciliata* by crossing with other species such as *B. stracheyi*, *B. cordifolia*, *B. crassifolia*, *B. purpurascens,* etc. (Yeo 1966). Now-a-days genetic markers are considered a valuable source for identification of potential genotypes for breeding purpose by studying genetic diversity within and among the species. There are only limited information available on genetic variability and population structure of species of the family Saxifragaceae, based on RAPD (Reisch et al. 2003), allozyme (Chang et al. 2007), microsatellite, internal transcribed spacer and chloroplast markers (Pietiläinen and Korpelainen 2013; van deer Meer and Jacquemyn 2015; Gao et al. 2017). Moreover, an earlier study specific to *B. ciliata* was restricted to Western Himalayas representing relatively a limited geographic area (Tiwari et al. 2015), though the species has a wide-spread distribution in IHR. The present study was aimed at examining the pattern of genetic variability and population structure in natural populations of *B. ciliata* found in IHR, using two SPAR markers namely, Directed Amplification of Minisatellite region DNA (DAMD; Heath et al. 1993) and Inter Simple Sequence Repeats (ISSR; Provost and Wilkinson 1999). These markers have been found suitable in investigating genetic variability and population structure in some medicinally important, endemic and threatened Himalayan species such as *Podophyllum hexandrum* (Naik et al. 2010; Nag et al. 2015), *Rheum tanguticum* (Hu et al. 2014) and *Roscoea procera* (Rawat et al. 2016). Investigating the genetic diversity and genetic structure in *B. ciliata* assumes importance because of its high demand as a valuable medicinal herb in Indian Systems of Medicine.

Materials and methods

Collection sites and extraction of DNA

Plant materials were collected from ecologically diverse populations across different forest regions in IHR (Fig. S1 and Fig. S2). A total of 11 populations comprising 111 accessions sampled from Jammu & Kashmir (15), Himachal Pradesh (28), Uttarakhand (43), West Bengal (13) and Sikkim (12) were analyzed for extent of genetic variability and population structure in *B. ciliata* (Table 1; Fig. 1). Each of the collection sites was geo-referenced for latitude (°N), longitude (°E) and altitude (m) with the help of a global positioning system receiver (Garmin, USA). While collecting, fresh leaf tissue samples were cleaned and stored dry at room temperature over self-indicating blue silica gel till extraction of genomic DNA. Voucher specimens of all samples were also collected for preparation of herbarium specimens and deposited in herbarium of CSIR-National Botanical Research Institute (LWG), Lucknow, India.

Total genomic DNA from silica-dried leaf tissues was isolated using conventional CTAB method (Doyle and Doyle 1990). The quality of the extracted DNA was checked on 0.8% agarose gel, stained with ethidium bromide and compared with a set of known concentration of lambda DNA (double digested with *EcoR*I and *Hind*III) and quantity was measured by UV spectroscopy using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., USA).

PCR amplification using SPAR markers

A set of twenty DAMD primers available in the public domain (Nakamura et al. 1988; Tourmente et al. 1994; Hu et al. 2011) were synthesized from Sigma Aldrich Chemicals Pvt. Ltd., New Delhi, India, and a set of 100 anchored ISSR primers was procured from University of British Columbia, Canada. All 20 DAMD and 100 ISSR primers were initially screened with two template DNAs of *B. ciliata*, of which 24 primers (9 DAMD and 15 ISSR) were selected for profiling of the complete set of 111 accessions of the species (Table S1). The amplification of primers was carried out according to Tiwari et al. (2015). The amplified PCR products were resolved on 1.5% agarose gel, stained with ethidium bromide, using $1\times$ TBE buffer at constant voltage of 5 V/cm. The size of each fragment for all the primers was determined using low range (100–3000 bp) size standards (Thermo Fisher Scientific India Pvt. Ltd). After electrophoresis, the gel was visualized using UV Tech Gel Documentation System (UK), and the patterns were photographed and documented as digital images. The

Table 1 Sample sites and habitat conditions of *B. ciliata* in Indian Himalayan Region

Population ID	Accession no's	Site	Habitat condition	Latitude (°N)	Longitude (°E)	Elevation (m)
DGF (24)	DGF (1–15)	Dhara Mohara Nallah, Dera Gali Forest, Rajouri, J&K	Moist, shady places, on rocks, among crevices and on big boulders along the water stream	33°35.349′	074°21.503′	2036
KUL (250)	KUL (16–30)	Kothi to Rahla, Kullu, HP	Moist, shady places, on rocks, among crevices and big boulders on gentle slopes	32°20.885′	077°13.303′	2277
SHM (150)	SHM (31–37)	Shimla, HP	Dry and open slopes	31°06.476′	077°10.105′	2135
KUF (250)	KUF (38–43)	Kufri, HP	Dry and partially shady rocky slopes	31°05.826′	077°16.380′	2529
GWS (100)	GWS (44–51)	Govind Wildlife Sanctuary, UK	Moist, on rocks and gentle slopes	31°04.498′	078°14.955′	2041
RNK (100)	RNK (52–58)	Ranikhet, UK	Dry, open rocky slopes under pine trees	29°39.917′	079°32.148′	1669
NTL (150)	NTL (59–64)	Kilbury Road, Nainital, UK	Dry, on almost vertical rocky slopes	29°25.138′	079°25.771′	2187
BWS (350)	BWS (65–77)	Binsar Wildlife Sanctuary, Almora, UK	Dry, on partially shady and gentle sandy slopes under pine forest	29°40.988′	079°43.789′	1923
PTH (150)	PTH (78–86)	Dewalthal, Pithoragarh, UK	Moist, on almost vertical slopes	29°41.089′	080°13.091′	1280
DRJ (70)	DRJ (87–99)	Darjeeling, WB	Dry, among the stone wall gaps	27°03.23.1′	088°15.16.1′	2081
PNL (50)	PNL (100–111)	Penlong, East Sikkim, SK	Moist, among the stone wall gaps and on rocky slopes	27°22.445′	088°37.328′	1646

DGF Dera Gali Forest, *KUL* Kullu, *SHM* Shimla, *KUF* Kufri, *GWS* Govind Wildlife Sanctuary, *RNK* Ranikhet, *NTL* Nainital, *BWS* Binsar Wildlife Sanctuary, *PTH* Pithoragarh, *DRJ* Darjeeling, *PNL* Penlong, *J&K* Jammu and Kashmir, *HP* Himachal Pradesh, *UK* Uttarakhand, *WB* West Bengal, *SK* Sikkim

representative gel profiles for each marker have been provided as supplementary data (Fig. S3 and Fig. S4).

Scoring of bands and statistical analysis

Reproducible profiles with consistent banding patterns generated by the two marker systems (DAMD and ISSR) were selected for data analysis. A binary data matrix for each primer was generated by scoring the data as presence (1) or absence (0) of a band. The data matrices (DAMD, ISSR and cumulative) generated by the two marker systems were analyzed statistically to evaluate relative informativeness of the two marker systems using percentage of polymorphism (%P), polymorphic information content (PIC), diversity index (DI), effective multiplex ratio (EMR) and marker index (MI) for each primer (Botstein et al. 1980; Powell et al. 1996). Mantel Z-statistics to test the goodness of fit between two marker systems was computed using NTSYS-pc software version 2.02e (Rohlf 1998).

Genetic diversity within and among populations and population genetic structure statistics were computed using cumulative data matrix. The pair wise Jaccard's genetic distances and similarity coefficient (Jaccard 1908) were calculated by Neighbour Joining (NJ) and unweighted pair group method with arithmetic mean (UPGMA)

respectively, in the FREETREE program 0.9.1.5 (Pavlicek et al. 1999). The genetic diversity coefficients were estimated assuming that the populations were in Hardy–Weinberg equilibrium through percentage of polymorphic bands (%P), mean number of observed alleles (N_a), mean number of effective alleles (N_e), Nei's genetic diversity (H) (Nei and Li 1979), Shannon information index (I) (Lewontin 1972), total genetic diversity (H_T), genetic diversity within populations (H_S), genetic differentiation coefficient (G_{ST}) and gene flow (N_m) using POPGENE program version 1.32 (Yeh et al. 1999). A population dendrogram was constructed based on Nei's unbiased genetic distances calculated in POPGENE program (Nei 1978) using UPGMA method. Analyses of molecular variance (AMOVA) to examine hierarchical partitioning of genetic variability at three strata i.e. within populations, among populations and among regions; principal coordinate analysis (PCoA) to provide spatial representation of the relative genetic distances among accessions and to determine the consistency of differentiation among populations; and Mantel test (Mantel 1967) to estimate correlation between genetic and geographic distances of populations to test isolation by distance (IBD), were performed using GenAlEx program version 6.5 (Peakall and Smouse 2012).
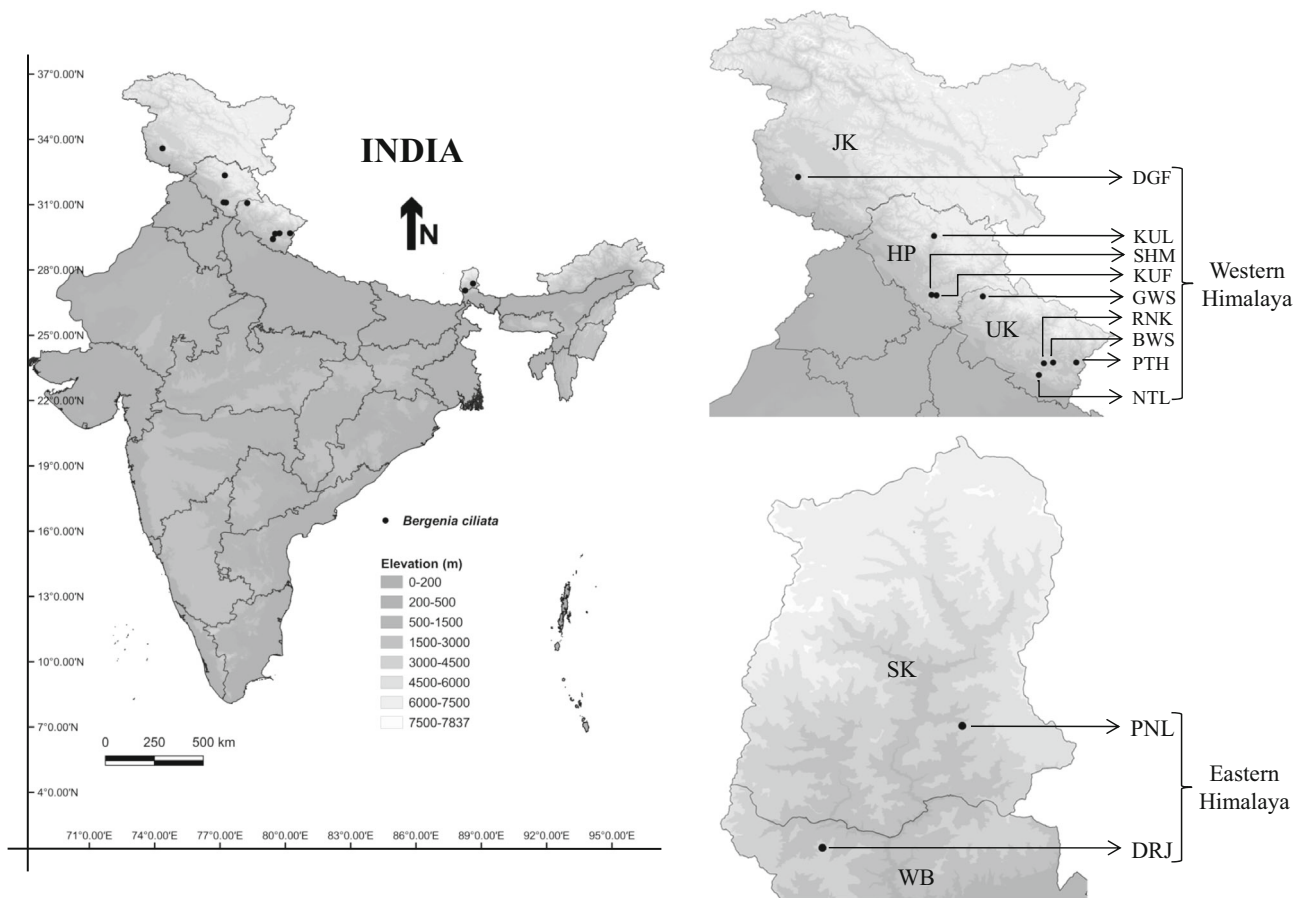
Fig. 1 Map showing the sampling locations of *Bergenia ciliata* populations in Indian Himalayan Region. The populations and state codes are same as in Table 1. [Drawn using QGIS 3.4.15 Madeira (2018)]

Bayesian clustering model implanted in STRUCTURE software version 2.3 was used to assess genetic stratification of multilocus dataset (Pritchard et al. 2000; Falush et al. 2003). Complexities of mixed ancestry in real populations can be resolved by using admixture model for analysis. Therefore, to infer the number of 'k' with prior population information, admixture model with correlated allele frequencies was taken as parameter. All analyses were performed with a burn-in time period of 50,000 and a Markov Chain Monte Carlo (MCMC) replication number set up to 100,000. The programme was run 20 times for each 'k', ranging from 1 to 11. The number of 'k' was obtained by implementing the method described by Evanno et al. (2005) in a web based python program STRUCTURE HARVESTER version 0.6.94 (Earl and vonHoldt 2012). Plots generated by STRUCTURE HARVESTER included mean of estimated Ln probability of data, Evanno results showing mean rate of change of the likelihood distribution, mean of the absolute value of second order rate of change of the likelihood distribution and the number of 'k' groups that best fit the data.

Results

DAMD and ISSR polymorphism

Amplification of nine DAMD markers with 111 *B. ciliata* accessions resulted into 131 bands with size range 100–2000 bp, of which 119 were polymorphic, revealing 90.27% polymorphism. Primer FVIIex8 resulted in the maximum number of polymorphic bands (16) along with maximum PIC (0.38) while primer URP9F resulted in the minimum number of bands (06) and minimum PIC (0.13). Out of nine DAMDs employed, six primers (6.2H+, 33.6, FvIIex8, FvIIex8C, HVR and URP2R) resulted in 100% polymorphic bands, whereas primer URP9F revealed the least levels of polymorphism (46.15%). The amplification of fifteen ISSR primers generated 240 bands with a size range 150–2000 bp, of which 215 were polymorphic, revealing 89.96% polymorphism. Primer UBC 807 produced maximum number of polymorphic bands (20) and maximum PIC (0.44) whereas primers UBC 840 and 891 resulted into minimum number of polymorphic bands (10) and three primers UBC 823, UBC 836 and UBC 842

showed minimum PIC (0.26) values. Five ISSR primers UBC 807, UBC 808, UBC 811, UBC 834 and UBC 835 produced 100% polymorphic bands, whereas UBC 842 revealed least polymorphism (70%). Comparatively, ISSRs revealed slightly higher values of mean PIC (0.32) than DAMD markers (0.28). However, both DAMD and ISSR markers revealed almost similar levels of polymorphism (90.27% and 89.96%, respectively) across 111 accessions of *B. ciliata*. The cumulative data analysis (9 DAMD and 15 ISSR) revealed a total 371 amplified bands, of which 334 were polymorphic with 90.11% average polymorphism and 0.30 mean PIC value. Comparative evaluation of the two marker systems in respect of polymorphic information content, diversity index, effective multiplex ratio and marker index values revealed that ISSR markers were more informative (PIC = 0.32, DI = 5.10, EMR = 12.84, MI = 4.56) than DAMD markers (PIC = 0.28, DI = 4.18, EMR = 12.01, MI = 3.78) (Table S1). The genetic distances among pair of accessions ranged from 0.11 (Bc93–Bc94) to 0.64 (Bc04–Bc86), with a mean distance 0.37 (data not shown).

Mantel test (*r*) performed using three data sets ISSR, DAMD and cumulative, showed that cumulative v/s ISSR data (0.92) are the best fit, following cumulative v/s DAMD (0.73), and DAMD v/s ISSR (0.41) (Table S2). Thus the correlation was significantly high between ISSR and cumulative data, while DAMD showed a weak correlation with cumulative data as well as with ISSR data, suggesting the use and suitability of both marker systems in estimating the genetic variability in *B. ciliata* populations.

Extent of genetic diversity at species and population levels

The level of polymorphism among populations ranged from 45.01% (SHM) to 73.05% (BWS) with an average 56.95%; whereas, estimates of polymorphism detected at species level (90.03%) were substantially higher. Relatively, number of observed alleles (N_a) were found maximum in BWS followed by KUL and DGF populations, while GWS, RNK, PTH, DRJ and PNL showed moderate and NTL, KUF and SHM the lowest numbers of alleles (Table 2). Effective number of alleles, Nei's gene diversity and Shannon's diversity index also followed similar diversity pattern and were found maximum in KUL (N_e = 1.42, H = 0.24, I = 0.37), while least in SHM populations (N_e = 1.29, H = 0.16, I = 0.24). At species level, the overall genetic diversity (N_a = 1.90, N_e = 1.49, H = 0.29, I = 0.43) was much higher than the mean diversity found at population level (N_a = 1.56, N_e = 1.34, H = 0.19, I = 0.29) in *B. ciliata* occurring naturally in IHR (Table 2).

Genetic differentiation and population structure

Estimates of total genetic diversity (H_T) and genetic diversity within populations (H_S) were 0.28 and 0.19, respectively. The extent of genetic variance contributed by differentiation among populations (G_{ST}) was 0.30, revealing 70% of total genetic variations within the populations. A G_{ST} derived gene flow (N_m = 1.16) showed moderate rate of migration of genetic material between different

Table 2 Intra-population diversity statistics of *B. ciliata* populations were computed using POPGENE (Ver. 1.32)

Population[a] (sample size)	PB	%P	Mean N_a (SD)	Mean N_e (SD)	Mean H (SD)	Mean I (SD)	Mean H_T (SD)	Mean H_S (SD)	G_{ST}	N_m
DGF(15)	247	66.58	1.66 (0.47)	1.38 (0.38)	0.22 (0.19)	0.33 (0.28)				
KUL(15)	266	71.70	1.71 (0.45)	1.42 (0.37)	0.24 (0.19)	0.37 (0.27)				
SHM(7)	167	45.01	1.45 (0.49)	1.28 (0.37)	0.16 (0.20)	0.24 (0.28)				
KUF(6)	171	46.09	1.46 (0.49)	1.30 (0.38)	0.17 (0.20)	0.25 (0.29)				
GWS(8)	226	60.92	1.60 (0.48)	1.35 (0.37)	0.20 (0.19)	0.31 (0.28)				
RNK(7)	200	53.91	1.53 (0.49)	1.30 (0.35)	0.17 (0.19)	0.27 (0.27)				
NTL(6)	184	49.60	1.49 (0.50)	1.31 (0.37)	0.18 (0.20)	0.27 (0.29)				
BWS(13)	271	73.05	1.73 (0.44)	1.41 (0.37)	0.24 (0.19)	0.36 (0.26)				
PTH(9)	199	53.64	1.53 (0.49)	1.35 (0.39)	0.20 (0.21)	0.29 (0.29)				
DRJ(13)	197	53.10	1.53 (0.49)	1.31 (0.37)	0.18 (0.20)	0.27 (0.28)				
PNL(12)	196	52.83	1.52 (0.49)	1.32 (0.38)	0.18 (0.20)	0.27 (0.29)				
Mean	211.27	56.95	1.56 (0.48)	1.34 (0.37)	0.19 (0.20)	0.29 (0.28)				
ALL LOCI(111)	334	90.03	1.90 (0.30)	1.49 (0.34)	0.29 (0.17)	0.43 (0.22)	0.28 (0.02)	0.19 (0.01)	0.30	1.16

Population—population code, PB—number of polymorphic loci, %P—percentage of polymorphic bands, N_a—observed number of alleles, N_e—effective number of alleles, H—Nei's gene diversity, I—Shannon's information index, H_T—total genetic diversity, H_S—genetic diversity within populations; G_{ST}—relative magnitude of genetic differentiation among populations, N_m—gene flow among populations, SD—standard deviation of mean values

[a]The numbers in parenthesis in each population are the number of accessions from that population used in the present study

Table 3 Inter-population Nei's genetic and geographical distances of *B. ciliata* populations

Population ID	DGF	KUL	SHM	KUF	GWS	RNK	NTL	BWS	PTH	DRJ	PNL
DGF	****	0.1018	0.1592	0.1335	0.1082	0.1097	0.124	0.0973	**0.1855**	0.1426	0.1713
KUL	300.64	****	0.0831	0.0711	0.0838	0.1123	0.0995	0.0896	0.1321	0.1530	0.1462
SHM	381.80	137.99	****	0.0723	0.1064	0.1488	0.1150	0.1202	0.1759	0.1847	0.1818
KUF	389.52	139.19	**10.03**	****	0.0823	0.1307	0.1036	0.0981	0.1772	0.1679	0.1721
GWS	460.15	171.72	102.98	93.00	****	0.0773	0.0850	0.0814	0.146	0.1579	0.1625
RNK	656.06	370.96	278.01	269.18	199.51	****	0.0775	0.0661	0.1615	0.1446	0.1623
NTL	667.76	387.88	287.09	278.77	216.24	29.26	****	**0.0600**	0.1367	0.1275	0.1413
BWS	668.56	380.68	292.31	283.27	210.05	18.85	41.31	****	0.0970	0.1099	0.1195
PTH	704.20	411.35	332.54	323.19	244.06	65.96	81.81	47.18	****	0.1679	0.1804
DRJ	1516.75	1216.25	1166.90	1157.13	1069.53	900.72	903.18	883.37	838.52	****	0.0902
PNL	**1529.59**	1229.64	1185.56	1175.70	1086.85	923.27	927.19	905.57	860.01	51.00	****

Above the diagonal are Nei's genetic distance and below the diagonal are pair-wise geographic distance values (km). The values in bold are either minimum or maximum values, and cells with **** are for the identical populations

Table 4 AMOVA analysis carried out using GenAlEx program (Ver. 6.5) for the cumulative* (DAMD and ISSR) data of 111 *B. ciliata* accessions

| Source of variations	Degrees of freedom*	Sum of squares	Mean of squares	Variance component	Percentage of variations (%)
Among regions	2	758.510	379.255	6.101	10
Among populations	8	1164.159	145.520	10.530	17
Within populations	100	4395.565	43.956	43.956	73
Total	110	6318.234			100

*Degree of freedom: independent values that can be assigned to a statistical distribution

populations of the species (Table 2). The inter-population genetic distances ranged from 0.06 (NTL v/s BWS) to 0.19 (DGF v/s PTH) (Table 3). The corresponding geographic distances ranged from 10.03 km (SHM v/s KUF) to 1530 km (DGF v/s PNL) among populations of *B. ciliata* sampled from different habitats in IHR (Table 3).

The hierarchical partitioning of variance at three strata as determined by AMOVA revealed higher proportion of genetic variance within populations (73%) followed by among populations (17%) and least among the two Himalayan regions (10%) (Table 4). The levels of genetic diversity based on G_{ST} and AMOVA corroborate with each other, and further support the highest proportion of variance found within populations.

The UPGMA dendrogram grouped the 11 natural populations into two major groups (I and II) (Fig. 2). All the populations sampled from Western Himalaya grouped together in Cluster I, while both the Eastern Himalayan populations (DRJ and PNL) grouped together into cluster II. PTH population clearly segregated out from rest of the Western Himalayan populations of subgroup Ia, showing its distant relationship to form an independent subgroup Ib.

Bayesian clustering method employed for analyzing population genetic structure revealed two genetic clusters ($k = 2$) in *B. ciliata* (Fig. S5). The cluster I comprising 25
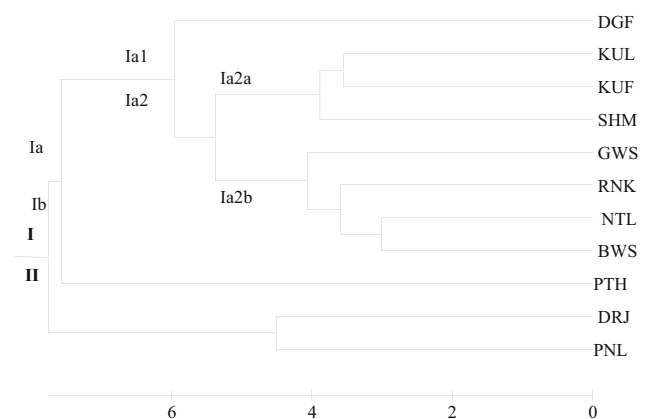
Fig. 2 UPGMA dendrogram showing relationships among 11 populations of *B. ciliata* in IHR and population codes are as given in the Table 1

individuals from two natural populations of Eastern Himalaya (DRJ and PNL), showed an average ancestry membership participation coefficient of 96.9% (DRJ = 96.3% and PNL = 96.55%). Cluster II comprising of 86 accessions of nine populations from Western Himalaya showed an average ancestry membership participation of 93.8% (DGF = 95.9%, KUL = 98%, SHM = 99.2%, KUF = 99.4%, GWS = 99.4%, RNK = 96.8%, NTL =
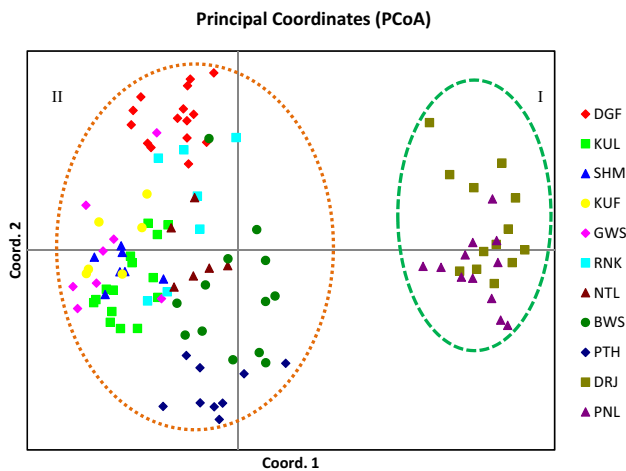
Principal Coordinates (PCoA)

Fig. 3 Principal coordinate analysis showing clustering of *B. ciliata* populations in IHR. The population codes are as given in the Table 1. PCoA was performed in the program GenAlEx (Ver. 6.5)

92.5%, BWS = 80.5%, PTH = 88.2%). Therefore, the assignment of the assumed 11 geographic populations indicates the existence of two natural genetic populations (nine populations of Western Himalayas and two populations of Eastern Himalayas) of *B. ciliata* in IHR.

On the basis of spatial relationships of genetic distances, PCoA plot grouped all the 111 accessions from 11 populations into two clusters (I and II) along the three axes of the plot (Fig. 3). Cluster I comprised all 25 accessions of DRJ and PNL populations from Eastern Himalayas, while cluster II contained rest 86 accessions belonging to nine geographic populations from Western Himalayas. The three axes of the plot accounted for 27.56%, 22.13% and 16.65% of the total variations, respectively. The results from the present study using the three methods of clustering of populations—neighbor-joining, Bayesian clustering and PCoA—were congruent and supported the admixture of individuals among the natural populations.

Isolation by distance (IBD) correlation analysis revealed a positively significant correlation among pair-wise genetic and geographic distances of populations ($r = 0.662$; $p = 0.001$), indicate that geographic isolation is contributing in shaping the present population structure in *B. ciliata* found in IHR (Fig S6).

Discussion

The analyses revealed high levels of average polymorphism for both the markers independently (DAMD-90.27% and ISSR-89.96%) as well as cumulatively (90.07%), suggesting a broad genetic base for *B. ciliata* in IHR. The significantly high levels of polymorphism detected in the present study is corroborating with earlier studies using

SPAR markers in taxa like *Bergenia ciliata* (DAMD = 87.8%; ISSR = 84.2% and Cumulative = 86.1%; Tiwari et al. 2015), *B. stracheyi* (DAMD = 86%, ISSR = 88%, Cumulative = 87.1%; Tiwari et al. 2017) and *Saxifraga paniculata* (RAPD = 91.2%; Reisch et al. 2003) of the family Saxifragaceae. Other medicinally important species such as *Podophyllum hexandrum* (92.37% with RAPD; 83.82% with ISSR and 84.40% with AFLP) (Naik et al. 2010), *Podophyllum hexandrum* (80% with AFLP primers) (Nag et al. 2015), *Rheum tanguticum* (82.19% with ISSR) (Hu et al. 2014) and *Roscoea procera* (94.68% with ISSR) (Rawat et al. 2016) occurring naturally in elevated locales of IHR also revealed similar levels of genetic polymorphism.

The analysis of genetic diversity at population level revealed that KUL and BWS populations harbour maximum levels of genetic diversity, whereas SHM population showed minimum levels of the diversity among the 11 populations. The higher levels of diversity among KUL and BWS populations may be due to comparatively larger population size and favourable habitat conditions for growth such as availability of soil moisture, gentle slopes and shady conditions (low light intensity). The SHM population grew at dry and exposed site, attenuated soil moisture, fragmentation and degradation of habitats and other anthropogenic pressures operating on the specific habitats could be the reasons for low genetic diversity in SHM population. The levels of genetic diversity found in *B. ciliata* were much higher at species level than the mean diversity observed at population level and these levels of polymorphism were further supported by the average genetic distances estimated among pair of accessions of the species. The levels of genetic diversity found in *B. ciliata* in the present study were comparatively higher than the levels found in widespread, long lived and perennial plants (Nybom 2004). Moreover, these levels were also comparable to limited number of populations studied earlier in case of *B. ciliata* (Tiwari et al. 2015). At population level, *B. ciliata* revealed higher levels of genetic diversity than the diversity found in *Saxifraga paniculata* using RAPD (Reisch et al. 2003) and *Kirengeshoma palmata* (family Hydrangeaceae) using allozyme markers (Chang et al. 2007), but these levels were lower than diversity found in *Saxifraga oppositifolia* (Pietiläinen and Korpelainen 2013) and *S. granulata* (van der Meer and Jacquemyn 2015) with polymorphic microsatellite markers. At population level, *B. ciliata* showed higher percentage of polymorphism than in *Saxifraga paniculata*, but at species level, polymorphism was comparatively low. However, these levels were comparable with other members of the family Saxifragaceae such as *Kirengeshoma palmata*, *Saxifraga granulata* and *S. oppositifolia*.

Genetic polymorphism in a plant species depends on many factors such as distribution range, life form, breeding system and dispersal mechanisms. It has been found that a species with long life span, high frequency of gene flow and more number of seeds tend to possess high genetic diversity (Hamrick and Godt 1990). In case of *B. ciliata,* anthropogenic pressures, fragmentation, degradation and loss of natural habitat may have been the additional driving factors that shaped the current levels of genetic diversity in the IHR. Kumar et al. (2012) reported the chromosome number in *B. ciliata*, 2n = 34, absence of polyploidy and high pollen fertility (96.35%).

A G_{ST} derived gene flow ($N_m = 1.16$) showed moderate levels of genetic exchange among populations and is indicating the current migration rates among the populations of *B. ciliata*. Present estimates of gene flow were slightly higher than in Western Himalayan populations ($N_m = 1.02$; Tiwari et al. 2015). For rare and endangered species, the higher estimates of gene flow are of great concern because they may reflect previous intermingling of populations (Godt et al. 2005); therefore it cannot be interpreted as an indication of the isolation of present populations. However, both the estimates of gene flow (Western Himalayan and IHR populations) suggest presence of enough exchange of genetic materials among populations to overcome any possibility of genetic drift and no immediate natural threat of extinction to *B. ciliata* populations found in IHR.

The moderate rate of genetic differentiation and gene flow detected in this study might be due to the anthropogenic pressures, habitat fragmentation and depleting population size. *B. ciliata* bears many hermaphrodite flowers on one sided raceme or corymbose inflorescence to attract pollinators, with high degree of self-incompatibility (Yeo 1971). These attributes of the species may have contributed significantly towards maintenance of high genetic diversity among natural populations of the species in IHR. Further studies on pollination biology are required to understand the genetic structure of *B. ciliata* comprehensively.

The hierarchical partitioning of the variance revealed that maximum diversity is preserved within populations, followed by among populations and least among the two Himalayan regions. These levels of genetic variance are in congruence with other high altitude plant species in the Himalayas such as *Rheum tanguiticum* (Hu et al. 2014), *Podophyllum hexandrum* (Naik et al. 2010; Nag et al. 2015) and *Roscoea procera* (Rawat et al. 2016). The partitioning of genetic variance within the populations is further corroborating with the magnitude of genetic differentiation ($G_{ST} = 0.30$) and the rate of gene flow ($N_m = 1.16$) among populations, suggesting that majority of genetic variance is preserved within populations and

exchange of genetic materials among the populations is not restricted.

The influence of altitudinal climatic gradients in structuring genetic variability within and among populations is highly complex and variable among species (Ohsawa and Ide 2008). Therefore, the structuring of genetic diversity depends on factors like population size and habitat conditions other than the temperature. The characteristics of population fragmentation have profound implications on the pattern of genetic diversity of a species and are very crucial while developing strategies for management and conservation of threatened species and populations. The presence of higher levels of genetic diversity in populations growing in moist and shady places than populations inhabiting dry and exposed regions indicate habitat preference by *B. ciliata* in IHR and is probably helpful in maintaining genetic diversity. The knowledge about structuring of genetic diversity helps to understand the evolutionary history of a species and to assess the future risks to diversity (Neel and Ellstrand 2003). Understanding the extent of genetic variability among populations is all the more important for developing strategies for conservation and management of threatened species and prioritizing their habitats. Greatly diverse or differentiated populations might be targeted for conservation, while genetically penurious populations might be targeted for management to restore the diversity (Petit et al. 1998).

Conclusion

B. ciliata is facing high threat in its natural habitats due to anthropogenic pressures such as over-exploitation, habitat fragmentation, depletion in population size, and habitat loss due to industrial demand, urbanization, construction and developmental activities. The populations like KUL, BWS and DGF which are maintaining high diversity in IHR should be prioritized for in situ conservation and populations such as SHM, KUF and RNK with low genetic diversity needs proper management for widening gene pool of the species. Furthermore, the propagules raised using conventional as well as in vitro methods for mass multiplication may also be reintroduced to enhance the level of genetic diversity in the impoverished populations. Besides, developing agro-techniques for commercial cultivation and standardised protocols based on qualitative and quantitative traits for harvesting would be helpful in collection of only mature stocks and desired materials, thereby pressure on natural populations will be reduced. Additionally more efforts are required to raise conservation awareness among locals, farmers, foresters and other stakeholders on sustainable collection and management of *B. ciliata*. In the present study, DAMD markers detected slightly higher

levels of polymorphism than ISSR and proved superior for investigating genetic diversity in *B. ciliata* accessions. However, both the markers provided means of rapid characterization of accessions and have enabled speedy and cost effective selection of appropriate accessions and populations for conservation of this highly important medicinal herb of IHR.

Compliance with ethical standards

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

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