Assessment of genetic diversity, population structure and phytochemical variations in *Polygonatum cirrhifolium* (Wall.) Royle: an endangered medicinal herb

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Abstract *Polygonatum cirrhifolium* (Wall.) Royle is an important medicinal herb of the family Asparagaceae used to cure several ailments. The rhizome of the species forms an important ingredient of “Chyavanprash” which is identified for its rejuvenating properties. However, *P. cirrhifolium* is least explored scientifically and systematically till date. Therefore, in this study genetic diversity and phytochemical variations along with antimutagenic activity of *P. cirrhifolium* populations were evaluated. Antimutagenic activity varied remarkably (p < 0.05) among population and Gagar population with significantly (p < 0.05) higher DNA recovery (84.95%) percentage. Higher genetic diversity (He) was recorded among populations using RAPD (He, 0.30–0.36) and ISSR (0.25–0.38) markers. High intra and low inter population variations were recorded in the species using both kinds of markers. Phenolics (p < 0.05; r = 0.924); tannins (p < 0.05; r = 0.897) and DNA damage inhibition efficiency displayed a highly positive correlation with genetic diversity (estimated using ISSR markers). The population structure analysis of *P. cirrhifolium* revealed that the greatest value of the K was 3 for studied populations. Gene flow among studied populations was found sufficient to encounter genetic erosion in the species. Therefore, it is recommended that the populations with higher ingredient and genetic diversity can be utilized for conservation priority and management plan of this species.

Keywords *Polygonatum cirrhifolium* · Threatened Himalayan plant · Population structure · Antimutagenic activity

Introduction

*Polygonatum* Mill. (Asparagaceae) is a genus of perennial herbs, owing to its wide array of health benefits; the species of this genus has been used traditionally. The genus comprises 71 species, of which 37 species are reported for medicinal value (Zhao et al. 2018). Most *Polygonatum* species comprises global curative properties and hence, consists of positions in Indian Ayurveda, traditional Chinese medicines, different pharmacopoeias, etc. *Polygonatum* herbs are
rich source of diosgenin, polysaccharides, steroidal saponins, homoisoflavanones, etc. (Liu et al. 2011; Khan et al. 2016; Wang et al. 2019) and hence, act as a cardio-tonic (Hirai et al. 1997) and possess anti-inflammatory (Khan et al. 2013), antispasmodic (Khan et al. 2016), antimutagenic (Suyal et al. 2019a) activities. Most of the important Polygonatum species lacks phytochemical, pharmacological and genetic diversity research. One such vital herb is Polygonatum cirrhifolium (Wall.) Royle generally recognized as Mahameda. The species is disseminated in the Himalayas from India, Bhutan, Nepal, and China to Pakistan (www.tropicos.org). Diverse plant parts of species like leaf, seeds, and rhizomes are utilized to treat kidney, rheumatism, and dermatological disorders. Rhizomes form the primary raw material of the Astavarga formulation, which is utilized in seminal weakness and as a cure for rakta, vata and pitta doshas (Balkrishna et al. 2012). The rhizome, stems and leaves of P. cirrhifolium are rich bioactive sources of steroidal saponins, flavonoids, anthraquinones, and their glycosides, coumarins, carbohydrates, etc. In addition, proteins, amino acids, and polyphenols are also recorded from roots and lactones in stems and leaves (Wang et al. 2006). 

P. cirrhifolium is adapted to several ecological habitats having prevalent variations in environmental conditions which ultimately affect the superiority and efficacy of the ultimate produce (Suyal et al. 2019a). Thus, evaluation of quality resources of P. cirrhifolium is essentially required. According to local and unofficial reports, huge amount of plant rhizome is collected annually from the Himalayan region for fulfilling the demand in domestic and global market and hence, depleted the species germplasm in wild. Few reports exist on diversity and distribution (Suyal et al. 2019b), propagation protocols (Lattoo et al. 2005) and pharmacological activity (Xu et al. 2017) of P. cirrhifolium. However, none of the studies reported the genetic diversity, phytochemical, and anti-mutagenic properties of the species. Studies on these aspects are necessary for elite identification and managing the species. Studies of genetic diversity are getting popularity across the world for setting priorities of conservation, especially to introduce the species in its natural habitats. Only few reports are available on the genetic multiplicity evaluation of herbal species from Himalayan region (Jugran et al. 2013; Giri et al. 2017; Fang et al. 2018; Ray et al. 2019).

This signifies an urgent need to quantify the genetic diversity among the populations of threatened and endemic medicinal plant species towards predicting future persistence. Random amplified polymorphic DNA (RAPD) and Inter simple sequence repeats (ISSR) markers are among such markers that are extensively employed for assessing genetic diversity and population structure studies of several plant species due to their cost effectiveness, robustness and high reproducibility (Fang et al. 2018). However, no prior information is available on the genetic diversity analysis and antimutagenic activity of P. cirrhifolium. Therefore, the major objectives of this investigation is to (i) characterize the genetic structure and differentiation of the studied populations, (ii) examine phytochemical, antioxidant and DNA damage inhibition efficiency of selected populations, and (iii) to establish relationship among phytochemicals, antioxidant properties and molecular attributes of P. cirrhifolium.

Material and methods

Exploration and collection of plant material

Extensive field investigation was carried in Kumaun region (28°44' to 30°49'N latitude & 78°45' to 81°05'E longitude) of IHR (Indian Himalayan Region) where P. cirrhifolium is predominantly found. A total of five different naturally occurring populations were selected and sampled randomly. Representative leaves of the samples from each population were collected, placed in the coarse silica gel and brought in the laboratory for further analysis. Rhizomes of P. cirrhifolium were utilized to measure the phytochemicals, DNA damage preventive or antimutagenic and antioxidant activities. The experiments in the current study were conducted in the CBCM (Centre for Biodiversity Conservation and Management), Govind Ballabh Pant National Institute of Himalayan Environment (GBP-NIHE), Kosi- Katarmal, Almora, India. Details of geographical coordinates and site characteristics were also recorded (Table 1).

Preparation of extract

Rhizomes of each population were separated and thoroughly washed with tap water followed by
ultra-pure water (Rions India Lab Water System, India). Rhizomes were dried at 40 °C in a hot air oven and ground into fine powder. Dried powder material (1 g) was added in 25 ml of methanol (80% v/v) and the suspension was incubated in water bath at 60 °C for 1 h. Further, the suspension was kept for incubation for 24 h at 25 °C and sonicated at 22 °C for 10 min (Model—ANIS 09001, Toshiba, Delhi, India). After filtering the solution it was centrifuged at 22 ± 1 °C at 10,000 rpm for up to 15 min. Thereafter, the supernatants were collected in distilled water (dH2O) and kept at 4 °C for use within 2 days.

### Determination of total phenols

Methanol extract derived from the rhizome of the species was used to measure total phenolic content by Folin- Ciocalteu’s colorimetric method (Singleton and Rossi 1965). Methanolic extract (0.50 ml) was diluted with dH2O (4.50 ml) followed by addition of 0.50 ml Folin–Ciocalteu’s reagent. The mixture was kept at temperature for reaction up to 5 min. Thereafter, 5.0 ml of sodium carbonate (7%) was used for neutralizing the reaction and the solution was placed in the dark at 25 °C for 90 min. A UV–vis spectrophotometer was used to record the absorbance of the solution at 765 nm using. The amount of total phenolics present in the extract was calculated as mg GAE (gallic acid equivalent)/g dw (per gram dry weight).

### Total tannins estimation

Total tannins in the plant methanol extract were measured by applying the method developed by Nwinnuka et al. (2005) with slight modifications. 5 ml of methanol extract was mixed with 0.5 ml of Folin-Dennis reagent. The mixture was than saturated with 7% of sodium carbonate (1.0 ml) followed by placing the solution in the dark at 22 ± 1 °C for 20 min. The absorbance of the solution was noted at 700 nm and the results were calculated as mg TAE (tannic acid equivalent) per g of DW.

### Determination of total flavonoids and flavonol

Aluminium chloride colorimetric method was used to measure total flavonoids in the methanolic extract of the species (Chang et al. 2002). Rhizome extract (0.50 ml) was thinned with dH2O (1.5 ml) and aluminium chloride (0.50 ml of 10% w/v) along with addition of 1 M potassium acetate (0.10 ml) and dH2O (2.80 ml). The absorbance of the mixture was recorded at 415 nm after 30 min of incubation at room temperature and the results were expressed in mg QE (quercetin equivalent)/g of dw.

An assay developed by Kumaran and Kaunakaran (2007) was applied to estimate total flavonol content in the methanol extract of the species rhizomes. Briefly, extract (2.0 ml), 2.0 ml of 2% ethanolic aluminium chloride (w/v) and sodium acetate solution (3.0 ml) were added. The mixture was placed at room temperature for reaction for 2.5 h at 20 °C. The absorbance was recorded at 440 nm and the results were calculated as mg QE (quercetin equivalent)/g dw.

### Antioxidant activity

#### Radical scavenging activity (ABTS assay)

Antioxidant properties in the rhizome of *P. cirrhifolium* were determined by the ABTS method (Cai et al., 2004). ABTS cation (ABTS•+) was produced by mixing potassium persulfate (2.45 mM) with ABTS salt (7.0 mM) followed by placing solution in the dark for 16 h at 23 °C. ABTS•+ solution
was thinned with ethanol (80%, v/v) to obtain an absorbance of 0.7 ± 0.005 at 734 nm. ABTS•+ solution (3.90 ml) was mixed with methanolic extract (0.10 ml) and vortexes to mix properly. The reaction mixture was placed in the dark for 6 min at 25 °C followed by noting absorbance at 734 nm. Samples were diluted with methanol (80%, v/v) to obtain 20–80% reduction in absorbance as compared to blank prepared with 0.1 ml methanol (80%, v/v). The results were shown as mM AAE (milli-mole ascorbic acid equivalent)/ 100 g dw.

Radical scavenging activity (DPPH assay)

A slightly modified DPPH method developed by Brand-Williams et al. (1995) was used to measure DPPH activity. Briefly, DPPH (25 ml, 400 mM) was mixed with MES buffer (25 ml of 0.2 M). The pH 6.0 was obtained with 1 N sodium hydroxide and ethanol (25 ml of 20% v/v). Sample extract (0.9 ml) was mixed with DPPH cation solution (2.7 ml) and placed in the dark for 20 min at room temperature. Decrease in the absorbance was recorded at 520 nm and the results were calculated as mM AAE/100 g dw (milli-mole of ascorbic acid equivalent per 100 g of dry weight).

Reducing power (FRAP) assay

Benzie and Strain (1996) method was employed to measure the ferric reducing antioxidant power (FRAP) of the methanol extract of the samples. FRAP solution was set by mixing 10 vol. of 300 mM acetate buffer (i.e., 3.1 g of NaOAC and 16 ml glacial CH3COOH per liter), 1 vol. of 10 mM TPTZ (2,4,6-tri-2-pyridyl-1,3,5-triazin) in 40 mM HCl and 1 vol. of 20 mM FeCl3. 3 ml of pre-warmed (at 37 °C) mixture was mixed to methanolic extract (0.10 ml) and placed at 37 °C for 8 min. The absorbance of the solution was recorded at 593 nm and the results were displayed as mM AAE (milli mole of ascorbic acid equivalent)/100 g dw.

Antimutagenic activity

Antimutagenic (DNA damage inhibition) property of P. cirrhifolium extract was screened using pBR322 plasmid DNA. DNA breakage is regarded as the alteration of the super-coiled (S) shape to the open-circular (OC) and/or linear (L) shape (Russo et al. 2011). Each population extracts was screened for DNA damage inhibition activity. Reaction mixture (15 µM) containing 2 µM of pBR322 plasmid DNA (180 ng), 2 µM of 7.5% H2O2, and plant extracts with positive control (+C) and negative control (−C) was prepared. The reaction was conducted at room temperature under UV emission for 20 min. Agarose gel electrophoresis was carried out using 0.8% agar in TBE buffer (0.5x) for 2 h at 45 V. The gel was analyzed for presence of fragments and density of the fragments was estimated using Uvi-pro Platinum 1.1, gel documentation system (Uvitech, Cambridge, UK). Based on band intensity, band area was calculated and finally converted into percent protection.

DNA isolation

One gram of leaf tissue was sliced into sections and grounded into fine powder in liquid nitrogen. DNA extraction was performed using CTAB (Cetyl trimethyl ammonium bromide) scheme with slight changes (Jugran et al. 2013). DNA was electrophoresed with 1% agarose gel to verify its purity and presence. The amount of genomic DNA was determined using a UV visible spectrophotometer (Hitachi, Japan). The absorbance ratio of DNA samples between 260 and 280 nm was measured and DNA quality was confirmed. The purified DNA sample was stored at 4 °C for further analysis.

PCR amplification

A total of 55 RAPD and 45 ISSR primers were initially screened to amplify the DNA samples of P. cirrhifolium. Only nine RAPD and ten ISSR markers exhibited clear, reliable and reproducible fragments (Table 2). The PCR reaction was performed using a reaction mixture of 20 µl in a 0.2 ml microfuge tube with 2 µl reaction buffer (10x), 2 µl, 25 mM MgCl2, 0.2 µM of each dNTPs, 0.2 µM primer, 20 ng DNA and 1 unit Taq polymerase (Genetix, India). The RAPD markers amplification programme was initiated with denaturation of 94 °C for 4 min 1 cycle, final denaturation at 94 °C for 1 min, primer annealing (37 °C) for 2 min followed by initial extension (72 °C) of 2 min and 45 cycle and final extension of PCR cycle was kept 72 °C for 7 min followed by reaction hold at 4 °C.
For ISSR, denaturation was conducted for 5 min at 95 °C, final denaturation for 1 min at 94 °C, primer annealing (46.5–55 °C) for 1 min followed by initial extension (72 °C) of 2 min and final extension of PCR cycle was kept for 7 min at 72 °C followed by reaction hold at 4 °C. The amplified DNA fragments were separated using 1 kb DNA ladder (Genetix, India) on agarose gel (1.5% (w/v) for RAPD marker and 2% for ISSR marker) using TAE buffer (0.5×) and marked with EtBr (ethidium bromide) (0.5 µg/ml). UVI-Pro platinum gel documentation system (Version 11.9, Cambridge, UK) was utilized to visualize the pattern of the gel.

Statistical analysis

Data generated for genetic diversity parameters analysis were scored in binary matrix format and used for statistical evaluation. The occurrence of the band was demarcated by 1 and absence by 0. Genetic variability attributes like frequency of the alleles, percent of polymorphic loci (Pp%), average allele number per locus (Na), effective number of alleles (Ne), genetic diversity index (He) and Shannon Information Index (I), was estimated by using POPGENE program version 1.31 (Yeh et al. 1999). Nei genetic distances (D) between populations were analyzed using Nei (1978) and rate of gene flow using standard method (Wright, 1951). An unrooted phylogenetic tree was constructed using Neighbor-joining methods (Saitou and Nei 1987) using the NEIGHBOR component of the PHYLIP program version 3.68 (Felsenstein 2006). The bootstrapping at 1000 replicates was applied to measure the statistical connotation of the clusters obtained using the SEQBOOT, GENEDIST, NEIGHBOR and CONSENSE programmes (Felsenstein 2006). GenAlex version 6.1 (Peakall and Smouse 2006) was used to investigate inter and intra-population genetic variations by analysis of molecular variance (AMOVA).

The admixture level and cluster number among populations were measured in combined RAPD and ISSR data by Bayesian assignment tests by means of STRUCTURE software version 2.3.4 (Pritchard et al. 2000). Different burning time length and number of MCMC (Markov chain Monte Carlo) replications were set to establish the appropriate set of both parameters initially. STRUCTURE was placed with a

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**Table 2** Details of RAPD and ISSR primers used in the analysis of genetic diversity of 90 genotypes of *P. cirrhifolium*

| Marker | Primer name | Sequence (5′-3′) | Annealing temperature | No. of fragment amplified |
|--------|-------------|-----------------|-----------------------|---------------------------|
| RAPD   | OPJ3        | TCTCCGCTTG      | 37 °C                 | 6                         |
|        | OPA3        | AGT CAG CCA C    | 37 °C                 | 3                         |
|        | OPA8        | GTG ACG TAG G    | 37 °C                 | 4                         |
|        | OPA9        | GGG TAA CGC C    | 37 °C                 | 3                         |
|        | OPA12       | TCG GCG ATA G    | 37 °C                 | 4                         |
|        | OPA13       | CAG CAC CCA C    | 37 °C                 | 4                         |
|        | OPA19       | CAA ACG TCG G    | 37 °C                 | 3                         |
|        | OPA20       | GTTGCGATCC       | 37 °C                 | 4                         |
|        | OPA14       | TCTGTGCTGG       | 37 °C                 | 2                         |
| ISSR   | UBC840      | GAGAGAGAGAGAGAYT | 55 °C                 | 7                         |
|        | HB12        | CACCCACACGC      | 46.5 °C               | 5                         |
|        | HB13        | GAGAGGAGGC       | 53 °C                 | 5                         |
|        | UBC860      | TGTTGTGTGTGTTGTRA | 55 °C                | 4                         |
|        | UBC880      | GAGAGAGAGAGAGAGA | 52 °C                 | 3                         |
|        | UBC859      | TGTTGTGTGTGTTGRC | 55 °C                 | 4                         |
|        | 17898B      | CACACACACACAGT   | 53 °C                 | 4                         |
|        | UBC810      | GAGAGAGAGAGAGAT  | 55 °C                 | 3                         |
|        | HB9         | GTTGTGTGTGTGG    | 46.5 °C               | 4                         |
|        | UBC888      | BDBCACACACACACA  | 52 °C                 | 3                         |

B = (C, G, T) (i.e., not A); D = (A, G, T) (i.e., not C); R = (A, G); Y = (C, T)
burning time length of 100,000 followed by number of MCMC reps after burning 500,000 replications by employing admixture model through previously assigned population as sampling location and with the among populations with linked alleles frequency. Number of K was established by conducting a total of ten runs (K = 1–10) with 3 interactions initially to decrease extra burden of simulation on computer. The maximum probable K-value was predicted by ΔK statistics using program Structure Harvester (Earl 2012) to estimates the utmost stage of changes between all succeeding values of K (Evanno et al. 2005). Concluding evaluation for each K value is conducted using K value from 1 to 5 with 20 interactions.

Data on phytochemicals and antioxidant properties were analyzed as an average value ± standard error (SE) of three separate replicates. Significant variations were estimated among average values of populations using DMRT (Duncan’s multiple range test; p < 0.05). Correlation coefficients (r) and coefficients of determination (R²) were calculated through SPSS Version 17.0 (SPSS Inc., Chicago, IL, USA). R studio was used to develop a correlogram using the CORRPLOT programme.

Results

Total phenol, tannin, flavonol and flavonoids

A significant variation was observed in total phenolics (1.80–3.47 mg GAE/g), tannins (1.41–2.50 mg TAE/g), flavonols (1.30–4.05 mg QE/g) and flavonoids (0.71–4.59 mg QE/g). Phenolic content was recorded higher (3.47 mg GAE/g) in Gagar population compared to other populations (Table 3). Total tannin content was found significantly (p < 0.05) higher (2.50 mg TAE/g) in Gagar and lower in Satbunga population (1.51 mg TAE/g).

Total flavonols was recorded significantly (p < 0.05) higher (4.05 mg QE/g) in Pandavkhuli population and lower in Mukteshwar (1.30 mg QE/g) and Gagar (1.31 mg QE/g) populations. Similarly, total flavonoid was found to be maximum (4.59 mg QE/g) in Mukteshwar population and minimum (0.71 mg QE/g) in Gagar population.

Antioxidant and DNA damage inhibition properties

Methanol extracts of the rhizomes of P. cirrhifolium evaluated for antioxidant properties by ABTS (3.26–5.12 mM AAE/100 g dw), DPPH (1.09–1.70 mM AAE/100 g dw) and FRAP (0.58–2.33 mM AAE/100 g dw) showed significant variations (Table 3). Considerably (p < 0.05) higher ABTS activity (5.12 mM AAE/100 g dw) found in Mukteshwar and lower in Pandavkhuli (3.26 mM AAE/100 g dw) population. FRAP activity was recorded maximum (2.33 mM AAE/100 g dw) in Thanidar population and minimum (0.58 mM AAE/100 g dw) in Satbunga population. DPPH was recorded significantly (p < 0.05) higher (1.70 mM AAE/100 g dw) in Thanidar comparing to remaining populations except Mukteshwar (1.65 mM AAE/100 g dw) population.

Antimutagenic activity of P. cirrhifolium populations was investigated (Fig. 1), which is an initial analysis of anti-mutagenic potential against oxidative stress. Gagar population showed significantly (p < 0.05) higher DNA recovery percentage (84.95%) compared to other populations (Table 3). Total tannin content was found significantly (p < 0.05) higher (2.50 mg TAE/g) in Gagar and lower in Satbunga population (1.51 mg TAE/g).

Total flavonols was recorded significantly (p < 0.05) higher (4.05 mg QE/g) in Pandavkhuli population and lower in Mukteshwar (1.30 mg QE/g) and Gagar (1.31 mg QE/g) populations. Similarly, total flavonoid was found to be maximum (4.59 mg QE/g) in Mukteshwar population and minimum (0.71 mg QE/g) in Gagar population.

Table 3 Phytochemical composition, antioxidant and anti-mutagenic activity in selected population of P. cirrhifolium

| Population | Phenol mg GAE/g | Tannin mg TAE/g | Flavonol mg QE/g | Flavonoid mg QE/g | ABTS mM AAE/100 g dw | FRAP mM AAE/100 g dw | DPPH mM AAE/100 g dw | AMA |
|------------|----------------|----------------|------------------|------------------|----------------------|----------------------|----------------------|-----|
| Thanidar   | 1.89 ± 0.05c   | 1.54 ± 0.02c   | 3.41 ± 0.02b    | 1.83 ± 0.01b    | 4.59 ± 0.08b        | 2.33 ± 0.01a         | 1.70 ± 0.02a          | 72.32 ± 1.55ab |
| Gagar      | 3.47 ± 0.01a   | 2.50 ± 0.01a   | 3.11 ± 0.07d    | 0.71 ± 0.04d    | 4.06 ± 0.03c        | 1.05 ± 0.01c         | 1.58 ± 0.01b          | 84.95 ± 7.27c |
| Satbunga   | 1.80 ± 0.04c   | 1.51 ± 0.01c   | 2.43 ± 0.01c    | 1.83 ± 0.01b    | 3.29 ± 0.07d        | 0.58 ± 0.02e         | 1.30 ± 0.01c          | 67.33 ± 1.58b |
| Mukteshwar | 2.80 ± 0.06b   | 2.34 ± 0.05b   | 1.30 ± 0.01d    | 4.59 ± 0.01a    | 5.12 ± 0.31a        | 1.18 ± 0.01b         | 1.65 ± 0.03a          | 82.92 ± 2.66a |
| Pandavkhuli| 2.00 ± 0.13c   | 1.41 ± 0.01d   | 4.05 ± 0.02a    | 0.79 ± 0.01c    | 3.26 ± 0.13d        | 0.85 ± 0.01d         | 1.09 ± 0.01d          | 79.03 ± 3.50ab |

ABTS-2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, FRAP ferric reducing antioxidant power assay, DPPH 2,2-diphenyl-1-picryl-hydrazyl-hydrate, AMA anti mutagenic activity

Means with different superscript letters are significantly different (p < 0.05) according to Duncan’s multiple-range test (DMRT)
Genetic diversity and analysis of molecular variance (AMOVA)

Significantly higher genetic diversity was noted in all studied populations of *P. cirrhifolium* using RAPD and ISSR markers. Out of 33 unambiguous and reproducible bands, 30.40 (mean across population) polymorphic RAPD loci were detected. The amplified fragments ranged from 02 (OPA14) to 06 (OPJ3), with an average of 3.6 fragments per primer (Fig. 2a). With an average of 92.12%, the polymorphic loci percent (Pp%) ranged from 87.88% (Thanidar) to 96.97% (Pandavkholi). Nei gene diversity index (He) ranged from 0.303 (Satbunga) to 0.362 (Gagar) 0.30 with an average value of 0.34 (Table 4). Shannon information

![Fig. 1](Image)

**Fig. 1** Antimutagenic activity of *P. cirrhifolium* extract. Lane 1—only pBR322; lane 2—pBR322, H2O2 and UV exposure; lane 3,4,5,6 and 7—damage pBR322 treated with extract

followed by Mukteshwar population (82.92%) whereas; lower recovery percentage was found in Satbunga population (67.33%).

![Fig. 2](Image)

**Fig. 2** PCR amplification pattern of *P. cirrhifolium* a obtained by OPA8 RAPD markers; b UBC 840 ISSR marker

![Table 4](Image)

**Table 4** Genetic diversity analysis of *P. cirrhifolium* in studied populations using RAPD and ISSR markers

| Marker | Populations | Np | Pp (%) | Na   | Ne   | I     | He    |
|--------|-------------|----|--------|------|------|-------|-------|
| RAPD   | Thanidar    | 29 | 87.88  | 1.82±0.09 | 1.59±0.05 | 0.51±0.04 | 0.34±0.03 |
|        | Gagar       | 30 | 90.91  | 1.85±0.09 | 1.64±0.05 | 0.53±0.04 | 0.36±0.03 |
|        | Satbunga    | 31 | 93.94  | 1.94±0.04 | 1.50±0.05 | 0.46±0.04 | 0.30±0.03 |
|        | Mukteshwar  | 30 | 90.91  | 1.91±0.07 | 1.56±0.06 | 0.49±0.04 | 0.33±0.03 |
|        | Pandavkholi | 32 | 96.97  | 1.97±0.03 | 1.62±0.05 | 0.53±0.03 | 0.36±0.02 |
|        | Mean        | 30.4| 92.12  | 1.89±0.03 | 1.58±0.02 | 0.50±0.02 | 0.34±0.01 |
| ISSR   | Thanidar    | 41 | 97.62  | 1.98±0.02 | 1.55±0.05 | 0.48±0.03 | 0.31±0.02 |
|        | Gagar       | 41 | 97.62  | 1.98±0.05 | 1.67±0.04 | 0.56±0.02 | 0.38±0.02 |
|        | Satbunga    | 38 | 90.48  | 1.91±0.06 | 1.41±0.05 | 0.39±0.04 | 0.25±0.03 |
|        | Mukteshwar  | 41 | 97.62  | 1.98±0.02 | 1.61±0.05 | 0.53±0.03 | 0.36±0.02 |
|        | Pandavkholi | 39 | 92.86  | 1.93±0.04 | 1.49±0.05 | 0.45±0.03 | 0.30±0.03 |
|        | Mean        | 40 | 95.24  | 1.95±0.02 | 1.54±0.02 | 0.48±0.01 | 0.32±0.01 |

*Np* Number of polymorphic loci, *Pp%* Percentage of polymorphic loci *Na* Observed number of alleles, *Ne* Effective number of alleles, *He* Nei’s (1973) gene diversity *I* Shannon’s Information index
index (I) ranged from 0.46 (Satbunga) to 0.53 (Gagar and Pandavkholi populations).

In the case of ISSR markers out of 42 unambiguous and reproducible bands, 40 polymorphic ISSR loci were detected using 10 selected primers. The amplified fragments ranged from 03 (UBC810, UBC880, UBC888) to 07 (UBC840) (Fig. 2b). With an average of 95.24%, Pp% ranged from 90.48% (Satbunga) to 97.62% (Mukteshwar, Gagar and Thanidar populations). He ranged from 0.25 (Satbunga) to 0.38 (Gagar) with a mean value of 0.32. I ranged from 0.39 (Satbunga) to 0.56 (Gagar) with a mean value of 0.48 (Table 4). AMOVA using RAPD marker demonstrated that the genetic variability was partitioned as 78% within populations and 22% among populations (Table 5). A value of 0.168 genetic differentiations (G_{ST}) between populations was detected. The gene flow (Nm) level measured among populations was 2.472. Genetic diversity was partitioned as 73% within populations and 27% between populations by AMOVA using ISSR markers (Table 5). A high value of 0.221 genetic differentiations (G_{ST}) was detected between populations and gene flow was 1.759.

**Cluster analysis and population structure**

RAPD markers based pairwise Nei genetic distance revealed minimum (0.069) genetic distance between Satbunga (2276 m, grassy slopes) and Pandavkholi (2521 m, rocky and boulder areas) populations and higher (0.187) genetic distance between Satbunga (2276 m, grassy slopes) and Mukteshwar (2319 m, grassy slopes) populations (Table 6). Nei genetic distance was observed to be lowest (0.113) between Thanidar (1984 m, open rocky hill slope) and Mukteshwar (2319 m, grassy slope) populations and highest (0.250) between Satbunga (2276 m, grassy slope) and Pandavkholi (2521 m, rocky and boulder areas) populations using ISSR marker (Table 6). Likewise, neighbor joining method based dendrogram separated all studied populations into 2 major groups (i.e. A and B). Group A includes Pandavkholi population

### Table 5 Analysis of molecular variance (AMOVA) based on RAPD and ISSR markers

| Marker | Source of variation | Degree of freedom (df) | Sum of square (SS) | MS | Variance component | Percentage of total variance |
|--------|---------------------|-----------------------|-------------------|----|-------------------|-----------------------------|
| RAPD   | Among Population    | 4                     | 148.510           | 37.128 | 1.730            | 22**                        |
|        | Within Population   | 85                    | 528.200           | 6.214 | 6.214            | 78**                        |
|        | Total               | 89                    | 676.710           | 7.944 | 7.944            | 100                         |
| ISSR   | Among Population    | 4                     | 226.733           | 56.683 | 2.731            | 27**                        |
|        | Within Population   | 85                    | 639.333           | 7.522 | 7.522            | 73**                        |
|        | Total               | 89                    | 866.067           | 10.253 | 10.253           | 100                         |

MS- mean square; Level of significance **p < 0.01

### Table 6 Nei’s Unbiased Measures of Genetic Identity and Genetic distance using RAPD marker

| Marker | Population  | Mukteshwar | Pandavkholi | Gagar    | Thanidar | Satbunga |
|--------|-------------|------------|-------------|----------|----------|----------|
| RAPD   | Mukteshwar  | 0          | 0           | 0        | 0.146    | 0.165    |
|        | Pandavkholi | 0.146      | 0           | 0.118    | 0.121    | 0.126    |
|        | Gagar       | 0.126      | 0.120       | 0.120    | 0.117    | 0.113    |
|        | Thanidar    | 0.121      | 0.120       | 0.078    | 0.187    | 0.132    |
|        | Satbunga    | 0.187      | 0.069       | 0.132    | 0.136    | 0.191    |
| ISSR   | Mukteshwar  | 0          | 0           | 0        | 0.165    | 0.131    |
|        | Pandavkholi | 0.165      | 0           | 0        | 0.129    | 0.113    |
|        | Gagar       | 0.131      | 0.225       | 0.150    | 0        | 0.150    |
|        | Thanidar    | 0.113      | 0.225       | 0.150    | 0        | 0.173    |
|        | Satbunga    | 0.191      | 0.250       | 0.140    | 0.173    | 0        |
The contribution of data, reflected the diverse distribution of the populations in the two coordinates is comparable to the clustering pattern. Similarly, the dendrogram revealed that log likelihood approximations increased regularly as K increased and started to decrease when K = 4 using both markers (Fig. 5a). A mean log likelihood ratio plot was prepared by placing values over 10 runs for K values ranging from 1 to 5. The value of K was optimum at 3 as evaluated by the ΔK statistic STRUCUTRE based on utilized markers (Fig. 5b, c). It was observed attractive that the best possible subgroups number was reasonably lower than the total studied populations using both kinds of markers, exhibited extensive amount of gene flow, either presently or historically. At probability threshold (Q) of 0.60 using ISSR markers structure analysis, the most of

Fig. 3  a Unrooted genetic distance tree showing the relationships among five populations of *P. cirrhifolium* using RAPD marker [The tree was constructed by using a neighbor-joining method (Felsenstein 2006). The numbers at the nodes of branches are the confidence values obtained from 1000 replications]. b Projection of 05 populations (90 individual) of *P. cirrhifolium* through PCoA based genetic distance using RAPD marker. 1: Mukteshwar; P2: Pandavkholi; P3: Gagar; P4: Thanidar; P5: Satbunga

The first two principal coordinates [PCo 1 (41.54%) and PCo 2 (26.84%)] explained 68.38% of the total variation when combined. The relationship among populations in the two coordinates is similar to the clustering pattern. Population structure of *P. cirrhifolium* relying on binary data obtained using both kinds of markers was investigated by Pritchard et al. (2000) method revealed that log likelihood approximations increased regularly as K increased and started to decrease when K = 4 using both markers (Fig. 4a). A mean log likelihood plot was prepared by placing values over 10 runs for K values ranging from 1 to 5. The value of K was optimum at 3 as evaluated by the ΔK statistic STRUCUTRE based on utilized markers (Fig. 5b, c). It was observed attractive that the best possible subgroups number was reasonably lower than the total studied populations using both kinds of markers, exhibited extensive amount of gene flow, either presently or historically. At probability threshold (Q) of 0.60 using ISSR markers structure analysis, the most of
the genotypes were visibly isolated to a definite cluster. Of which, 16 individuals (17.78%) were allocated to cluster-1 and cluster-2 each. Cluster-1 comprises the individuals, mainly collected from Pandavkholi population, while cluster 2 possesses the individuals gathered from Gagar population. Cluster 3 consist of 55 individuals (61.11%) including the samples from Thanidar, Satbunga and, Mukteshwar populations in majority; whereas only 3 (3.33%) individuals were belongs to the admixed group depending on the threshold of 60%.

**Fig. 5** STRUCTURE analysis of natural populations of *Polygonoatum cirrifolium* using combined inter-simple sequence repeat (ISSR) and Random amplified polymorphic DNA (RAPD) markers data. a K=3 appeared to be the optimal number of clusters by showing the ΔK at its peak; b Estimated genetic structure based on K=3 using a Bayesian framework implemented in the STRUCTURE programme across 3 subpopulations of 165 individuals.
Relationship among antioxidant, antimutagenic and genetic diversity characteristics

Phytochemical parameters correlated with genetic diversity using RAPD and ISSR markers revealed a considerable (p < 0.05) positive correlation (Fig. 6a, b). Phenol (p < 0.05; r = 0.924) and tannin content (p < 0.05; r = 0.897) attributed a noteworthy positive correlation) with Nei genetic diversity (He) using ISSR marker. Similarly, antimutagenic activity displayed a considerable positive correlation (p < 0.05; r = 0.898) with He using ISSR marker. DPPH demonstrated a strong negative correlation (p < 0.01; r = −0.968) with percent of polymorphism (Pp%) using RAPD marker. No other parameter showed relationship with genetic diversity. However, phenol revealed a noteworthy positive relationship (p < 0.05; r = −0.959) with tannin whereas, tannin, revealed a remarkable negative correlation (p < 0.05; r = −0.903) with flavonol. ABTS displayed a considerable positive correlation (p < 0.05; r = 0.885) with DPPH.

Discussion

Medicinal plants serve as a universal remedy for several ailments because of the potent secondary metabolites such as alkaloids, polyphenolic compounds, terpenes, carotenoids etc. These metabolites help plants to cope up with the adverse effects of oxidative stress generated through environmental variables (Suyal et al. 2019a). Phenolic compounds, tannins, flavonols and flavonoids of Himalayan medicinal herbs have been identified for their antioxidant (Giri et al. 2017), anti-inflammatory and anti-cancer activity (Singh and Patra 2019) etc. Therefore, in present study we have investigated total phenolics, tannin, flavonoid, anti-oxidant and, anti-mutagenic activity of *P. cirrhifolium* which might be responsible for its medicinal properties. A significant variation was observed in total phenols (1.80–3.47 mg GAE/g), tannins (1.41–2.50 mg TAE/g), flavonols (1.30–4.05 mg QE/g) and flavonoids (0.71–4.59 mg QE/g). Antioxidant properties of rhizomes derived methanolic extract of *P. cirrhifolium* showed significant variation. These findings were in the agreement of Rawat et al. (2013) who reported tannins (2.24 mg TAE/g) and flavonoids (3.05 mg QE/g) in root extract of *Platanthera edgeworthii* (Hook.f. exCollett) R.K.Gupta. Similarly Giri et al. (2017) reported similar range of phenol, tannin, flavonol and, antioxidant properties in Astavarga species including...
P. cirrhifolium (2.41 mg/g flavonoid; 3.11 mg/g tannins; 1.85 mg/g flavonol; 4.42 mM, AAE/100 g dw ABTS; 1.19 mM, AAE/100 g dw FRAP). Likewise, a similar range of total tannin (2.34 mg CE/g dw) and FRAP activity (0.27–1.78 mM, AAE/100 g dw) was reported in Polygonatum verticillatum (L.) All. (Singh and Patra 2018; Suyal et al. 2019a).

The antioxidant property of flavonoid from diverse natural resources in the prevention of cellular DNA from damage through inhibition of ROS is documented (Azqueta and Collins 2016). Inhibition of DNA damage is evaluated to detect UV tolerance as a defense arrangement of high-altitude medicinal plants (Suyal et al. 2019a). Ultraviolet radiation produces hydroxyl (OH) radical by peroxidizing H₂O₂, which can damage the plasmid DNA, especially the supercoiled form. Methanolic extracts of diverse rhizome parts of P. cirrhifolium in different populations were studied for DNA prevention activity. The rhizome extracts of different populations showed significant (p < 0.05) DNA recovery percentage, which is ranged from 67.33 to 84.95%. Therefore, P. cirrhifolium extracts and other high value medicinal plants could either check or dwindle the process of DNA damage and thus contribute to eliminate main disorders linked to DNA. Reports on the functionality of a few Himalayan herbs to inhibit oxidative pressure due to DNA harm are available (Jugran et al. 2016; Giri et al. 2017; Suyal et al. 2019a). Information pertaining to genetic diversity in P. cirrhifolium is currently lacking. However, reports are available on genetic diversity studies in other species of the genus Polygonatum (Kramp et al. 2009; Chung et al. 2014; Meng et al. 2014; Feng et al. 2020). Recently, genetic variations of Polygonatum verticillatum populations were evaluated using ISSR markers (Suyal et al. 2021). P. cirrhifolium has been categorized as an endangered species in the entire Himalayan region due to its limited population size coupled with poor regeneration (Suyal et al. 2019b), continuous harvesting, habitat degradation and, grazing in the study area.

However, low variation at genetic level is expected as a general reported trend in endangered plants with smaller population size (Manners et al. 2013). In the present study, higher genetic diversity was reported in P. cirrhifolium using RAPD (He = 0.34; Pp = 92.12%) and ISSR (He = 0.32; Pp = 95.24%) markers. Likewise, many other threatened and endemic plant species despite of their small population size and restricted distribution, comprise high genetic diversity. For instance, Suyal et al. (2021) reported high (He = 0.32; Pp% = 85.49%) genetic variations in P. verticillatum using ISSR marker. Similarly, Naik et al. (2010) reported higher level (He = 0.34; Pp% = 92.37) of genetic variations in endangered Sinopodophyllum hexandrum (Royle) T. S. Ying using RAPD marker; Tabin et al. (2016) in Rheum webbianum Royle (He = 0.34; Pp% = 87%) using ISSR markers; Chaudhary et al. (2012) in Hedychium spicatum Sm. (RAPD- He = 0.44; Pp = 89.58%). The higher genetic diversity in P. cirrhifolium might be due to its population distribution in different geographical conditions. In different geographical conditions, the plant species may differ in their content due to diverse environmental variables (altitude, temperature, rainfall, humidity, etc.) and the breeding system of the species (Zhang et al. 2020). Understanding the genetic variations inside and among populations is critical for the basis of powerful and efficient conservation practices for rare species.

Most of the phenotypic variations, in P. cirrhifolium allotted inside the population [RAPD—78%; ISSR—73%]. These results of within population variations are in line with reported variation for some endangered orchids and other threatened herbs e.g., Dendrobium catenatum Lindl. [RAPD—78.88%; ISSR—78.84%]; Fritillaria tubiformis subsp. moggridgei (Boiss. & Reut. ex Planch.) Rix [RAPD—82.91%], P. edgeworthii [ISSR—74%] respectively (Ding et al. 2009; Mucciarelli et al. 2014; Giri et al. 2017). The higher intra population variability of P. cirrhifolium can be elucidated based on its life history traits, particularly due to its breeding system. The breeding system of a plant species was found to influence the distribution of cistronic diversity in plant populations. Additionally, factors like habitat disintegration, gene flow, and tiny population size have additionally been reported to contribute to the present difference (Nybom 2004).

The constant of genetic discrimination (GST) and gene flow (Nm) are two important parameters by which the genetic structure of a population is often measured. Low genetic discrimination was recorded using RAPD and ISSR markers in P. cirrhifolium [RAPD: GST = 0.168; ISSR: GST = 0.221] although it was comparable to the average coefficients i.e. GST = 0.22 reported for out-crossing species (Nybom 2004).
2004) and $G_{ST}=0.231$ for monocots (Hamrick and Godt 1990). Similar low genetic differentiation as reported elsewhere (Naik et al. 2010; Tiwari et al. 2015).

Factors like geographical isolation, small population size of target species, habitat fragmentation and genetic drift could be responsible for low genetic differentiation (Tiwari et al. 2015; Suyal et al. 2019b). A relatively high correlation between chemotypic and genetic markers was identified. Giri et al. (2017), observed similar results in *P. edgeworthi*, where ISSR marker revealed a noteworthy correlation with total phenolics ($t=3.196; p<0.09$); Jugran et al. (2016) in *Valeriana jatamansi* Jones [ABTS with (DPPH, $r=0.482; p<0.05$); FRAP, $r=0.637; p<0.01$]; and Suyal et al. (2019a) in *P. verticillatum* [ABTS (DPPH, $r=0.544; p<0.05$)].

**Conclusion**

This study is the first account of the phytochemical properties and genetic diversity of *P. cirrhifolium*. Populations of *P. cirrhifolium* are restricted to specialized small patches and are threatened in the wild due to destructive harvesting. Considering habitat specificity, low plant density, and increasing extraction trends, the species needs immediate conservation intervention. Genetic diversity assessment, therefore, is important to define conservation interventions for the survival of genetic resources, particularly those of threatened plants with low population density. The Gagar population, with the highest genetic variation, phenolic, tannin and antimutagenic properties should be considered by priority as an important reservoir of potentially useful genes as the species shows enormous potential to adapt to altering environmental conditions.

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**Author contribution** RS, AKJ, and IDB conceptualized the study. RS and AKJ standardized methodology and conducted the experiments. AKJ performed data analysis. IBD and RSR contributed in management of resources and funds. RS and AKJ contributed in Writing—Original Draft of the MS. All authors contributed in Writing—Review & Editing of the manuscript.

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**Declarations**

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