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RESEARCH ARTICLE

Morphological, phytochemical and genetic diversity of threatened *Polygonatum verticillatum* (L.) All. populations of different altitudes and habitat types in Himalayan region

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Abstract *Polygonatum verticillatum* (L.) All. is an important medicinal herb that belongs to the family *Asparagaceae*. The rhizome of the species is used in Chyavanprash preparation and several other ayurvedic formulations. Numerous active constituents like saponins, alkaloids, phytohormones, flavonoids, antioxidants, lysine, serine, aspartic acid, diosgenin, β-sitosterol, etc. have been reported from this species. In this study, morphological, phytochemical, antioxidant and genetic variations of 11 distant populations of *P. verticillatum* were measured. Considerably ($P < 0.05$) higher variations were recorded among different populations of *P. verticillatum* using morphological, phytochemical and genetic diversity parameters. AGFW (above ground fresh weights); flavonols, FRAP (Ferric ion reducing antioxidant power) and NO (Nitric Oxide scavenging activity) were recorded maximum in Kafni population. Similarly, a significantly higher above and below ground dry weight was recorded in Mayawati and Surmoli populations respectively. Maximum phenolic content, tannins, and DPPH (2,2-diphenyl-1-picrylhydrazyl) activity were recorded in Milam population. A total of 165 individuals from 11 populations were assessed for genetic diversity using inter-simple sequence repeats (ISSR) marker. High genetic diversity (He = 0.35) was recorded in Himkhola and Surmoli populations while it was observed minimum (0.28) in the Mayawati population. Altitude showed a significant positive correlation with tannins (r = 0.674; $P < 005$) and DPPH (r = 0.820; $P < 0.01$). Phenol content exhibited a considerably positive relationship with He (r = 0.606; $P < 0.05$) and BGFW (r = 0.620; $P < 0.05$), flavonol displayed a positive correlation with Pp% (r = 0.606; $P < 0.05$). The population structure of *P. verticillatum,* exhibited that the optimal value of the K was 3 for its populations as determined by the ΔK statistic structure. Among populations, the amount of gene flow is higher (Nm = 1.717) among all sites. Hence, it can be concluded that *P. verticillatum* populations possess considerable variability in the collected populations. Likewise, the populations from Kafni, Satbunga and Himkhola with higher morphological, phytochemicals and genetic variability were prioritized and therefore recommended for cultivation and mass multiplication to meet the industrial demand for target species.

Keywords Medicinal plants · Indian Himalayan Region · Population structure · Altitude · Habitat types · Conservation

Introduction

Polygonatum verticillatum commonly known as *Meda* is a tall, erect, perennial rhizomatous geophytes that belong to the family *Asparagaceae* (formerly *Liliaceae*) and are growing in the Himalayas upto 4000 m asl. The species is 80–150 cm in height with the angled or grooved stem. The shoot of the species comprises of 1–2 scaly leaves and 4–8 whorls of green leaves. The leaves are linear or narrowly lanceolate. The fruits of *P. verticillatum* are known as berries which are globose, green when unripe and purple–

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black on ripening. The above ground shoot produced from the terminal bud dies after one growth season and leaves behind a distinct scar and the rhizome segment stretches from one scar to the next (Tybjerg and Vestergaard 1992). The flowers of the species are hermaphrodite and pollinated by bees (Kramp et al. 2009). The flowering and fruiting period for the species is from May–October (Lohani et al. 2013). The species preferentially found to grow in different altitudes and diverse habitats like moist shady places, open grassy slopes, Oak-Rhododendron forest, on slopes near big boulders, etc. where different ecological conditions are prevalent which might be responsible for the variability in phytochemical, morphological and genetic makeup of plants (Suyal et al. 2019a). Also, the species occurs in varied geographical locations and possess diverse morphological and genetic features which are responsible for the variation in active constituents of the species. The rhizome of *P. verticillatum* contains 1.17-12-15.35 mg GAE/g phenolics, 1.30–10.35 mg TAE/g tannins and 0.09–13.85 mg QE/g flavonoids (Suyal et al. 2019a). Being a part of Astavarga plant (a group of eight medicinal plants), *P. verticillatum* is used in the treatment of vata, pitta and rakta doshas. The rhizome of the species is used as an ingredient for "Chyavanprash" and other formulations of Ayurveda (Dhanwantharam Kashayam and Dhanwantharam Kuzhambu). In the Northwest Himalaya, the dried and baked leaves/tubers were taken with milk to increase sexual potency; in Chhota Bhangal region, Western Himalaya, solution made of fresh roots and water is taken to cure spermatorrhaea and piles (Uniyal et al. 2006); in Niti valley, Uttarakhand, root powder mixed with water has been used against leucorrhea (Phondani et al. 2010). The curative property of the *P. verticillatum* is credited to its active constituents like saponins, alkaloids, phytohormones, flavonoids, antioxidants, lysine, serine, aspartic acid, diosgenin, β-sitosterol, etc. (Sagar 2014). Similarly the studies are available on different biochemical attributes of *P. verticillatum* elsewhere. Khan et al. (2013), reported phytochemicals (saponins, alkaloids, flavonoids, glycosides, phenols, terpenoids, and tannins), antibacterial and antifungal activity from aerial parts of *P. verticillatum*. Suyal et al. (2019a) studied phytochemical, antioxidant and antimutagenic activity in rhizomes of 16 different populations of *P. verticillatum* across different altitude and habitat types. Sharma et al. (2019) analysed flavonols, flavonol glycoside and homoisoflavonoids in underground part of *P. verticillatum* using ultra high–performance liquid chromatography diode array detector quadrupole time-of–flight (DAD-QTOF-IMS). Suyal et al. (2021) studied phenol, tannin, flavonol, flavonoids and antioxidant properties of *P. verticillatum*, *Polygonatum cirrhifolium* and *Polygonatum multiflorum* using rhizome extracts.

In recent years, variety of DNA based markers such as amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), random amplified polymorphic DNA (RAPD) markers etc., are widely employed to study genetic diversity in large groups of plants (Kramp et al. 2009). Among others, inter simple sequence repeats (ISSR) markers have been broadly used for assessing genetic diversity and variability due to its quick and high polymorphism rate along with its cost effectiveness. ISSR markers have been efficiently used in variety of medicinal plants like *Podophyllum hexandrum* (Naik et al. 2010), *Artemisia annua* (Kumar et al. 2011), *Valeriana jatamansi* (Jugran et al. 2013a, b), *Acorus calamus* (Kasture et al.2016), *Hedychium spicatum* (Rawat et al. 2017), *Saussurea obvallata* (Semwal et al. 2020). Szczecinska et al. (2006) studied genetic variability between *Polygonatum multiflorum, Polygonatum odoratum* and *P. verticillatum* in Poland using DNA marker and reported 21.9% (111 RAPD), 9.5% (35 semi-random ISJ marker), 25% (IS6110) and 5.3% (KatG gene) polymorphism respectively. Kramp et al. (2009) carried out genetic diversity analysis of 198 individual of *P. verticillatum* using AFLP marker and reported 96.8% polymorphism. However, previous marker based studies on *P. verticillatum* are restricted to a limited number of markers and samples thus, it might not provide the actual genetic diversity available in the genetic makeup of this species. Further, to the best of our understanding, this is first report in which ISSR markers have been employed for diversity and population structure analysis in *P. verticillatum*. Also, information on species performance and genetic diversity in relation to different altitude zones and varied habitat types has not yet been evaluated.

Though, the species has extremely high potential for its uses in improving health of human being, unscientific and over exploitation has become main reason behind species endangerment (Lohani et al. 2013). The state medicinal plant board of Uttarakhand has forbidden the collection of this species from wild, however, the illegal collection of the *P. verticillatum* rhizome is still uncontrolled. Hence, the species is considered under endangered (Lohani et al., 2013) and vulnerable categories (Ved et al. 2003). Several factors like habitat alteration, heavy grazing and population bottleneck contribute towards its rare occurrence (Lohani et al. 2013). Therefore, it is urgently required to develop strategies for conservation and effectively utilize the potential of *P. verticillatum* using molecular and biochemical approaches.

Therefore, in the present investigation, individuals of *P. verticillatum* gathered from varied locations and habitats has been evaluated to measure (i) the extent of morphological, phytochemical and genetic variability (ii) to investigate the genetic structure of studied populations and

(iii) relationship between morphological, phytochemical and genetic diversity across altitudinal zones and habitat types.

Materials and methods

Study area and plant material collection

The present study was conducted in four districts i.e., Bageshwar, Champawat, Nainital and Pithoragarh region of Kumaun Himalaya (28° 44′–30° 49′ N and 78° 45′–81° 5′ E) covering an area of 21,032 Km^2 in the state of Uttarakhand, India. Altitude wise the area is separated into subtropical (300–1500 m), temperate (1500–3500 m) and alpine (> 3500) zones (Saxena et al., 1985). Individual samples of *P. verticillatum* were collected from 11 distantly located populations during 2016–2018 representing diverse habitat and altitudes. For detailed morphological and phytochemical evaluations, 5–10 individual samples were collected and for analysis of genetic diversity, 165 individuals (leaf samples) were collected from 11 distantly located populations. The plant specimens were also collected and conserved for the botanical authenticity of the species. Botanical identity of the species was validated at G.B.Pant National Institute of Himalayan Environment (GBP-NIHE), and specimen were deposited at the GBP-NIHE herbarium. The site details of the identified populations are presented in Table 1; Fig. 1.

Morphological variability

For morphological attributes, plant height (PH), length of rhizome (RL), above and below ground fresh (AGFW and BGFW) and dry weight (AGDW and BGDW) were recorded from randomly selected mature individuals from each population (n = 5). The sampled individuals were brought to the laboratory and washed to remove soil particles, etc. The water that remained at the surface was removed by pressing the plant carefully on tissue paper. Thereafter, the entire sample was oven dried (40 °C; Narang Scientific Works, Pvt. Ltd. Serial no/37096) to a constant weight. The dried samples were then weighed on an electronic balance (Citizen Scale India, Pvt. Ltd. Serial no/187547/07). Rhizome length was analyzed using a scale.

Phytochemical and antioxidant activity

The leaves of the plants gathered from diverse populations were carefully washed with tap water followed by cleaning with distilled water. The leaves were grounded into fine powder after drying in hot air oven at 40°C. Dried powdered material (1 g) mixed in 25 mL 80% (v/v) methanol was stirred gently and sonicated at 22°C for 10 min (Model-ANIS 09001, Toshiba, New Delhi, India), the extract was then centrifuged at 10,000 rpm at 22 ± 1 °C for 15 min. The supernatants were collected, filtered and stored at 4 °C prior to usage for chemical evaluation. Total phenolics, tannins and flavonols were measured using regular methods (Singleton and Rossi 1965; Bhatt et al. 2012; Chang et al. 2002). Different standard curves (gallic acid–phenols; tannic acid-tannins; and quercetin-flavonols) were employed and prepared in 80% methanol (v/v) for quantification of phytochemicals. The results were presented in mg Gallic acid equivalent (GAE)/g dry weight (dw); mg Tannic acid equivalent (TAE)/g dw; and mg Quercetin equivalent (QE)/g dw, respectively.

For determining antioxidant activity different in vitro methods such as ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay (Cai et al. 2004), DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical

Table 1 Details of studied populations of *P. verticillatum* in Uttarakhand, India

Location	Code	District	Altitude (m asl)	Latitude (°N)	Longitude (°E)	Collection number
Mayawati	P1	Champawat	1932	29° 22′ 24.10″	80° 3.0′ 24.10″	GBP 5340
Kilbury	P2	Nainital	2088	29° 25′ 16.98″	79° 26′ 17.94″	GBP 4335
Gagar	P3	Nainital	2151	29° 28′ 55.20″	79° 38′ 59.64″	GBP 4339
Satbunga	P4	Nainital	2272	29° 26′ 2.04″	79° 36′ 5.40″	GBP 4351
Surmoli	P5	Pithoragarh	2306	30° 4.0′ 35.04″	80° 13′ 53.04″	GBP 5341
Mukteshwar	P6	Nainital	2319	29° 28′ 30.00″	79° 38′ 43.80″	GBP 4359
Himkhola	P7	Pithoragarh	2368	30° 0.0′ 11.88″	80° 37′ 57.36″	GBP 4365
Balati	P8	Pithoragarh	3100	30° 3.0′ 51.84″	80° 12′ 35.28″	GBP 5336
Khaliyatop	P0	Pithoragarh	3450	30° 3.0′ 0.72″	80° 12′ 0.00″	GBP 5012
Kafni	P10	Bageshwar	3490	30° 11′ 22.56″	80° 2.0′ 38.04″	GBP 5303
Milam	P11	Pithoragarh	3500	30° 26′ 3.48″	80° 9.0′ 5.76″	GBP 5319

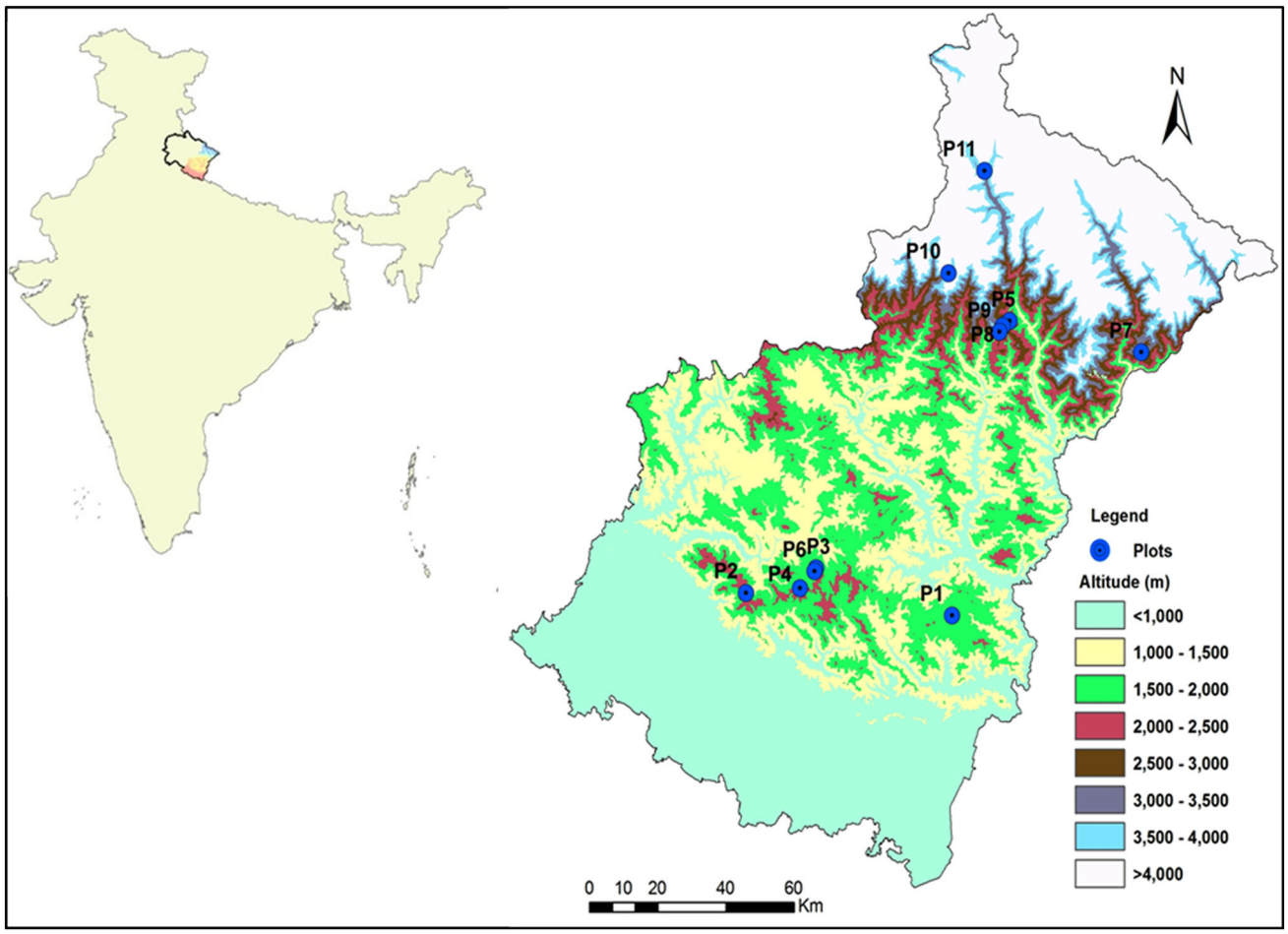


Fig. 1 Location map of *P.verticillatum*. P1-Mayawati; P2-Kilbury; P3-Gagar; P4-Satbunga; P5-Surmoli; P6-Mukteshwar; P7-Himkhola; P8-Balati; P9-Khaliatop; P10-Kafni; P11-Milam

scavenging assay (Bhatt et al. 2012), FRAP (Ferric reducing antioxidant power) assay (Benzie and Strain 1996), nitric oxide (NO) scavenging activity (Kumaran 2006), and hydroxyl ion (OH) scavenging activity (Halliwell 1987; Singh and Rajini 2004) were applied. Standard curves of ascorbic acid were prepared in 80% (v/v) methanol and results were expressed in mM ascorbic acid equivalent (AAE)/100 g dw.

DNA extraction and PCR amplification

The leaf sample was collected from the individuals of the selected populations and kept separately in plastic bags containing coarse silica crystal. The samples were instantly brought to the laboratory to protect them from heat, air and light exposure and stored at -20°C till DNA extraction. Leaf samples (1 g) of each genotype (individual) were taken and homogenized in liquid nitrogen for DNA extraction. Genomic DNA was isolated from leaf tissue using cetyl trimethyl amonium bromide (CTAB) method with minor modification (Jugran et al. 2013a, b). The purity and concentration of DNA was investigated using ultraviolet (UV) visible spectrophotometer (Hitachi, Japan) and verified through DNA electrophoresis with 1% agarose gel. 45 ISSR primers were primarily tested for amplification with selected populations, out of 45 ISSR primers only 10 primers [8 primers (Merck Bio-sciences, Germany; http://www.merk4biosciences.com) and 2 primers (University of British Colombia; series 800–900)] shows consistent and reproducible bands (Table2). Genomic DNA (50–100 ng) were amplified in 20 µl reaction volume comprising 2 µl of 10 × reaction buffer, 2 µl of 25 mM MgCl2, 0.2 µM of each dNTPs, 0.2 µM primer (inter simple sequence repeats, ISSR), and 1 U *Taq* polymerase (Genetix, India) using a T-gradient thermal cycler (Biometra, Goettingen, Germany). The amplification programme was initiated with denaturation of 95 °C for 5 min per cycle, with final denaturation of 1 min, and primer annealing (46–53 °C) for 1 min followed by extension (72 °C) of 1 min and the process continued for 35 cycles. Final extension of PCR cycle was kept at 72 °C for 7 min followed by reaction held at 4 °C. Gel electrophoresis was conducted to separate

Table 2 Details of ISSR primers used in the analysis of genetic diversity of *P. verticillatum*

Primer name	Sequence (5′–3′)	Annealing temperature	No. of amplified fragments
17898B	CACACACACACAGT	46 °C	6
17898A	CACACACACACAAC	46 °C	5
17899A	CACACACACACAAG	46 °C	8
814	CTCTCTCTCTCTCTCTTG	51 °C	4
UBC810	GAGAGAGAGAGAGAGAT	53 °C	5
UBC840	GAGAGAGAGAGAGAGAYT	53 °C	8
HB8	GAGAGAGAGAGAGG	51 °C	3
HB9	GTGTGTGTGTGTGG	48 °C	2
HB10	GAGAGAGAGAGACC	51 °C	3
HB12	CACCACCACGC	46 °C	8

(Y = C,T)

the PCR products using 2% agarose. The resulting gel pattern was visualized using the UVI Pro Platinum Gel Imaging system (Version 11.9, Cambridge, UK). Gene ruler DNA ladder mix (Genetix, India) was utilized as a marker to measure size for DNA fragment.

Statistical analysis

The data produced by 10 ISSR primers on 165 individuals were scored into a binary matrix format and utilized for statistical analysis. The presence of the band was demarcated by 1 and absence by 0. Genetic diversity attributes such as allele frequencies, Pp% (percentage of polymorphic loci), Na (mean number of alleles per locus), Ne (effective number of alleles per locus), H_e (genetic diversity index) and I (Shannon Information Index) were calculated using POPGENE version 1.31 (Yeh et al. 1999). Nei genetic dissimilarity (D) between populations was calculated using Nei (1978) and gene flow rate was analyzed using Wright (1951). A phylogenetic tree was prepared using PHYLIP version 3.66. Neighbor-joining method (Saitou and Nei 1987) was employed to construct unrooted dendrogram by using NEIGHBOR program of PHYLIP package (Felsenstein 2006). The statistical significance of the groups obtained was measured by bootstrapping (1000 replicates) by applying the SEQBOOT, GENEDIST, NEIGHBOR and CONSENSE programme (Felsenstein 2006). The partition of genetic inconsistency among studied populations was studied by estimating the analysis of molecular variance (AMOVA) using GenAlex software version 6.1 (Peakall and Smouse 2006). Principal component analysis (PCA) was performed using the relationship among altitude and genetic characteristics by SPSS version 17 (SPSS Inc., Chicago, IL, USA).

The admixture level and cluster number among population was investigated with ISSR markers by Bayesian assignment tests by means of STRUCTURE software version 2.3.4 (Pritchard et al. 2000). STRUCTURE was set with a length of burning time of 100,000, followed by 500,000 MCMC repetitions by applying admixture model through already assigned population as sampling localities and among populations in which frequency of alleles is linked. The 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 utmost probable K value was estimated by ΔK statistic with STRUCTURE HARVESTER software (Earl 2012) which determines the greatest level of alterations between each subsequent K value (Evanno et al. 2005). Final analysis is carried out for each K value ranging from 1 to 5 with 20 interactions.

For better understanding, development performance and genetic diversity of species, populations were grouped into four habitat types, i.e. Oak-Rhododendron forest, grassy slopes, Oak mix forest, and rocky/boulder/under Juniper species (Fig. 2) and three altitude range (1900–2400, 2401–2900, > 2900 m asl). For morphological parameter, phytochemical and antioxidant analysis, data are shown as mean values ± standard error (SE) of five replicates. Significant variations were evaluated among mean values of populations using Duncan's multiple- range test (DMRT; $P < 0.05$). The correlation coefficient (r) among studied parameters was investigated using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). R studio was used to develop a correlogram using CORRPLOT programme.

Results

Morphological variability

The morphological variation's investigation in *P. verticillatum* exhibited significant ($P < 0.05$) differences across the populations. Among the studied parameters, maximum

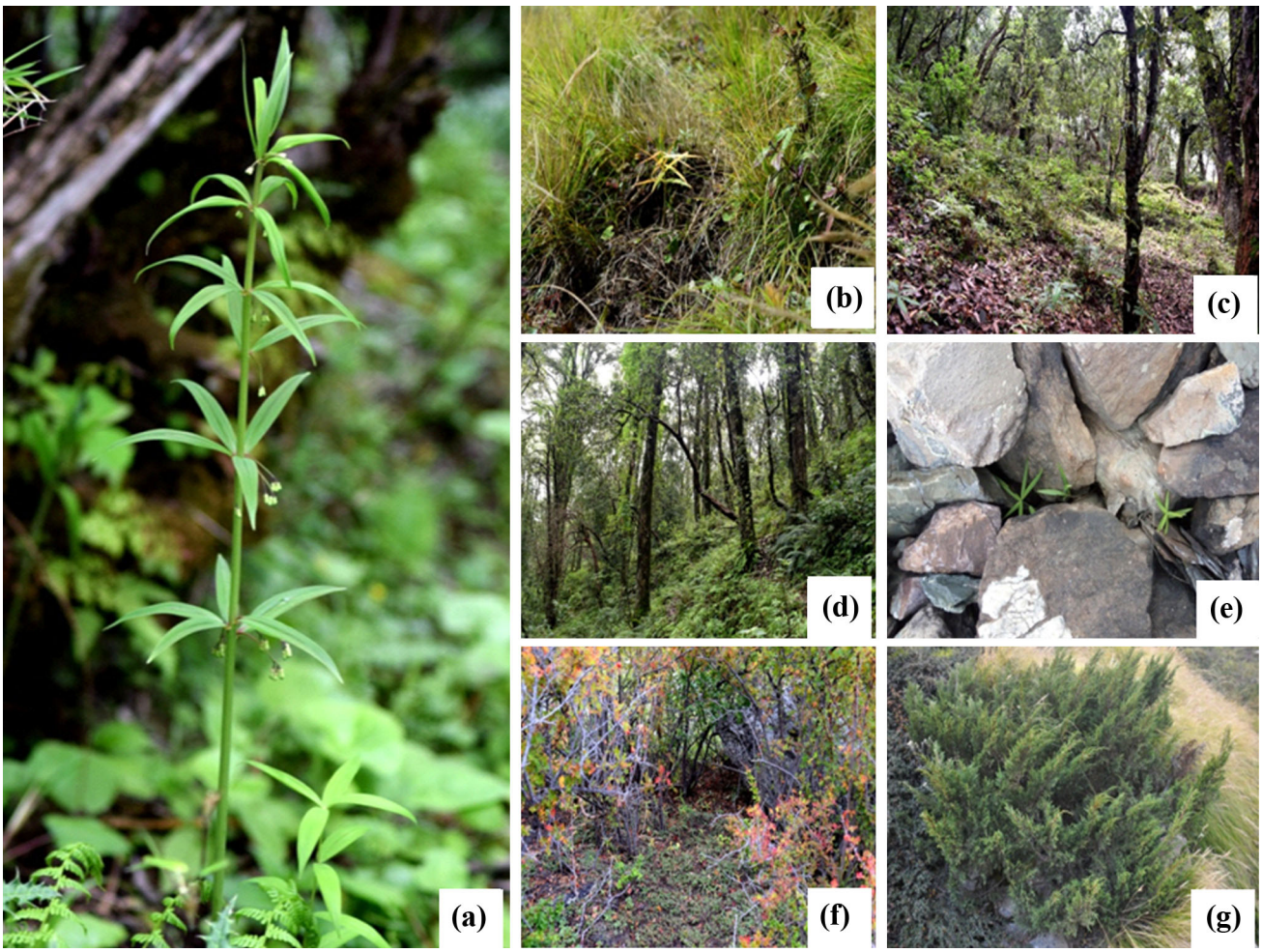


Fig. 2 Different habitat types of *P. verticillatum*-(**a**) Species; (**b**) Grassy habitat; (**c**) Oak mixed; (**d**) Oak-Rhododendron; (**e**) Bouldery/rocky; (**f**) undergrowth of Berberis and (**g**) Juniperus species

plant height (59.66 cm) was recorded in Kafni population, whereas, minimum plant height (15.60 cm) was observed for Himkhola population. The leaf number was found highest (31.80) in Himkhola compared to other populations and lower (8.40) in Balati population. Rhizome length was found maximum (11.10 cm) in Satbunga population and minimum (1.84 cm) in Himkhola population. Similarly, remarkably ($P < 0.05$) higher AGFW was observed in Kafni (3.20 g) and lower in Balati (1.04 g) population. BGFW was recorded considerably higher ($P < 0.05$) in Kafni (5.34 g) population whereas, minimum was recorded in Milam (1.68 g) population. AGDW was found highest (0.75 g) in Mayawati and Kafni populations which was significantly ($P < 0.05$) more than the other populations. Maximum BGDW was observed in Surmoli (2.74 g) population compared to the other populations (Fig. 3).

Phytochemical and antioxidant variability

Significant ($P < 0.05$) variations were observed in studied phytochemical parameters among leaf samples collected from different populations of *P. verticillatum* (Fig. 4a). Phenolic content ranged from 1.14 (Gagar)– 5.46 mg GAE/g dw (Himkhola), total tannin content was

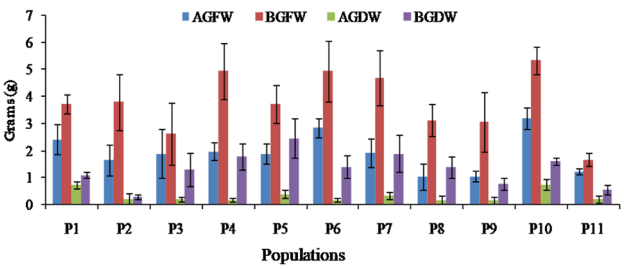


Fig. 3 Biomass details of different populations of *P. verticillatum*. AGFW: Above ground fresh weight; BGFW: Below ground fresh weight; AGDW: Above ground dry weight; BGDW: Below ground dry weight

considerably ($P < 0.05$) higher in Milam population (5.47 mg TAE/g dw) followed by Kafni (5.01 mg TAE/g dw) population and lower in Mayawati (1.17 mg TAE/ g dw) population. Similarly, highest flavonol content was obtained in Kafni population (5.21 mg QE/ g dw), followed by Satbunga (4.35 mg QE/ g dw) population and lowest in Balati population (1.37 mg QE/g dw). Antioxidant activity in leaf samples of *P. verticillatum* was determined by ABTS, DPPH, FRAP, NO and OH scavenging activity assays (Fig. 4b). A remarkable ($P < 0.05$) variations was detected among selected populations in these parameters. ABTS activity was found higher (5.43 mM AAE/ 100 g dw) in Khaliatop population as compared to other population except Surmoli (5.0 mM AAE/ 100 g dw). Antioxidant activity measured through DPPH assay revealed significantly ($P < 0.05$) higher activity in Milam (3.98 mM AAE/100 g dw) and lower in Mayawati (0.76 mM AAE/ 100 g dw) population. FRAP activity ranged from 2.38 (Kafni)–0.44 mM AAE/100 g dw (Himkhola population). Likewise, NO scavenging property was detected considerably ($P < 0.05$) higher in Kafni population (2.97 mM AAE/100 g dw) and lower in Gagar (0.46 mM AAE/100 g dw), Milam (0.49 mM AAE/100 g dw) and Balati (0.61 mM AAE/100 g dw) populations. OH scavenging activity ranged from 0.22 (Satbunga population)–1.28 mM AAE/100 g dw (Mayawati population).

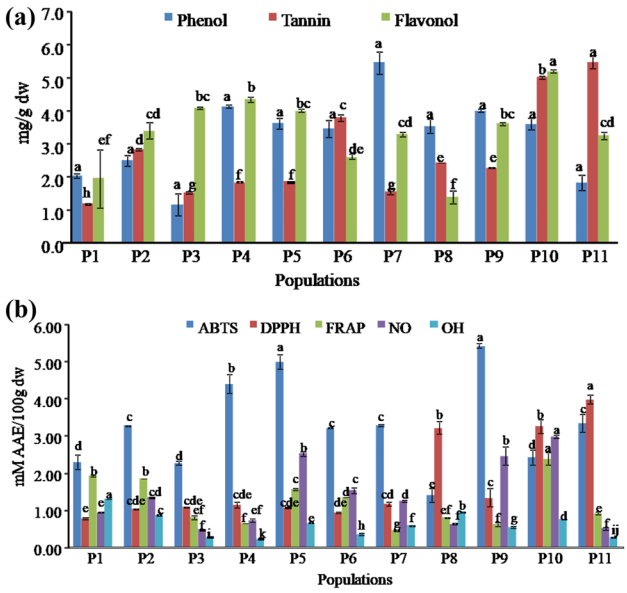


Fig. 4 **a** Phytochemical assessment of *P. verticillatum*. **b** Antioxidant activity assessment of *P. verticillatum*. Means with different letters are significantly different ($P < 0.05$) according to Duncan's multiple-range test (DMRT)

Genetic diversity estimates

A total of 52 unambiguous and reproducible fragments using 10 ISSR markers were obtained of which 44.45 (mean recorded across population) polymorphic ISSR loci were identified. The amplified DNA bands ranged from 2 to 8 with a mean of 5.2 bands per primer (Supplementary Figure S1). The number of polymorphic loci (Np) ranged from 39–47 and with a mean value of 85.49% percentage of polymorphic loci (Pp %) ranged from 75% (Kilbury population) to 90.38% (Kafni population). Nei genetic diversity index (He) ranged from lowest (0.28) in Mayawati to highest (0.35) in Himkhola and Surmoli populations with a mean value of 0.32 (Table 3). Shannon information index (I) was ranged from 0.42 (Mayawati) to 0.51 (Himkhola and Surmoli) with a mean value of 0.47.

Analysis of molecular variance (AMOVA) and population structure

AMOVA using ISSR marker in *P. verticillatum* populations exhibited that the hereditary variability was partitioned as 78% intra and 22% inter population (Supplementary Table S1). A value of 0.226 genetic differentiations (G_{ST}) between populations was detected. Among populations, the level of gene flow recorded was 1.717. Cluster analysis based on Nei genetic distance (pairwise) revealed lowest (0.050) genetic distance between Milam (3500 m, Under Juniperus and Berberis sp.) and Khaliatop (3450 m, Open grassy habitat) populations and highest (0.277) genetic distance between Balati (2821 m; Oak-Rhododendron forest) and Gagar (2151 m; Oak mix forest) (Supplementary Table S2).

Neighbor joining method based dendrogram separated 11 populations of *P. verticillatum* into 2 major groups i.e. A and B. Group A includes Satbunga population while group B was divided into 07 sub groups, i.e. (i) Mayawati; (ii) Himkhola; (iii) Balati and Kafni; (iv) Khaliatop and Surmoli; (v) Milam; (vi) Kilbury and Mukteshwar; (vii) Gagar (Fig. 5a). Genetic dissimilarity based PCA analysis positioned the majority of genotypes of the populations into two dissimilar groups which revealed the similarity among population relationship in two components as detected in clustering pattern (Fig. 5b).

P. verticillatum population structure based on binary data was determined by Pritchard et al. (2000) method exhibited that the log likelihood approximations enhanced gradually as K enhances and start to decline when K = 4 by using ISSR markers (Fig. 6a, b). Average log likelihood plot is developed by keeping values over 10 runs for K values ranging from 1 to 5. The optimum value of K was found to be 3 as evaluated by the ΔK statistic STRUCTURE based on utilized markers (Fig. 6a, b). It was found

Table 3 Genetic variation of *P. verticillatum* populations using ISSR primers

Location	Altitude (m asl)	N*p*	No. of Individual	Pp (%)	Na	Ne	He	I
Mayawati	1900	44	15	84.62	1.85	1.47	0.28	0.42
Kilbury	2088	39	15	75.00	1.75	1.51	0.29	0.43
Gagar	2151	46	15	88.46	1.88	1.54	0.32	0.47
Satbunga	2272	46	15	88.46	1.88	1.57	0.33	0.48
Surmoli	2300	46	15	88.46	1.88	1.64	0.35	0.51
Mukteshwar	2319	43	15	82.69	1.83	1.58	0.32	0.47
Himkhola	2400	46	15	88.46	1.89	1.62	0.35	0.51
Balati	2821	41	15	78.85	1.79	1.52	0.30	0.44
Khaliyatop	3450	45	15	86.54	1.87	1.59	0.34	0.49
Kafni	3490	47	15	90.38	1.90	1.57	0.33	0.49
Milam	3500	46	15	88.46	1.89	1.56	0.32	0.48
Mean		44.46	165	85.49	1.86	1.56	0.32	0.47

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

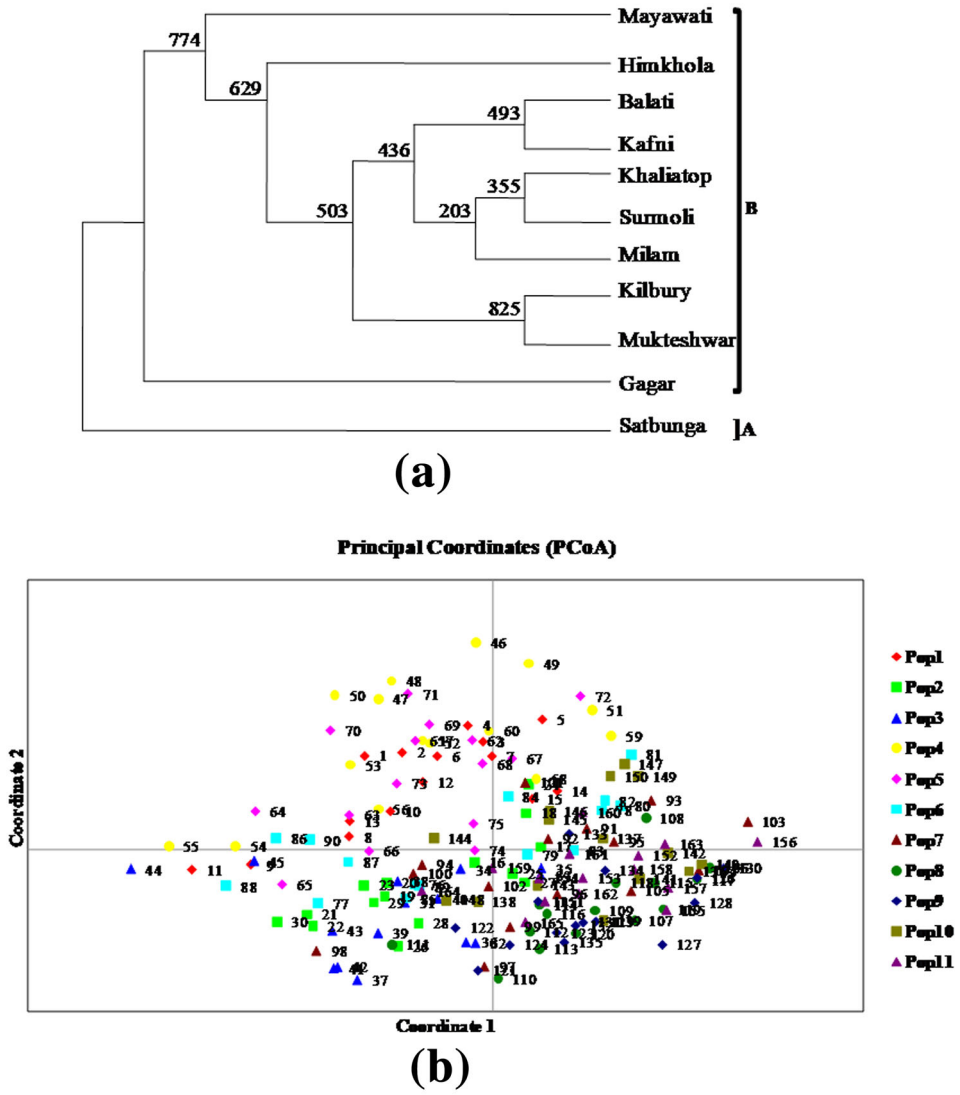
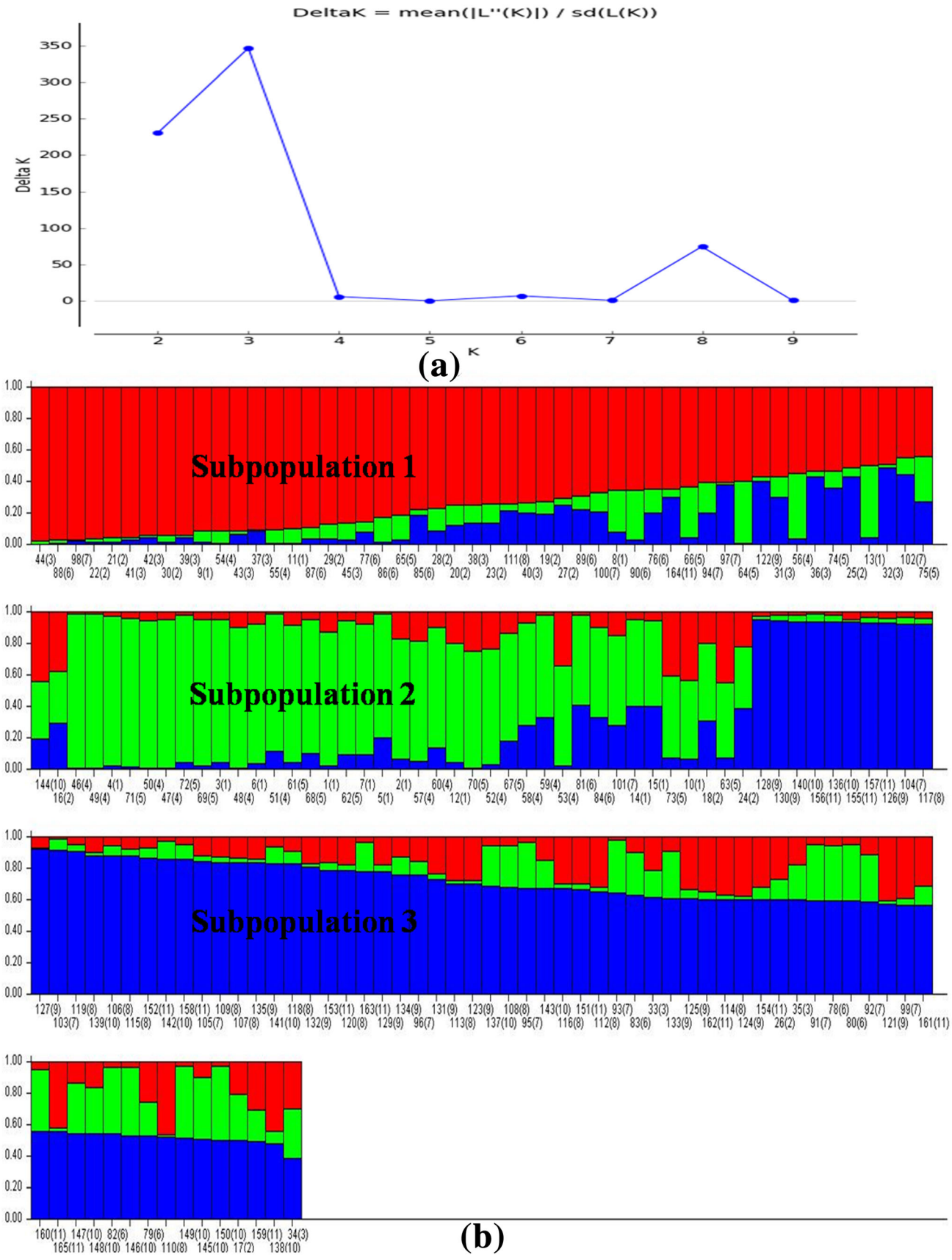


Fig. 5 **a** Unrooted genetic distance tree showing the relationships among eleven populations of *P. verticillatum* using ISSR markers [The tree was constructed by using a neighbor-joining method (Felsenstein 2006). The numbers at the nodes of branches are the confidence values obtained from 1000 replications]. **b** Projection of 11 populations *of P. verticillatum* through PCA based genetic distance. 1-Mayawati; 2-Kilbury; 3-Gagar; 4-Satbunga; 5-Surmoli; 6-Mukteshwar; 7-Himkhola; 8-Balati; 9-Khaliyatop;10-Kafni; 11-Milam


DeltaK = mean(|L''(K)|) / sd(L(K))
Delta K
350
300
250
200
150
100
50
0
2
3
4
5
6
7
8
9
K
(a)
Subpopulation 1
Subpopulation 2
Subpopulation 3
1.00
0.80
0.60
0.40
0.20
0.00
(b)

◀**Fig. 6** STRUCTURE analysis of natural populations of *P. verticillatum* sampled to assess inter-simple sequence repeat markers. **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

interesting that the optimal subgroups number was comparatively low compared to the number of studied populations using ISSR markers, displayed broad gene flow level, either in present or historically. Structure analysis using ISSR markers at 0.60 level of probability threshold (Q), most of the genotypes were clearly dispersed to a specific group. Of which, 38 individuals (23.03%) were allocated to cluster-1 with the genotypes, largely collected from Kibury, Ramgarh and Mukteshwar populations; 28 individuals (16.97%) were allotted to cluster-2 comprising the individuals gathered from Mayawati, Satbunga and Surmoli populations and cluster 3 comprised 52 individuals (31.52%) containing the samples from Himkhola, Balati, Khaliatop, Kafni and Milam populations in majority; whereas 47 (28.48%) individuals were included in the admixed group based on the threshold of 60%.

Morphological, phytochemical and genetic diversity across altitude zones and habitat types

Across altitude, highest genetic diversity (He = 0.330), Shannon information index (I = 0.489), plant height (PH = 35.41 cm), AGDW (0.37 g) was recorded at an altitude above 2900 m asl (Supplementary Table S3). Similarly, tannin (4.25 mg TAE/g dw), flavonols (4.02 mg QE/g dw), ABTS (3.73 mM AAE/100 g dw), DPPH (2.86 mM AAE/100 g dw), and NO scavenging activity (1.97 mM AAE/100 g dw) were observed maximum at higher altitude i.e. above 2900 m asl (Supplementary Table S4). Whereas, higher values for rhizome length (RL = 7.26 cm), AGFW (2.11 g), BGFW (3.95 g), and FRAP activity (1.35 mM AAE/100 g dw) were observed in lower altitude (1900–2400 m asl). BGDW (1.65 g), phenolic content (4.50 mg GAE/g dw) and OH scavenging activity (0.76 mM AAE/100 g dw) were recorded higher at mid altitude (2401–2900 m asl) Minimum genetic diversity (He = 0.314), Shannon information index (I = 0.464), BGDW (1.40 g), phenols (2.81 mg GAE/g dw), and DPPH activity (1.0 mM AAE/100 g dw) was observed at lower altitude (1900–2400 m asl). While, minimum values for rhizome length (RL = 3.13 cm), AGFW (1.48 g), AGDW (0.26 g), tannins (1.97 mg TAE/ g dw), flavonols (2.32 mg QE/ g dw), ABTS (2.35 mM AAE/100 g dw), FRAP (0.61 mM AAE/100 g dw) and NO scavenging activity (0.92 mM AAE/100 g dw) were recorded in mid altitude (2401–2900 m asl).

Across habitats, maximum genetic diversity, Shannon information index (He = 0.34; I = 0.49), plant height (35.89 cm), tannins (3.65 mg TAE/g dw), ABTS (4.05 mM AAE/100 g dw), DPPH (2.41 mM AAE/100 g dw), and NO scavenging activity (2.11 mM AAE/100 g dw) were recorded in rocky-boulder/Juniperus/Berberis habitat (Supplementary Figure. S2a, b, c, d & e). AGFW (2.13 g), BGFW (4.47 g), and phenols (3.80 mg GAE/ g dw) were observed maximum in grassy slope habitat. OH scavenging property was observed maximum (1.11 mM AAE/100 g dw) in Oak-Rhododendron forest. However, FRAP activity was recorded maximum (1.36 mM AAE/100 g dw) in Oak-Rhododendron and rocky-boulder/Juniperus/Berberis habitats. Rhizome length (RL = 7.71 cm), BGDW (1.56 g), and flavonols (4.22 mg QE/100 g dw) were observed higher in Oak mix forest. Whereas, minimum values of genetic diversity (He = 0.29), Shannon information index (I = 0.43), plant height (PH = 21.51 cm), rhizome length (RL = 4.36 cm), AGFW (1.73 g), BGFW (3.42 g), and ABTS (1.85 mM AAE/100 g dw) were detected in Oak-Rhododendron habitat similarly, lowest values for phenols (2.64 mg GAE/ g dw), tannins (1.67 mg TAE/g dw), FRAP (070 mM AAE/100 g dw), NO (0.59 mM AAE/100 g dw), and OH scavenging activity (0.25 mM AAE/100 g dw) were recorded in Oak mix forest.

Prioritization and identification of promising populations

PCA based on a correlation matrix of investigated morphological, phytochemical and genetic diversity attributes highlighted the presence of two major components, i.e. PC1 and PC2 (Supplementary Figure. S3). The score plot of first principal component (PC1) accounts up to 82.11% of total variance of all the data and exhibited a significantly positive correlation with flavonols (r = 0.604; $P < 0.05$), NO (r = 0.737; $P < 0.01$), plant height (r = 0.999; $P < 0.01$), AGFW (r = 0.704; $P < 0.05$), and BGFW (r = 0.616; $P < 0.05$). Component 1 represented the presence of the populations of Satbunga, Surmoli, Mukteshwar and Kafni and these populations might be considered as a major group affected by morphological and phytochemical attributes. PC2 accounts up to 10.92% of total variance and displayed a significantly positive correlation with He (r = 0.628; $P < 0.05$) and Pp% (r = 0.946; $P < 0.01$). Component 2 represented by populations of Surmoli, Himkhola, Kafni and this group is considered as major group affected by genetic diversity attributes. Hence, results of PCA analysis demonstrated that population of Satbunga, Surmoli, Milam and Kafni could be prioritized

for higher morphological, phytochemical and genetic variability attributes. The population from Mukteshwar considered as promising based on morphological attributes and Himkhola population on the basis of higher genetic variability.

Relationship between altitude and studied parameters

Considerably positive correlations of altitude with tannins and DPPH activity were recorded while no other parameters exhibited any relationship with altitude (Fig. 7). A significantly strong and positive correlation of altitude was detected with tannins (r = 0.674; $P < 005$) and DPPH (r = 0.820; $P < 0.01$). Phenol showed a significant positive correlation with He (r = 0.606; $P < 0.05$) and BGFW (r = 0.620; $P < 0.05$), flavonol exhibited a positive correlation with Pp% (r = 0.606; $P < 0.05$). ABTS activity revealed a positive correlation with He (r = 0616; $P < 0.05$), whereas, FRAP displayed significant positive correlation with plant height (r = 0.607; $P < 0.05$); AGFW (r = 0.645; $P < 0.05$) and AGDW (r = 0.744; $P < 0.01$). NO showed a strong positive correlation with plant height (r = 0.738; $P < 0.01$) and OH with AGDW (r = 0.616; $P < 0.05$). Nei's genetic diversity (He) showed a significant positive correlation with Pp% (r = 0.686; $P < 0.05$) and BGDW (r = 0.603; $P < 0.05$).

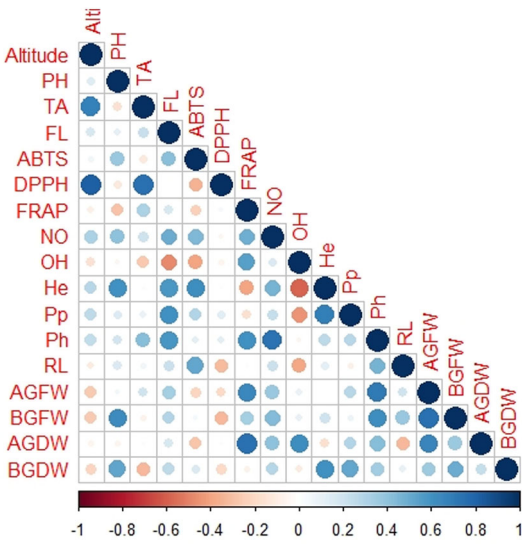


Fig. 7 Correlogram showing relationship between altitude and all studied parameters.PH-Phenol; TA-Tannin; FL-Flavonol; ABTS-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; DPPH-2,2-diphenyl-1-picrylhydrazyl; FRAP-Ferric ion reducing antioxidant power; NO-Nitric Oxide scavenging activity; OH-Hydroxyl ion scavenging activity; He-Nei's genetic diversity; Pp-percent polymorphism; Ph-Plant height; RL-Rhizome length; AGFW-Above Ground Fresh Weight; BGFW-Below ground Fresh Weight; AGDW-Above ground dry weight; BGDW-Below ground dry weight

Discussion

Biological, chemical and molecular variability in plants depend on various factors like altitude (Suyal et al. 2019a; Adhikari et al. 2020), growing seasons and growth conditions (Guo et al.2011; Gouvinhas et al. 2020), different phenological stages (Rawat et al. 2016), soil (Zargoosh et al.2019), rainfall and temperature (Chelghoum et al. 2021), different solvent types (Suyal et al. 2021), etc. These factors strongly influenced the growth of a plant and quality and the quantity of active metabolites present in the samples such as alkaloids, steroids, and essential oils including phenol and flavonoids (Rawat et al. 2020). Apart from the ecological factors, the production of active ingredients and growth performance in medicinal plants is also guided by genetic processes. Recently, the global and international communities have highlighted the concerns on genetic diversity conservation as a part of terrestrial and aquatic environment conservation (Shen and Yue 2019; Chao et al. 2019). Meanwhile, the knowledge about the genetic variation is also vital for breeder to produce better germplasm with enhanced yield and value of industrial product (Deperi et al. 2018). Among the several markers-dependent methods like morphological biochemical and molecular markers, the molecular markers are the most advanced approach applied for genetic variations measurement because of their higher efficiency, reliability along with no alterations with changing environment (Ray et al. 2019a). However, there is no information available on the genetic diversity assessment of *P. verticillatum* using molecular markers in different altitudes and habitat types till date. Likewise, no or less information about the *P. verticillatum* genome is available; therefore, random molecular markers like ISSR were used in this study. Molecular markers particularly ISSR have been extensively applied for investigating genetic variations in diverse medicinal plants like *Valeriana jatamansi* (Jugran et al. 2013a, b; Jugran et al. 2015), *Roscoea procera* (Rawat et al. 2016), *Houttuynia cordata* etc. (Gupta and Bharalee 2020), *Pandanus odorifer* (Nasim et al. 2020), *Thymus vulgaris* (Gyorgy et al. 2020). In this study, ISSR markers are employed for genetic diversity evaluation due to their robust nature, low cost requirement, high reproducibility and their ability for not getting affected by the environmental factors (Das et al. 2017; Ray et al. 2019a). Further, these markers do not require prior information about the genome (Kaur et al. 2015). Moreover, conservation and effective utilization of plant genomic resources required the evaluation of genetic differences in a species (Sun et al. 2008). Sample collected from diverse locations (11 populations), different altitudes (1900–3500 m asl) and diverse habitat types (Oak-rhododendron forest, oak mix

forest, open grassy slopes and rocky/boulder and under Juniperus species) demonstrated significant differences among morphological, phytochemical and genetic diversity attributes. Similar reports were available for different threatened medicinal plants such as *Valeriana jatamansi* (Jugran et al. 2013a); *Habenaria edgeworthii* (Giri et al.2016); *Polygonatum cirrhifolium*, *Malaxis muscifera* (Suyal et al. 2019b), *Polygonatum verticillatum* (Suyal et al.2019a). Causes might be attributed to source specific variability i.e. (Suyal et al. 2019a; Rawat et al. 2020). Furthermore, patchy distribution including small population size and geographical barriers also accelerates small genetic variations which create variability and better adaptability in such quantitative traits for specific habitat conditions (Rawat et al. 2017).

Small population size also leads to poor genetic differences which is a common tendency in endangered plants (Loveless and Hamrick 1984; Cai et al. 2011; George et al. 2009). However, *P. verticillatum* being of low population size possess unexpectedly high level of average genetic diversity (He = 0.320; Pp% = 85.49). Considering the individual populations maximum genetic diversity and Shannon information index (He = 0.35; I = 0.51) were recorded for Surmoli and Himkhola populations. The higher genetic diversity in threatened target species might be due to their different geographical conditions. Similar results were also recorded in other threatened taxa using different ISSR markers. For instance, Jugran et al. (2011) reported high genetic diversity in *Hedychium spicatum* (He = 0.372; Pp% = 78.08), Naik et al. (2010) in *Podophyllum hexandrum* (He = 0.29; Pp% = 83.82); Kasture et al. (2016) in *Acorus calamus* (He = 0.33), Warghat et al. (2013) in *Dactylorhiza hatagirea* (He = 0.2967; Pp% = 97.73); Ray et al. (2019b) in *Hedychium coronarium* (Pp% = 86.30). Clustering of the diverse populations of *P. verticillatum* in three major clusters as demonstrated in the STRUCTURE and neighbor joining cluster analyses supported the admixture of individuals among the assumed geographical populations. The flowers of *P. verticillatum* are hermaphrodite and pollination occurs with the help of the bees. The genetic differentiation in flowering plants is generally affected by its breeding system (Hamrick and Godt 1989). Therefore, it is assumed here that breeding systems of *P. verticillatum* may play a vital role in maintaining the genetic structure of its populations. This is also strengthened by karyology of *P. verticillatum* as 2n = 28 chromosomes have been reported from this species in many sources, but 2n = 58 were detected in the population from east (Tamura 1993).

AMOVA showed most of the phenotypic variation in targeted species was allocated within the population in *P. verticillatum* (78%). Similar pattern of variation within the population was observed for *Valeriana jatamansi* (ISSR-89%), *Habenaria edgeworthii* (ISSR-74%); *Malaxis monophyllos* (AFLP-87%), *Cymbidium tortisepalum* (nSSR-89.25%), *Lilium cernuum* (allozymes-84%), respectively [Jugran et al. 2013a; Giri et al. 2016; Zhao et al. 2017; Chung et al. 2018]. The high intra population diversity of this species could be explained on account of its life history traits, especially the breeding system, which strongly influence distribution and magnitude of the genetic diversity in plant populations. Ji et al. (2020) also reported that perennial, outcrossing plant species retains most of their genetic variability within the population compared to annual, selfing species. This holds true in case of targeted species where species reproduces both sexually (seeds) and asexually (rhizomes) and pollinated by insects preferably bumble bees (Kramp et al. 2009).

Low genetic differentiation recorded in *P. verticillatum* [G_{ST}-0.226; Nm = 1.717] in this study was comparable to the average coefficients reported for long-lived perennial (G_{ST} = 0.19), out-crossing species (G_{ST} = 0.22) (Nybom 2004), and monocots (G_{ST} = 0.231) (Hamrick and Godt 1989). Similar reports of low genetic differentiation are reported elsewhere [Rawat et al. 2016 (*Roscoea procera* (G_{ST} = 0.202)); Naik et al. 2010 (*Podophyllum hexandrum* (G_{ST} = 0.20)) etc.]. The low genetic differentiation might be resulted due to the fact that the targeted species were locally restricted and declining due to habitat loss and over harvesting and grazing.

The higher values of tannin, flavonol, NO scavenging activity, DPPH and ABTS activity at higher altitude are indicative of higher stress mediated by higher UV-B radiation (Cirak et al. 2017). Reports are available where, total phenols, rutin, proteins, and proline were observed as increased with incline in elevation (Guo et al. 2011; Sharaf et al. 2013). The higher genetic diversity compared to lower and mid altitude at higher altitude findings are not in agreement to the altitudinal decline in genetic diversity observed by Jugran et al. (2013a) in *Valeriana jatamansi*, where maximum genetic diversity was found in lower populations. An altitudinal enhancement in genetic diversity was however previously reported in 48 populations of *Sorghum bicolor* using 4 enzyme loci (Ayana et al. 2001); 7 populations of *Lilium longiflorum* using 9 RAPD primers (Wen and Hsiao 2001); 10 populations of *Primula farinosa* using 9 RAPD primers (Reisch et al. 2005) etc. This might be because of harder climatic conditions and more heterogeneous habitats. The population at high altitude experience greater climatic variations and are under greater environmental stress due to rapid changes in temperature, low light intensity, strong wind and coverage of snow and ice (Reisch et al. 2005). Similarly, the habitats at higher altitude are characterized by rocky/ boulder paths, scrub lands etc. In the present case, the higher genetic diversity, tannins, DPPH, ABTS and NO scavenging activity in

rocky/boulder and Juniper scrub might be ascribed to the better opportunities of survival and growth. In open exposed sites, wherever a lack of forest vegetation occurs, direct UV radiation affects the plants, and therefore, plants deposits higher level of secondary metabolites for defense. These phyto-constituents then acts as antioxidants and may vary to improve the effects of potentially damaging UV radiation efficiently before the radiation reaches the sensitive chromophores (Ruhland et al. 2007). The lack of forest vegetation barriers, affects positively to higher levels of gene flow between populations, which increases genetic diversity within high-altitude populations (Reisch et al. 2005).

Conclusion

The genetic variability studies at intra species level are a potential tool to devise genetic advancement and strategies for germplasm conservation. The genetic differentiation among studied population was high (Gst = 0.226; $P < 0.001$) with relatively high gene flow (Nm = 1.717). Based on our finding of this study, we suggest ex situ conservation could be an appropriate measure to adequately capture and conserve the total genetic diversity of *P. verticillatum* populations of Uttarakhand, India by choosing few genotypes from diverse populations. Meanwhile conserving the sites with these species and promoting the cultivation at these sites would be appropriate for in situ conservation of this valuable species. Further, studies revealed that *P. verticillatum* is restricted to some specialized habitats and subject to destructive harvesting and heavy grazing in the wild. Therefore, conservation measures should be taken to protect species habitat to maintain the genetic variations. The result demonstrated that the genetic diversity of plant populations can be exaggerated by alterations in micro environment as well as that driven by animal grazing. Grazing increases the genetic diversity, while an excessive disturbance lessens the plant density. So, it is important to protect the alpine meadows from excessive grazing. Based on PCA analysis, populations of Kafni, Satbunga, Himkhola, Milam and Surmoli could be prioritized for higher morphological, phytochemical and genetic variability and used as elite germplasm for mass multiplication and for market demand of target species. Also these populations are important reservoir of useful genes. Additionally, efforts should be made to conserve this species through seed germination, vegetative propagation or tissue culture techniques.

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Authors contribution The study was conceptualized by AKJ, RSR and IDB. RS & AKJ standardized and performed the experiments and analyzed data. RS and AKJ wrote initial draft of the Manuscript. AKJ, RSR and IDB edited the final version of the MS. RSR and IDB acquired funds for the study and facilitated the chemicals and glassware's used in the study.

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