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DNA Profiling of the Threatened Himalayan Herb *Polygonatum Verticillatum* L. using Cross-Transferred *Betula* SSR Markers

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Abstract

Polygonatum verticillatum is an important Himalayan herb that is used in different medicine systems for improving health and curing many diseases. Herein, simple sequence repeat (SSR) marker characterization of this plant species was performed using cross-transferred SSR markers of a distantly related species *Betula utilis*. Among the 25 SSR markers tested, 13 generated clearly distinguishable alleles. Of these, 12 SSR primers were polymorphic and 1 was monomorphic. All the 12 markers collectively amplified 42 alleles. The average value of 3.5 alleles was observed. The size of alleles ranged from 100 - 600 bp. The mean polymorphism information content (PIC) was 0.459, and mean marker index was 1.61. The dendrogram clustered all the studied accessions into three groups according to geographical locations. The results showed high genetic diversity in the populations of *P. verticillatum* in Indian Himalayan region. SSR marker exhibited good amplification in distantly related species. The SSR markers used in the present work can help diversity and breeding research of *P. verticillatum* in coming days. The results of present work will be helpful for characterization, conservation, management and improvement of the germplasm of this plant in the future.

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Betula Utilis.

Introduction

Polygonatum verticillatum L. is a highly valued medicinal herb of Indian Himalayan region (IHR) and occurs from 2000 -3000 m asl. It is a member of family Asparagaceae. It is known as “Meda” in Sanskrit and “Salam Mishri” in Hindi. This herb yield important metabolites and is one of the constituents of many ayurvedic formulations.[1-3] Morphologically, plants are slender and generally, unbranched with leaves arranged in verticillaster manner. Leaves are lanceolate, and stem bear flowers near the base of leaves. Flower colour is generally pale yellow and greenish. The fruits are round berries that are initially green in colour and become red when ripe. It is a plant of high importance. However, unscientific exploitation by local traders and anthropogenic

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activities has resulted in habitat destruction of its natural populations, which has resulted in the loss of the different wild genetic stocks of this valuable plant species. It has been reported from Uttarakhand that plant is vulnerable due to overuse by pahemaceutical companies, less awareness about the plant among local people, habitat destruction and habitat fragmentation in addition to unscientific harvesting and other anthropogenic activities.[4,5] Hence, it is also listed in threatened plants category.[6,7] In this situation, supreme importance should be given for characterizing the existing germplasm at molecular level for its genetic diversity and conserving its diverse germplasm. Genetic diversity data can be useful in identifying the diverse accessions which can be selected for conservation and for breeding experiments on priority basis. Diversity data can also be used for better management of the germplasm. Further, molecular characterization should be carried out at genetic level to identify elite germplasm stocks. This will help maintain diverse germplasms in the future and consequently will help in their conservation and management. Molecular markers, specifically DNA markers, are the highly preferred for the genetic characterization of any type of germplasm and plant collection. There are various types of DNA markers such as RFLP, RAPD, AFLP, ISSR, SSR, and SNP. Of these, SSR markers are currently the most commonly used DNA markers due to their desirable features such as the potential to resolve heterozygosity, easy laboratory procedures, cross-transferable nature and evenness in genomes.[8-15] Hence, these markers are widely used in plant genetic diversity research.

Table 1: List of Accessions Characterized in the Present Study using Cross-Transferred SSR Markers

S. No.	Sample code	Location	District	State
1.	JH	Jhungi	Mandi	Himachal Pradesh
2.	JL	Jalori	Kullu	Himachal Pradesh
3.	TU-1	Tunga Dhar	Mandi	Himachal Pradesh
4.	TU-2	Tunga Dhar	Mandi	Himachal Pradesh
5.	KP	Kataula	Mandi	Himachal Pradesh
6.	KS	Fatehpura	Anantnag	Kashmir
7.	TU-3	Tunga Dhar	Mandi	Himachal Pradesh
8.	TU-4	Tunga Dhar	Mandi	Himachal Pradesh
9.	SR-1	Sarahan	Shimla	Himachal Pradesh
10.	SR-2	Sarahan	Shimla	Himachal Pradesh
11.	SN	Solang Nullah	Kullu	Himachal Pradesh
12.	JL-1	Jalori	Kullu	Himachal Pradesh
13.	KT-1	Kala Top	Chamba	Himachal Pradesh
14.	KT-2	Kala Top	Chamba	Himachal Pradesh
15.	TU-5	Tunga Dhar	Mandi	Himachal Pradesh
16.	TU-6	Tunga Dhar	Mandi	Himachal Pradesh
17.	JL-1	Jalori	Kullu	Himachal Pradesh
18.	KTH-1	Kainthley	Chamba	Himachal Pradesh
19.	KTH-2	Kainthley	Chamba	Himachal Pradesh
20.	KTH-3	Kainthley	Chamba	Himachal Pradesh
21.	KTH-4	Kainthley	Chamba	Himachal Pradesh
22.	KTH-5	Kainthley	Chamba	Himachal Pradesh

Moreover, the cross-transferability of SSR markers across species and genera is of great importance for exploring plant species for which SSR markers are not available. Many researchers have utilized the SSR markers from closely related and distantly related plant species to characterize and study the

genetic diversity of germplasms of other species.[7,12-15] Few biochemical and genetic studies have been conducted in different species of *Polygonatum*, and more such studies are required for proper utilization and maintenance of this important genetic resource. Researchers categorized *Polygonatum* as an important genus with immense potential.[16,17] This plant contains many vital compounds, which exhibits curative and health promoting effects. Some important chemical constituents have been isolated and identified in this species. The researchers have developed method for identifying adulterants in mixtures of P. *verticillatum*.[18] As it is a threatened plant, some conservationists have tried to establish tissue culture methods for its rapid propagation and availability.[19] However, genetic characterization studies on this species are lacking. In other species of the genus *Polygonatum*, such investigations have been performed. In *P. sibiricum,* attempt has been made to explore genes involved in biosynthetic pathways.[20] Other researchers identified some phytochemicals and antioxidants from this species[21] *P. cyrtonema* Hua, which is an endemic species of this genus in China, was evaluated for its genetic diversity and structure in Anhui with the help of SSRs and morphological traits.[22] Genetic diversity of *P. multiflorum* and *P. odoratum* was also explored by Chinese researchers.[23] Cross-species SSR markers were evaluated for their cross- transferability in P. *verticillatum*.[24] A comparative study on clones and genetic structure was also undertaken in *Polygonatum*.[25] Different DNA markers were used for studying the genetic similarity of three species of *Polygonatum*, including P. *verticillatum*, occurring in Poland.[26] In India only one study using ISSR markers was conducted in P. *verticillatum*.[27] Therefore, In the present study, SSR markers of an alpine species, namely, *Betula utilis* (Betula) were checked for cross-transferability in P. *verticillatum* and unambiguously amplified markers were utilized to characterize and study the genetic diversity of P. *verticillatum*. The rationale for using *Betula utilis* SSR was overlapping habitat of both the species. Both these species occurs in temperate and alpine regions of Himalaya and it was assumed that at genetic level these may contain similar genomic regions which help in adaptation of these species in their habitats. The results of current research work can be important for future research pertaining to conservation, breeding and management of this species.

Materials and Methods

Plant Sampling and DNA Extraction

Our sampling sites included state of Himachal Pradesh and Union Territory of Kashmir, India. Leaf samples from twenty two accessions of P. *verticillatum* were collected from different regions of four districts of Himachal Pradesh and one district of Kashmir. Young and fresh leaf samples were collected in plastic bags containing silica gel and transported to the laboratory at room temperature. DNA isolation was performed according to the CTAB method[28] using liquid nitrogen. A detailed description of accessions is given in Table 1.

PCR Reactions

In total, twenty-five SSR primers were checked for amplification in a pooled DNA sample. Among the 25 SSR primers of B. *utilis*,[29] 13 SSR primers were clearly amplified. Finally, these 13 unambiguously amplified primers were chosen for the SSR diversity study. The final volume of SSR reaction mixture was consisted of 10 µl. The composition of this included 4.8 µl deionized water, 2.0 µl template DNA of 13 ng/ µl quantity, 0.5 µl of each forward and reverse primer with 5 µM concentration, 0.5 µl $MgCl_2$ (25 mM), 1.0 µl 10 X buffer containing 10 mMTris-Hcl, 50 mMKcl having pH 8.3, 0.5 µl dNTP mix consisting of 0.2 mM each dATP, dGTP, dCTP and dTTP, and lastly 0.2 µl *Taq* polymerase with 5U/ µl. The PCR conditions were set as: First stage- 1 cycle of 5 min at 94 ºC, Scond stage- 35 cycles of 1 min at 94 ºC, 1 min at annealing temperature of each primer, 1 min at 72 ºC and third stage- 7 min at 72ºC. PCR amplified products were run on 3% agarose gel in 1 X TBE buffer for visualization of fragments, and size of each fragment was determined with 100 bp DNA ladder (Genei, Bangalore). Ethidium bromide dye was used for detecting DNA fragments. Photo of gel was taken with the help of gel documentation system (Bio-print, Vilber Eppendorf, France).

Data Analysis

For analysis, DNA bands detected in the agarose gel were manually scored. The clearly amplified alleles were considered for scoring. After scoring, a binary data file was created in an Excel sheet, and all downstream analyses were performed using this binary file. Polymorphism Information Content (PIC) was determined with the formula as per Botstein and his group.[30] PIC is the indicatore

of the level of polymorphism which the samples exhibit and can be employed to select polymorphic samples and primers. Similarly, Marker Index (MI) is the indicator of the efficiency of a marker and can be used to differentiate most informative versus least informative markers. Cluster analysis was done using distance based method and Jaccards similarity coefficient with UPGMA was used to generate dendrogram with DARwin.[31] The Groupings shown in the dendrogram were observed, and inferences and interpretations were made to reach conclusions.

Results

SSR Data and Diversity Indices

Thirteen SSR primers generated clear prominant alleles. In total, 12 primers were polymorphic, and generated 42 alleles. The average value of allele was 3.5. The size of alleles ranged from 100 bp to 600 bp. A representative gel image is given in Figure 1.

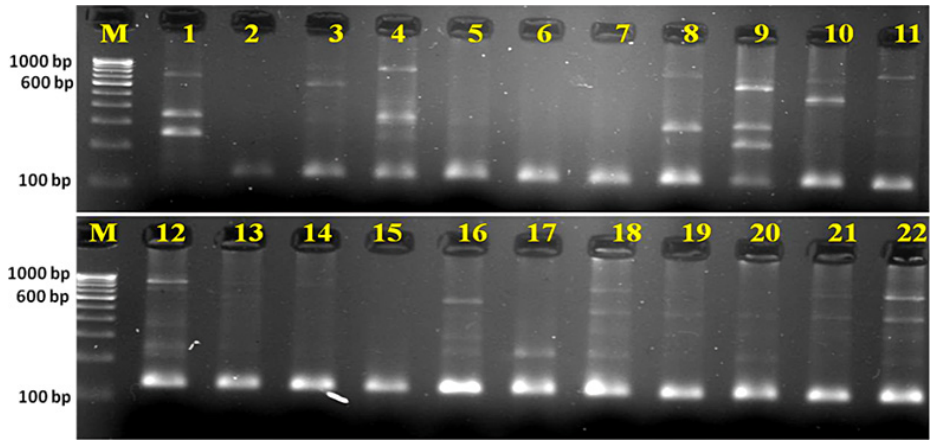

Fig. 1: Representative gel showing the amplification the of SSR primer BUMS-5 in twenty-two accessions of *Polygonatum verticillatum*.

Table 2: Features of Primers used to Characerize Polygonatum Verticillatum Accessions PIC: Polymorphism Information Content, MI: Marker Index, *BUMS-14 was not included for finding the mean value of No. of Alleles as it was monomorphic.

S.No.	Name of SSR primer	Amlified or not (Yes/ No)	No. of Alleles	Sige range	PIC	MI
1.	BUMS-03	Yes	5	160-500	0.404	2.02
2.	BUMS-05	Yes	4	150-700	0.490	1.96
3.	BUMS-06	Yes	2	200-330	0.5	1
4.	BUMS-08	Yes	4	170-500	0.456	1.82
5.	BUMS-10	Yes	4	180-420	0.493	1.97
6.	BUMS-14	Yes	1	400	-	-
7.	BUMS-15	Yes	5	150-500	0.486	2.43
8.	BUMS-16	Yes	3	150-500	0.444	1.33
9.	BUMS-18	Yes	3	200-500	0.454	1.36
10.	BUMS-21	Yes	4	100-400	0.479	1.91
11.	BUMS-22	Yes	4	190-600	0.5	2
12.	BUMS-24	Yes	2	160-250	0.375	0.75
13.	BUMS-25	Yes	2	220-370	0.433	0.867
	Mean		**3.5***	**-**	**0.459**	**1.61**

The lowest number (2) of alleles were amplified by two primer pairs, i.e. BUMS-24 and BUMS-25 while the maximum number of alleles was 5 and amplified by two primers i.e. BUMS-03 and BUMS-15, as shown in Table 2. Maximum value of PIC was 0.500, exhibited by the primer BUMS-6 and the primer BUMS-22. Minimum value of PIC (0.375) was observed in case of primer BUMS-24. The mean PIC was 0.459. Maximum marker index was detected by the primer BUMS-15 (2.43), and lowest marker index was observed in case of the primer BUMS-24 (0.75). The mean marker index was 1.61. The dendrogram clustered studied accessions into three groups (Figure 2). Group-I consisted of two accessions i.e. SR-2 and KP. Group-II consisted of JA, JH, KS, SN, SR-1, TU-1, TU-2, TU-3, TU-4, TU-5 and TU-6. Group-III contained accessions KTH-1, KTH-2, KTH-3, KTH-4, KTH-5, JL, JL-1, KT-1 and KT-2.

Discussion

Genetic diversity indicates allele polymorphism in an observed plant species and can be an excellent measure for estimating the dynamics of alleles through time. Change in alleles of the existing populations may be driven by forces such as selection, habitat destruction, overexploitation, anthropogenic and geographical disturbances. On the other hand, the detected polymorphisms can be utilized to manage and manipulate the existing germplasm for improvement through different plant breeding approaches. Hence, detecting DNA diversity is a prerequisite for different types of research. Previously, Suyal and her co-authors investigated the morphological, phytochemical and genetic diversity of P. *verticillatum* from Uttarakhand using ISSR markers.[26] However, there study did not include any sample from Kashmir or Himachal Pradesh. In addition, ISSR markers are supposed to be dominant markers and may not reveal genetic aspects as the SSR markers can. Furthermore, they also suggested a high genetic diversity in the studied samples. In the present study, SSR markers of a distantly related plants species namely, B. *utilis* were successfully employed. Among 12 markers, 11 had PIC value greater than 4. Hence, it is suggested that the markers used in the present study can also be helpful for profiling and assessing population structure at larger levels with more samples. In the past, several authors have studied different species of *Polygonatom*, but the lines of work and methods used differed. Sheng and his group analyzed P. cyrtonema and showed normal levels of genetic diversity, and accessions clustered into three distinct genetic groups.[21] Other workers evaluated the phenotypic variation and population genetic structure of *P. multiflorum* and *P. odoratum* and their results revealed the effect of light availability to flowering intensity and population structure.[22] Sharma with his co-authors checked the cross-transferability of SSR markers of T. *govanianum* in P. *verticillatum* and oberseved that 10 SSRs showed reliable amplification.[23] Chung and his team used twenty-one allozyme loci in *P. stenophyllum* and *P. inflatum.*[24] Their data suggest that populations of *P. stenophyllum* have been mainly founded by a single seed or rhizome by river water or by few seeds, whereas populations of *P. inflatum* would have been established through multiple, repeated seedling recruitment. Szczecińska with his group determined the genetic similarity of three species i.e. *P. multiflorum*, *P. odoratum* and P. *verticillatum*, and concluded that P. *verticillatum* showed no considerable diversity.[25] When compared to some earlier studies which were done on different Himalayan herbs by various authors, it was found that mean value (0.459) of PIC and mean MI value (1.61) was higher than observed in *Trillium govanianum* by Dhyani and his co-workers.[32] On the other, Rana *et al*. observed 0.513 value of PIC which was higher than observed by us in current study.[33] A high level of genetic diversity was reported in another Himalayan herb Rheum australefrom western Himalayan region.[34] Pant *et al*. also recorded 0.46 value of PIC which was almost equal to the value detected by us herein.[35] These diversity values indicated that considerable genetic diversity exists in P. *verticillatum* which is comparable to diversity detected in other Himalayan herbs.

The diversity detected in the present study was high, and the dendrogram clustered all the twenty-two accessions into three groups. The samples were grouped according to geographical locations; however, few samples were mixed within different geographical locations. Furthermore, groupings of the dendrogram revealed that the samples from Chamba district (KTH-1, KTH-2, KTH-3, KTH-4, KTH-5, KT-1 and KT-2) of Himachal Pradesh were conserved and grouped into a single group with two accessions (JL and JL-1) from Kullu district. Other accessions

seemed to be mixed within the three groups; however, the accessions of disctrict Mandi (JH, KS, SN, SR-1, TU-1, TU-2, TU-3, TU-4, TU-5 and TU-6) remained in one group except for KP. These exceptions in clustering may be attributed to low number of markers used. However, it is interesting that the distantly related SSR markers have the ability to distinguish and generate enough polymorphisms for genetic diversity studies in P. *verticillatum*. Group-II also showed subclustering to some extent, and three sub-groups were detected. Except for one accession from Kashmir and one accession from Shimla, the other accessions from district Mandi and district Kullu almost grouped in single groups, which indicate that every population has some conserved alleles which resulted in such clustering. Based on this diversity data, the populations belonging to diverse accessions such as TU4, SR2, KP and KS can be selected for conservation in their natural strands. Moreover, the germplasm of identified populations can be raised through tissue culture techniques for its large scale propagation for even commercial cultivation. Furthermore, a detailed study with a large sample size could be helpful. The limited number of SSR markers and small sample size can be the limitation of the present study, however, the results are encouraging. In the future, studies with large number of samples and more SSR markers are needed for the clarity of allele arrangements in different populations of this species.

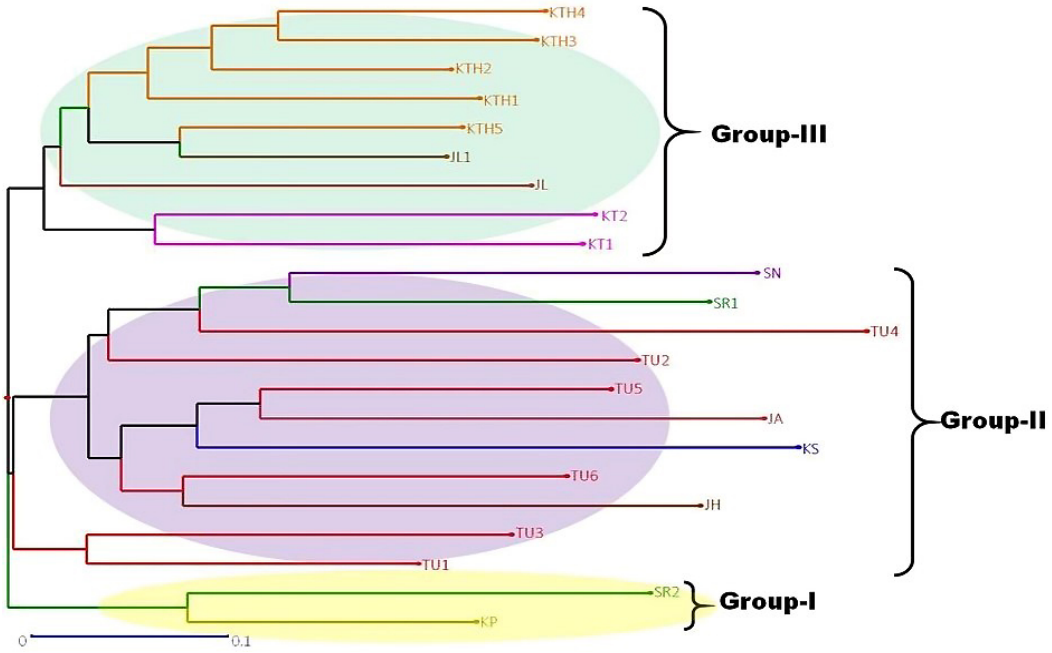

Fig. 2: Dendrogram showing twenty-two accessions of *Polygonatum verticillatum* clustered based on thirteen SSRs marker data.

Prospective Conservation and Management Plans

At present, natural populations of P. *verticillatum* are found in patches of Indian Himalayan regions and extracted from these natural populations to meet the demand for the different purposes, such as medicine production and other dietary items by folklore and industrial systems. Hence, this threatened plant species need conservation and management for its proper utilization. Urgent attention and steps are required in this direction to safeguard this important germplasm resource. For *in-situ* conservation, cultivation in native regions with the help of neighboring dwellers and forest departments can be highly beneficial. This can help increase its population size and be utilized economically in a sustainable way. For *ex-situ* conservation, introduction and propagation at different locations with the help of scientific

advisors from nearby organizations is necessary. Tissue culture strategies should be employed for large scale plantations. Furthermore, it can also be introduced in those Himalayan regions that are suitable for its natural growth so that its distribution may be widened. Natural extraction should be monitored scientifically to ensure the collection of roots and plant parts at appropriate stage of growth. At least extraction should not be done before the seed set. Government bodies and other organizations involved in the conservation of flora and environment needs to take care of these types of activities. Proper documentation of the quantity of the plant material harvested and supplied should be maintained at the native production site.

Conclusion

In conclusion, the present study reported the diversity of P. *verticillatum* using cross-transferred SSR markers of B.*utilis*. The results of this study showed that considerably high genetic diversity prevails in the populations of P. *verticillatum* in IHR. However, record of the population distribution and spreading trends in this region are not available. Proper conservation and management of this plant species is urgently needed for sustainable utilization. Large-scale studies involving maximum populations of IHR should be initiated for the identification of diverse accessions for improvement in the future. The SSRs used in this study can help exploring genetic variations and identifying important alleles in P. *verticillatum*.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statements

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Authors' Contribution

PDS done sampling, experimental work and VS analyzed the data and wrote manuscript.

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