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SHORT REPORTS

Development and characterization of novel microsatellite markers in *Trillium govanianum*: a threatened plant species from North-Western Himalaya

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Abstract *Trillium govanianum* is a temperate forest understory plant species of high value belonging to the family Melanthiaceae. It is endemic to Himalayan region and facing a bottleneck situation due to reckless extractions from its natural strands. In the present study, 21 microsatellite markers were developed and characterized in 20 accessions of *T. govanianum*. Collectively, the polymorphic markers amplified 31 alleles in a range of 2–4 with an average of 2.6 alleles per marker. The mean observed heterozygosity (H_o), expected heterozygosity (H_e), and Shannon information index (I) were 0.46, 0.48, and 0.73, respectively. Average polymorphism information content (PIC) was 0.385. The cross-transferability in a related species, namely, *Polygonatum verticillatum*, showed amplification of ten markers. The newly developed microsatellite markers efficiently distinguished the different accessions on the basis of their geographic origin. Thus, these microsatellites can be useful in exploring genetic diversity in various existing populations of *T. govanianum* in north-western Himalaya, which may be useful for their conservation, management, and improvement in future.

Keywords *Trillium govanianum* · Microsatellite markers · Genetic diversity · Cross-transferability · *Polygonatum verticillatum* · Polymorphism information content (PIC)

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Introduction

Trillium govanianum Wall. ex D. Don [Syn. *Trillidium govanianum* (Wall. ex D. Don) Kunth], locally known as Nagchhatri, is a native species of the Himalayan region and found distributed from 2400 to 4000 m asl (Vidyarthi et al. 2013). It is a temperate forest understory species and generally inhabits the shady moist places. The plant consists of an unbranched stem reaching up to 15–25 cm in height. The plant bears characteristic three leaves which originate from a single central point of main stem. From this point of leaf origin, arises a solitary reddish brown colored flower on a stalk. The plant perennates through underground rhizome. The rhizome is the main plant part utilized in therapeutic purposes. The rhizome is used to cure stomachic diseases by folks and also used in the preparation of steroidal and sex hormones (Uniyal and Datta 2012). The overexploitation and unscientific uprooting of this herb in recent years from north-western Himalayan region, specifically from Himachal Pradesh, has left it in the verge of extinction (Vidyarthi et al. 2013). Hence, the different populations of *T. govanianum* are under high anthropogenic pressure, which is causing their diminution. Although, a plant species of immense importance, its germplasm remained uncharacterized, and no report on genetic diversity exist in this species. However, the information on genetic diversity and structure of this species is required to identify diverse accessions for conservation priority. Therefore, we developed the microsatellite markers or simple sequence repeat (SSR) markers in this species for the first time. The polymorphic microsatellites detected in this study can be useful in future genetic analyses in *T. govanianum* and in other related species.

Materials and methods

The nucleotide sequence data of *Trillium* species available at National Centre for Biotechnology Information (NCBI) were utilized for designing microsatellite primers. The nucleotide sequences were downloaded and redundancy was removed by assembling these sequences using EGassembler online software (Masoudi-Nejad et al. 2006). Microsatellite search and primers designing was performed as per Sharma et al. (2009). The markers were validated in 20 accessions of *T. govanianum* collected from various geographical locations of the North-Western Himalaya of Indian region (Table 1). Cross-transferability of these markers was also checked in *Polygonatum verticillatum*. The genomic DNA was extracted and PCR for all the markers was performed in 10 µL reaction volume as per Sharma et al. (2015). The thermal cycles were set as initial cycle of 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at respective annealing temperature (Table 2), 1 min at 72 °C, and the final extension of 7 min at 72 °C. The amplified products were separated on 6% denaturing polyacrylamide gels in 1 X TBE buffer and visualized by silver staining. Alleles were scored manually and the data were analyzed by computing various diversity indices. Expected heterozygosity (H_e), observed heterozygosity (H_o), Shannon information index (I), and Hardy–Weinberg equilibrium were obtained using POPGENE version 1.32 (Yeh and Boyle 1997), and polymorphism information content (PIC) was calculated using CERVUS version 3.0 (Kalinowski et al. 2007). Dendrogram was constructed using Jaccard's coefficient with the DARwin software (Perrier and Jacquemoud-Collet 2006).

Results and discussion

The routine scientific explorations of natural bioresources, both at basic or advanced levels, can lead to their new products and utilities. The same has happened in case of *T. govanianum*. It was lesser known as medicinal up to the last decade, but has become important due to its new uses as medicine. At present, it is facing the danger of extinction due to the overexploitation. To assess its genetic diversity, we developed the microsatellite markers utilizing the

Table 1 List of accessions characterized in the present study

S. no.	Species	Sample code	Location	State
1.	*Trillium govanianum*	TG-FTP-01	Fatehpura, Kashmir	Jammu and Kashmir
2.	*T. govanianum*	TG-FTP-02	Fatehpura, Kashmir	Jammu and Kashmir
3.	*T. govanianum*	TG-FTP-03	Fatehpura, Kashmir	Jammu and Kashmir
4.	*T. govanianum*	TG-FTP-04	Fatehpura, Kashmir	Jammu and Kashmir
5.	*T. govanianum*	TG-FTP-05	Fatehpura, Kashmir	Jammu and Kashmir
6.	*T. govanianum*	TG-FTP-06	Fatehpura, Kashmir	Jammu and Kashmir
7.	*T. govanianum*	TG-GLM-01	Gulmarg, Kashmir	Jammu and Kashmir
8.	*T. govanianum*	TG-GLM-02	Gulmarg, Kashmir	Jammu and Kashmir
9.	*T. govanianum*	TG-GLM-03	Gulmarg, Kashmir	Jammu and Kashmir
10.	*T. govanianum*	TG-GLM-04	Gulmarg, Kashmir	Jammu and Kashmir
11.	*T. govanianum*	TG-GLM-05	Gulmarg, Kashmir	Jammu and Kashmir
12.	*T. govanianum*	TG-GLM-06	Gulmarg, Kashmir	Jammu and Kashmir
13.	*T. govanianum*	TG-CHV-01	Chauhar Valley, Mandi	Himachal Pradesh
14.	*T. govanianum*	TG-CHV-02	Chauhar Valley, Mandi	Himachal Pradesh
15.	*T. govanianum*	TG-CHV-03	Chauhar Valley, Mandi	Himachal Pradesh
16.	*T. govanianum*	TG-HDJ-01	Hathidhar, Janjehli	Himachal Pradesh
17.	*T. govanianum*	TG-HDJ-02	Hathidhar, Janjehli	Himachal Pradesh
18.	*T. govanianum*	TG-HDJ-03	Hathidhar, Janjehli	Himachal Pradesh
19.	*T. govanianum*	TG-CDS-01	Churdhar, Sirmaur	Himachal Pradesh
20.	*T. govanianum*	TG-CDS-02	Churdhar, Sirmaur	Himachal Pradesh
21.	*Polygonatum verticillatum*	PV-SDJ-01	Shikari Devi, Janjehli	Himachal Pradesh
22.	*P. verticillatum*	PV-SDJ-02	Shikari Devi, Janjehli	Himachal Pradesh
23.	*P. verticillatum*	PV-SDJ-03	Shikari Devi, Janjehli	Himachal Pradesh
24.	*P. verticillatum*	PV-SDJ-04	Shikari Devi, Janjehli	Himachal Pradesh

Table 2 Characteristics of 14 microsatellite markers developed and characterized in the present study

Marker name	Primer Sequence (5′–3′)	Repeat motif	T_a (°C)	Size range (bp)	N_a	H_o	H_e	P	I	PIC
TGSSR-01	F-CTACCGATGTCCCGATCAGT R-TTAGAGCGAATGCAACAACG	$(TTCC)_3$	48	160–230	3	0.500	0.714	0.053	1.03	0.555
TGSSR-02	F-ACGACCCGAATCGATATTTG R-TGGGCTTTTTGTCAGTTTTG	$(TA)_6$	47	225	1	–	–	–	–	–
TGSSR-03	F-AATTCTGATGCAATCCTTGG R-ATTTCGCTGCGTTCTTCATC	$(AC)_5$	49	170–210	3	0.750	0.700	0.000*	1.08	0.582
TGSSR-04	F-TAGGCACTGGGTGAACTGTG R-CCGCTAACACAGGCAAAGA	$(TTA)_4$	47	210	1	–	–	–	–	–
TGSSR-07	F-GGTCGAGAAAGATGGGCTCT R-GGTCGAACGACCGGTACATA	$(ATAG)_3$	49	300, 310	2	0.500	0.409	0.518	0.56	0.305
TGSSR-08	F-TTCCCGATTCACCAATTCTT R-TGGATACTATGACCCACATTCG	$(TA)_5$	48	190, 200	2	0.833	0.530	0.122	0.67	0.368
TGSSR-09	F-TATAAAGGACCCGCCGAGTT R-TGGAGAAACGACAGAAACTATG	$(TTA)_4$	47	220–245	3	0.333	0.451	0.593	0.72	0.371
TGSSR-13	F-TGGGGTTCCAAAATTGTTTC R-GTCATTGGGTCGAACGGTAT	$(ATAG)_3$	51	170, 175	2	0.167	0.167	1.000	0.28	0.141
TGSSR-14	F-TATAAAGGACCCGCCGAGTT R-TGGAGAAACGACAGAAACTATG	$(TTA)_5$	47	220, 225	2	0.667	0.485	0.300	0.63	0.346
TGSSR-15	F-CCCCTTTCGTTTGTCCACTA R-AAGGAATGGTCGGGGTAAAC	$(ATT)_4$	49	150–210	3	0.500	0.667	0.008*	1.03	0.555
TGSSR-16	F-CCCAAAAGGACATTCATTCA R-CGGATTTCGTTTTGCTTCTC	$(CTT)_4$	47	190–200	3	0.200	0.484	0.004*	0.80	0.410
TGSSR-17	F-GACCCCGTCGTAGTTCTCAA R-GCGAGATGGTGGTTTTTGTT	$(GAAG)_3$	49	205–230	4	0.600	0.695	0.151	1.22	0.610
TGSSR-18	F-AGCAATTGACCGACCCCTAC R-TCTATTAACCCCGGGCTCTT	$(TAA)_5$	47	330, 370	2	0.250	0.233	0.781	0.37	0.195
TGSSR-19	F-TTTTGGCGTGATTGATAGGA R-CAATGCTATTCCAGATACACATGC	$(ATAACA)_3$	47	180, 190	2	0.250	0.233	0.781	0.37	0.195
Mean					2.35	0.462	0.480		0.73	0.385

Ta annealing temperature, *bp* base pairs, *Na* number of alleles, *Ho* observed heterozygosity, *He* expected heterozygosity, *P* probability that genotype proportions conform to Hardy–Weinberg equilibrium, *I* Shannon information index, *PIC* polymorphism information content

* Deviation from Hardy–Weinberg equilibrium

sequence data available at public domain. Of the 870 nucleotide sequences available at NCBI, 31 sequences showed the presence of microsatellites. From these 31 microsatellite containing sequences, 21 microsatellite primer pairs were designed. Screening of these designed primers in selected samples showed that 14 microsatellites amplified unambiguously. Finally, these 14 microsatellites were used for the characterization of 20 different accessions of *T. govanianum*. Of these, 12 microsatellites showed polymorphism, while two microsatellites showed monomorphism among the characterized accessions. The polymorphic markers showed an average of 2.6 alleles. The mean H_o and H_e were 0.46 and 0.48, respectively. The highest value of PIC was 0.610 shown by TGSSR-17, while lowest was 0.141 shown by TGSSR-13 with an average of 0.385. Only 4 (33%) markers were having PIC value more than 0.5 (Table 2). Highest (1.22) Shannon information index was detected by TGSSR-17, while lowest (0.37) was detected by two markers (TGSSR-18 and TGSSR-19) with a mean value of 0.73. Dendrogram showed that the new microsatellite markers efficiently resolved the different geographic accessions and clustered all the accessions into three major groups (Fig. 1). The genetic diversity estimates reported here were higher than reported by Li et al. (2005), who detected very low genetic diversity in *T. tschonoskii.* Overall, the genetic diversity recorded in this study was low. The low levels of genetic diversity were in contrast to Gonzales and Hamrick (2005), and Tomimatsu and Ohara (2003), who observed high genetic diversity in *T. reliquum* and *T. camschatcense,*

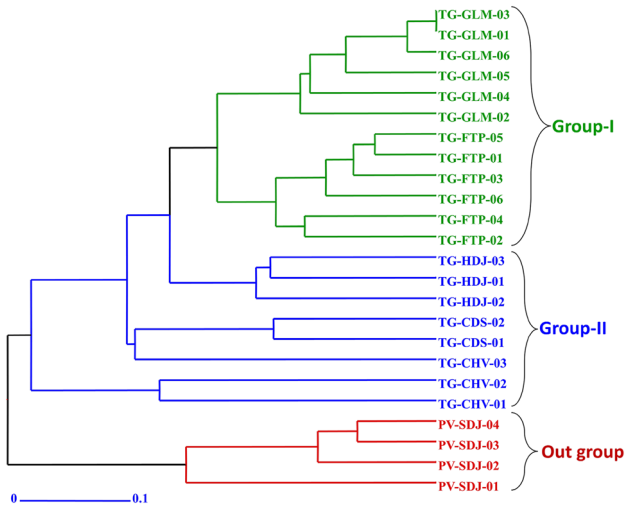

Fig. 1 Dendrogram constructed based on the alleles amplified by polymorphic microsatellite markers. Different geographic populations/species were clearly distinguished into three major clusters

respectively. The low level of diversity detected in the present study may be attributed to its vegetative multiplication. Although, it also produces seeds, but the limited seed dispersal, geographical barriers and specificity of habitat for germination may restrict its growth and genetic diversity, as also reported by Wallace and Doffitt (2013) in *T. cuneatum* and *T. stamineum*. The low genetic diversity was also in agreement to Griffin and Barrett (2004) in *T. erectum*, and Walker et al. (2009) in *T. maculatum*. The cross-transferability showed that 10 (71.4%) microsatellite markers were amplified in *P. verticillatum* (Table 3). Thus, most of the markers developed in the present study were also conserved in *P. verticillatum* and can be used to study its genetic characteristics. The cross-transferability of newly developed markers also suggests their wider utility for characterization purposes in the other related species. Furthermore, it was observed that accessions from Himachal Pradesh, which include the most anthropogenically disturbed habitats of *T. govanianum*, exhibited higher diversity than the accessions from undisturbed habitats of Kashmir Himalaya. The results of the present work highlight the need for investigating genetic diversity in various existing populations of *T. govanianum* in north-western Himalaya for the identification of diverse genotypes. The microsatellite markers developed in this study can be useful for diversity evaluation which is a prerequisite for designing effective conservation and management strategies necessary for sustainable utilization of this species.

Table 3 Details of microsatellite markers cross-amplified in *Polygonatum verticillatum*

Marker name	Amplification	N_a	Size of alleles (bp)
TGSSR-01	+	2	160, 180
TGSSR-02	+	1	225
TGSSR-03	+	1	205
TGSSR-04	+	1	210
TGSSR-07	–	–	–
TGSSR-08	+	2	190, 200
TGSSR-09	+	2	220, 245
TGSSR-13	+	1	170
TGSSR-14	–	–	–
TGSSR-15	+	1	1
TGSSR-16	+	1	1
TGSSR-17	+	2	2
TGSSR-18	–	–	–
TGSSR-19	–	–	–

+ represent amplification, – represent no amplification or null allele, N_a number of alleles, *bp* base pairs

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