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Genetic diversity analysis in the *Hypericum perforatum* populations in the Kashmir valley by using inter-simple sequence repeats (ISSR) markers

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Assessment of genetic variability among the *Hypericum perforatum* populations is critical to the development of effective conservation strategies in the Kashmir valley. To obtain accurate estimates of genetic diversity among and within populations of *H. perforatum,* inter-simple sequence repeats (ISSR) markers were used. The study was aimed to check, whether ISSR fingerprinting may be a useful tool for studying genetic variations among *H. perforatum* populations in the Kashmir valley (India). A total of 15 ISSR primers were tested with the 20 genotypes of *H. perforatum*. The ten informative primers were selected and used to evaluate the degree of polymorphism and genetic relationships within and among all the *H. perforatum* populations. ISSR of 20 genotypes analysis yielded 98 fragments that could be scored, of which 71 were polymorphic, with an average of 7.1 polymorphic fragments per primer. Number of amplified fragments varied in size from 150 to 1650 bp. Percentage of polymorphism ranged from 60% to a maximum of 100%. Resolving power ranged from a minimum of 7.7 to a maximum of 14.3. Shannon indexes ranges from 0.166 to 0.389 with an average of 0.198 and Nei's genetic diversity (h) ranges from 6.98 to 9.8. Estimated value of gene flow (Nm = 0.579) indicated that there was limited gene flow among the populations. The genetic diversity (Ht) within the population of 0.245 was clearly higher than that of among population genetic diversity (Hs= 0.115), indicating an out-crossing predominance in the studied populations. Analysis of molecular variance by ISSR markers indicated that over half of the total variation in the studied populations (58%) could be accounted for by differences among the 8 divisions, with a further 42% being accounted for by the variation among populations within a division.The dendrogram grouping the populations by unweighted pair-group method with arithmetic averages (UPGMA) method revealed eight main clusters. In conclusion, combined analysis of ISSR markers and hypericin content is an optimal approach for further progress and breeding programs.

Key words: *Hypericum perforatum* (St. John's Wort), inter-simple sequence repeats (ISSR) markers, unweighted pair-group method with arithmetic averages (UPGMA), Nei's genetic diversity.

INTRODUCTION

Hypericum perforatum L. (St. John's wort) is one of the most important medicinal plants, which has been used as a medical herb for over 2000 years (Patooka, 2003). *H. perforatum* (St. John's wort) is a widespread Eurasian perennial plant species with remarkable variation in its morphology, ploidy and breeding system, which ranges from sex to apomixes (Koch et al., 2013). The genus *Hypericum L.* (St. John's wort, Hypericaceae) includes more than 450 species that occur in temperature or tropical mountain regions of the world. Monographic work on the genus has resulted in the recognition and description of 36 taxonomic sections, delineated by specific combinations of morphological characteristics and biogeographic distribution (Nürk et al., 2011). One of the top-selling medicinal products worldwide is *H. perforatum* (St. John's Wort). Despite its cosmopolitan distribution and utilization, little is known regarding the relationship of the bioactive compounds in *H. perforatum* to the plants from which they are purportedly derived and it produces pharmaceutically important metabolites with antidepressive, anticancer and antiviral activities (Zanoli, 2004; Kubin et al., 2005; Percifield et al., 2007). A number of *Hypericum* species are well known for their therapeutic efficacy and use in traditional medicine.

H. perforatum L. has been used in herbal medicine for the treatment of burns, skin injuries, neuralgia, fibrositis, sciatica and depression (Ghasemi et al., 2013; Barnes et al., 2001). The existence of distinct multilocus genotypes in apomictic *H. perforatum* populations were reported by Ellstrand and Roose (1987). Also, Noyes and Soltis (1996), reported the existence of different clones in apomictic plant populations. Hısil and Sahin (2005) performed various experimental studies and reported the existence of diverse clones in *H. perforatum* populations but the source of this genetic diversity remains largely unexplained. Meirmans and Van Tienderen (2004) documented that the clonal diversity within a population reflects the sexual genetic pool from which the clones originated, the frequency of clonal origin, and the somatic mutations that subsequently accumulate in established clones. The diversity arising from the clonal origin is directly dependent on reproductive mode, and hence varying degrees of apomixis between *H. perforatum* landraces may have a significant impact on the diversity of local populations. The mode of reproduction was also investigated using the FCSS analysis to reconstruct the patterns of seed formation and to infer the sources of genetic variation at the population level (Percifield et al., 2007; Arnholdt-Schmitt, 2000). Earlier studies demonstrated that randomly amplified polymorphic DNA (RAPD) analysis would enable the elucidation of genetic diversity in wild populations and cultivars of *H. perforatum*. Reproduction modes of *H. perforatum*, which is facultative apomict with apospory and pseudogamy have been one of the most examined subjects by different molecular markers (Skyba et al., 2010; Arnholdt-Schmitt, 2002) such as RAPD restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) (Mayo and Langridge, 2003) and inter-simple sequence repeats (ISSR). Recently, RAPD and simple sequence repeat (SSR) were performed to estimate correlations between secondary metabolite contents and genetic profile of *H. perforatum* and other *Hypericum* species, which demonstrated variable rates of correlation (Smelcerovic et al., 2006; Verma et al., 2008, Joshi et al., 2004).

The *H. perforatum* clones examined in this study were improved by Ceylan et al. (2005) with clonal selection method from the seeds of *H. perforatum* accessions collected from eight provinces and 35 locations in the flora of Aegean Region of Turkey. Genetic distance analysis of the AFLP data (Ryan et al., 2007) revealed that the cultivated populations studied share higher genetic identity with the Western and Central European populations (0.925) than with populations from East Europe and Asia (0.828). This could be attributed to the fact that the cultivated varieties used in this study were developed in Germany and Denmark. Crockett et al. (2004) study provides a genetic method for authentication of commercial *H. perforatum* preparations and allows a preliminary assessment of phylogenetic relationships within the genus, revealing three strongly supported monophyletic clades, plus several secondary monophyletic groupings. Using internal transcribed spacer (ITS) gene sequences, they were able to distinguish *H. perforatum* from all other species of *Hypericum* included in this study. Our study demonstrates that there is a great deal of genetic diversity among *Hypericum* species as well as within *H. perforatum*, and that this diversity is structured phylogenetically and geographically. These data provide the foundation for future work characterizing the genetic relationships and recent domestication of St. John's wort and its closely related species. The use of ISSR markers gave hints for the occurrence of sexual recombination in *H. perforatum* plants. In comparison to other molecular

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Abbreviations: RAPD, Randomly amplified polymorphic DNA; **RFLP,** restriction fragment length polymorphism; **AFLP,** amplified fragment length polymorphism; **ISSR,** inter-simple sequence repeats; **SSR,** simple sequence repeat; **Et-Br**, ethidium bromide; **PCR**, polymerase chain reaction; **ITS**, internal transcribed spacer; **Rp**, resolving power; **PIC**, polymorphic information content; **MI**, marker index; **Hav**, average heterozygosity; **EMR**, effective multiplex ratio; **DI**, diversity index; **HPLC**, high performance liquid chromatography.

Table 1. Twenty populations of *H. perforatum* collected from different sites at different altitudes covering eight divisions in Kashmir valley.

Division	Population	Accession code	North (‘N)	East (′E)	Altitude (ft)
Ganderbal	Ganderbal hill	GH-1	34°10	74.39	5390
	Prangh hill	PH-2	34°16	74.40	5496
	Arhoma hill	AH-3	34°14	74.41	5505
	Sumbal hill	SB-4	34°13	74.39	5777
	Sonmarg hill	SH-5	34°18	75°17	8901
	Mansbal hill	MH-6	34°13	74.34	5312
Srinagar	Shalimar	SM-7	34°09	74°52	5249
	Hazaratbal	HB-8	34°07	74°50	5226
	Botanicalgarden	BG-9	34°06	74°50	5255
Baramulla	Gulmarg	GM-10	34°03	74°23	8816
	Khilanmarg	KM-11	34°02	74°21	11000
	Tangmarg	TM-12	34°03	74°25	7039
Anantnag	Pahalgam	PG-13	34°00	75°18	7048
	Kokarnag	KN-14	33°35	75°18	6299
	Pombai	PB-15	33°38	75°′20	6300
Badgam	Yusmarg	YM-16	34°01	74°47	5204
Shopian	Aharbal	AB-17	33°43	74°49	6655
	Darogabagh	DB-18	33°45	74°48	7932
Bandipore	Aaloosavillage	AV-19	34°25	74°38	5548
Kupwara	Lolabvalley	LV-20	34°31	74°15	5326

markers studies, ISSR analysis is easier to handle and can be performed with different primers that cover not only one but several sites of a genome. Till date, limited work has been carried out to study the genetic diversity in *H. perforatum* by molecular markers in the Kashmir diversity and population structure as well as their relationship with hypericin content in the diminishing wild populations of *H. perforatum* with the aim to providing insight to facilitate conservation management of the remaining populations.

MATERIALS AND METHODS

The study was conducted in the Division of Floriculture, Medicinal and Aromatic Plants (FMAP)**,** Sher-e-Kashmir University of Agricultural Sciences and Technology Kashmir, Srinagar, J and K (India) in collaboration with Medical Phytochemistry Lab, Department of Biochemistry, Maulana Azad Medical College, New Delhi. The plant material from different populations was authenticated in the division of FMAP.

Plant materials

The *H. perforatum* clones used in the study of genetic diversity and population structure was obtained wild from 20 populations (20 sites), covering 08 geographical locations (Forest Divisions) with altitude ranging 5000-13000 feets from the from the Kashmir valley, India as depicted in Table 1. Each population of *H. perforatum* consist of about two to three plants with different age groups (1st, 2nd, 3rd and 4th year). Whereas, for analysis of genetic diversity, representative samples of three plants (3 replicates for each population) were used and designated with accession code with respect to the site of collections. The interval between samples was 2-5 m, the pair wise distance between populations was 0.5 - 20 Km, whereas, the pair wise distance between forest divisions was 10-200 Km. Morphological feature of each plant sample as well as the environmental factors for each sampling site were also documented. About 5 g of young leaves from each representative plant samples were obtained and placed in a ziplock plastic bag containing silica gel which sped up the drying. The samples were stored at -80°C until use.

DNA extraction from *H. perforatum* without using liquid nitrogen

There were minor modifications in the protocol already used by Sharma et al. (2003). Approximately, 0.4 g of leaf tissue was collected from healthy plants (*H. perforatum)* and stored at -80°C. DNA was extracted without using liquid Nitrogen stored in TE buffer at -20°C until use. The quality was checked on 0.8% agarose gel after staining with ethidium bromide (Et-Br, 5 mg/ml) as shown in Figure 1.

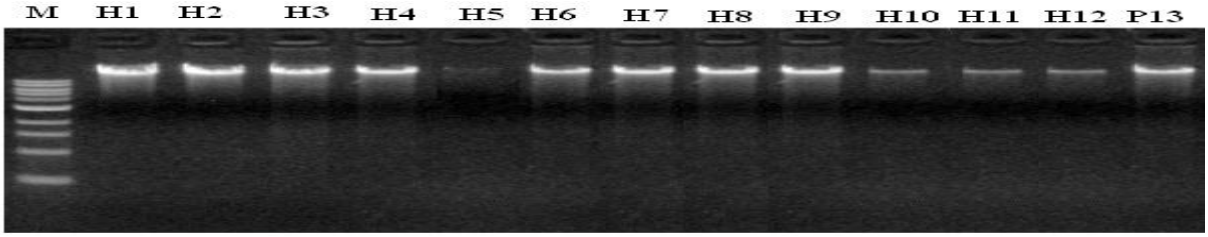

Figure 1. Gel picture showing DNA extracted from *H. perforatum.* Lane: DNA present in H1 TO H13 except H5; M, marker.

Table 2. List of 10 selected ISSR primers.

Primer	ISSR-PCR	GC content (%)	Tm (°C)
ISSR1	GTGCTCTCTCTCTCTCTC	56	50.3
ISSR2	GTGTGTGTGTGTCG	57.1	40.23
ISSR3	GTGTGTGTGTGTCA	50.0	37.2
ISSR4	AGCAGCAGCAGCC	69.2	36.4
ISSR5	AGCAGCAGCAGCAC	64.3	38.0
ISSR6	CCAGCTGCTGCTGCT	66.7	42.2
ISSR7	CTCTCTCTCTCTCTCTGAC	65	51
ISSR8	CTGCTGCTGCTGCTGCTGCTGCTGCTGG	68	68.7
ISSR9	GCAGCAGCAGCAGCAGCAGCAGCAAG	65	65.8
ISSR10	CTGCTGCTGCTGCTGCTGCTGCTGG	68	65

260/280 Ratio

The DNA was quantified by taking the optical density (OD) at λ 260 with a Thermo Scientific NanoDrop™ 1000 Spectrophotometer. The ratio of absorbance at 260 and 280 nm was used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as "pure" for DNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The ISSR polymerase chain reaction (PCR) was performed only in those DNA samples in which absorbance were between 1.75 to 1.85. An optical density (or "OD") of 1 corresponds to a concentration of 50 µg/ml for double-stranded DNA.

Evaluation of primers

A total of 20 ISSR primers were screened with 10 plant samples. A gradient PCR was performed to figure out what annealing temperatures work best. The primer sequences, GC content and melting temperatures of ISSR primers is depicted in Table 2 and were commercially synthesized by Sigma. Similar ISSR primers were used by Barcaccia et al. (2006).

PCR amplification of ISSR primers in *H. perforatum*

The PCR cocktail was prepared by using HotStarTaq Master Mix Kit (Qiagen), ready-to-use solution. Twenty five (25 ul) reaction volume was used containing 12 ul of master mix, 50 pmole of 1 ul of primer, 10 ul of molecular grade water and finally added 2 ul of DNA. The thermocycling conditions were initial denaturation for 5 min at 94°C followed by 45 cycles of 1 min at 94°C, 55 s at specific annealing temperature, 45 min at 72°C and a 10 min final extension step at 72°C. The annealing temperature for each primer was determined. Amplification products were electrophoresed on 2.0% agarose gels run at constant voltage and 1X TBE for approximately 2 h, visualized by staining with ethidium bromide and photographed under ultraviolet light (using Gel Doc, Biorad). Molecular weights were estimated using DNA markers (100 bp) purchased from Gene Aid. Gel-Pro analyzer version 3-1 software was used to score ISSR profile. Each genomic DNA sample was scored for the presence or absence of specific PCR bands generated by a primer as depicted in Figure 2. The presence (1) or absence (0) of the amplified bands was scored in all genotypes for each primer. Faintly stained bands were not considered.

Marker properties

The ability of the most informative primers to differentiate between the clones was assessed by calculating their resolving power (Rp) according to Prevost and Wilkinson (1999). Polymorphic information content (PIC) or average heterozygosity was calculated using the formula of Roldan-Ruiz et al. (2000). Marker index (MI) was calculated by multiplying the average heterozygosity (Hav) with effective multiplex ratio (EMR) (Powell et al., 1996). EMR and MI were calculated for ISSR analysis to measure the usefulness of the marker system as depicted in Table 3.

Primer resolving power

Primer resolving power (Rp) was calculated according to Prevost and Wilkinson formula:

$Rp = \sum Ib_i$

Where, $Ib_i = 1-(2 \times | 0.5 - p_i |)$. '$p_i$' is the proportion of accessions containing the i^{th} band and Ib_i is the informativeness of the i^{th}

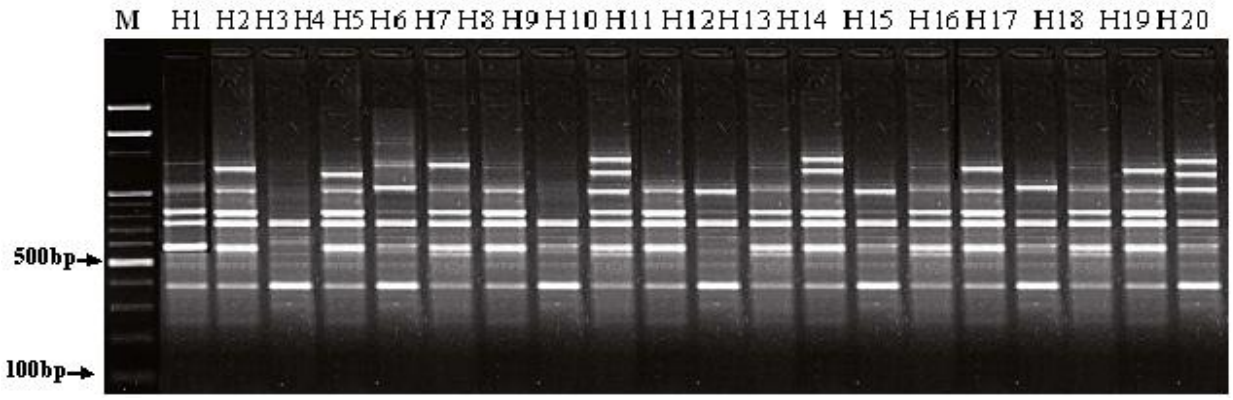

Figure 2. PCR amplification of ISSR primers in *H. perforatum*. Typical ISSR profiles obtained for 20 Hypericum perforatum genotypes. M, Marker.

Table 3. Total number (n) and number of polymorphic bands (np), percentage of polymorphic bands (%P), main values of proportion of clones containing band (mp), main values of band informativeness (mIb), resolving power (Rp) and polymorphic information content (PIC) of ISSR primers among the clones of *H. perforatum.*

Marker	Resolving power	Effective multiplex ratio (EMR)	Marker Index	PIC or diversity index
ISSR1	12.3	2.0	0.6	0.30
ISSR2	14	2.0	0.66	0.33
ISSR3	12.9	3.6	1.22	0.34
ISSR4	16.6	2.5	1.02	0.41
ISSR5	11.8	9.09	2.09	0.23
ISSR6	19	5.0	2.55	0.51
ISSR7	9.2	7.0	3.15	0.45
ISSR8	10	9.0	3.78	0.42
ISSR9	12.2	9.0	3.69	0.41
ISSR10	14	11.0	4.4	0.40

PIC, Polymorphism information content.

band.

According to Prevost and Wilkinson (1999), the resolving power (Rp) of a primer is: Rp = IB where IB (band informativeness) takes the value of: 1-[2x (0.5 - P)] P being the proportion of the n number of genotypes containing the band.

Polymorphism information content (PIC) or diversity index

Diversity index/genetic diversity (DI) is the expected heterozygosity and was calculated according to Weir as:

$$PIC_i = 1 - 1/L \sum_{j=1}^{n} Pi^2$$

Where, PICi is the polymorphic information content of a marker i; Pij is the frequency of the jth pattern for marker i and the summation extends over n patterns. Where, pi is the frequency of ith allele at the l locus and L is the number of loci. This formula is equivalent to an average of PIC. The PIC obtained for ISSR markers are as depicted in Table 3.

Effective multiplex ratio (EMR)

It is the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay. The EMR obtained for ISSR markers are as depicted in Table 3.

Marker index (MI)

MI of each primer was determined according to Powell et al. (1996). Marker index is the product of two functions: Diversity index (DI) and EMR.

$DI = 1-\Sigma pi^2$

Where, pi is the frequency of ith band; EMR, the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay. The MI obtained for ISSR markers are as depicted in Table 3.

Table 4. Hypericin contents among the *H. perforatum* populations with respect to altitude by HPLC.

Division	Accession code	Sampling site	Number of leaves	Percentage polymorphism (%)	Altitude (ft)	Hypericin (% of dry weight) (mean ± SD) (mg/g)
Ganderbal	GH-1	Ganderbalhill	3	68.60	5390	0.60 ± 0.10
	PH-2	Pranghill	3	65.82	5496	0.71 ± 0.03
	AH-3	Arhomahill	3	69.66	5505	0.82 ± 0.04
	SB-4	Sumbalhill	3	46	5777	0.89 ± 0.01
	SH-5	Sonmarghill	3	53.44	8901	0.96 ± 0.026
	MH-6	Mansbalhill	3	63.48	5312	0.78 ± 0.06
Srinagar	SM-7	Shalimar	3	68.91	5249	0.77 ± 0.08
	HB-8	Hazaratbal	3	57.14	5226	0.87 ± 0.09
	BG-9	Botanicalgarden	3	63.01	5255	0.68 ± 0.02
Baramulla	GM-10	Gulmarg	3	61.97	8816	0.94 ± 0.02
	KM-11	Khilanmarg	3	68.60	11000	0.97 ± 0.01
	TM-12	Tangmarg	3	66.25	7039	1.37 ± 0.04
Anantnag	PG-13	Pahalgam	3	63.01	7048	0.89 ± 0.09
	KN-14	Kokarnag	3	61.97	299	0.98 ± 0.008
	PB-15	Pombai	3	67.85	6300	0.92± 0.04
Badgam	YM-16	Yusmarg	3	66.25	5204	1.16 ± 0.05
Shopian	AB-17	Aharbal	3	67.07	6655	0.93 ± 0.07
	DB-18	Darogabagh	3	68.23	7932	0.92 ± 0.08
Bandipore	AV-19	Aaloosa Village	3	59.09	5548	0.92 ± 0.08
Kupwara	LV-20	Lolab valley	3	63.01	5326	1.22± 0.03

HPLC analysis for hypericin content

The air-dried *H. perforatum* leaves and roots (50 g) were broken into small pieces (2-6 mm) by using a cylindrical crusher, and extracted with ethanol: water solution (80:20) (180 ml) using a Soxhlet apparatus. The mixture was filtered through a paper filter (Whatman, No.1) and evaporated. Crude extract (20 µl) was used for injection into the high performance liquid chromatography (HPLC) system.

The residue (5.8 g) was stored in a dark glass bottle for further processing. The resultant residue was dissolved in 10 ml acetonitrile (HPLC grade) and filtered with 0.22 µm durapore membrane filter (Millipore). HPLC analysis was carried out using Nova Pack C18 cartridge column (250 x 4.6 mm) in HPLC system (Water). Acetonitrile: water: methanol (37:58:5) was used as a mobile phase with a flow rate of 1.5 mlmin-1. Hypercin (0.1mg/L Sigma, P-4405) was used as a standard for calculating Hypericin content in the samples on the basis of peak heights. Phytoextracts were prepared by removing solvents under vacuum.

RESULTS

Hypericin content

Among the different locations of Baramulla, the highest hypericin contents were obtained from clones of *H. perforatum* (leaves) collected from Tangmarg (1.37% on dry weight basis). Also, the highest hypericin contents were obtained from clones of *H. perforatum* collected from Kupwara division (1.22% on dry weight basis). In the different locations of Ganderbal division, the highest hypericin contents were obtained from clones of *H. perforatum* collected from Sonmarg (0.96% on dry weight basis). Among the different locations of Srinagar, the highest hypericin contents were obtained from clones of *H. perforatum* collected from Hazaratbal (0.87% on dry weight basis).

The hypericin contents obtained among the different locations of Anathnag was comparatively more (0.89 to 0.97% on dry weight basis) than that of the samples collected from other forest divisions of Badgam, Shopian and Bandipore. The hypericin content was correlated with altitude and genetic diversity among the *H. perforatum* populations. Similar results were reported by Aziz et al. (2002). The Hypericin contents obtained among the *H. perforatum* populations with respect to altitude by HPLC are as depicted in Table 4.

ISSR polymorphism

The number of scored bands, number of polymorphic

Table 5. Total number of scored bands, number of polymorphic bands, percentage of polymorphism, and total fragments amplified by using ISSR primers among the clones of *H. perforatum*.

Primer	Scored band	Polymorphic loci/band	Polymorphic loci (%)	Monomorphic band	Monomorphic band (%)	Fragments amplified
ISSR1	8	4	50	4	50	123
ISSR2	8	4	50	4	50	141
ISSR3	10	6	60	4	40	131
ISSR4	10	5	50	5	50	170
ISSR5	11	10	90.9	1	9.1	119
ISSR6	16	7	43.7	9	56.2	220
ISSR7	7	7	100	0	0	95
ISSR8	8	8	100	0	0	105
ISSR9	9	9	100	0	0	124
ISSR10	11	11	100	0	0	142
	98	71	74	27		1370

bands, percentage of polymorphism and total fragments amplified were shown in Table 5. A total of 20 ISSR primers were screened with 10 plant samples. Ten pairs of ISSR primers were used to screen 60 randomly selected plant samples from 20 sites (populations) covering eight geographical locations (regions). A total of 68 ISSR loci were detected. Out of the 98 loci surveyed, 71 were polymorphic (74%). The amplified PCR fragment size ranged from 150 to 1650 bp with an average of 9.8 bands per primer. Out of these 10 primers, ISSR1 to ISSR 07 revealed 24 monomorphic loci existed in all of the 20 populations. Among the divisions by ISSR markers, the numbers of polymorphic bands varied in between 23 (sumbalhill) and a maximum of 74 (Shalimar). Also polymorphism differed substantially and was found to be between minimum 46% and a maximum of 69.66%. Out of the 98 loci surveyed, 71 were polymorphic (74%). The total number of polymorphic alleles were 71, thereby giving an estimate of profound (>74%) polymorphism. The high reproducibility of ISSR markers may be due to the use of longer primers and higher annealing temperature.

The ISSR primers possess the highest Rp values (>10) ISSR1 to ISSR 10 except ISSR7 and ISSR8. These primers are able to distinguish between all the 20 populations of *P. hexandrum* collected from the different locations. A particular band would have optimal discriminating power when it is scored in the 50% of the genotypes. The band discriminating powers and a primer's discriminating power increases with the increasing number of bands of that primer. The high reproducibility of ISSR markers may be due to the use of longer primers and higher annealing temperature than those used for RAPD. The highest DI or PIC (0.51) was found in ISSR6 primer and lowest DI (0.23) was found in ISSR5. The ability of different assay to infer genetic relationship was also observed. The PIC value of ISSR primers varied from 0.23 to 0.51 with an average of 0.38.

The primer ISSR-6 showed the highest value of average Rp and PIC. The suitability of each ISSR system was examined in terms of number of loci revealed EMR and the amount of polymorphism detected (DI). For the ISSR markers used in this study, the EMR ranged from 2.00 to 11. Based on EMR, ISSR markers used were efficient tools for *H. perforatum* accessions.v The MI was used to evaluate the overall utility of each marker system. The high MI is the reflection of efficiency of ISSR markers to simultaneously analyze a large number of bands, rather than level of polymorphism detected. The ISSR resulted in the highest marker indices ranged from 0.60 to 4.40 (average 2.31) as depicted in Table 6.

Phylogenetic analysis

Genetic distance based on the Jaccard's coefficient was calculated by making a pairwise comparison between 20 *H. perforatum* clones by ISSR marker using the NTSYS-pc software version 2.01e. Based on the Jaccard similarity coefficient between individuals, a cluster analysis was carried out and a dendrogram was generated that represented the genetic relationship among 20 populations or 60 individuals as depicted in the Figure 3. In the dendrogram, all the individuals in each population clustered together. Based on ISSR markers, the similarity index values ranged from 0.54 to 0.84 as depicted in Table 7. These values were used to construct a dendrogram using UPGMA. Based on the presence or absence of the amplification of alleles, the pair-wise genetic similarity according to Jaccard's coefficient was analyzed ranging from 0.54 to 0.84.

The cluster analysis of ISSR data based on similarity matrix among the populations with respect to their geographical location generated a dendrogram with 5 clusters. Cluster I represents Ganderbalhill, Arhomahill, Khilanmarg, Pombai,Yusmarg, Tangmarg, Darogabagh

Table 6. Average value of total number of scored bands, number of polymorphic bands, percentage of polymorphism, and total fragments amplified by using ISSR primers among the clones of *H. perforatum.*

Properties of markers	ISSR
Number of primers used	10
Total number of polymorphic bands	71
Total number of monomorphic bands	27
Total number of scored bands	98
Total number of bands amplified	1370
Percentage polymorphism (%)	74
Average number of bands/primer	9.8
Resolving power(Average)	13.2
Effective multiplex ratio (EMR) (Average)	6.019
Marker Index	2.316
Diversity index/PIC	0.38

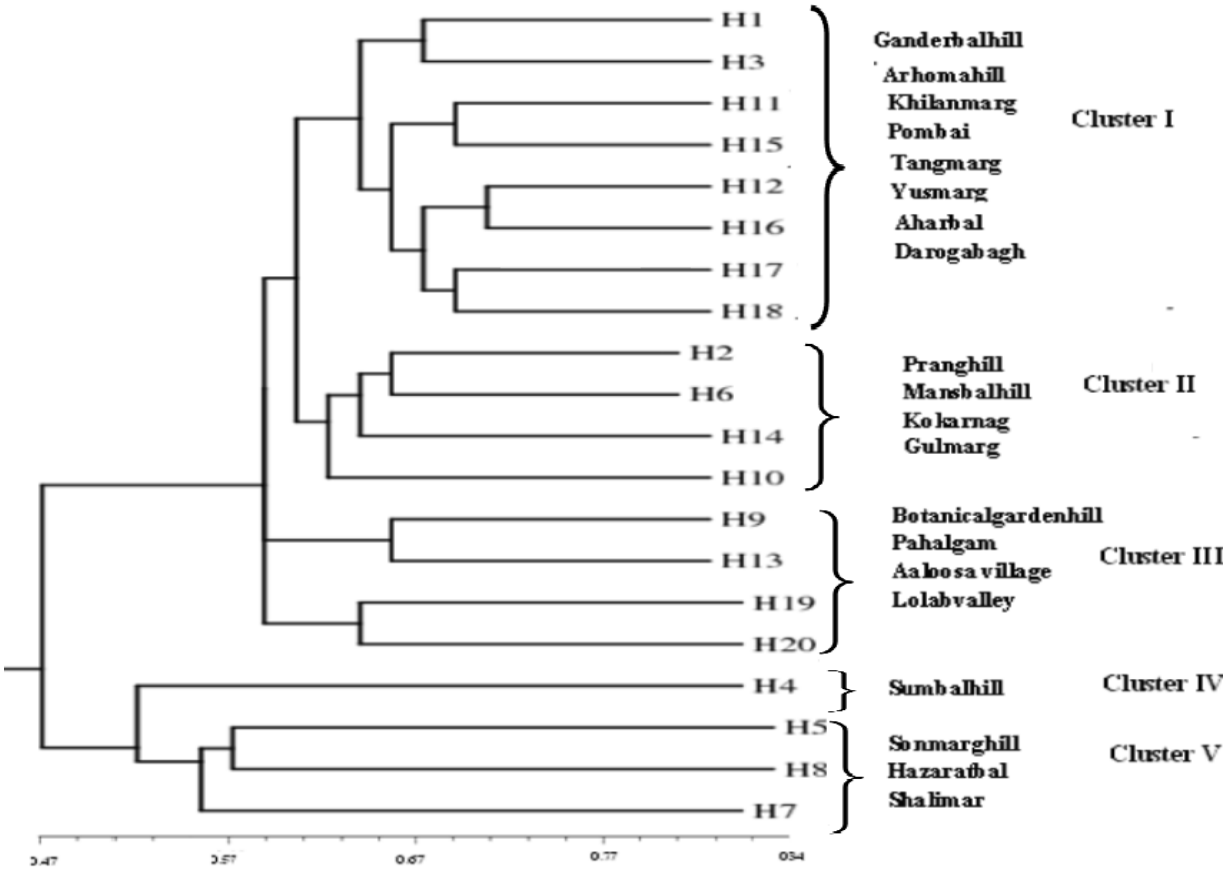

Figure 3. Dendrogram illustrating genetic relationships among 20 *H.perforatum* populations in population diversity study generated by UPGMA cluster analysis calculated from 1090 ISSR bands produced by 10 primers.

and Aharbal; Cluster II represents Pranghill, Mansbalhill, Kokarnag and Gulmarg; Cluster III included Botanicalgarden, Pahalgam, Alosavillage and Lolabvalley; cluster IV represents Sumbalhill; cluster V represents Hazaratbal, sonamarg and Shalimar. Hence from our observations, the clones of *H. perforatum* collected from Ganderbalhill, Arhomahill, Khilanmarg, Pombai were more phylogenetically related with that of

Table 7. Distance matrix for *H. perforatum* clones by Jaccard's coefficient based on ISSR bands.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20
H1	0																			
H2	0.21	0																		
H3	0.17	0.18	0																	
H4	0.43	0.45	0.43	0																
H5	0.51	0.44	0.43	0.43	0															
H6	0.3	0.18	0.25	0.53	0.44	0														
H7	0.46	0.48	0.52	0.39	0.35	0.52	0													
H8	0.4	0.38	0.37	0.34	0.33	0.42	0.33	0												
H9	0.22	0.27	0.27	0.48	0.43	0.3	0.47	0.47	0											
H10	0.31	0.22	0.26	0.52	0.47	0.21	0.49	0.49	0.3	0										
H11	0.24	0.21	0.23	0.49	0.51	0.31	0.46	0.35	0.3	0.26	0									
H12	0.23	0.2	0.18	0.52	0.48	0.25	0.52	0.4	0.25	0.17	0.19	0								
H13	0.25	0.32	0.3	0.45	0.48	0.31	0.46	0.49	0.2	0.28	0.33	0.24	0							
H14	0.3	0.18	0.29	0.53	0.44	0.24	0.46	0.44	0.33	0.25	0.27	0.23	0.31	0						
H15	0.24	0.18	0.21	0.44	0.47	0.26	0.46	0.32	0.29	0.27	0.16	0.18	0.26	0.24	0					
H16	0.26	0.23	0.23	0.53	0.48	0.31	0.51	0.39	0.34	0.25	0.24	0.14	0.29	0.17	0.19	0				
H17	0.2	0.19	0.17	0.48	0.44	0.26	0.48	0.35	0.24	0.23	0.18	0.17	0.23	0.24	0.17	0.2	0			
H18	0.21	0.22	0.2	0.42	0.47	0.29	0.43	0.35	0.29	0.26	0.23	0.22	0.27	0.25	0.18	0.16	0.17	0		
H19	0.31	0.3	0.33	0.43	0.42	0.31	0.28	0.38	0.35	0.3	0.29	0.34	0.27	0.31	0.26	0.39	0.29	0.31	0	
H20	0.22	0.21	0.27	0.46	0.47	0.28	0.41	0.41	0.28	0.32	0.3	0.29	0.29	0.26	0.23	0.28	0.26	0.2	0.24	0

clones obtained from Yusmarg, Tangmarg, Darogabagh and Aharbal and were clustered together in Cluster V. Similarly, plants collected from Tangmarg, Yusmarg were similar in genetic makeup and were clustered together. The genotypes of *H. perforatum* collected from Pranghill, Mansbal, Kokarnag and Gulmarg were similar in genetic makeup and were clustered together in Cluster V. The clones of *H. perforatum* collected from Yousmarg were more phylogenetically related with that of clones obtained from and Tangmarg. Clusters obtained from ISSR marker were less overlapping and indicated comparatively good extent of genetic diversity assessment by ISSR marker.

Genetic diversity and hypercin content

There was a significant correlation between hypericin contents and ISSR data of the clones. In the dendrogram (Figure 3), clones 1, 3, 11, 15, 12, 16, 17 and 18 were grouped in same sub-cluster. A close relationship among these clones could be seen in both genetic and hypericin data.

Furthermore, close relationship among clones 4, 5, 8 and 7 could be seen in both dendrograms constructed from genetic and hypericin data. Clone-12 and clone-14 had the highest hypericin content (0.137 and 0.98%, respectively) with respect to the mean hypericin content as shown in Table 8.

Relationships between populations

To assess the overall distribution of diversity within and among these populations, analysis of molecular variance (AMOVA) was completed.

Table 8. Genetic variation and polymorphic features estimated using ISSR markers among the *H perforatum* populations.

Division	Population	No of leaves	Total bands	Polymorphic bands	Polymorphism (%)	Hypericin (Mean ± SD) (mg/g)
Ganderbal	Ganderbalhill	1-3	86	59	68.60	0.60
	Pranghill	1-3	79	52	65.82	0.71
	Arhomahill	1-3	89	62	69.66	0.82
	Sumbalhill	1-3	50	23	46	0.89
	Sonmarghill	1-3	58	31	53.44	0.96
	Mansbalhill	1-3	71	45	63.48	0.78
Srinagar	Shalimar	1-3	51	74	68.91	0.77
	Hazaratbal	1-3	63	36	57.14	0.87
	Botanicalgarden	1-3	73	46	63.01	0.68
Baramulla	Gulmarg	1-3	71	44	61.97	0.94
	Khilanmarg	1-3	86	59	68.60	0.97
	Tangmarg	1-3	80	53	66.25	1.37
Anantnag	Pahalgam	1-3	73	46	63.01	0.89
	Kokarnag	1-3	71	44	61.97	0.98
	Pombai	1-3	84	57	67.85	0.92
Badgam	Yusmarg	1-3	80	53	66.25	1.16
Shopian	Aharbal	1-3	88	61	69.31	0.93
	Darogabagh	1-3	82	55	67.07	0.92
Bandipore	Aaloosa village	1-3	66	39	59.09	0.92
Kupwara	Lolab valley	1-3	73	46	63.01	1.22

Table 9. Summary of nested analysis of molecular variance (AMOVA) based on ISSR genotypes of *Hypericum perforatum*.

Source of variation	Primer	d.f	SSD	Variance component	Percentage	P-Value
Among groups	ISSR	8	399.974	6.789	58	<0.001
Among populations within groups		15	311.223	5.974	42	<0.003
		23	711.197	12.763		

d.f, Degree of freedom; SSD, sum of square deviation; P-value, probability of null distribution.

Highly significant ($P < 0.000\ 2$) genetic differentiation was observed among the 20 populations. The summary of nested analysis of molecular variance (AMOVA) based on ISSR genotypes obtained in *H. perforatum* is depicted in Table 9. The levels of significance were based on 1000 interation steps.

Analysis of molecular variance indicated that over half of the total variation in the studied populations (58%) could be accounted for by differences among the 8 divisions, with a further 42% being accounted for by the variation among populations within a division (Table 9). All components of molecular variance were significant (P < 0.001). This is helpful in making strategy for germplasm collection and evaluation.

Population genetic structure

However, with the hypervariable nature of ISSR, asymmetrical patterns of gene flow are easily detected. Low frequency markers are sometimes more useful in detecting these patterns than species-typical markers.

After the identification of particular marker alleles, the frequency of occurrence of the markers in the popula-

Table 10. Genetic variation and polymorphic features estimated using ISSR markers among the *H. perforatum* populations.

Population	Sample size	Na*	Ne	H	I	Ht	Hs	Gst	Estimate of gene flow	Number of polymorphic bands	Percentage of polymorphism
Mean	1.775	1.421	0.265	0.399	0.253	0.157	0.451	0.304	0.304	985	66.82%
St. Dev	0.336	0.328	0.169	0.2406	0.035	0.018					

Na*, Observed number of alleles; Ne, effective number of alleles; H, Nei's genetic diversity; I, Shannon's information index; Ht, total gene diversity.

lation of interest is measured, and the hypothesis of gene flow is evaluated based on the patterns detected. The data for observed number of alleles, effective number of alleles, Nei's genetic diversity, Shannon's information index, for all the twenty populations were analysed using ten ISSR markers and their respective values were found as 1.775, 1.421, 0.265 and 0.399 as depicted in Table 10. The value for total genotype diversity among population (Ht) was 0.253 while within population diversity (Hs) was found to be 0.157. Mean coefficient of gene differentiation (Gst) value 0.451. Estimate of gene flow in the population was found as 0.30432.

A close relationship among the clones in dendrogram generated by ISSR markers, that there is a stronger correlation between hypericin contents and genetic structure in *H. perforatum*. The existing variation in hypericin content among the *H. perforatum* populations was proved to be coupled with geographical altitude but rarely with genetic variations by ISSR as shown in Figure 4. The genetic relationship of different populations in *H. perforatum* studied by ISSR markers is equally important. Based on this, we suggest that *in situ* conservation be an important and practical measure for maintaining the genetic diversity of this species.

DISCUSSION

Research reports on medicinal and aromatic plants are increasing day by day because of the search for new active molecules, for improvement of the plant production systems and new species or molecules for the herbal pharmaceutical industries (phytotherapy or allopathy). However, one of the most important problems related to medicinal plants is that there is an extreme variability in the phytochemical content, mostly resulting from environmental factors and other contaminations during cropping. It has been reported that one obstacle facing breeders, horticulturists, researchers, and oversight agencies working with medicinals is the inability to genetically determine the source of plant material. Markers generated in this study may aid in overcoming this obstacle. Collectively, the 10 unique *H. perforatum* markers may aid breeders in determining genetic identity and source, can be employed as a tool by producers to accurately diagnose the identity of individual plant lots, and could be useful to agencies or consumer groups as a means to evaluate end-user *H. perforatum* "St. John's wort" preparations. Additionally, this molecular marker study provides the foundation for future work focused on developing species-specific primers that could be used to identify material purported to be *H. perforatum* with a single PCR reaction.

The present study utilized ISSR markers for analysis that was successful in detecting genetic diversity and relationships among the *H. perforatum* clones. A moderate level to high level genetic diversity was found in the clones as was expected since the *H. perforatum* clones subjected to this study originated from only eight provinces of Kashmir valley of India. As a result of genetic variation studied in this by ISSR markers, 98 loci were reported, 71 were polymorphic (74%) and 29 were monomorphic. The numbers of polymorphic bands varied in between 23 (Sumbalhill) and a maximum of 74 (Shalimar). Also, polymorphism differed substantially and was found to be in between minimum of 46% and a maximum of 69.66%. The total number of polymorphic alleles were 71, thereby giving an estimate of profound (>74%) polymorphism. Ellstrand and Roose (1987), reported the existence of distinct multilocus genotypes in apomictic *H. perforatum* populations. Also, Noyes and Soltis (1996), reported the existence of different clones in apomictic plant populations. Similarly, Chapman et al. (2000) documented the genetic diversity in *H. perforatum*. Similar results were reported by Hısil et al. (2005) who performed various experimental studies and reported the existence of diverse clones in *H. perforatum* populations but the source of this genetic diversity remains largely unexplained. Meirmans and Van Tienderen (2004) documented that the clonal diversity within a population reflects the sexual genetic pool from which the clones originated, the frequency of clonal origin, and the somatic mutations that subsequently accumulate in established clones. The diversity arising from the

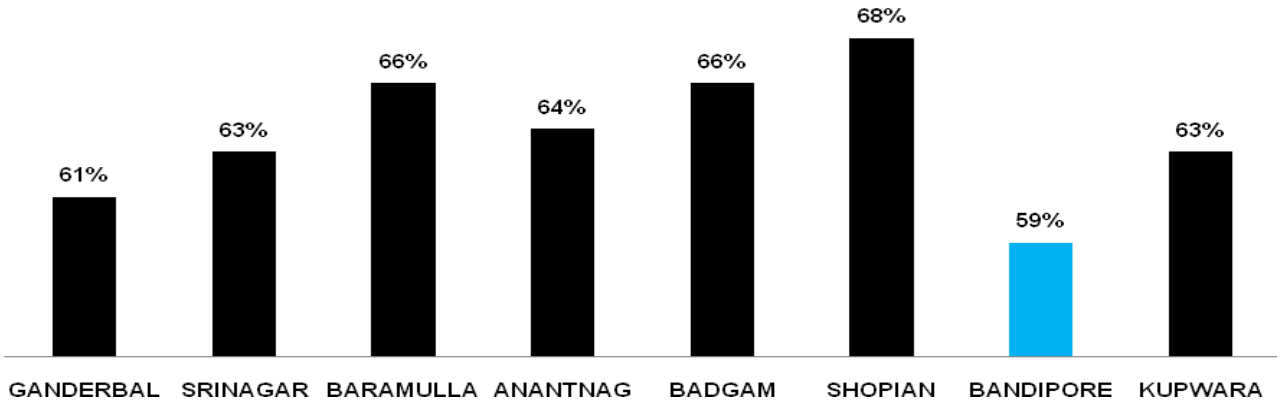

Figure 4. Diagrammatical representation of genetic variation and polymorphic features estimated using ISSR markers among the *H. perforatum* populations with respect to their divisions.

clonal origin is directly dependent on reproductive mode, and hence varying degrees of apomixis between *H. perforatum* landraces may have a significant impact on the diversity of local populations. The mode of reproduction was also investigated using the FCSS analysis to reconstruct the patterns of seed formation and to infer the sources of genetic variation at the population level. The EMR and MI were calculated for RAPD analysis to measure the usefulness of the marker system according to Powell et al. (1996).

The PIC or average heterozygosity (Hav) is estimated by taking the average of PIC values obtained for all the markers. MI was calculated by multiplying the average heterozygosity with EMR (Powell et al., 1996). MI and Rp were two different methods used to measure the ability of primers or techniques to distinguish between genotypes (Prevost and Wilkinson, 1999; Fatma et al., 2011). In comparison to the work of Sarwat et al. (2008), the marker index in the present study was higher (4.4) and reported a marker index for molecular markers of 3.3 in *Tribulus terrestris*. This difference may be explained by higher average heterozygosity and EMR values, which were measured as 0.34 and 12 in the study, respectively. The resolving power values of ISSR primers in the present study (9.2 to 19) were found to be higher than that of Fernandez et al. (2002) (1.38-8.88) but lower than that of Prakash and Van Staden (2007) (9.4-26.8).

ISSR analysis was successful in detecting genetic diversity and relationships among the *H. perforatum* clones. A low level of genetic diversity was found in the clones of Ganderbal division as was expected since the *H. perforatum* clones used originated from six sites of only one district of Kashmir Valley (59.47%). High level of genetic similarity in *H. perforatum* accessions collected from different locations in India was previously reported by Verma et al. (2008). According to similarity coefficients of cluster analysis of the *H. perforatum* clones and variety Topaz, expected results were found. Clustering or similarity coefficient showed that there was a partial relationship on a regional basis of the clones. Hence from our observations, the clones of *H. perforatum* collected from *G*anderbal, Arhomahill, Khilanmarg, Pombai were more phylogenetically related with that of clones obtained from Yusmarg, Tangmarg, Darogabagh and Aharbal and were clustered together in Cluster V. Similarly, plants collected from Tangmarg, Yusmarg were similar in genetic makeup and were clustered together. The genotypes of *H. perforatum* collected from Pranghill, Mansbal, Kokarnag and Gulmarg were similar in genetic makeup and were clustered together in Cluster V. The clones of *H. perforatum* collected from Yousmarg were more phylogenetically related with that of clones obtained from and Tangmarg. Clusters obtained from ISSR marker were less overlapping indicating comparatively good extent of genetic diversity assessment by ISSR marker. The distribution pattern of clones of diverse origin in cluster I indicated that genetic diversity observed within *H. perforatum* clones was not related to geographical origin. In general, the results of ISSR analysis confirmed the efficacy of ISSR markers for detecting DNA polymorphism among *H. perforatum* clones.

Similarly, several studies have exploited RAPD and ISSR markers to characterize *H. perforatum* accessions and populations (Arnholdt-Schmitt, 2000; Arnholdt-Schmitt, 2002; Halušková and Košuth, 2003; Barcaccia et al., 2006; Verma et al., 2008) and confirmed effectiveness of molecular markers. Significant similarities were observed between the dendrograms constructed from hypericin and ISSR data. A close relationship among the clones in both dendrograms demonstrated that there is a stronger correlation between hypericin contents and genetic structure in *H. perforatum*. Contrary to our results, Verma et al. (2008) found a partial correlation between secondary metabolite contents and molecular data in *H. perforatum* accessions. This result could be explained by differences in chemical composition and genetic profile among the accessions of one species, which show less significance compared to

different species.

AMOVA revealed that there was significant variation arising from habitat-correlated genetic difference (58%) suggesting that, besides the effects of gene flow and genetic drifts, local ecological conditions (altitude, distance, temperature, rainfall, humidity, soil, pH among others) also played an important role in the variation of the genetic structure in the study populations of *H. perforatum*. Considering the high genetic differentiation among the wild populations of *H. perforatum,* presservation of only a few populations may not adequately protect the genetic variation within the species in Himalayan region. AMOVA indicated that over half of the total variation in the studied populations (58%) could be accounted for by differences among the eight divisions, with a further 42% being accounted for by the variation among populations within a division. All components of molecular variance were significant ($P < 0.001$). This is helpful in making strategy for germplasm collection and evaluation.

The hypericin contents and genetic structures of superior *H. perforatum* clones were identified by HPLC or spectrophotometric analysis and ISSR markers. Cluster analyses of hypericin contents and ISSR markers grouped the clones in five major clusters and significant correlations were observed between them. The combined analysis of ISSR markers and hypericin content is an optimal approach for further progress and breeding programs. There was a significant correlation between hypericin contents and ISSR data of the clones. Clone-12 and clone-14 had the highest hypericin content (0.137% and 0.98%, respectively) with respect to the mean hypericin content. The observed increase in genetic diversity as well as hypericin contents with increase in altitude is an interesting phenomenon that requires further research.

Conclusion

This is the first report on the genetic profile of *H. perforatum* originating from Kashmir valley of India. There is a stronger correlation between hypericin contents and genetic structure in *H. perforatum*. The existing variation in hypericin content among the *H. perforatum populations* was proved to be coupled with geographical altitude but rarely with genetic variations by ISSR. Based on this, we suggest that *in situ* conservation be an important and practical measure for maintaining the genetic diversity of this species. Especially, this knowledge should be considered in the breeding of superior hybrid genotypes for adaptation to different environments and utilization systems.

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