Genetic diversity in populations of Girardinia diversifolia from Nepal Himalaya using ISSR markers

Article *in* Ecological Genetics and Genomics · April 2022

DOI: 10.1016/j.egg.2022.100120

4 authors:

Bijay Raj Subedee
Tribhuvan University
14 PUBLICATIONS **101** CITATIONS
SEE PROFILE

Giri Raj Tripathi
Tribhuvan University, Kirtipur, Kathmandu
12 PUBLICATIONS **74** CITATIONS
SEE PROFILE

Nabin Narayan Munankarmi
Biotechnology Soceity of Nepal
17 PUBLICATIONS **52** CITATIONS
SEE PROFILE

Ram Prasad Chaudhary
Tribhuvan University
13 PUBLICATIONS **16** CITATIONS
SEE PROFILE

Ecological Genetics and Genomics 23 (2022) 100120

Contents lists available at ScienceDirect

Ecological Genetics and Genomics

journal homepage: www.elsevier.com/locate/egg

Genetic diversity in populations of *Girardinia diversifolia* from Nepal Himalaya using ISSR markers

Bijay Raj Subedee [a,*], Giri Raj Tripathi [b], Nabin Narayan Munankarmi [c], Ram Prasad Chaudhary [a]

[a] Research Centre for Applied Science and Technology, Tribhuvan University, Kirtipur, Kathmandu, Nepal
[b] Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal
[c] Department of Botanical Sciences, Biotechnology Society of Nepal, Kausaltar, Kathmandu, Nepal

ARTICLE INFO

Keywords:
Himalayan giant nettle
Girardinia diversifolia
Genetic variation
Bast fibers
Nepal Himalaya

ABSTRACT

Girardinia diversifolia (Link) Friis from Urticaceae family is a perennial herb, commonly known as the Himalayan giant nettle. It has cultural, medicinal, and economic values among the indigenous peoples and local communities living in the mountains of Nepal and India. Prior knowledge of genetic diversity in plant species may contribute to conservation and sustainable utilization of important genotype. The aim of this study is to assess the genetic diversity within and among different populations of the 45 accessions of *G. diversifolia* collected from Far-western, Central, and Eastern regions of Nepal. The amplification of genomic deoxyribonucleic acid (DNA) with ten inter-simple sequence repeat (ISSR) primers yielded 131 clear DNA bands, of which 98.09% were found to be polymorphic. The mean effective number of alleles (ne), Nei's gene diversity (h) and Shannon's information index (I) were 1.598, 0.349, and 0.523, respectively. The total genetic diversity Ht (0.350 ± 0.04), low intra-population genetic diversity Hs (0.141 ± 0.03) and low estimated gene flow Nm (0.355 ± 0.11) reflected high genetic differentiation among population Gst (0.594 ± 0.08). The analysis of molecular variance among and within five populations of *G. diversifolia* showed that the value of genetic variation among the population was 60% whereas the value of genetic variation within the population was 40%. The results show that the ISSR markers are informative for the study of genetic diversity, which may help in the conservation and sustainable utilization of *G. diversifolia* and allied taxa.

1. Introduction

Natural fiber harvested in an environment-friendly and sustainable way is becoming popular among textile industries, scientists and consumers [1]. *Girardinia diversifolia* (Link) Friis is a perennial herb that belongs to the Urticaceae family and is commonly known as the Himalayan giant nettle. *G. diversifolia* is an important natural fiber-producing plant with great cultural, economic, and medicinal value among the indigenous peoples and local communities (Rais, Gurung, Sherpa, Magar, Tamang, Lepcha, etc.) living in the Hindu Kush Himalayan region of Nepal and India [2–5]. This plant is widely distributed in subtropical and temperate regions of the Himalayas between the altitudes of 1,200 to 3,000 m above sea level [6–8]. The fiber obtained from the stem of this plant is among the important livelihood options for the indigenous peoples and local communities of Nepal from which clothes, fishing nets, bags, coats, and many other textile products are prepared [9]. Different products (textiles and souvenirs) from the fiber of *G. diversifolia* are in increasing demand in national and international markets [4,10,11].

Many bioactive compounds have been found in plants such as β-sitosterol, 7- hydroxysitosterol and 3-hydroxystigmast-5-en-7-one [12] trans syringin, linoleic and linolenic acid [13]. The plant is used in traditional medicine for the treatment of gastritis, joint pain, headache, and skin allergies [2,3,9]. *G. diversifolia* has potential application in pharmaceuticals showing significant ability to inhibit acetylcholinesterase, downregulation of low-density lipoprotein receptor affecting the hepatocarcinoma cells, a crucial regulator of cellular cholesterol homeostasis [13].

Morphological study of *G. diversifolia* showed high level of variation mostly on its leaf lobes [6,8,14]. Leaves are alternate, petiolate, and elliptic to ovate, but consist of varying degrees of division in the same plant or within the population [2,6,8,15,16]. Genetic analysis of plants provides broader knowledge on their diversity and basis to study important metabolites that they produce [17]. Genetic diversity can be

* Corresponding author.
E-mail address: bijayrajsubedee@gmail.com (B.R. Subedee).

https://doi.org/10.1016/j.egg.2022.100120
Received 17 July 2021; Received in revised form 8 March 2022; Accepted 10 April 2022
Available online 14 April 2022

determined based on morphological, biochemical, and molecular information of the plant [18]. Molecular markers detect genetic diversity at the DNA level and help to determine the differences not shown by morphological and biochemical markers [19].

Molecular markers have advantages over other kinds of markers because they show genetic differences on a more detailed level without interferences from environmental factors [20]. Molecular markers based on polymerase chain reaction (PCR) are widely used to study genetic diversity of plants because they are effective, and require a small amount of DNA. The popular markers are random amplified polymorphic DNA (RAPD) [21], simple sequence repeat (SSR) [22], and inter-simple sequence repeat (ISSR) [23]. ISSR markers have shown high potential for assessing the genetic diversity of wild species which are based on di-, tri-, tetra-, or penta-nucleotide repeats and used for various purposes including genetic diversity study [24,25]. The ISSR markers were also utilized to study genetic diversity in some of the natural fiber-producing plants like *Boehmeria* species [26], *Cannabis sativa* [27,28] which have become revolutionary tools for both applied and basic studies. ISSR molecular markers have been selected for this study where ISSR follows relatively simple procedure with high reproducibility and stability [24,25]. Generic phylogeny and character evaluation in Urticaceae including *G. diversifolia* have been studied based on analysis of nuclear ribosomal internal transcribed spacer (nrITS) and two plastid DNA regions *rbcL* exon and *trnL-F* spacer [16]. However, there have been no studies so far on the genetic diversity of *G. diversifolia* from the Himalayan region. Thus, there is a need to study genetic diversity of the plant species.

Despite the multipurpose utility of *G. diversifolia*, existing genetic diversity requires conservation and sustainable utilization, the plant has not received adequate attention from conservation biologists. Therefore, the objective of the study is to determine genetic relationship and diversity among and within the populations of *G. diversifolia* occurring in Himalayan region using ISSR markers which can facilitate conservation and sustainable utilization of *G. diversifolia* and allied taxa.

2. Materials and methods

2.1. Plant materials

A total of forty-five germplasms have been collected from the population of *G. diversifolia* at an interval of approximately 900 m altitudinal gradients beginning from 1000 m to 2700 m based on uniform distribution in selected districts. The forty-five accessions representing five populations of the *G. diversifolia* collected from different districts representing four regions: Far-Western region (Darchula District); Western region (Kaski District); Central region (Kathmandu District and Dolakha District); and Eastern region (Panchthar District) of Nepal (Table 1; Fig. 1). The herbarium was prepared in the field and brought to Kathmandu. The herbarium specimen was first identified with the help of Prof. Dr. Ram Prasad Chaudhary, Professor of Botany (plant systematics), who is also one of the co-authors. The collected herbarium specimens were later reconfirmed by comparing morphological characteristics of herbarium specimens deposited at National Herbarium and Plant Laboratories (KATH), Department of Plant Resources, Nepal. During the DNA extraction process, large amount of exudates were observed, which inhibited PCR reaction. The same plants were planted and maintained at Truffle Research Centre, Coronation Garden of Tribhuvan University, Kirtipur, Kathmandu, Nepal (27°40′50″N, 85°17′26.5″E). DNA was extracted from fresh leaf. The samples were collected from August to October 2019 during autumn which is the plant's flowering season. Herbarium specimens were deposited at Tribhuvan University Central Herbarium (TUCH), Kathmandu, Nepal.

2.2. DNA isolation and quantification

Young leaf samples (100 mg) from each forty-five accessions were

Table 1
Sample summary of *G. diversifolia* used in the study.

SI. No	Collection site	District	Plant accession no.	Latitude/Longitude
1	Hopari, Dhulighad	Darchula	Dar1	29° 46′ 4.5″ N 80° 39′ 31″ E
2	Dhulighad	Darchula	Dar2	29° 46′ 51″ N 80° 38′ 40″ E
3	Okhaldhar	Darchula	Dar3	29° 47′ 0.6″ N 80° 37′ 28″ E
4	Malephar	Darchula	Dar4	29° 47′ 13″ N 80° 37′ 02″ E
5	Godhyan	Darchula	Dar5	29° 47′ 06″ N 80° 36′ 52″ E
6	Godhyan	Darchula	Dar6	29° 47′ 12″ N 80° 36′ 37″ E
7	Pangdhunga	Darchula	Dar7	29° 47′ 17″ N 80° 36′ 21″ E
8	Pangdhunga	Darchula	Dar8	29° 47′ 16″ N 80° 36′ 11″ E
9	Pangdhunga dhar	Darchula	Dar9	29° 47′ 20″ N 80° 36′ 08″ E
10	Pangdhunga dhar	Darchula	Dar10	29° 47′ 34″ N 80° 36′ 23″ E
11	Ghandruk	Kaski	Kas1	28° 23′ 14″ N 83° 48′ 45″ E
12	Ghandruk	Kaski	Kas2	28° 22′ 31″ N 83° 48′ 12″ E
13	Ghandruk	Kaski	Kas3	28° 22′ 29″ N 83° 48′ 08″ E
14	Panchase	Kaski	Kas4	28° 14′ 03″ N 83° 49′ 27″ E
15	Panchase	Kaski	Kas5	28° 13′ 51″ N 83° 49′ 08″ E
16	Machhegau	Kathmandu	Kat1	27° 39′ 22″ N 85° 14′ 51″ E
17	Machhegau	Kathmandu	Kat2	27° 39′ 21″ N 85° 14′ 45″ E
18	Machhegau	Kathmandu	Kat3	27° 39′ 08″ N 85° 14′ 45″E
19	Machhegau	Kathmandu	Kat4	27° 39′ 07″ N 85° 14′ 34″ E
20	Machhegau	Kathmandu	Kat5	27° 39′ 05″ N 85° 14′ 23″ E
21	Machhegau	Kathmandu	Kat6	27° 39′ 02″ N 85° 14′ 22″ E
22	Machhegau	Kathmandu	Kat7	27°39′ 02″ N 85° 14′ 14″ E
23	Nagarjun	Kathmandu	Kat8	27° 44′ 10″ N 85° 15′ 56″ E
24	Nagarjun	Kathmandu	Kat9	27° 44′ 23″ N 85° 15′ 37″E
25	Nagarjun	Kathmandu	Kat10	27° 44′ 30″ N 85° 15′ 14″ E
26	Jiri	Dolakha	Jir1	27° 36′ 57″ N 86° 12′ 48″ E
27	Jiri	Dolakha	Jir2	27° 37′ 03″ N 86° 12′ 34″ E
28	Jiri	Dolakha	Jir3	27° 37′ 13″ N 86° 11′ 59″ E
29	Jiri	Dolakha	Jir4	27° 37′ 36″ N 86° 11′ 30″ E
30	Jiri	Dolakha	Jir5	27° 37′ 47″ N 86° 11′ 24″ E
31	Jiri	Dolakha	Jir6	27° 38′ 06″ N 86° 11′ 19″ E
32	Malung	Dolakha	Jir7	27° 31′ 05″ N 86° 02′ 56″ E
33	Malung	Dolakha	Jir8	27° 30′ 59″ N 86° 02′ 42″ E
34	Dandakharkha	Dolakha	Jir9	27° 31′ 08″ N 86° 00′ 28″ E
35	Dandakharkha	Dolakha	Jir10	27° 31′ 10″ N 86° 00′ 11″ E
36	Siddin	Panchthar	Phi1	27° 09′ 41″ N 87° 54′ 01″ E
37	Siddin	Panchthar	Phi2	

(*continued on next page*)

Table 1 (*continued*)

SI. No	Collection site	District	Plant accession no.	Latitude/Longitude
				27° 09′ 25″ N 87° 53′ 43″ E
38	Siddin	Panchthar	Phi3	27° 09′ 08″ N 87° 53′ 28″ E
39	Siddin	Panchthar	Phi4	27° 08′ 47″ N 87° 53′ 08″ E
40	Siddin	Panchthar	Phi5	27° 08′ 31″ N 87° 52′ 47″ E
41	Siddin	Panchthar	Phi6	27° 08′ 19″ N 87° 52′ 35″ E
42	Prangbung	Panchthar	Phi7	27° 10′ 15″ N 87° 54′ 59″ E
43	Prangbung	Panchthar	Phi8	27° 10′ 36″ N 87° 56′ 07″ E
44	Prangbung	Panchthar	Phi9	27° 10′ 11″ N 87° 58′ 15″ E
45	Prangbung	Panchthar	Phi10	27° 10′ 01″ N 87° 58′ 26″ E

taken, inserted into liquid nitrogen, and ground until a fine homogenous powder was obtained. Total genomic DNA was extracted using a modified Doyle and Doyle (1984) method [29]. Varying concentrations of sodium chloride (NaCl), β-mercaptoethanol, and polyvinylpyrrolidone (PVP) were used in the modified DNA isolation method utilized [29]. The concentration and purity of the extracted DNA was quantified in Nano Spectrophotometer (Bio-spec nano, Shimadzu, Japan). Final concentration of the isolated DNA was adjusted to 50 ng μL^{-1} for polymerase chain reactions. All extracted DNA samples were kept at −20 °C until PCR amplification.

2.3. ISSR PCR optimizations and amplifications

All 18 ISSR primers used in this research, including the ISSR-18 and ISSR-19 markers were obtained from the University of British Columbia (UBC), and were initially screened using five randomly selected accessions. Ten ISSR primers were found more polymorphic with strong reproducible bands and were used for amplification of PCR (Table 2). The total reaction volume of 15 μL for PCR amplifications consisting of the concentration of 1X buffer (Vivantis, Malaysia), (0.6) mM dNTPs (Vivantis, Malaysia), 4 mM $MgCl_2$ (Vivantis, Malaysia), 0.7 μM primer (Vivantis, Malaysia), 2 unit *Taq* polymerase (Vivantis, Malaysia), and 50 ng μL^{-1} of DNA template. The thermocycler (Biorad T100) was programmed and optimized by testing various conditions: 3 min at 93 °C, followed by 45 cycles for 30 s at 93 °C, 45 s at different annealing temperature (45–52) °C, extension at 72 °C for 2 min, final extension at 72 °C for 10 min, and finally holding temperature at 4 °C [27]. After PCR reaction, electrophoresis of the PCR products were carried out in 1.8% (w/v) agarose gel containing 10 mg/mL of ethidium bromide, 1X TAE buffer at 80 V for 1 h. The 100 base pair (bp) plus DNA ladder (New England biolabs) was used for determining the molecular weight. The DNA bands were observed under ultraviolet light using unified gel documentation system WGD 30 - POA (Fig. 2).

2.4. Statistical analysis

Clear, unambiguous, and strong bands were used to score DNA bands (Fig. 2). Experiments have been performed twice for the purpose of scoring strong and clear DNA bands. Bands obtained from the ISSR markers were scored in a binary matrix as 1 for presence and 0 for absence of all the bands obtained relative to 100 bp plus DNA ladder. The obtained binary data matrix was investigated using MS-Excel 2007 for assessment of total number of bands (TNB), number of polymorphic

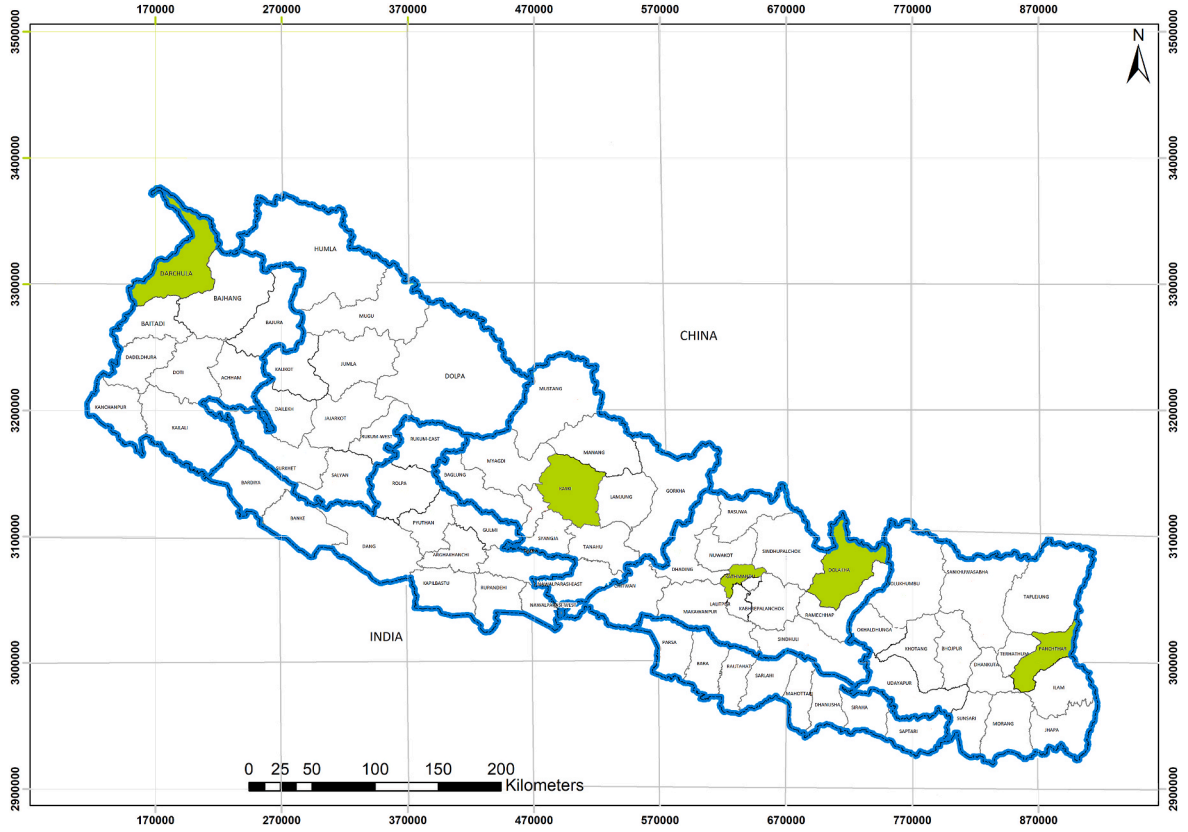

Fig. 1. Map of Nepal showing five sampling sites from Far-western region to Eastern region: Darchula District, Kaski District, Kathmandu District, Dolakha District and Panchthar District.

Table 2
Primer code, sequence and annealing temperature of selected ten ISSR primers.

S.N	Primers	Primer sequence (3′-5′)	Annealing temperature (°C)	TNB	NPB	P(%)	PIC	Rp
1	UBC 812	(GA)8A	52	18	18	100	0.266	8.177
2	UBC 834	(AG)8 YT	45	18	18	100	0.252	7.733
3	UBC 808	(AG)8C	45	14	14	100	0.381	9.288
4	UBC 824	(TC)8G	45	12	12	100	0.414	9.244
5	UBC 811	(GA)8C	48	14	14	100	0.396	10.400
6	UBC 828	(TG)8A	45	15	15	100	0.428	11.911
7	UBC 826	(AC)8C	50	11	10	90.91	0.272	9.822
8	UBC 817	(CA)8A	52	11	11	100	0.388	10.044
9	ISSR-18	(ATC)6T	45	8	8	100	0.409	8.711
10	ISSR-19	(ATC)6C	45	10	9	90	0.295	9.511
	Total			**131**	**129**			
	Mean			**13.1**	**12.9**	**98.09**	**0.350**	**9.484**

TNB = Total number of bands; NPB= Number of polymorphic bands; P(%) = Polymorphism percentage; PIC= Polymorphic information content; Rp = Resolving power; Single letter abbreviation for mixed base Y= (C,T).

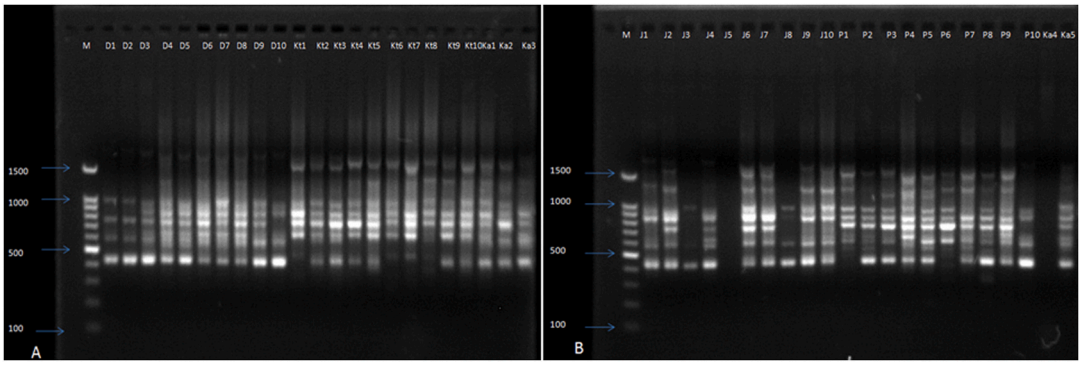

Fig. 2. ISSR amplification results of 45 accessions (A and B) of *G. diversifolia* with primer UBC 824. Lane M = DNA markers (from bottom to top, the bands represent 100 to 1500bp).

bands (NPB), percent polymorphism (P%) (Table 2), polymorphic information content (PIC), band informativeness (BI), and resolving power (RP) for each primer (Table 2). PIC was calculated using the formula [30] given below:

$$PIC_i = 2f_i(1\text{-}f_i)$$

Where PIC*i* is the polymorphic information content of marker *i*, *fi* is the frequency of the marker bands which were present, and *1-fi* is the frequency of marker bands which were absent [30]. Resolving power was calculated according to the formula

$$Rp = \sum I_b$$

Where, I_b is the band informativeness with $I_b = 1 - [2 \times (0.5\text{-}p)]$ and where '*p*' is the proportion of clones containing the band [31].

Cluster analysis was performed using Unweighted Pair-Group Method with Arithmetic means (UPGMA) and similarity indices were computed using a Similarity for Qualitative Data (SIMQUAL) computer algorithm via NTSYS-PC (Numerical Taxonomy and Multivariate System, version 2.02i, Exeter software; Setauket, New York, USA). Genetic similarities between *G. diversifolia* accessions were estimated using Jaccard coefficients and the corresponding similarity matrix [32]. PopGene ver. 1.32 [33] was applied to compute genetic diversity indices, effective number of alleles, Nei's (1973) gene diversity, Shannon's information index, total heterozygosity, mean heterozygosity, genetic differentiation and gene flow (Table 3). Analysis of Molecular variance (AMOVA) was investigated using GenAlEx 6.5 considering variation (Table 7) among and within the population [34].

Table 3
Genetic differentiation and diversity within and between the populations of *G. diversifolia*.

S.N.	Primers	ne	H	I	Ht	H_S	G_{ST}	Nm
1	UBC 812	1.406	0.265	0.423	0.265	0.142	0.462	0.581
2	UBC 834	1.387	0.252	0.404	0.266	0.094	0.646	0.273
3	UBC 808	1.649	0.381	0.565	0.37	0.164	0.559	0.393
4	UBC 824	1.713	0.405	0.592	0.397	0.112	0.718	0.195
5	UBC 811	1.648	0.385	0.571	0.384	0.182	0.524	0.453
6	UBC 828	1.642	0.378	0.561	0.364	0.136	0.625	0.299
7	UBC 826	1.559	0.322	0.484	0.332	0.101	0.695	0.218
8	UBC 817	1.649	0.364	0.536	0.363	0.170	0.531	0.440
9	ISSR-18	1.744	0.416	0.603	0.408	0.180	0.558	0.395
10	ISSR-19	1.581	0.327	0.489	0.347	0.130	0.623	0.302
	Mean	**1.598**	**0.349**	**0.523**	**0.350**	**0.141**	**0.594**	**0.355**
	SD	**0.118**	**0.056**	**0.069**	**0.049**	**0.032**	**0.080**	**0.119**

ne = effective number of alleles; h = Nei's (1973) gene diversity; I = Shannon's information index.; Ht = Total heterozygosity/diversity; Hs = mean heterozygosity/gene diversity within population; Gst = genetic differentiation between population; Nm = gene flow.

3. Results

3.1. Markers analysis and genetic diversity in G. diversifolia

Genetic diversity assessment of 45 accessions of *G. diversifolia* using 10 ISSR primers generated 131 bands of which 98.09% were found polymorphic. The average amplification was 13 bands per primer using 45 *G. diversifolia* accessions. The highest number of DNA bands 18 was generated by primer UBC 812 and primer UBC 834 showing 100% polymorphism. The lowest number of DNA bands i.e. eight DNA bands was produced by ISSR-18. The PIC ranged from 0.252 to 0.428. The primer UBC 828 showed the maximum PIC value of 0.428 and was found to be the most informative primer revealing a good amount of polymorphism information content (Table 2).

The obtained mean value of effective number of alleles was 1.598. The highest number of effective alleles was obtained from the primer ISSR-18 (1.744) while the lowest value was obtained from the primer UBC 834 (1.387). The mean value of Nei's gene diversity was 0.349, with the highest value for the primer ISSR-18 (0.416) and the lowest value for the primer UBC 834 (0.252). The mean value of Shannon's information index as a measure of genetic diversity was 0.523 for all the primers, with the highest value for the ISSR-18 (0.603) and the lowest value for the primer UBC 834 (0.404) (Table 3).

Total heterozygosity/diversity (0.350), mean heterozygosity/gene diversity within population (0.141), genetic differentiation between population (0.594) and gene flow (0.355) were obtained indicating greater efficiency of ISSR primers (Table 3).

The genetic similarity coefficient based on the Jaccard's similarity ranged from 0.98 to 0.16. The highest similarity coefficient was found between accessions from Jiri 6 and Jiri 7, whereas the lowest similarity was found between accessions Phi 8 and Dar 2 showing genetic variations among 45 accessions of *G. diversifolia* (Fig. 3).

3.2. Dendrogram analysis by ISSR primers

Genetic relationships among the 45 accessions of *G. diversifolia* were observed based on the Jaccard's coefficients from 131 amplified loci and a dendrogram was constructed using UPGMA method. A cluster analysis was performed on the basis of Jaccard's similarity (Fig. 3). The genetic similarity calculated from 45 accessions of *G. diversifolia* ranged from 0.24 to 1.00. Jaccard's coefficient generated five major clusters (i.e. A, B, C, D, E) and has the similarity coefficient of 0.50. Cluster A compared to the accessions from Darchula was further sub-clustered at the similarity coefficient level of 0.74 and 0.90, Cluster B from Kathmandu was further sub-clustered at 0.75 and 0.89, Cluster C from Dolakha at 0.56 and 0.83, Cluster D from Kaski at 0.75 and 0.74, and Cluster E from Panchthar at 0.61 and 0.53.

UPGMA tree construction methods were used to construct the dendrogram of five populations. The dendrogram obtained from UPGMA analysis of ten ISSR primers revealed five major groups of *G. diversifolia* (Fig. 3).

The result obtained from Mantel test using NTSYSpc version 2.02i showed the highest and most significant correlation between Jaccard and Dice similarity matrices 0.99094 (Table 4). The highest correlation value, comparison of standard chart of goodness of fit and Jaccard's coefficient of similarity with UPGMA clustering method was found most suitable for studying relationship among *G. diversifolia* accessions.

3.3. Genetic variability in G. diversifolia

Among the populations, the highest degree of variation was recorded in the population of Dolakha with Nei's genetic diversity of 0.192 and Shannon's information index of 0.285. The lowest degree of variation was recorded on the population of Darchula with Nei's genetic diversity of 0.110 and Shannon's diversity of 0.162 (Table 5).

The highest genetic identity was (0.756), and found between the populations of Dolakha and Panchthar, with the shortest genetic distance (0.278). The maximum genetic distance was found between the populations of Darchula and Panchthar (0.448) with the minimum genetic identity (0.638) (Table 6).

3.4. Analysis of molecular variance (AMOVA) of G. diversifolia

The partitioning of variations within and among the populations was

Table 4
Correlation coefficients from Mantel test of original matrices (2 way).

	Simple Matching	Jaccard	Dice
Simple matching	******	–	–
Jaccard	0.96536	******	–
Dice	0.96213	0.99094	******

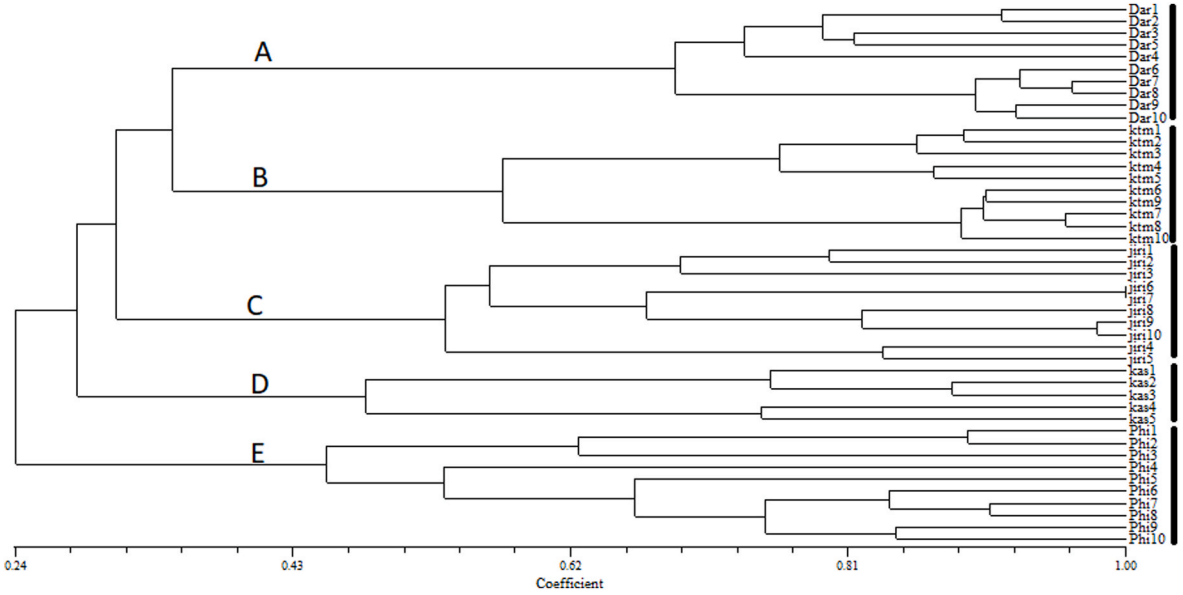

Fig. 3. UPGMA based dendrogram showing the genetic relationship among 45 accessions of *G. diverisifolia* using 10 ISSR primers.

Table 5
Genetic variability within the population of *G. diversifolia*.

Population	Sample size	No of polymorphic band	PPB %	h	I
Darchula	10	38	29.01	0.111	0.162
Kaski	5	45	34.35	0.125	0.186
Kathmandu	10	44	33.59	0.126	0.187
Dolakha	10	68	51.91	0.192	0.285
Panchthar	10	62	47.33	0.153	0.232

Percentage of polymorphic bands/loci (PPB), Nei's (1973) genetic diversity (h), Shannon's information index (I).

Table 6
Nei's unbiased measure of genetic identity (above diagonal) and genetic distance (below diagonal).

Population	Darchula	Dolakha	Kathmandu	Panchthar	Kaski
Darchula	****	0.728	0.721	0.638	0.697
Dolakha	0.316	****	0.714	0.756	0.746
Kathmandu	0.326	0.335	****	0.682	0.714
Panchthar	0.448	0.278	0.382	****	0.731
Kaski	0.360	0.292	0.336	0.312	****

analyzed by AMOVA, which revealed occurrence of 60% total genetic variation among the different populations and 40% total genetic variation within populations (Table 7). The difference between the individuals within and among the populations was statistically significant with the P value < 0.001 (Table 7).

3.5. Principal Coordinate Analysis (PCoA)

All the data obtained using ten ISSR primers were used in principal coordinate analysis (PCoA) using Jaccard's coefficient of similarity. The first PC1, PC2, PC3 explained total variation of 52.73 (22.07, 17.28, 13.38, respectively). The grouping of individuals is indicated in Fig. 4 using two coordinates. PCoA analysis categorized genotypes into four different groups without following their genetic information (Fig. 4).

4. Discussions

Knowledge on the genetic diversity of plant species will help to select genotypes for conservation and development programs [35]. Molecular characterization is one of the reliable tools which have shown important role in estimating relation between cultivars [36]. It uses molecular markers like ISSR [23,25]. ISSR markers are highly reproducible and sensitive markers [25]. The selected ISSR primers (Table 2) in this study indicated the existence of microsatellite regions of (GA)n, (TC)n, (AT)n, (AC)n in *G. diversifolia* which were also found in *Urtica dioica* [37] and *Boehmeria nivea* [26]. Genetic polymorphism observed from 10 ISSR markers for *G. diversifolia* was high (98.09%) in comparison to the results observed for *Urtica dioica* (68%) [37], *Cannabis sativa* (85.8%) [26], *Boehmeria nivea* (96.3%) [26], and lower than *Chenopodium quinoa* (99%) [38]. Since the selected ISSR primers showed high genetic polymorphism, these primers appear to be useful in determining the genetic diversity of *G. diversifolia*.

Polymorphism information content (PIC) is a widely used metric of usefulness of molecular markers and calculated based on the number of alleles and their distribution in population [39–41]. The maximum value of PIC in dominant marker is 0.5 [42]. The average value of PIC of the primers was found to be 0.350 in this study. The ten primers selected in this study had relatively proper distribution in population of *G. diversifolia*. UBC primer 828 gave relatively high PIC value (0.428) in the study. A study conducted in one of the highly used natural fiber producing plant *Linum usitatissimum* showed the PIC value of 0.367 [43]. The obtained values of PIC also suggested that high level of genetic diversity is present in analyzed germplasm.

ISSR markers system evaluated genetic variation and similarities among and within the five populations of *G. diversifolia* from Far-western, Western, Central and Eastern Nepal. The results obtained from the similarity coefficient indicated that genetic variation ranged from 0.98 to 0.16. High level of genetic diversity was recorded in the population of Dolakha PPB (51.91%), H (0.1928), I (0.2852), and lower level was recorded in the population of Darchula PPB (29.01%), H (0.111), I (0.162) (Table 5).

The study obtained the effective number of alleles (1.38–1.74) (Table 3). Nei and Li classify genetic differentiation between population (Gst) into three classes: low (Gst<0.05), moderate (0.05< Gst<0.15) and high (Gst>0.15) [44]. The genetic structure obtained in the study suggested that the differentiation coefficients (Gst 0.594) is higher than the average coefficients (Table 3). The Shannon's index varies from 0 to 1 and the values closer to zero represent lower genetic diversity [45]. The Shannon's diversity index obtained in the study was in the average of 0.349. The Shannon's diversity index from Dolakha (0.285) was the most diverse population compared to Panchthar (0.232), Kathmandu (0.187), Kaski (0.186) and Darchula (0.162). High genetic differentiation in this species suggests that the individual populations have been reproductively isolated and have little current gene flow between them. The value of Nm was found to be 0.355. The value of gene flow (Nm) < 1 which denotes less than one migrant per generation into a population is the threshold value at which the differentiation occurs in population in a significant amount [46]. An Nm of less than one suggests that the diversity maintained in the population is prone to genetic drift [47].

It is generally agreed that plant genetic diversity changes with time and distance [48]. The extent and distribution of genetic diversity in a plant species depends on different factors like its evolution and breeding system, ecological and geographical factors, past bottlenecks, and time and again, by many human factors [49].

Analysis of molecular variance (AMOVA) of *G. diversifolia* showed that higher distribution of genetic variation is present among the populations (60%) compared to within the populations (40%) with the significance value of $p < 0.001$. The genetic diversity is closely correlated with different factors like effective population size; breeding system; natural selection; and life history traits (including life form, ecological tolerance, seed dispersal, and gene flow) [46]. For *G. diversifolia*, the plant harbors shady moist habitat, commonly found in high altitudes above 1,000 m to 3,000 m between mountain gorges. A similar analysis was conducted on *Stylosanthes scabra* [50]; *Zingiber officinale* [51]; *Oryza granulata* [52]; and *Cypripedium japonicum* [53]. Genetic relationship of *Boehmeria* species from Urticaceae Family using 37 accessions showed ISSR markers are more informative in assessment of ramie diversity collected from different agroecological conditions [26]. The study showed that there is clear association between

Table 7
Analysis of molecular variance (AMOVA).

Source of variation	DF	SS	MS	Estimated variance	Total variance	P value
Among populations	4	593.856	148.464	15.540	60%	<0.001
Within populations	40	413.300	10.333	10.333	40%	<0.001
Total	44	1007.156	–	25.872	100%	

Degree of freedom (DF), Sum of Square (SS), Mean Sum of Square (MS), Estimated Variance, Percentage of Variation and Significance based on permutation across the full data set.

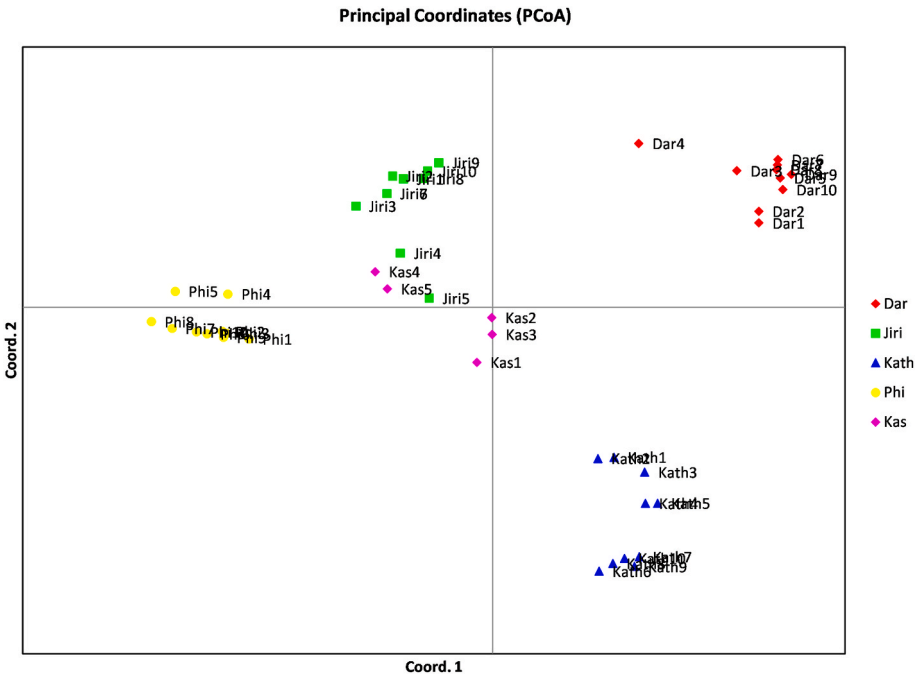

Fig. 4. Principal coordinates analysis (PCoA) among 45 *G. diversifolia* accessions using 10 ISSR primers: Dar (Darchula); Kas (Kaski); Kath (Kathmandu); Jiri (Dolakha); Phi (Panchthar).

geographical origin and genetic similarity in populations distributed in different regions. The present study also revealed that high level of genetic differentiation is present among populations of *G. diversifolia* and increase with an increase in geographical distance. Gst value 0.594 also strengthens the result of the study. Principal coordinate analysis (PCoA) confirmed the clustering of 45 *G. diversifolia* accessions into five main populations using 10 ISSR primers.

The Nm value less than 1, Gst value more than 0.15, and clear cluster formation depending upon geographic distance suggested that high level of genetic variance is present among the population of *G. diversifolia*. Further, overexploitation and massive destruction of its natural habitat [5] indicate that conservation and sustainable utilization of *G. diversifolia* is essential for cultural identity and economic benefits for the indigenous peoples and local communities living in the Hindu Kush Himalayan region of Nepal and India.

5. Conclusions

ISSR markers allowed determination of the genetic variability in *G. diversifolia* by grouping them according to their geographical locations in Nepal Himalaya. Comprehensive molecular analysis reveal that *G. diversifolia* has low genetic diversity within population and high genetic differentiation among the population. This genetic variability potential provides scientific basis for sustainable utilization and conservation efforts.

CRediT authorship contribution statement

Bijay Raj Subedee: Conceptualization, Methodology, Software, Data curation, Writing – original draft. **Giri Raj Tripathi:** Writing – review & editing, Supervision, Methodology. **Nabin Narayan Munankarmi:** Visualization, Investigation. **Ram Prasad Chaudhary:** Supervision, Conceptualization, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

This study was supported by Kailash Sacred Landscape Conservation and Development Initiative (KSLCDI) Nepal, a collaborative programme between Ministry of Forests and Environment (MoFE), Government of Nepal; Research Centre for Applied Science and Technology (RECAST), Tribhuvan University; and International Centre for Integrated Mountain Development (ICIMOD); the German Federal Ministry for Economic Cooperation and Development (BMZ) through the Deutsche Gesellschaft für INTERNATIONALE ZUSAMMENARBEIT (GIZ) GmbH, and the United Kingdom's Department for International Development (DFID) – UK Aid, as well as by the core funds of ICIMOD contributed by the governments of Afghanistan, Australia, Austria, Bangladesh, Bhutan, China, India, Myanmar, Nepal, Norway, Pakistan, Switzerland, and the United Kingdom. The views and interpretation in this publication are those of the authors and should not be ascribed to MoFE, RECAST, ICIMOD or their donors.

References

[1] A. Mohanty, S. Vivekanandhan, J. Pin, M. Misra, Composites from renewable and sustainable resources: challenges and innovations, Science 362 (2018) 536–542, https://doi.org/10.1126/science.aat9072.
[2] T.P. Barakoti, K.P. Shrestha, Commercial utilization of allo (*Girardinia diversifolia*) by the rais of sankhuwasabha for income generation, Banko Janakari 18 (2008) 18–24, https://doi.org/10.3126/banko.v18i1.2162.
[3] A. Gurung, H. Flanigan, A.K. Ghimeray, R. Karki, R. Bista, O.P. Gurung, Traditional knowledge of processing and use of the himalayan giant nettle (*Girardinia diversifolia* (Link) Friis) among the Gurungs of Sikles, Nepal, Ethnobot. Res. Appl. 10 (2012) 167–174, https://doi.org/10.17348/era.10.0.167-174.
[4] A. Pandey, U.P. Lepcha, K.S. Gaira, R. Joshi, N. Chettri, Nettle plant (*Girardinia diversifolia*) fibre extraction and yarn making, NIHE (2020) 1–18.
[5] B.R. Subedee, R.P. Chaudhary, Y. Uprety, T. Dorji, Socio-ecological perspectives of himalayan giant nettle (*Girardinia diversifolia* (link) Friis) in Nepal, J. Nat. Fibers 17 (2018) 9–17, https://doi.org/10.1080/15440478.2018.1458684.
[6] I. Friis, A synopsis of *girardinia* (Urticaceae), Kew Bull. 36 (1981) 143–157, https://doi.org/10.2307/4119014.
[7] O. Polunin, A. Stainton, Flowers of the Himalaya, Oxford University Press, Delhi, 1984.
[8] S.C. Singh, R. Shrestha, *Girardinia diversifolia* (Urticaceae), a non-conventional fiber resource in Nepal, Plants, Econ. Bot. 42 (1988) 445–447.
[9] B.R. Subedee, R.P. Chaudhary, T. Dorji, A.J. Shrestha, Indigenous and local knowledge of conservation and sustainable use of himalayan giant nettle

(*Girardinia diversifolia* (link) Friis) in eastern and far-western regions of Nepal, in: M. Karki, R. Hill, W. Alangui, K. Icikawa, P. Bridgewater (Eds.), In Knowing Our Lands and Resources, United Nations Educational, Scientific and Cultural Organization (UNESCO), France, 2017, pp. 191–198.
[10] L. Adhikari, A.J. Shrestha, T. Dorji, E. Lemke, B.R. Subedee, Transforming the lives of mountain women through the himalayan nettle value chain: a case study from Darchula, far west Nepal, Mt. Res. Dev. 38 (2018) 4–13, https://doi.org/10.1659/MRD-JOURNAL-D-17-00074.1.
[11] P.L. Bhandari, Value chain analysis of Allo (*Girardinia diversifolia*) in Solukhumbhu district of Nepal, AAES 4 (2019) 410–423, https://doi.org/10.26832/24566632.2019.040407.
[12] P.M. Njogu, G.N. Thoithi, J.W. Mwangi, F.N. Kamau, I.O. Kibwage, S.T. Kariuki, A. Yenesew, H.N. Mugo, J.M. Mwalukumbi, Phytochemical and antimicrobial investigation of *girardinia diversifolia* (Link)Friis (Urticaceae), east cent, Afr. J. Pharm. Sci. 14 (2011) 89–94.
[13] S.S. Shrestha, S. Sut, I. Ferrarese, S.B. Marco, G. Zengin, M.D. Franco, D.R. Pant, M. F. Mahomoodally, N. Ferri, N. Biancorosso, F. Maggi, S.D. Acqua, S. Rajbhandary, Himalayan nettle *Girardinia diversifolia* as a candidate ingredient for pharmaceutical and nutraceutical applications—phytochemical analysis and in vitro bioassays, Molecules 25 (2020) 1563, https://doi.org/10.3390/molecules25071563.
[14] J. Chen, Q. Lin, I. Friis, C.M. Wilmot-Dear, A.K. Monro, Urticaceae, in: Z.Y. Wu, P. H. Raven, D.Y. Hong (Eds.), Flora of China: Ulmaceae through Basellaceae, Science Press, Beijing and Missouri Botanical Garden Press, St. Louis, 2003, pp. 76–189.
[15] C. Kim, T. Deng, M. Chase, D.-G. Zhang, Z.-L. Nie, H. Sun, Generic phylogeny and character evolution in Urticeae (Urticaceae) inferred from nuclear and plastid DNA regions, Taxon 64 (2015) 65–78, https://doi.org/10.12705/641.20.
[16] Z.-Y. Wu, A.K. Monro, R.I. Milne, H. Wang, T.-S. Yi, J. Liu, D.-Z. Li, Molecular phylogeny of the nettle family (Urticaceae) inferred from multiple loci of three genomes and extensive generic sampling, Mol. Phylogenet. Evol. 69 (2013) 814–827, https://doi.org/10.1016/j.ympev.2013.06.022.
[17] A.R. Fernie, H.J. Klee, The use of natural genetic diversity in the understanding of metabolic organization and regulation, Front. Plant Sci. 2 (2011) 59, https://doi.org/10.3389/fpls.2011.00059.
[18] A. Mohammadi, B.M. Prasanna, Analysis of genetic diversity in crop plants—salient statistical tools and considerations, Crop Sci. 43 (2003) 1235–1248, https://doi.org/10.2135/cropsci2003.1235.
[19] G.S. Erzurumlu, N. Sultana, M. Vural, S. Serce, Genetic and phenotypic variation among Turkish terrestrial orchid species as revealed by RAPD and morphological characteristics, Turk. J. Agric. For. 42 (2018) 227–236, https://doi.org/10.3906/tar-1711-37.
[20] J. Baruah, S.K. Pandey, T. Begum, N. Sarma, M. Paw, M. Lal, Molecular diversity assessed amongst high dry rhizome recovery Ginger germplasm (*Zingiber officinale* Roscoe) from NE-India using RAPD and ISSR markers, Ind. Crop. Prod. 129 (2019) 463–471, https://doi.org/10.1016/j.indcrop.2018.12.037.
[21] Z. Li, R.L. Nelson, RAPD marker diversity among cultivated and wild soybean accessions from four Chinese provinces, Crop Sci. 42 (2002) 1737–1744, https://doi.org/10.2135/cropsci2002.1737.
[22] X. Qi, T.S. Pittaway, S. Lindup, H. Liu, E. Waterman, F.K. Padi, C.T. Hash, J. Zhu, M.D. Gale, K.M. Devos, An integrated genetic map and a new set of simple sequence repeat markers for pearl millet, *Pennisetum glaucum*, Theor. Appl. Genet. 109 (2004) 1485–1493, https://doi.org/10.1007/s00122-004-1765-y.
[23] M.P. Reddy, N. Sarla, E.A. Siddiq, Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding, Euphytica 128 (2002) 9–17, https://doi.org/10.1023/A:1020691618797.
[24] J.G.K. Williams, A.R. Kubelik, K.J. Livak, J.A. Rafalski, S.V. Tingey, DNA polymorphism amplified by arbitrary primers are useful as genetic markers, Nucleic Acids Res. 18 (1990) 6531–6535, https://doi.org/10.1093/nar/18.22.6531.
[25] E. Zietkiewicz, A. Rafalski, D. Labuda, Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification, Genomics 20 (1994) 176–183, https://doi.org/10.1006/geno.1994.1151.
[26] L. Liu, Z.Q. Meng, B. Wang, X. Wang, J. Yang, D. Peng, Genetic diversity among wild resources of the genus *Boehmeria* jacq. From west China determined using inter-simple sequence repeat and rapid amplification of polymorphic DNA markers, Plant Prod. Sci. 12 (2009) 88–96, https://doi.org/10.1626/pps.12.88.
[27] L.G. Zhang, Y. Chang, X.F. Zhang, F.Z. Guan, H.M. Yuan, Y. Yu, L.J. Zhao, Analysis of the genetic diversity of Chinese native *Cannabis sativa* cultivars by using ISSR and chromosome markers, Genet. Mol. Res. 13 (2014) 490–500, https://doi.org/10.4238/2014.December.12.10.
[28] Z.K. Punja, G. Rodriguez, S. Chen, Assessing genetic diversity in *Cannabis sativa* using molecular approaches, in: S. Chandra, H. Lata, M. Elsohly (Eds.), *Cannabis Sativa* L.-Botany and Biotechnology, Springer, Cham, 2017, pp. 395–418, https://doi.org/10.1007/978-3-319-54564-6_19.
[29] B.R. Subedee, G.R. Tripathi, R.P. Chaudhary, DNA isolation and optimization of PCR protocol for ISSR analysis of *Girardinia diversifolia*: a medicinal and economic plant species from Nepal Himalaya, Afr. J. Biotechnol. 19 (2020) 747–753, https://doi.org/10.5897/AJB2020.17228.
[30] I. Roldan-Ruiz, J. Dendauw, E.V. Bockstaele, A. Depicker, M.D. Loose, AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.), Mol. Breed. 6 (2000) 125–134, https://doi.org/10.1023/A:1009680614564.
[31] A. Prevost, M.J. Wilkinson, A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars, Theor. Appl. Genet. 98 (1999) 107–112, https://doi.org/10.1007/s001220051046.
[32] F.J. Rohlf, NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, New York, 2000.
[33] F.C. Yeh, R.C. Yang, T.B.J. Boyle, Z. Ye, J.M. Xiyan, PopGene32, Microsoft Windows-Based Freeware for Population Genetic Analysis, Version 1.32. Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, Alberta, Canada, 2000.
[34] R. Peakall, P.E. Smouse, GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update, Bioinformatics 28 (2012) 2537–2539, https://doi.org/10.1093/bioinformatics/bts460.
[35] M.A. Monfared, D. Samsampour, G.R. Sharifi-Sirchi, F. Sadeghi, Assessment of genetic diversity in *Salvadora persica* L. based on inter simple sequence repeat (ISSR) genetic marker, J. Genet Eng. Biotechnol. 16 (2018) 661–667, https://doi.org/10.1016/j.jgeb.2018.04.005.
[36] J. Baruah, B. Gogoi, K. Das, N.M. Ahmed, D.K. Sarmah, M. Lal, B.S. Bhau, Genetic diversity study amongst *Cymbopogon* species from NE-India using RAPD and ISSR markers, Ind. Crop. Prod. 95 (2017) 235–243, https://doi.org/10.1016/j.indcrop.2016.10.022.
[37] M. Haghpanah, S.K. Kazemitabar, S.H. Hashemi, S. Alavi, Comparison of ISSR and AFLP markers in assessing genetic diversity among Nettle (*Uritica dioica* L.) populations, J. Plant Mol. Breed. 4 (2016) 10–16, https://doi.org/10.22058/JPMB.2016.19659.
[38] M.C. Ana-Cruz, M.E. Helena, M.C. Yacenia, Molecular characterization of *Chenopodium quinoa* Willd. using inter-simple sequence repeat (ISSR) markers, Afr. J. Biotechnol. 16 (2017) 483–489, https://doi.org/10.5897/AJB2017.15925.
[39] J.A. Anderson, G.A. Churchill, J.E. Autrique, S.D. Tanksley, M.E. Sorrells, Optimizing parental selection for genetic linkage maps, Genome 36 (1993) 181–186, https://doi.org/10.1139/g93-024.
[40] D. Botstein, R.L. White, M. Skolnick, R.W. Davis, Construction of a genetic linkage map in man using restriction fragment length polymorphisms, Am. J. Hum. Genet. 32 (1980) 314–331.
[41] S. Nagy, P. Poczai, I. Cernák, A.M. Gorji, G. Hegedűs, J. Taller, PICcalc: an online program to calculate polymorphic information content for molecular genetic studies, Biochem. Genet. 50 (2012) 670–672, https://doi.org/10.1007/s10528-012-9509-1.
[42] Y.V. Chesnokov, A.M. Artemyeva, Evaluation of the measure of polymophism information of genetic diversity, Agriculture Biology 50 (2015) 571–578, https://doi.org/10.15389/agrobiology.2015.5.571eng.
[43] A. Kumari, S. Paul, V. Sharma, Genetic diversity analysis using RAPD and ISSR markers revealed discrete genetic makeup in relation to fibre and oil content in *Linum usitatissimum* L. genotypes, Nucleus 61 (2018) 45–53, https://doi.org/10.1007/s13237-017-0206-7.
[44]] Z. Hashemifar, M. Rahimmalek, Genetic structure and variation in *Perovskia abrotanoides* Karel and *P. atriplicifolia* as revealed by molecular and morphological markers, Sci. Hortic. 230 (2018) 169–177, https://doi.org/10.1016/j.scienta.2017.11.029.
[45] M.A. Monfared, D. Samsampour, G.R.S. Sirchi, F. Sadeghi, Assessment of genetic diversity in *Salvadora persica* L. based on inter simple sequence repeat (ISSR) genetic marker, J. Genet Eng. Biotechnol. 16 (2018) 661–667, https://doi.org/10.1016/j.jgeb.2018.04.005.
[46] M. Slatkin, Rare alleles as indicators of gene flow, Evolution 39 (1985) 53–65, https://doi.org/10.1111/j.1558-5646.1985.tb04079.x.
[47] S. Wright, The genetical structure of populations, Ann. Eugen. 15 (1949) 323–354, https://doi.org/10.1111/j.1469-1809.1949.tb02451.x.
[48] M.D. Loveless, J.L. Hamrick, Ecological determinants of genetic structure in plant populations, Annu. Rev. Ecol. Systemat. 15 (1984) 65–95, https://doi.org/10.1146/annurev.es.15.110184.000433.
[49] V.R. Rao, T. Hodgkin, Genetic diversity and conservation and utilization of plant genetic resources, Plant Cell Tissue Organ Cult. 68 (2002) 1–19, https://doi.org/10.1023/A:1013359015812.
[50] J.C. Costa, G.G.M. Fracetto, F.J.C. Fracetto, T.C. Souza, M.V.F. Santos, M.A. L. Junior, Genetic diversity in natural populations of *Stylosanthes scabra* Fabaceae using ISSR markers, Genet. Mol. Res. 17 (2018) 1–9, https://doi.org/10.4238/gmr18219.
[51] A. Das, M. Gaur, D.P. Barik, E. Subudhi, Genetic diversity analysis of 60 ginger germplasmcore accessions using ISSR and SSR markers, Plant Biosyst. 151 (2016) 822–832, https://doi.org/10.1080/11263504.2016.1211197.
[52] W. Qian, S. Ge, D.Y. Hong, Genetic variation within and among populations of a wild rice *Oryza granulata* from China detected by RAPD and ISSR markers, Theor. Appl. Genet. 102 (2001) 440–449, https://doi.org/10.1007/s001220051665.
[53] H.Z. Tian, L.X. Han, J.L. Zhang, X.L. Li, T. Kawahara, T. Yukawa, J.L. Pujol, P. kumar M.G. Chung, M.Y. Chung, Genetic diversity in the endangered terrestrial orchid *Cypripedium japonicum* in East Asia: insights into population history and implications for conservation, Sci. Rep. 8 (2018) 1–13, https://doi.org/10.1038/s41598-018-24912-z.