Saudi Journal of Biological Sciences 29 (2022) 3528–3538

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Assessment of variability among morphological and molecular characters in wild populations of mint [*Mentha longifolia* (L.) L.] germplasm

Anjina Devi [a], Tasir Iqbal [a], Ishfaq Ahmad Wani [b], Geeta Sharma [a,*], Susheel Verma [a,*], Ahmed Noureldeen [c], Hadeer Darwish [d]

[a] Department of Botany, University of Jammu, Jammu 180006, Jammu and Kashmir, India
[b] Conservation and Molecular Biology Lab. Department of Botany, Baba Ghulam Shah Badshah University, Rajouri 185234, Jammu and Kashmir, India
[c] Department of Biology, College of Sciences, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia
[d] Department of Biotechnology, College of Sciences, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia

ARTICLE INFO

Article history:
Received 6 January 2022
Revised 7 February 2022
Accepted 13 February 2022
Available online 17 February 2022

Keywords:
Mentha longifolia
Molecular diversity
AMOVA
Polymorphism
Hierarchical cluster analysis
Conservation

ABSTRACT

Mentha longifolia is an important medicinal and aromatic perennial herb that exhibits wide distribution range from sub-tropical to temperate regions. In the present study, agro-morphological traits and genetic differences in 19 different populations of *M. longifolia* were studied to evaluate the level and extent of its diversity. Analysis of variance (ANOVA) showed that the different phenotypic characters show considerable differences among various populations and was significant at $p < 0.05$. Molecular diversity analysis performed by using arbitrary amplified eleven ISSR primers generated a total of 121 amplicons that range within the size of 200–2500 base pairs (bp). Each primer on average generated 11 amplicons with percentage polymorphism being 100. The analysis of molecular variance (AMOVA) showed more (64%) among population genetic diversity and less (36%) within the populations. Greater genetic differentiation (Gst = 0.6852) among these populations occurs due to low gene flow (*Nm* = 0.2297) and greater habitat variability. Geographic and genetic distances were positively correlated according to Mantel's test. In order to remove any kind of biases, we used R software to perform cluster and redundancy analysis to analyse the extent of relatedness among studied populations. In terms of morphological and molecular aspects, the populations were grouped into four and five clusters respectively based on hierarchical clustering method. The results demonstrated that *M. longifolia* displays a great degree of morphological and genetic variation and can be utilized in breeding, genetic improvement, and gene bank conservation programmes in future.

1. Introduction

Mentha longifolia (L.) L., belonging to family Lamiaceae, is an important herbaceous perennial with medicinal as well as aromatic value which grows mostly in cool and moist places (Ahmad et al., 2011; Shinwari et al., 2011). The plant has a creeping stolon along with underground rootstock and upright stems with height ranging from 30 to 120 cm. It has paired, simple, opposite and decussate, elliptical to oblong, lanceolate, with sparse to dense hairs, greyish-green petiolated leaves. The flowers are 3–5 mm long, white to purple, found densely clustered on long elongated spikes (Mikaili et al., 2013; Panjeshahin et al., 2018; Araghi et al., 2019). This species exhibits widest natural geographic distribution of all the *Mentha* species and grows extensively in the Mediterranean region, Europe, the middle East, South and North Africa, Australia and central Asia including India (Mikaili et al., 2013; Panjeshahin et al., 2018). In India, *M. longifolia* is reported from Western Himalayan states and union territories including Jammu and Kashmir (Sobti, 1971), Himachal Pradesh, Uttarakhand and Ladakh (Singh et al., 2017; Srivastava and Saggoo, 2018).

* Corresponding authors.
E-mail addresses: geetaji@yahoo.com (G. Sharma), eremurus@rediffmail.com (S. Verma).

Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2022.02.013
1319-562X/

Jammu province of J&K exhibits altitudinal, climatic and ecological variation which support rich floristic diversity and hosts many medicinally important species. *M. longifolia* is one of these and occurs naturally in a wide geographical distribution i.e. from Jammu (300 m.a.s.l.) to Bhaderwah (1820 m.a.s.l.) (Sharma and Kachroo, 1981; Bhellum and Magotra, 2012). Locals have been using this species in fresh and dried form as a spice, for adding flavour to dishes and for medicinal purposes. The species possesses high medicinal value as extracts of different parts of this species have anti-microbial, anti-spasmodic properties and is used to treat gastrointestinal and nervous system related effects. Besides, the species is utilised for treating headache, rheumatism, neuralgia, gall and bladder stone, jaundice, toothache and digestive disorders (Mikaili et al., 2013; Panjeshahin et al., 2018). Among the various biochemicals present in the essential oil of *M. longifolia*, menthol plays an important role in various pharmacological applications (Mikaili et al., 2013). This species is highly variable with respect to plant habit, leaf shape and its extent of hairiness, inflorescence lengths and floral colour and therefore ensembles number of subspecies and varieties (Vining et al., 2005; Mohammadi and Asadi-Gharneh, 2018) which are mostly are diploid (2n = 2x = 24) and in rare cases are tetraploid (2n = 4X = 48) forms (Sobti, 1971; Harley and Brighton, 1977; Chambers and Hummer, 1994; Malik et al., 2017).

The genus *Mentha* has undergone frequent interspecific hybridization between wild and cultivated populations which leads to varying basic chromosome count, occurrence of polyploids and aneuploids, cytomixis, variation in morphology and composition of essential oils under different environmental conditions (Jedrzejczyk and Rewers, 2018). To discriminate different *Mentha* species, besides morphological, biochemical and cytological tools (Jedrzejczk and Rewers, 2018) different molecular markers such as RFLP, CAPS, RAPD, AFLP, ISSR, SSR and limonene synthase gene polymorphism have been used to establish phylogenetic relationships among species (Khanuja et al., 2000; Gobert et al., 2002; Shasany et al., 2005; Jabeen et al., 2012; Wang et al., 2013; Kumar et al., 2015; Ibrahim, 2017). Recently, there has been a substantial increase in the use of both morphological as well as molecular markers for the assessment of genetic diversity. ISSR is an efficient PCR-based molecular marker technique for studying genetic diversity. ISSR are regions on DNA that are flanked by microsatellite sequences. With the use of a single primer, higher stringency of amplification due to long primers (16–25 bp) and elevated annealing temperatures (45–60 °C) makes ISSR markers more reproducible and reliable.

For comprehensive characterisation of diversity existing in Indian horsemint germplasm, morpho-variability need to be coupled with that at molecular level. As this species grows extensively in Jammu province at different altitudinal ranges (300–1821 m.a.s.l.), morphological studies are expected to bring to light morpho-variants. Some of these variants can be more suited for economic exploitation. Additionally, the molecular data in conjunction with data generated on morphological variability of current taxa will also be helpful in establishing inter- and intra-population phylogenetic relationships. Though extensive work in this line has been carried out for horsemint germplasm of Iran, Egypt, France, Belgium, Netherlands, Switzerland, Denmark (Harley and Brighton, 1977; Panjeshahin et al., 2018; Choupani et al., 2019; Soilhi et al., 2020; Yaghini et al., 2020; Heylen et al., 2021), limited data are on record regarding genetic diversity of Indian horsemint accessions (Khanuja et al., 2000; Shasany et al., 2005), however nothing is on record from Jammu province. Present study is a first attempt of assessing genetic diversity among different *M. longifolia* populations from varying altitudinal ranges (sub-tropical to temperate) of Jammu province and for having proper programme on breeding and conservation of its elite genotypes (producing more herbage).

2. Materials and methods

2.1. Study area

Present study has been from the Jammu and Kashmir UT of India. A total of 19 different populations were located and studied for morphological and molecular characters (Fig. 1). These populations were located 2 (Laddan and Sangoor) to 302 kms (Khellaini top and Rajouri) of distance from each other. Geo-coordinates of all the sites were determined using Magellan Professional Mobile Mapper (990603–50) (Table 1).

2.2. Diversity and differentiation of morphological traits

A total of 45 individuals per population were selected for the morphometric analysis. For phenotypic study, 15 morphological traits (foliar and floral) of *M. longifolia* were taken into consideration such as plant height (PH), internode length (IL), leaf length (LL) and width (LW), petiole length (PL), inflorescence length (IFL), number of leaves and inflorescences per offshoot (LOS and IOS), number of whorls/inflorescence (WI), number of flowers/whorl (FW) and inflorescence (FI), calyx (CLX) and corolla (CRL) length as well as pistil (SYL) and stamen (STL) length. Measurements were taken during full blooming season (July-August) with the help of ruler (30 cm) in both field as well as laboratory conditions.

2.3. Diversity and differentiation of molecular traits

The young and healthy leaves of individual horsemint populations were collected during the growing season (May and June). The leaf samples were collected and preserved in liquid N_2 (in cryocan) before transporting to Molecular Biology Laboratory of Department of Botany, Baba Ghulam Shah Badshah University (BGSBU), Rajouri (J&K).

2.4. Isolation of DNA and PCR amplification

DNA isolation has been performed following the procedure of Doyle and Doyle, 1990 with some modifications. Preserved leaf samples (3 gm) were finely powdered in liquid nitrogen using mortar and pestle. Finely powdered leaves were suspended in 15 ml of buffer composed of 2% Cetrimonium bromide (CTAB, w/v), Tris base (100 mM, pH 8), 1.4 M Sodium Chloride, 20 mM Ethylenediaminetetraacetic acid (EDTA, pH = 8), 2% β-mercaptoethanol (v/v) and 2% Polyvinylpyrrolidone (PVP, w/v). This was followed by mixing of the suspension and incubation at 65 °C in water bath for half an hour. 15 ml of Chloroform:Isoamyl alcohol (24:1) was added to the cooled mixture followed by vortexing for few seconds and then mixing gently by inverting in the form of 8 for 10 min. The mixture was centrifuged at 13,000 rpm for 14 min (25 °C) and the supernatant was collected in another tube and precipitated with an equal amount of chilled isopropyl alcohol for 1 h at 4C. The DNA pellet formed was washed with 70% ethanol and kept for drying until the smell of ethanol vanishes. The pellet was resuspended in TE (10:1, pH 8) for further use. DNA was further subjected to gel electrophoresis in 0.8% agarose for quantification and quality check. A total of 18 14mer-16mer oligonucleotide primers (Supplementary file 1) synthesized by Promega were screened and 11 chosen for further studies.

The present study is based on utility of PCR amplification of ISSR markers. The final PCR mixture volume (25 µl) contained 1x buffer,

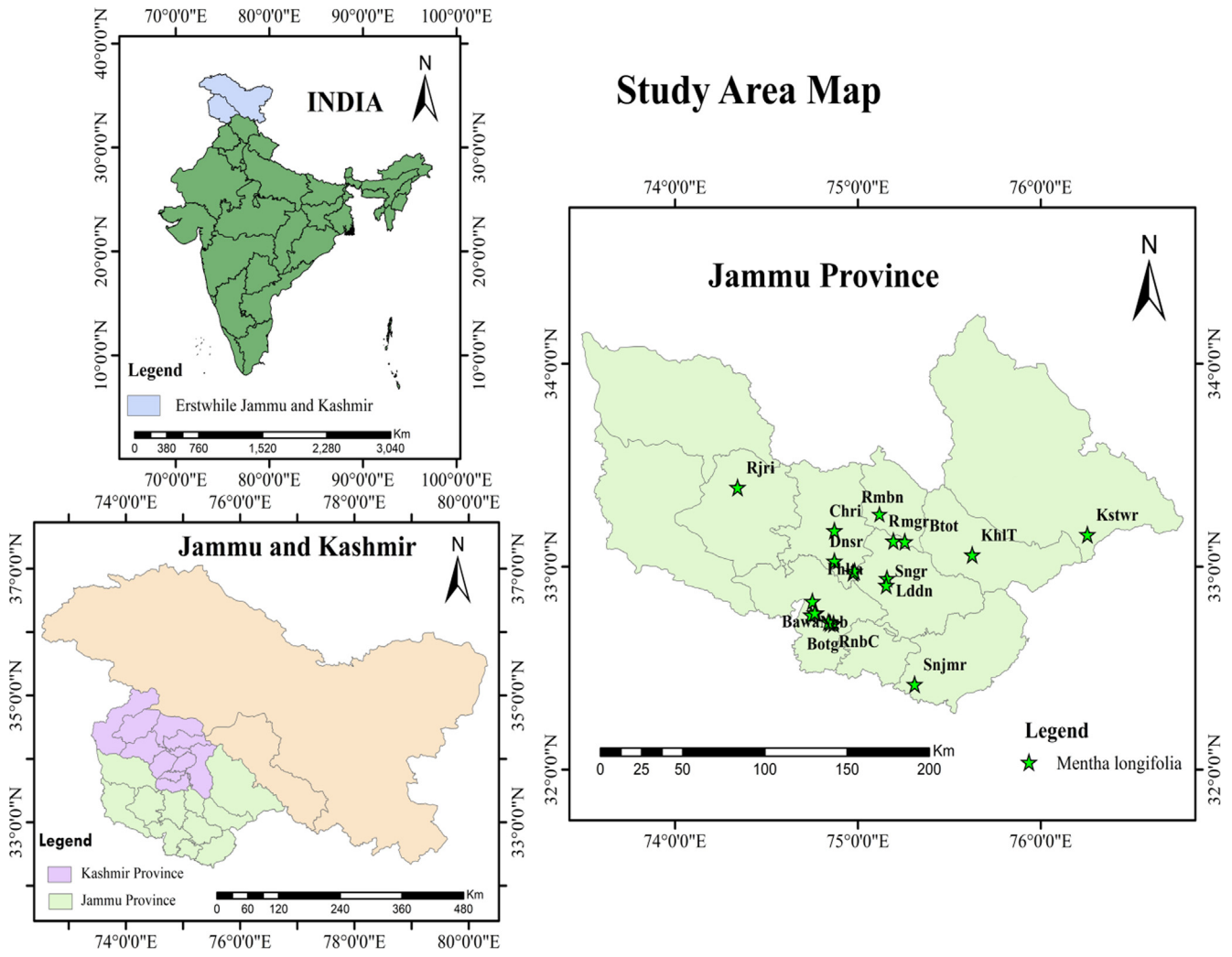

Fig. 1. Study area map depicting different populations of *Mentha longifolia*.

2 ng of gDNA, dNTPs (0.2 mM), 0.5 μM primer, 3 mM $MgCl_2$, Taq polymerase (2.5U). Amplification was carried out in thermocycler (Eppendorf, nexus gradient, Germany) under the following conditions: initial denaturation at 95 °C (5 min) followed by 37 cycles, each with denaturation at 94 °C (1 min), annealing at 44–58 °C (1 min), extension at 72 °C (2 min) and final extension at 72 °C (8 min). The amplified products were kept at 4 °C for further studies. The products were electrophoresed on 1.8% agarose gel (with 0.5 μg/ml EtBr in 0.5% TAE) followed by visualizing in UV and photography by Gel documentation system (Alpha Innotech, San Fransisco). Only the unambiguous bands scored were considered for further analysis.

2.5. Data analysis using statistical softwares

The data obtained from morphological and molecular studies were subjected to cluster analysis based on UPGMA (Unweighted Pair Group Method with Arithmetic mean) to evaluate the similarity/dissimilarity among the studied populations. Redundancy analysis was performed on both morphological and molecular datasets separately for analyzing the morphologically and genetically structured populations using the *Biodiversity R* package (Kindt, 2020). In addition, we also looked for the variables and loci, explaining 40% or more variation in both morphological and molecular data sets respectively. To analyse the correlation between the studied morphological variables, we performed the Pearson's multiple correlation using the *Performance Analytics* package (Carl et al., 2013). All these data were analysed in R statistical software v. 4.0.2 (Carl et al., 2013).

From ISSR profiles of *M. longifolia*, presence of band was scored as 1 whereas its absence was marked as 0. POPGENE 1.32 software was used for measuring the parameters such as observed (*Na*) and effective number of alleles (*Ne*), Nei's genetic diversity (*H*) (Nei, 1978), polymorphic loci (PL), %age polymorphic loci (PPL) and Shannon's information index (*I*) (Shannon and Weaver, 1949). Besides, total (*Ht*), and intrapopulation genetic diversity (*Hs*) as well as differentiation (*Gst*) was determined to analyze genetic diversity in subdivided populations. Interpopulation gene flow was determined by the formula [(Nm = 0.5(1 − *Gst*)/*Gst*] proposed by McDermott and McDonald, 1993). Molecular variance was analysed using GenAlex software v6.51 (Peakall and Smouse, 2006). The statistical parameter studied include mean, standard deviation and standard error. For assessing the variability means of different populations were compared using one way ANOVA.

3. Results

3.1. Morphological diversity

Mentha longifolia is a stoloniferous, perennial, pubescent and herbaceous plant with quadrangular stem which differentiates paired opposite leaves with serrated margins, bearing white,

Table 1
Location, climatic conditions, geographical coordinates and altitude of the areas under study.

Pop. Code	Accession name & code	District	Climatic zone	Latitude (N)	Longitude (E)	Altitude (m.a.s.l.)
1.	ChakHira (ChkH)	Jammu	Sub-tropical	32°46′21.34″	74°45′49.76″	270 ± 2
2.	Nagbani (Ngb)	Jammu	Sub-tropical	32°46′35 ”	74°46′08″	284 ± 3
3.	Ranbir Canal (RnbC)	Jammu	Sub-tropical	32°43′20.82″	74°50′49.89″	299 ± 2
4.	Bawa-da-talab (Bawa)	Jammu	Sub-tropical	32°49′44.26″	74°45′04.86″	304 ± 2
5.	Akhnoor (Aknr)	Jammu	Sub-tropical	33°1′38.52″	74°52′17.4″	333 ± 6
6.	Garden (Botg)	Jammu	Sub-tropical	32°43′9.18″	74°52′0.96″	320 ± 5
7.	Sanji-morh (Snjmr)	Kathua	Sub-tropical	32°25′13.24″	75°18′37.10″	317 ± 3
8.	Dhansar (Dnsr)	Reasi	Sub-tropical	33°0′1′38.29″	74°52′17.87″	546 ± 3
9.	Panthal (Pnthl)	Reasi	Sub-tropical	32°58′13.8″	74°58′22.2″	654 ± 8
10.	Laddan (Lddn)	Udhampur	Sub-tropical	32°56′10.8″	75°09′33.12″	766 ± 2
11.	Sangoor (Sngr)	Udhampur	Sub-tropical	32°54′30.24″	75°09′17.76″	790 ± 2
12.	Phalata (Phlta)	Udhampur	Sub-tropical	32°53′30″	75°03′5.4″	941 ± 9
13.	Chauri (Chri)	Udhampur	Temperate	33°1′38.52″	74°52′17.4″	2155 ± 3
14.	Ramban (Rmbn)	Ramban	Temperate	33°14′45.59″	75°1′38.84″	952 ± 2
15.	Ramgarh (Rmgr)	Ramban	Temperate	33°07′36.70″	75°1′38.84″	1148 ± 4
16.	Batote (Btot)	Ramban	Temperate	33°06′18.15″	75°20′31.41″	1434 ± 3
17.	Rajouri (Rjri)	Rajouri	Temperate	33°23′26.4″	74°20′30.6″	1163 ± 2
18.	Khellaini Top (KhT)	Doda	Temperate	33°03′29.82″	75°37′29.39″	1428 ± 3
19.	Paddar (Pddr)	Kishtwar	Temperate	33°1′38.52″	74°52′17.4″	2275 ± 5

pink/purple flowers clustered in spikes with pentamerous hermaphrodite flower having 5 sepals and 5 fused petals, 4 epipetalous stamens and a single bicarpellary syncarpous pistil having gynobasic style. However, the studied populations differed from each other with respect to their vegetative and/or floral traits (Table 2).

While plants of Akhnoor population are the tallest (89.51 cm), those of Paddar are the smallest (28.89 cm). Amongst these populations, variations also existed in the number of leaves per offshoot with its value ranges from 13 (Paddar) to 56.56 (Nagbani). At population level, variations have also been witnessed in the overall size of the leaves. While Ranbir Canal population differentiates smallest sized leaves (5.66 × 1.50 cm), leaves borne by the plants of Akhnoor population were the largest (8.76 × 2.11 cm).

Among floral traits, marked variation was witnessed in inflorescence length and number of flowers per inflorescence. Among different populations analysed, the longest inflorescences with maximum flowers (678) were borne by plants of Chauri (30.31 cm) whereas the smallest ones with least flowers (113) were produced by the plants of Paddar (5.67 cm).

M. longifolia is hermaphrodite and contains both male and female reproductive organs. Reproductive organs of *M. longifolia* are enclosed within calyx and corolla tube. Length of calyx ranges from 1 to 2.6 mm and that of corolla varies between 3 and 5.5 mm. Differences between lengths of pistil and stamen were meagre, with their values ranges between 4 and 5.8 mm and 4–5.6 mm respectively. Interestingly, the plants of Botanical garden population were male sterile with aborted stamens.

3.2. Statistical analysis

Pearson's correlation analysis showed that among all the morphological variables studied, the significant positive correlation was observed between calyx (CLX) and corolla (CRL) length; style (SYL) and filament (STL) length ($r = 0.99$, $p < 0.001$); no. of flowers (FI) and whorls (WI) per inflorescence ($r = 0.89$, $p < 0.001$); no. of flowers per whorl (FW) and inflorescence (FI) ($r = 0.60$, $p < 0.01$); inflorescence length (IFL) and no. of whorls per inflorescence (WI) ($r = 0.53$, $p < 0.05$), corolla (CRL) and style length (SYL) ($r = 0.49$, $p < 0.05$); corolla (CRL) and filament length (STL) ($r = 0.48$, $p < 0.05$) and FI and PSL ($r = 0.47$, $p < 0.05$); number of inflorescences per offshoot (IOS) and Petiole length (PL) ($r = 0.63$, $p < 0.01$); number of leaves per offshoot (LOS) and internode length (IL) ($r = 0.48$, $p < 0.05$) and Plant height (PH) ($r = 0.59$, $p < 0.01$), while a significant -ve correlation was seen between plant height (PH) and calyx length (CXL) ($r = -0.50$, $p < 0.05$) and plant height (PH) and corolla length (CRL) ($r = -0.51$, $p < 0.05$ (Fig. 2).

Quantitative traits have been further analysed by applying one-way ANOVA to determine statistical significance of differences in traits among populations. All the analysed morphological quantitative traits of *M. longifolia* portrayed statistically significant differences at $p = 0.05$ ($p < 0.05$).

With respect to morphological variables, the 19 studied populations were clustered into four groups (Fig. 3). Group 1 (blue) is the largest containing 12 populations in two sub-groups A and B with former containing 2 sub-tropical (Garden and Akhnoor) and latter encompassing 10 (Sangoor, Nagbani, Rajouri, Ramgarh, Ramban,

Table 2
Phenotypic matrices of different *M. longifolia* populations from Jammu province.

Chr. Pop.	PH (cm)	IL (cm)	LL (cm)	LW (cm)	LOS	P L (cm)	Inflorescence (In)					CLX (mm)	CRL (mm)	SYL (mm)	STL (mm)
							IL (cm)	IOS	WI	FW	FI				
Chk H	72.18 ±1.02	3.08 ±0.1	7.62 ±0.09	2.38 ±0.03	39.02 ±0.29	0.93 ±0.02	6.28 ±0.19	5.52 ±0.13	12.51 ±0.44	21.67 ±0.64	393.82 ±15.03	1.2 ±0.01	5.6 ±0.01	4.8 ±0.1	4.7 ±0.1
Ngb	80.10 ±2.8	3.52 ±0.14	8.13 ±0.19	1.78 ±0.02	56.56 ±0.68	0.23 ±0.01	12.3 ±4.4	13.82 ±0.43	24.6 ±1.25	22.87 ±0.42	345.07 ±8.49	1.33 ±0.02	4.5 ±0.01	4 ±0.03	4.2 ±0.04
RnbC	48.5 ±0.72	3.6 ±1.34	5.66 ±2.03	1.50 ±0.52	23.5 5 ± 0.58	0.5 ±0.19	12.44 ±0.31	12.27 ±4.36	15.09 ±5.34	15.09 ±5.34	218.51 ±88.1	1.3 ±0.01	5.3 ±0.01	5.0 ±0.04	4.8 ±0.04
Bawa	71.12 ±1.61	3.24 ±0.17	7.51 ±0.11	2.04 ±0.03	34.52 ±1.29	0.54 ±0.0	12.6 3 ± 0.22	15.13 ±0.37	22.87 ±0.42	24.6 ±1.25	263.47 ±11.31	1.33 ±0.02	5.55 ±0.01	4.9 ±0.1	5.6 ±0.1
Aknr	89.51 ±1.13	3.47 ±0.13	8.76 ±0.08	2.11 ±0.02	43.64 ±1.61	1.13 ±0.03	6.83 ±0.29	18.36 ±0.67	21.6 7 ± 0.64	29.18 ±0.59	404.98 ±2.33	1.15 ±0.01	5.8 ±0.01	4.2± 0.1	4.3± ±0.1
Bot G	84.60 ±1.11	2.74 ±0.11	7.60 ±0.11	2.07 ±0.01	46.0 ±0.30	1.26 ±0.01	8.78 ±0.17	17.4 ±0.35	29.18 ±0.59	20.53 ±0.38	315 ±2.50	0.84 ±0.01	3.18 ±0.01	5.2 ±0.2	Male sterile
Snjmr	43.42 ±0.58	2.58 ±0.14	7.54 ±0.12	2.29 ±0.03	23.0 ±0.82	0.8 5 ± 0.03	8.7 8 ± 0.17	3.3 ±0.05	10.51 ±0.24	18.64 ±0.22	191.09 ±5.77	1.15 ±0.01	5.5 ±0.01	4.8 ±0.1	4.7 ±0.1
Dnsr	70.27 ±1.56	3.71 ±0.08	7.07 ±0.11	1.99 ±0.04	49.82 ±1.10	0.34 ±0.02	15.55 ±0.35	4.69 ±0.10	11.67 ±0.26	15.38 ±0.34	167.78 ±3.60	1.34 ±0.01	5.6 ±0.01	5.8 ±0.1	4.5 ±0.1
Pnthl	59.41 ±6.58	3.30 ±0.38	5.87 ±1.06	1.51 ±0.25	29.80 ±0.74	1.08 ±0.03	12.78 ±2.42	11.4 0 ± 0.45	13.13 ±1.88	24.51 ±3.42	299.96 ±66.97	1.34 ±0.01	5.4 ±0.15	5.4 ±0.1	4.9 ±0.1
Ldn	66.85 ±1.35	3.70 ±0.16	6.78 ±0.13	2.05 ±0.02	55.96 ±0.46	1.26 ±0.01	11 ±0.22	15.76 ±0.15	12.0 6 ± 0.31	16.58 ±0.39	188.22 ±4.57	1.34 ±0.01	5.4 ±0.01	4.5 ±0.1	4.6 ±0.1
Sngr	78.33 ±0.76	2.42 ±0.09	8.63 ±0.16	1.66 ±0.01	47.57 ±1.77	1.27 ±0.04	7.29 ±0.38	10.86 ±0.40	8.69 ±0.23	11.62 ±0.22	168.98 ±4.57	1.34 ±0.01	5.6 ±0.01	5.2 ±0.01	5.0 ±0.01
Phlta	82.33 ±1.47	3.75 ±0.15	7.99 ±0.08	2.10 ±0.03	43.71 ±0.62	0.83 ±0.02	11.63 ±0.21	3.9 ±0.05	12.48 ±0.38	19.37 ±0.53	226.75 ±2.47	1.25 ±0.01	4.8 ±0.01	4.3 ±0.1	4.3 ±0.1
Chri	77.27 ±2.15	3.62 ±0.19	7.18 ±0.21	2.21 ±0.06	52.4 ±0.47	1.23 ±0.04	30.31 ±0.37	11.40 ±0.54	28.67 ±0.56	20.67 ±0.69	678.87 ±11.12	2.27 ±0.58	5.6 ±0.01	4.89 ±0.02	5.0 ±0.1
Rmbn	58.2 ±0.51	2.77 ±0.12	6.37 ±0.11	2.32 ±0.03	30.45 ±1.35	0.44 ±0.01	10.81 ±0.22	4.02 ±0.14	10.58 ±0.23	22.2 ±0.37	232.49 ±4.78	1.05 ±0.01	5.2 ±0.01	5.0 ±0.03	4,0 ±0.02
Rmgr	46.93 ±1.69	2.59 ±0.17	7.04 ±0.11	1.99 ±0.03	45.05 ±0.40	0.88 ±0.02	16.59 ±1.02	3.95 ±0.20	15.93 ±0.29	23.8 ±0.42	264.44 ±14.45	2.3 ±0.5	5.6 ±0.01	5.2 ±0.03	4.6 ±0.02
Btot	63.33 ±0.69	3.12 ±0.16	7.04 ±0.09	2.13 ±0.02	27.30 ±1.01	0.78 ±0.01	7.28 ±0.13	4.85 ±0.14	11.69 ±0.28	12.38 ±0.30	168.4 ±1.89	1.54 ±0.01	4.6 ±0.01	4.8 ±0.01	4.0 ±0.01
Kh T	79.36 ±0.10	3.10 ±0.05	6.76 ±0.03	1.41 ±0.01	47.91 ±2.78	0.59 ±0.02	5.90 ±0.03	4.16 ±0.11	11.16 ±0.31	21.51 ±0.53	252.69 ±5.05	1.26 ±0.01	5.3 ±0.01	4.2 ±0.02	4.0 ±0.02
Rjri	63.60 ±2.77	3.17 ±0.11	6.30 ±0.2	2.2 8 ± 0.06	45.22 ±0.56	1.04 ±0.04	6.98 ±0.22	4.29 ±0.17	9.87 ±0.31	17.29 ±0.60	189.9 ±2.18	1.15 ±0.01	5.0 ±0.01	4.3 ±0.02	4–3 ±0.02
Pddr	28.89 ±0.53	1.82 ±0.01	8.28 ±0.05	1.55 ±0.01	13.0 ±0.47	0.59 ±0.01	5.67 ±0.04	4.07 ±0.15	6.8 ±0.10	17.87 ±0.17	113.07 ±0.73	1.27 ±0.01	5.2 ±0.01	4.0 ±00	4.0 ±00
P>F	303.49*	17.50*	62.38*	47.05*	303.49*	107.11*	185.09*	239.98*	70.75*	133.07*	210.63*	42.55*	37.59*	43.11*	13.35*

*denotes significant difference at p = 0.05, Fcritical = 1.58.
PH = plant height, IL = internode length, LL = leaf length, LW = Leaf width, L/OS = leaves per offshoot, PL = petiole length, In L = Inflorescence length, In/OS = no. of inflorescence per offshoot, W/In = no. of whorls per inflorescence, F/W = no. of flowers per whorl, F/In = no. of flowers per inflorescence, Ca = Calyx tube length, Co = Corolla tube length, St = style length and Fl = filament length, -=absent/reduced anther

Khellaini Top, Batote, Dhansar, Phalata and Laddan) populations from sub-tropical and temperate regions, Group 2 (green) and 3 (yellow) contains a lone population from Chauri and Paddar and group 4 ensembles 5 populations again in two sub-clusters i.e A and B with first one (A) containing 3 (Bawa-da-talab, Chack Hira and Sanjhi-morh) and 2 (Panthal and Ranbir Canal) populations of sub-tropical zone. The Redundancy analysis revealed that 47.4% of the variation was explained by axis 1 (RDA 1) whereas 2.5% of the variation in the data was described by axis 2 (RDA2) respectively (Fig. 4). The variables that explained more than 40% variation in the data include plant height (PH, r = 0.96), number of flowers per inflorescence (FI, r = 0.99) and number of whorls

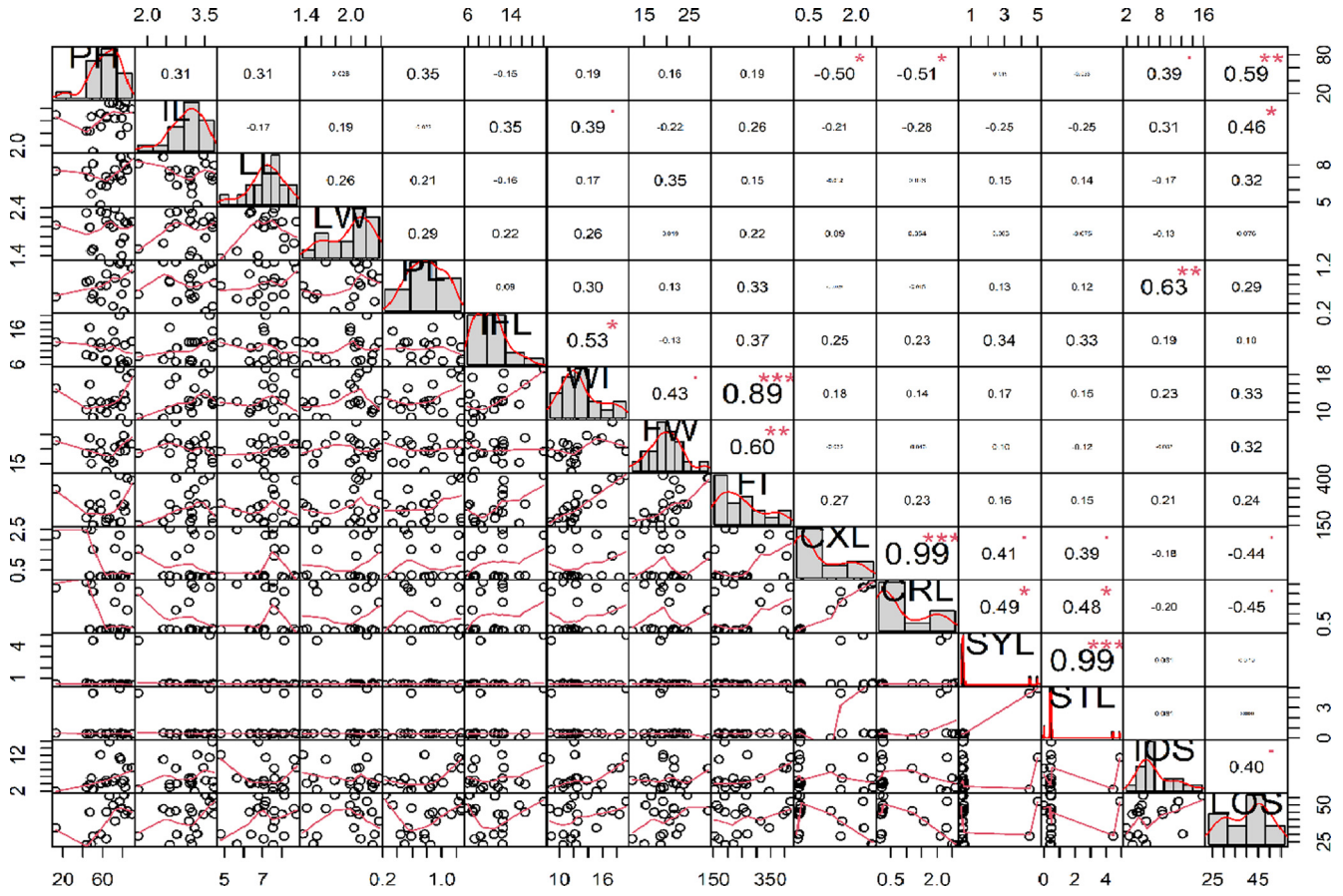

Fig. 2. Correlation plot between the studied morphological variables. The lower half denotes the scatter plot, the upper half the correlation coefficient along with the statistical significance and the middle diagonal the distribution of variables. Statistical significance - $p < 0.001^{***}$ $p < 0.01$ ** and $p < 0.05$ *

per inflorescence (WI, r = 0.64) (Supplementary file 2), with flowers number per inflorescence (FI) as the variable contributing maximum towards phenotypic variation as evident from smoothened surface plot (Supplementary file 3).

3.3. Molecular analysis using inter-simple sequence repeats

From 18 initially screened primers, only 11 yielded unambiguous and reproducible bands which lead to amplification of 121 loci in 57 samples. All 121 loci obtained were polymorphic with percentage polymorphism being 100, reflected a rich diversity of alleles in different samples. ISSR profile obtained for different populations of *M. longifolia* is shown in Fig. 5. The values of the different parameters such as *Na*, *Ne*, *H*, *I*, PL and PPL (Supplementary file 4) revealed that among studied populations, highest values of *Na* (1.0341), *Ne* (1.3810), PL (56), PPL (38.10%), *H* (0.1693) and *I* (0.2425) were witnessed in Chauri whereas lowest values of these parameters (*Na* = 1.0272, *Ne* = 1.3048, PL = 5, PPL = 3.4, *H* = 0.01 51 ± 0.0808 and *I* = 0.0217) were exhibited by Batote population. *Gst* (genetic differentiation) and *Nm* (gene flow) figured as 0.6852 and 0.2297 respectively. The higher values of *Ht* (0.3230) lower *Hs* (0.1017) values (Supplementary file 5) revealed high genetic diversity among populations and low within population.

Highest genetic distance (0.4742) was recorded between Chack Hira (Jammu) and Ramgarh (Ramban) whereas its lowest value (0.1307) was witnessed between Panthal (Reasi) and Phalata (Udhampur) populations. Results of AMOVA depicted high genetic diversity among populations (64%) and low but significant (36%) within populations (Supplementary file 6).

3.4. Statistical analysis

Based on the molecular data, the above studied populations were clustered into five groups (Fig. 6) with Group 1 (dark green) encompassing 8 populations in two sub-clusters i.e A and B with former containing 2 sub-tropical (Akhnoor and Sanjhi-morh), latter including 6 sub-tropical as well as temperate (Panthal, Phalata, Nagbani, Bawa-da-talab, Rajouri and Khellaini Top) populations. Group 2 (blue) again bifurcates into two sub-clusters i.e A and B with sub-cluster A encompassing a lone population from Ranbir Canal (growing in stressed condition) and sub-cluster B having 5 sub-tropical populations (Garden, Chak Hira, Sangoor, Dhansar and Laddan). Group 3 (light green) contained a single population from Chauri, Group 4 (brown) incorporates 3 temperate populations (Batote, Ramgarh and Ramban) and Group 5 (pink) again includes a lone population from Chauri. RDA based on molecular data reveals that first RDA axis (RDA 1) described nearly 9% of the variation, while second RDA axis (RDA 2) explained about 8.5% of variation in the molecular dataset (Fig. 7).

The examination of the loci contribution revealed that 15 loci (A4, A8, A32, A39, A42, A46, A48, A52, A53, A64, A85, A87, A89, A109 and A122) explained more than 40% variation in the data (Supplementary file 7), with A42 (r = 0.7103) having maximum contribution towards genetic diversity as revealed by smoothened surface plot of RDA (Supplementary file 8).

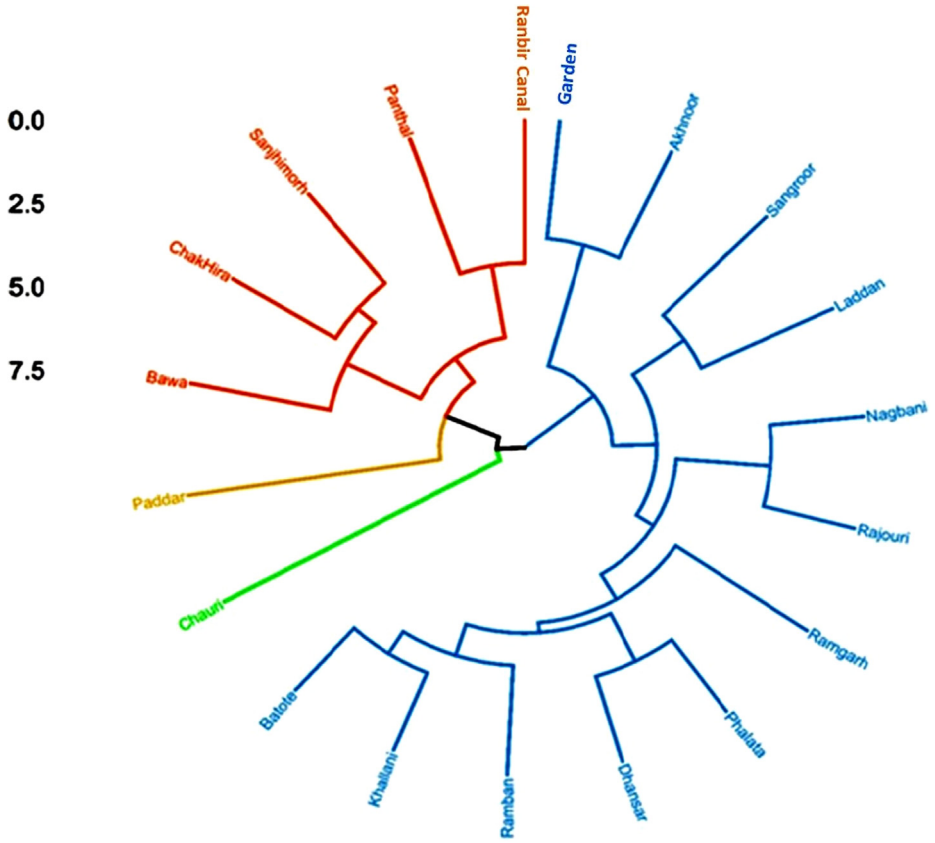

Fig. 3. Cluster dendrogram of the 19 studied populations based on morphological variables.

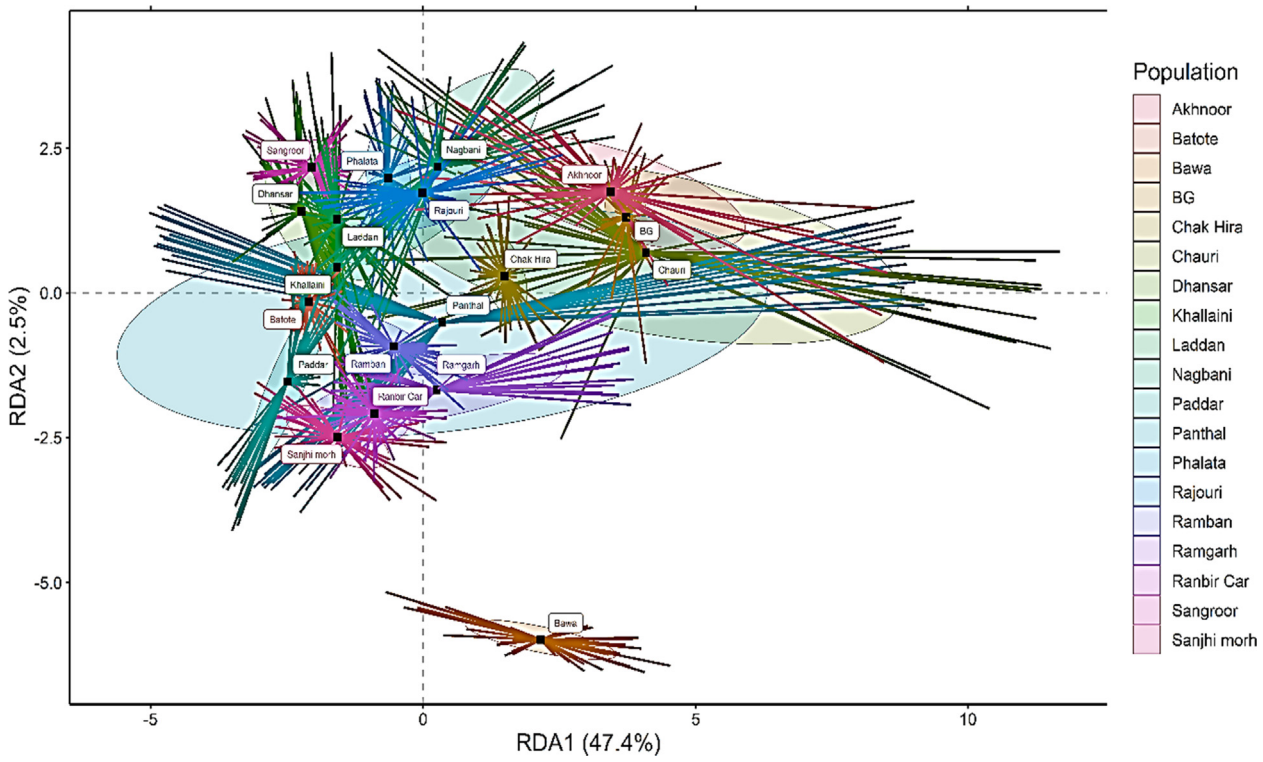

Fig. 4. RDA plot of the 19 studied populations based on morphological variables.

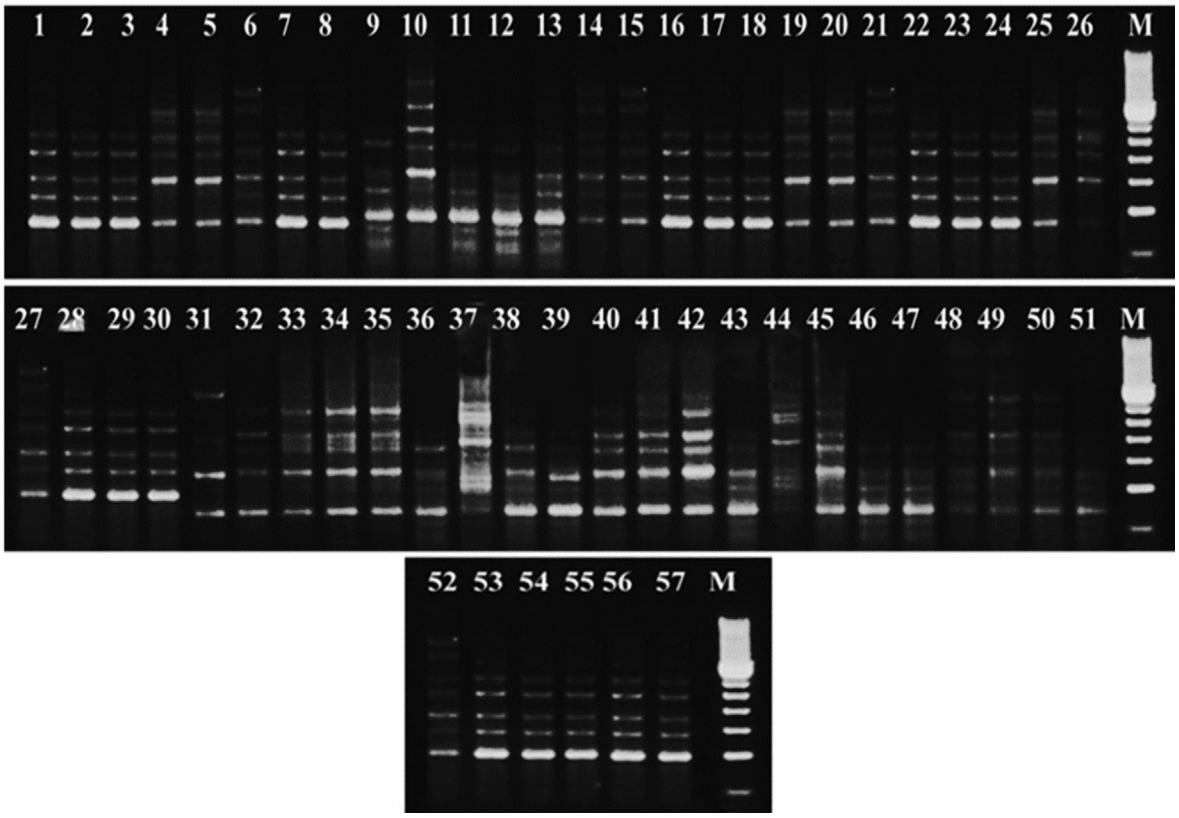

Fig. 5. ISSR marker profile generated by primer 18. l = 250 bp DNA ladder, Thermo Fisher); Lanes 1–24 (Dnsr, Lddn, ChkH, Sngr, RnbC, Botg, Chri, Rmbn), 25–48 (Pnthl, Ngb, Phlta, Rjri, Snjmr, Bawa, Aknr, KhT) and 49–57 (Pddr, Rmgr, Btot).

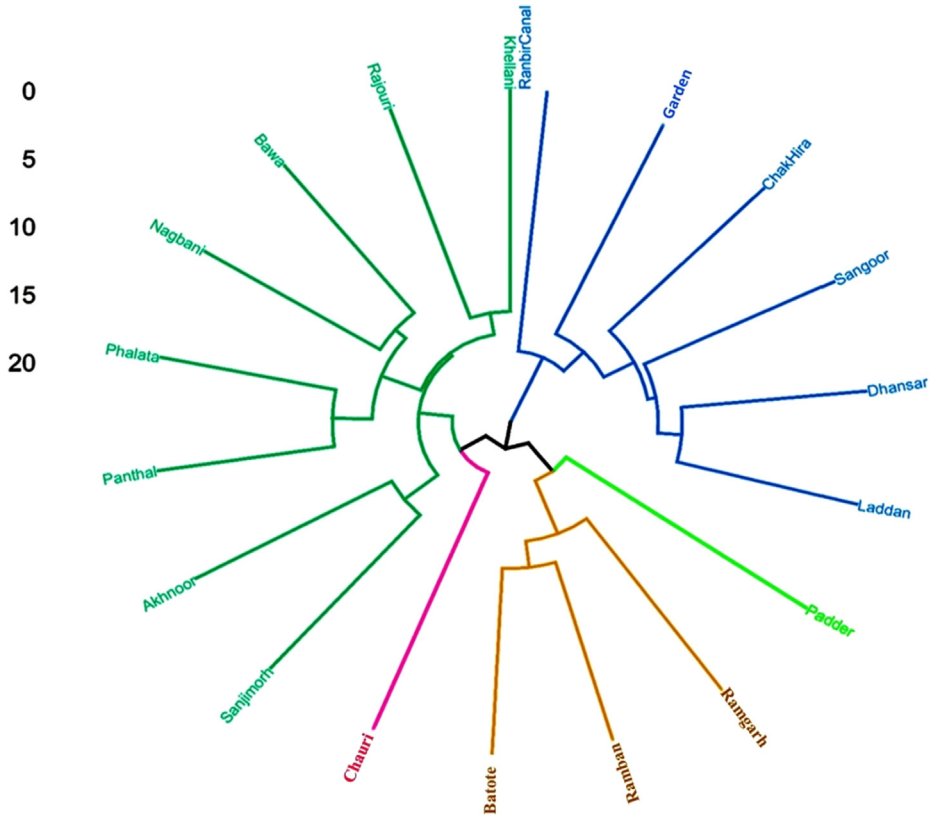

Fig. 6. Cluster dendrogram of the 19 studied populations based on molecular variables.

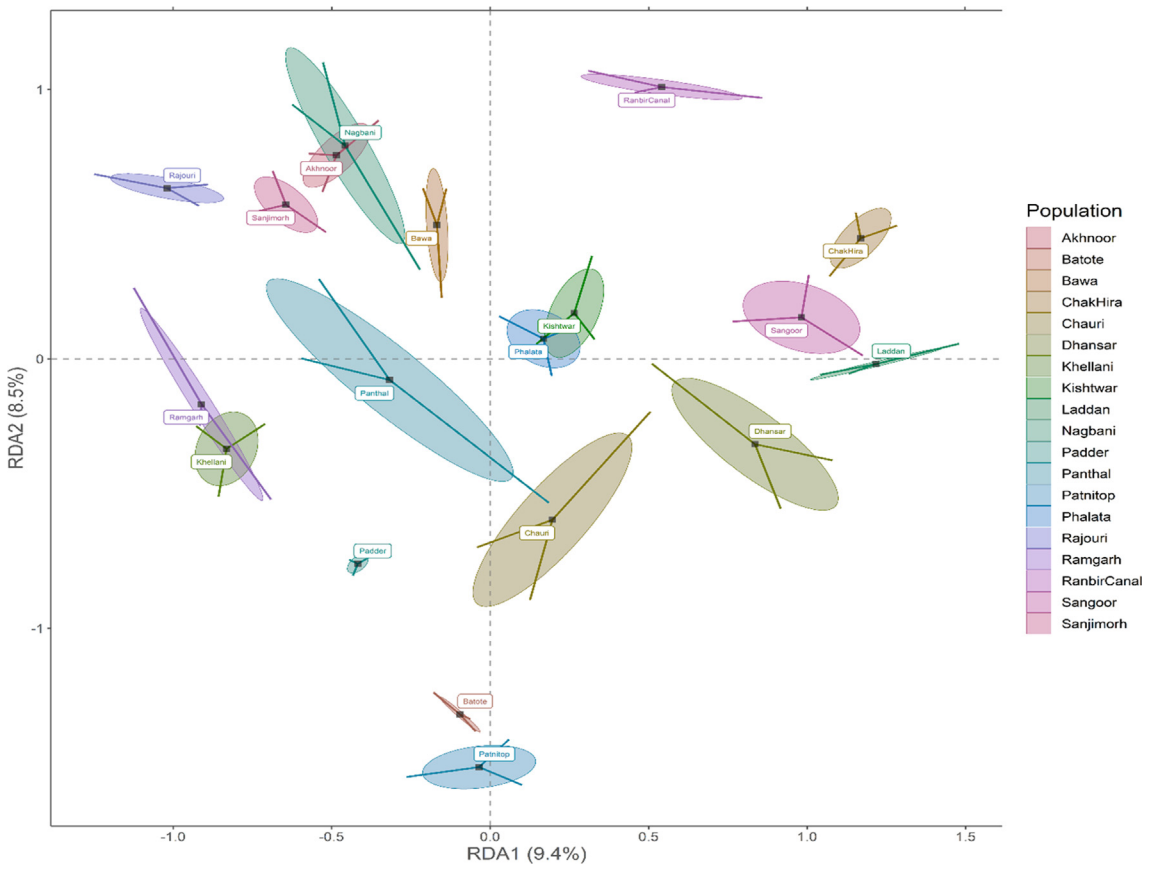

Fig. 7. RDA plot of the 19 studied populations based on molecular data.

3.5. Genetic structure

The relationship among *Mentha* populations as revealed by clustering is somewhat in line with their geographical distances and climatic similarity. Genetic distance of some of the populations showed slight positive correlation with their geographic distance (Mantel test; Supplementary file 9) which means populations from the closely located geographic regions were placed in the same cluster (e.g. Panthal and Phalata; Bawa-da-talab and Nagbani) whereas some populations from closely distanced regions were placed in different clusters [e.g. Pnthal and Dhansar (Reasi); Phalata and Sangoor (Udhampur) and vice-versa (Rajouri and Khellaini Top)].

4. Discussion

Assessment of intra-specific morphological characterization is important in studying the genetic diversity and phenotypic variability within a species and is indicative of taxonomic heterogeneity and evolutionary mechanism (Blinova, 2012; Shinwari et al., 2011). In *M. longifolia*, morphological and molecular techniques have been proved more useful for tagging desired parents for breeding purposes in order to produce elite genotypes with more yield and high quality of oil (Yaghini et al., 2020). During the present studies large amount of morphological variability was witnessed among the studied populations. As these traits are of agro-economic importance, plants exhibiting these can be considered more vigorous and can be implicated in various breeding programmes. The high morphological and genetic diversity during the present study reflects towards the role of environmental and genetic factors in controlling these traits.

Clustering analysis based on UPGMA revealed 4 and 5 major clusters in morphological and molecular datasets, respectively with populations growing in similar climatic zone may/may not fall in same cluster, however, phenotypically and/or genetically distinct populations were placed in separate clusters. The sampled individuals from Chauri and Paddar exhibiting higher phenotypic variation were grouped into two distinct cluster (Fig. 3) on account of former having distinct topography with extreme temperature gradient along the slope and latter having its location in an altogether distinct habitat having different climatic conditions with sub-zero degree temperature in most part of the year due to heavy snowfall. As this population inhabits higher reaches large geographic and climatic barrier may pose restriction in gene flow exchange with other populations, thus increasing its uniqueness. It is possible that environmental barriers can lead to species isolation which may result into species diversification and accumulation of infraspecific geneaology which can trigger allopatric speciation (Španiel et al., 2017). Besides, hierarchical clustering based on molecular data also set apart Chauri and Paddar population from the rest, thus authenticating their genetic distinctness. Furthermore, the low genetic diversity witnessed in Batote is probably because of same selection pressure operating over a large geographic area which prevents accumulation of intrapopulation variability due to similar environment.

While RDA 1 explains 47.4% variation, RDA 2 accounts for 2.5% of the total phenotypic variation with flower per inflorescence (FI) being the most contributing phenotypic variable (Fig. 4), thus explaining the flexibility of this vegetative trait in different envi-

ronments. Zeinali et al. (2004) while analyzing the morphological characters of twelve Iranian mint accessions put forth leaf length, an important trait and store house of essential oil as the most contributing variable (53%) towards phenotypic variability.

During the present study, a high degree of genetic polymorphism (100%) was detected in *M. longifolia*. This is in accordance with the findings of Panjeshahin et al. (2018) and Araghi et al. (2019) for different Iranian horsemint accessions. Similarly, RAPD analysis of 14 NCGR (National Clonal Germplasm Repository) accessions of *M. longifolia* revealed considerable genetic variation among them (Vining et al., 2005). Based on overall Nei's genetic diversity (H = 0.3230) and Shannon's information index (I = 0.4901), considerable amount of genetic diversity was witnessed in the present studied *M. longifolia.* Compared to present study, low values of these indices were noted in *M. longifolia* accessions from Iran (Panjeshahin et al., 2018). Further, high value of Gst (Gst = 0.6852 greater than 0.15) obtained in present study indicates high genetic differentiation between populations and low *Nm* (i.e 0.2297 < 1) values signify low gene flow leading to distinctness and uniqueness among populations. The average gene flow (Nm) values obtained in 8 *M. longifolia* populations from Iran figured as 0.625 with medium gene exchange between these populations (Panjeshahin et al., 2018).

AMOVA revealed that though among population genetic diversity was more (64%), less but significant (36%) proportion of this was due differences within individual population, an indicator towards the species exhibiting both self- and cross-pollination (Soilhi et al., 2020). Further field experiments on self and cross pollination vs seed set revealed seed formation in both the cases, pointing towards mixed type of mating system operating in the species. These results are supported by the findings of Nazem et al. (2019). On contrary, more variance within populations (59%) and less among populations (41%) were put on record by Panjeshahin et al. (2018) in 8 Iranian horsemint accessions. Patchy distribution of the populations by increasing their isolation and restricting reduces gene flow aids in more genetic differentiation (Salama et al., 2019). Yaghini et al. (2020) while analyzing ISSR banding patterns of Iranian *M. spicata* and *M. longifolia* recorded high degree of polymorphism (98.71%).

Results of Redundancy Analysis reveals that RDA axis 1 and 2 while explaining 9.4 and 8.5% of the total variation also put forth locus A42 as the most contributing variable for genetic diversity. Therefore, one can assume the probable role of this locus in regulating the most contributing phenotypic trait i.e flower number per inflorescence. The strong positive correlation between stamen (STL) and style lengths (SYL) in Pearson's correlation plot further indicate selfing being promoted over outcrossing as reproductive phases are simultaneous as well as physically overlapping.

Although closely located (within 2Km), Laddan and Sangoor exhibited more genetic distance (0.2648) as compared to Rajouri and Khellaini Top with a genetic distance of 0.2548 and separated by a geographic distance of 302 Km (Supplementary file 10). The low value of genetic distance in the latter pair (Rajouri and Khellaini Top) can be attributed to gene flow by migration causing genetic exchanges and thus changes in gene frequency whereas in former pair (Laddan and Sangoor), these results can be attributed to differential selection at two sites due to different selection pressures. Although the populations growing in Phalata and Sangoor were close to each other, they existed as single large clusters with no plants growing in between them. A similar kind of results between physical and genetic distance some of the genotypes from similar geographic region were grouped in same cluster while others were not were documented by Panjeshahin et al. (2018) and Bahmani et al. (2012).

Because of *M. longifolia* exhibiting high genetic diversity in Jammu province, this species is capable of establishing and proliferating at different altitudinal ranges (270 to 2275 amsl) and climatic zones (sub-tropical and temperate) which is testimony of their evolutionary adaptability. Besides, plants growing in Akhnoor, Nagbani and Chauri because of producing more herbage, can be selected for domestication or using for breeding purpose. The high genetic diversity in studied horsemint accessions can be attributed to selfing and inbreeding leading to adaptation to a specific geographic region in the absence of gene flow making all the populations unique.

5. Conclusions

The present study indicates rich morphological as well as allelic diversity in wild *M. longifolia.* Besides having a high degree of variation among population, genetic diversity pointing towards the selfing nature of the species, a significant proportion of within population genetic diversity has been observed indicating towards the occurrence of certain degree of outcrossing. Therefore, the species exhibits mixed type of mating system. Most of the studied populations exhibiting morphological variability also came out to be genetically distinct. It points out that the variation is under greater genetic control, though the influence of environmental factors in its manifestation cannot be neglected. This high genetic diversity could be applied in various breeding and conservation programmes of this high value medicinal and aromatic plant species. During the present study, as ISSR technique was found to be highly reproducible, discriminative and reliable requiring small amounts of genomic DNA. Therefore, they can be used for crop improvement, marker-assisted selection, gene mapping as well as QTL analysis of mint species.

6. Consent to participate

All authors consent to participate in the manuscript publication

7. Consent for publication

All authors approved the manuscript to be published

8. Availability of data and material

The data supporting the conclusions of this article are included within the article. Any queries regarding these data may be directed to the corresponding author.

Author contributions

GS and SV designed the research objectives; IAW helped in Methodology and Project administration; AD and TI conducted experiments and wrote the manuscript., AN and HD revised the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

All authors are thankful to the Head of Department of Botany, BGSBU (Baba Ghulam Shah Badshah University), Rajouri for providing necessary Lab facilities. Thanks are due to UGC (University Grants Commission), New Delhi for providing assistance under DRS SAP II. Besides, first author (Ms. Anjina Devi) is thankful to

CSIR-HRDG, New Delhi for providing Senior Research Fellowship (File no. 09/100(0210)/2018-EMR-I). The authors would like to thank the Deanship of Scientific Research at Taif University for supporting this work through Researchers Supporting Project number (TURSP - 2020/141), Taif University, Taif, Saudi Arabia.

References

Ahmad, I., Ahmad, M.S.A., Ashraf, M., Hussain, M., Ashraf, M.Y., 2011. Seasonal variation in some medicinal and biochemical ingredients in Mentha longifolia (L.) Huds. Pak J. Bot. 43, 69–77.

Araghi, A.M., Nemati, H., Azizi, M., Moshtaghi, N., Shoor, M., Hadian, J., 2019. Assessment of phytochemical and agro-morphological variability among different wild accessions of Mentha longifolia L. cultivated in field condition. Ind. Crops Prod. 140, 111698.

Bahmani, K., Izadi-Darbandi, A., Jafari, A.A., Noori, S.A.S., Farajpour, M., 2012. Assessment of genetic diversity in Iranian fennels using ISSR markers. J. Agric. Sci. 4, 79.

Bhellum, B., Magotra, R., 2012. A catalogue of flowering plants of Doda, Kishtwar and Ramban districts (Kashmir Himalayas), Bishen Singh Mahendra Pal Singh.

Blinova, I., 2012. Intra-and interspecific morphological variation of some European terrestrial orchids along a latitudinal gradient. Russ. J. Ecol. 43, 111–116.

Carl, P., Peterson, B., Boudt, K., Zivot, E., 2013. Econometric tools for performance and risk analysis.

Chambers, H.L., Hummer, K.E., 1994. Chromosome counts in the Mentha collection at the USDA-ARS National Clonal Germplasm Repository. Taxon 43, 423–432.

Choupani, A., Shojaeiyan, A., Maleki, M., 2019. Genetic relationships of Iranian endemic mint species, Mentha mozaffariani Jamzad and some other mint species revealed by ISSR markers. BioTechnologia 100.

Gobert, V., Moja, S., Colson, M., Taberlet, P., 2002. Hybridization in the section Mentha (Lamiaceae) inferred from AFLP markers. Am. J. Bot. 89, 2017–2023.

Harley, R., Brighton, C., 1977. Chromosome numbers in the genus Mentha L. Bot. J. Linn. 74, 71–96.

Heylen, O.C., Debortoli, N., Marescaux, J., Olofsson, J.K., 2021. A Revised Phylogeny of the Mentha spicata Clade Reveals Cryptic Species. Plants. 10, 819.

Ibrahim, H.M., 2017. Assessment of genetic diversity and relationships of five mentha species using RAPD marker. Curr. Sci. Int. 6, 271–277.

Jabeen, A., Guo, B., Abbasi, B.H., Shinwari, Z.K., Mahmood, T., 2012. Phylogenetics of selected Mentha species on the basis of rps8, rps11 and rps14 chloroplast genes. J. Med. Plant Res. 6, 30–36.

Jedrzejczyk, I., Rewers, M., 2018. Genome size and ISSR markers for Mentha L. (Lamiaceae) genetic diversity assessment and species identification. Ind. Crops Prod. 120, 171–179.

Khanuja, S., Shasany, A., Srivastava, A., Kumar, S., 2000. Assessment of genetic relationships in Mentha species. Euphytica 111, 121–125.

Kindt, R., 2020. BiodiversityR: Package for community ecology and suitability analysis.

Kumar, B., Kumar, U., Yadav, H.K., 2015. Identification of EST–SSRs and molecular diversity analysis in Mentha piperita. Crop J. 3, 335–342.

Malik, R.A., Gupta, R.C., Singh, V., Bala, S., Kumari, S., 2017. New chromosome reports in Lamiaceae of Kashmir (northwest Himalaya) India. Protoplasma 254, 971–985.

Mikaili, P., Mojaverrostami, S., Moloudizargari, M., Aghajanshakeri, S., 2013. Pharmacological and therapeutic effects of Mentha Longifolia L. and its main constituent, menthol. Anc. Sci. Life. 33, 131.

Mohammadi, M., Asadi-Gharneh, H.A., 2018. How the morphological properties of Mentha longifolia. (L.) Huds. may be affected by geographical differences. J. Photochem. Photobiol B Biol. 178, 237–242.

Nazem, V., Sabzalian, M.R., Saeidi, G., Rahimmalek, M., 2019. Essential oil yield and composition and secondary metabolites in self-and open-pollinated populations of mint (Mentha spp.). Ind. Crops Prod. 130, 332–340.

Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89, 583–590.

Panjeshahin, Z., Sharifi-Sirchi, G.-R., Samsampour, D., 2018. Genetic and Morphological Diversity of Wild Mint Mentha longifolia (L.) Hudson subsp. noeana (Briq.) Briq.“ in South and Southeastern Iran. JMPB 7, 105–115.

Peakall, R., Smouse, P.E., 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes 6, 288–295.

Salama, A.M., Osman, E.A., El-Tantawy, A.A., 2019. Taxonomical studies on four mentha species grown in Egypt through morpho-anatomical characters and scot genetic markers. Plant Arch. 19, 2273–2286.

Sharma, B.M., Kachroo, P., 1981. Flora of Jammu and plants of neighbourhood.

Shasany, A., Shukla, A., Gupta, S., Rajkumar, S., Khanuja, S., 2005. AFLP analysis for genetic relationships among Mentha species. Plant Genet. Resour. Newsl. 144, 14–19.

Shinwari, Z.K., Sultan, S., Mehmood, T., 2011. Molecular and morphological characterization of selected Mentha species. Pak. J. Bot. 43, 1433–1436.

Singh, P., Kumar, R., Prakash, O., Kumar, M., Pant, A., Isidorov, V., Szczepaniak, L., 2017. Reinvestigation of Chemical Composition, Pharmacological, Antibacterial and Fungicidal Activity of Essential oil from Mentha longifolia (L.) Huds. Res. J. Phytochem. 11, 129–141.

Sobti, S., 1971. Interspecific Hybrids in the Genus Mentha. Cytologia 36, 121–125.

Soilhi, Z., Trindade, H., Vicente, S., Gouiaa, S., Khoudi, H., Mekki, M., 2020. Assessment of the genetic diversity and relationships of a collection of Mentha spp. in Tunisia using morphological traits and ISSR markers. J. Hortic. Sci. Biotechnol. 95, 483–495.

Španiel, S., Marhold, K., Zozomová-Lihová, J., 2017. The polyploid Alyssum montanum-A. repens complex in the Balkans: a hotspot of species and genetic diversity. Plant Syst. Evol. 303, 1443–1465.

Srivastava, D.K., Saggoo, M.I.S., 2018. Morpho-meiotic study in Mentha longifolia from cold desert regions of Lahaul-Spiti and adjoining areas of Himachal Pradesh (India). Acta Biol. Szeged 62, 131–139.

Vining, K., Zhang, Q., Tucker, A., Smith, C., Davis, T., 2005. Mentha longifolia (L.) L.: a model species for mint genetic research. HortScience 40, 1225–1229.

Wang, C., Li, G.-R., Zhang, Z.-Y., Peng, M., Shang, Y.-S., Luo, R., Chen, Y.-S., 2013. Genetic diversity of castor bean (Ricinus communis L.) in Northeast China revealed by ISSR markers. Biochem. Syst. Ecol. 51, 301–307.

Yaghini, H., Sabzalian, M.R., Rahimmalek, M., Garavand, T., Maleki, A., Mirlohi, A., 2020. Seed set in inter specific crosses of male sterile Mentha spicata with Mentha longifolia. Euphytica 216, 1–14.

Zeinali, H., Arzani, A., Razmjo, K., 2004. Morphological and essential oil content diversity of Iranian Mints (Mentha spp). Iran. J. Sci. Technol. Trans A Sci. 28, 1–9.