Morphometric and Microsatellite Diversity in Seabuckthorn (*Hippophae rhamnoides* L.) Natural Populations Originating from the Different Geographical Regions of the Indian Himalayas

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Abstract

Seabuckthorn (*Hippophae rhamnoides* L.), an upcoming superfood plant, has attracted researchers' attention worldwide for its medicinal, nutritional, and socio-economic value, along with its characteristic features to sustain extreme climatic conditions. We have studied microsatellite marker-based genetic and morphometric diversity in 93 collections of *H. rhamnoides* from different geographic sites representing two regions, namely Leh and Lahaul of the Indian Himalayas. Microsatellite markers were isolated using two different approaches, including screening of microsatellite-enriched genomic library, and in silico screening of in-house developed seabuckthorn EST database and whole transcriptome assembly. In Leh and Lahaul collections, 32 and 30 microsatellite markers were found polymorphic, respectively. All the markers developed for *H. rhamnoides* showed cross-species transferability to *H. salicifolia* and *H. tibetana*. Two to six alleles were recorded in the two sets of collections with an average of 3.71 and 3.53 alleles per locus in Leh and Lahaul collections, respectively. Mean polymorphic information content (PIC) values for microsatellite markers were 0.39 and 0.41 for Leh and Lahaul collections, respectively. The average expected heterozygosity was less than the observed heterozygosity. Wright's fixation index (F_{IS}) varied from (–)0.2045 to 1.0 and (–)0.1688 to 1.0 for Leh and Lahaul collections, respectively. Shannon's informative index (I) remained in the range of 0.6745 to 1.8621, and 0.6824 to 1.6308 for Leh and Lahaul collections, respectively. The UPGMA-based combined dendrogram showed clear demarcation between Leh and Lahaul collections, although a few ecotypes were regrouped with collections from the other region. No significant relationship was observed between the morphological distance matrix and molecular marker distance matrix. The findings of the present study may prove helpful in future breeding and conservation strategies aiming for seabuckthorn improvement.

Keywords Genetic diversity · *Hippophae rhamnoides* · Microsatellites · Molecular markers · Morphometry · Seabuckthorn

Key Message

- We report microsatellite marker-based genetic and morphometric diversity in 93 collections of seabuckthorn representing different geographic sites in the Indian Himalayas.
- In Leh and Lahaul collections, 32 and 30 microsatellite markers were found polymorphic.
- The UPGMA-based combined dendrogram showed clear demarcation between Leh and Lahaul collections.
- Our findings are useful for seabuckthorn breeding and conservation programs.

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Introduction

Seabuckthorn (*Hippophae* sp.; family Elaeagnaceae), is a multipurpose, outcrossing plant distributed in geographically diverse regions of Euro-Asia, including the Indian Himalayas. In addition to being a traditional source of fodder and food, seabuckthorn holds immense potential in curing many human diseases and imparting many health benefits (Wang et al. 2021). In ancient traditional medicine systems, seabuckthorn products have been used extensively in treating a variety of ailments such as bowel irregularities, gastric ulcers to skin infections, and influenza (Korekar et al. 2011; Suryakumar and Gupta 2011; Wang et al. 2021). The medicinal properties of seabuckthorn are attributed to the presence of a variety of secondary metabolites, i.e., different terpenoids, flavonoids, phenolics, fatty acids, amino acids, vitamins, etc., in different

parts of the plant (Zeb 2004; Suryakumar and Gupta 2011; Fatima et al. 2012). Many of the pharmacological benefits of different seabuckthorn preparations have been clinically established, including anti-tumor, immunomodulatory, anti-carcinogenic, anti-atherogenic, and anti-microbial activities, and radio-protective properties (Zeb 2004, 2006; Suryakumar and Gupta 2011). Recently, researchers have identified probiotic bacteria from fermented seabuckthorn berries, which are known to inhibit coronavirus multiplication by repressing the activation of purines (Fanos et al. 2020). Considering overall benefits, seabuckthorn is now being considered as a potential superfood worldwide (Lentzou et al. 2020).

In addition to immense nutritional and medicinal value, seabuckthorn also plays a role as an effective ecosystem restorer and has been used to improve the nutritional quality index of the soil due to the presence of *Frankia*, a soil actinomycete in its root nodules (Sharma et al. 2019). Moreover, the ability of seabuckthorn plant to withstand the extreme environmental conditions has also attracted the interest of a large number of researchers to delineate the underlying mechanism of stress tolerance (Guo et al. 2007; Xu et al. 2009; Ghangal et al. 2012; Fatima et al. 2012; Ghangal et al. 2013). Unfortunately, there are very limited studies to document the morphometric and genetic diversity prevailing in the natural resources of this wonder plant. The common morphological variations in the natural populations can be adaptations to compete with other plants for nutrition and to sustain extreme environmental conditions (Yao 1994).

Present-day activities, including increasing tourism, urbanization, and exploitation for economic value, are resulting in the loss of genetic diversity of natural populations. There exists a possibility of a loss of agronomically important alleles. Evaluation of genetic variations existing in diverse Indian ecotypes could be helpful in designing effective conservation and breeding programs.

For a successful breeding program, it is important to decipher the existing natural diversity. Molecular markers provide a useful tool to characterize the extent of existing genetic diversity in a given set of germplasm. Cost-effectiveness, reproducibility, and easy-to-use markers for genotyping are the key criteria for the researchers while selecting a marker system for the proposed study (Erhardt and Weimann 2007; Pratap et al. 2013). For decades, microsatellites have been markers of choice as they qualify the above-mentioned criteria with functional significance. Due to a lack of genome sequence information in seabuckthorn until the recent past, the majority of the earlier reports on the characterization of genetic diversity exploited RAPD, ISSR, ITS, and AFLP markers (Kalia et al. 2011; Ruan et al. 2012; Jain et al. 2013). Of the different marker systems, microsatellite markers being codominant, highly polymorphic, and reproducible have been the markers of choice for diverse applications, including genome mapping, gene

tagging, and characterization of genetic diversity (Grover and Sharma 2016). Availability of limited marker resources especially reproducible ones remains a key challenge for the economically important medicinal plant, seabuckthorn. Furthermore, earlier genetic diversity studies have been mainly conducted on the Chinese seabuckthorn germplasm. Only a few studies have been initiated to assess genetic diversity in seabuckthorn populations growing in the Indian Himalayas (Raina et al. 2012; Srihari et al. 2013). Nevertheless, there have been some meaningful efforts in the recent past to identify molecular markers linked with important plant characteristics (Ruan et al. 2009; Li et al. 2020). Thus, it becomes important to enrich the marker resources with reproducible markers holding the potential to become functional markers as well.

The present study aims to enrich the marker resources with reproducible, robust codominant markers like microsatellites for seabuckthorn. We estimate the utility of these markers derived from genomic library, i.e. anonymous microsatellite markers, markers derived from in-house generated transcriptome data, i.e. genic markers, for the assessment of genetic diversity existing in the collected natural populations, as well as cross transferability to other species, viz. *H. salicifolia* and *H. tibetana*. Genic markers developed in the current study further hold the potential to become functional markers as many of these fall under the coding region(s) of the gene. We further undertake the assessment of these markers for the genetic analysis of morphologically varying seabuckthorn ecotypes collected from 36 different sites in two diverse geographical regions of the Indian Himalayas, namely Leh (Jammu and Kashmir) and Lahaul (Himachal Pradesh).

Materials and Methods

Sample Collection and Morphometric Analysis

Leaf samples were collected from seabuckthorn plants growing in two districts, Leh (Jammu and Kashmir) and Lahaul-Spiti (Himachal Pradesh), known to have an abundance of naturally growing seabuckthorn populations. Twenty-two geographically distinct locations in Leh and 16 locations in Lahaul were shortlisted for collecting plant samples. Each geographical location was at a minimum distance of 5 km from the neighboring location. These locations differ in terms of altitude, water availability, and sunlight exposure. Sample collection sites were geographically tagged using a GPS device (Garmin) and latitude, longitude, and elevation were recorded (Supplementary Tables 1a and 1b).

For the morphometric analysis, data were recorded on site on the 27 morphological characters, including plant habit, branching habit, degree of thorniness, branching of thorns,

leafiness on thorns, stem shape, immature stem color, mature stem color, silvery scales on stem, hardness of mature stem, stellate hairiness on young branch, number of leaves attached at single point, bulging of axillary bud, dorsal leaf surface color, leaf tip shape, upper leaf surface, silver scales on leaves, leaf density, stellate hairs beneath leaves, color of ventral midrib, phyllotaxy, color of leaf on ventral side, and attachment of leaves to stem. The description of these characters is given elsewhere (Singh et al. 2005). Characters like degree of thorniness and leaf density were assessed on the basis of number of thorns per 10 cm of the branch and number of leaves per 10 cm of branch. The mean values of each of the following characters were also recorded as given below:

plant height (cm): height from ground level to topmost portion of the canopy center exactly above the main trunk; canopy width (cm): the maximum canopy diagonal length in a horizontal plane; inter-branch distance (cm): average distance between 5 mature secondary branches from the base of the plant; number of leaves per 10-cm branch: average of number of leaves per 10 cm from 5 different branches; number of thorns per 10-cm branch: average number of thorns per 10 cm from 5 different branches; leaf length (cm): average of length of 10 leaves from the base of secondary branches; leaf width: average width (widest portion of the leaf) of the same leaves used for the measurement of leaf length.

Genomic DNA Isolation

Collected leaf tissue samples were snap-freeze in liquid nitrogen, transported to the laboratory, and stored at -80°C until further use.

The presence of secondary metabolites in leaf tissue samples hampered isolation of good-quality DNA using the traditional CTAB-based DNA isolation method and commercially available kits. To overcome the problem, modifications were made in CTAB-based protocol (Doyle and Doyle 1990) to isolate genomic DNA. Addition of 1.5% polyvinylpyrrolidone (PVPP) and enhanced concentrations of β -mercaptoethanol (0.5%) in CTAB extraction buffer (100 mM Tris-Cl (pH 8.0), 25 mM EDTA, 1.4 M NaCl, 2% CTAB) yielded high-quality genomic DNA.

Primer Designing, Validation of Primers, and Microsatellite Allele Detection

Microsatellite markers developed earlier in our laboratory using different approaches, i.e., (i) EST-SSR markers (Jain et al. 2010); (ii) unigene specific microsatellite markers (USMM; Jain et al. 2014); and (iii) markers developed by sequencing microsatellite-positive clones from microsatellite-enriched libraries (Jain 2015). PCR primers

complementary to flanking regions of microsatellite loci were designed using Primer 3 (Rozen and Skaletsky 2000). The primer designing parameters included annealing temperature between 50 and 60 $^{\circ}\text{C}$ with a maximum permitted difference of 5 $^{\circ}\text{C}$ between the reverse and forward primers of a pair, GC content in the range of 40–60%, primer length in the range of 18–25 bp, and desired amplicon size in the range of 100–500 bp. Primer pairs showing a minimum number of secondary structures were considered for the final synthesis. The details of the microsatellite markers used in the present study are given in Table 1. A PCR with the volume of 25 μL , containing 5 pmol of primer pair each, 100 μM of each of dNTPs, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.5 U of *Taq* polymerase (Kapa2G Robust, Kapa Biosystems), and 50 ng of seabuckthorn genomic DNA, was set up. Amplifications were carried out in Mastercycler (Eppendorf, Germany) with thermal profile kept as 5 min of pre-amplification denaturation at 95 $^{\circ}\text{C}$ followed by 35 cycles of 30 s at 95 $^{\circ}\text{C}$, 30 s at primer-specific annealing temperature, and 40 s at 72 $^{\circ}\text{C}$. As a final step, PCR products were extended for 5 min at 72 $^{\circ}\text{C}$ and stored at 4 $^{\circ}\text{C}$ until further use. Genomic DNA of seabuckthorn (*H. rhamnoides*) collections and other two species, namely *H. salicifolia* and *H. tibetana*, was used as the template DNA for the study. Resulted amplicons were electrophoresed on 4% Metaphor agarose (Lonza, Switzerland) in 1 $\times$ TAE buffer and recorded using a gel documentation system (Alpha Imaging) after ethidium bromide staining. The presence and absence of each allele (band) in the gel images were scored in a binary data format as 1 and 0, respectively.

Statistical Analysis and Genetic Diversity Estimation

Based on the selected morphometric characters and genotyping data generated from 32 microsatellite markers, phylogenetic trees were constructed following the UPGMA method by using DARwin 5.0.158 software (Perrier et al. 2003). To determine allele diversity at each locus, polymorphism information content (PIC) was calculated as per Roldan-Ruiz et al. (2001):

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

where f_i is the frequency of the amplified allele (allele present) and $(1 - f_i)$ is the frequency of a null allele (allele absent) of the i th marker.

Genetic distances were calculated among the collections according to Jaccard's coefficient (Jaccard 1908) using DARwin 5.0.158. The observed heterozygosity (H_o), Nei's average gene diversity (Nei 1973), Wright's fixation index (F_{IS}), and Shannon's informative index (I) were calculated using POPGENE 1.32 software (Yeh and Boyle 1997). The product-moment correlation (r) based on Mantel Z value

Table 1 Details of microsatellite markers used to assess the genetic diversity in Leh and Lahaul collections of seabuckthorn (*H. rhamnoides*)

Primer ID	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Microsatellite motif	Location	Tm (°C)
<i>HrMS 3</i>	GCT CTC ATC CGA TTT GAT CC	GTC GCA GTC TTC TTG GGT TC	(TCA) ₆	CDS	56
<i>HrMS 4</i>	GTT TGA GGT CGG TGC TGA GT	GGG TAA CCC AAC TCC TCC TT	(TC) ₈	3'-UTR	57
<i>HrMS 10</i>	GGA AGC GTG AGG AAA TGT C	GAA CAG ACA GAC CAT TAG AGA AC	(TGG) ₅	3'-UTR	56
<i>HrMS 12</i>	CTC CAT CTC AAT CAT CAC TGC	TTA GGG ATC CGG ATG AAG	(CTT) ₁₁	CDS	58
<i>HrMS 14</i>	ATA CCT AGC TCG GCA ACA AG	ACG ACC CAT GGC ATA ATA GTA C	(TG) ₆ (TA) ₈	3'-UTR	57
<i>HrMS 18</i>	CAA CAT TGT TTC GTG CAG	ACT CAC ATA ATC GAT CTC AGC	(ATG) ₅	3'-UTR	51
<i>HrMS 23</i>	TCA GTT GGT AGG ATG CTT C	ACA CCA CTC TGT CCT CAA TC	(AAAAT) ₄	3'-UTR	53
<i>HrMS 25</i>	GTA CTG TGA CCA CGC TGC	GGG TTC AAA GTA ATG GCA AG	(AG) ₈	3'-UTR	53
<i>HrMS 26</i>	ATG ATG ACG ACG ACA ACG	AGT GGT GGT GAC GAT AGT ATC	(CAC) ₅	5'-UTR	53
<i>USMM 1</i>	GGC GAA ACT TGA CTT GTT GC	ACC GAT CAA TAC CGT TCT GC	(TAC) ₁₆	3'-UTR	55
<i>USMM 3</i>	AAG GAT GTG GTC GAT CCA AG	GTT TGC AGG CAT TCC TTT GT	(TTC) ₁₀	CDS	54
<i>USMM 5</i>	TTC GAT CGG ATA AGG TCA TTG	GCA GTC GAG GAG GTT TGA AG	(AC) ₉ (AT) ₆	CDS	56
<i>USMM 7</i>	TCG CCG TCT GTT TCA GAT AA	GCT GAT CCA ACG GTC TCA TT	(AG) ₁₈	3'-UTR	50
<i>USMM 12</i>	CGT CAC CGT CAG TTC AGA GA	AAG GAG ACG CAG ACG AAG AG	(AAG) ₁₀	5'-UTR	50
<i>USMM 13</i>	GAT ATG CCG GCG GTA AAC TA	TGT ACC ACT TCC TGC TGC TG	(CAG) ₅ (CAC) ₆	CDS	57
<i>USMM 16</i>	CCA ACC AAC CTC ATC GTT TC	ATC GGA GGG ATC GTT GAA AG	(TC) ₁₄	5'-UTR	55
<i>USMM 24</i>	TAG CAT TGC AGG CTC AGA GA	ATC CGT GGT TAA GGT TGC AC	(AG) ₁₁	3'-UTR	55
<i>USMM 25</i>	CGA GGT CCG AGT AGG AAG A	CAT TGG CCT TCA ATC TCC TC	(AAG) ₁₀	CDS-3'	55
<i>USMM 26</i>	TTC CCT CTC CGT GAC AAG TT	GCT TGA GGT GAA GAG GAA AGT C	(CT) ₁₆	3'-UTR	55
<i>USMM 30</i>	GAA AGT CCT CGT CTT CCT CT	AGA GGA AGG GAG ATA GAT GG	(TTCTC) ₆	5'-UTR	54
<i>USMM 31</i>	GTG CAG CAG CAG CTA TTA AG	GTG GAT GCA GTG GAA TAG AC	(TTG) ₈	3'-UTR	53
<i>USMM 34</i>	CAG CAT GAT ACA GAG CTC CA	AGT CAG ATG AGG AAG GCT CT	(TCA) ₆	5'-UTR	57
<i>USMM 66</i>	TGA CCT ACA TTG ACC CTT GG	GTA GGA CTC ACC ACC CAC AT	(CTCCC) ₅	3'-UTR	56
<i>USMM 50</i>	GGT CAG CCA AAG CAG TAA GT	CGT GTT AGG CTA GCT TTA GG	(CTCCTT) ₅	3'-UTR	55
<i>USMMii_2</i>	GTC CAC CAC CAA TCA CTA GA	GAT GAT GGT CCT TGG TCT GA	(TTC) ₆	CDS	55
<i>USMMii_7</i>	GAG AGA TGA ACG GCC TGA TT	GGC CTG ATG GAA CTA AGA AG	(AT) ₅	CDS	55
<i>USMMii_9</i>	GCA GGT CAA GAG AAG TGG TA	GCT ATG TTT CAG CCC TCA AG	(AGT) ₅	CDS	55
<i>USMMii_12</i>	GAT TCC ACC TGA AAC TCC AC	CCT CTC AAG ACC TAA ACT GC	(CT) ₈	CDS	55
<i>GLC 12</i>	ATT CGA AGA AGC AGG GAT A	GCA ATG AAG TCC TTG TTA TC	(GA) ₁₂	Anonymous	54
<i>GLC 2–5</i>	GTG ATG GCC CTA GTT ATA GA	CGT AAT ATC CAA ACA GAC CA	(GA) ₁₃	Anonymous	53
<i>GLC 2–6</i>	GGT ATG TAG ATA AGG CCA CAA	CCA ATG ATA TAA ACC CTC CAC	(CT) ₇	Anonymous	56
<i>GLC 2–14</i>	GAC AAC ACG ACA GAA GAG GT	GTT TAC TAC TAC CAG GCA TGA	(GA) ₁₁	Anonymous	55

(Mantel 1967) was computed using XLSTAT to measure the degree of relationship between matrices produced by morphological and microsatellite data.

Results and Discussion

Abundant populations of seabuckthorn (*H. rhamnoides* L.) are present in the different regions of the Indian Himalayas, including Leh (Jammu and Kashmir) and Lahaul-Spiti (Himachal Pradesh) regions that witness the major distribution of seabuckthorn. Huge diversity is known to exist in these two diverse geographical regions as indicated by previous studies also (Singh et al. 2005). In the current study, we attempted to study the morphometric

and microsatellite-based genetic variation existing in the seabuckthorn natural collections growing in the Leh and Lahaul regions.

Morphometric Analysis of the Seabuckthorn Collections

There were marked differences between the two selected major geographical regions in the current study in terms of various environmental parameters such as annual rainfall (Leh: 80–200 mm; Lahaul: 100–400 mm), temperature variation (Leh: –30 to 35 °C; Lahaul: –18 to 38 °C), and soil texture (Leh: loamy clay; Lahaul: sandy loam), average soil pH (Leh: 7.3; Lahaul: 6.7). In total, 52 plants were selected from 20 different sites in Leh for marker analysis

(Supplementary Table 1a). Similarly, 41 plant samples from 16 different collection sites in Lahaul were collected (Supplementary Table 1b). The morphometric data were recorded on these collections.

The study of twenty-seven different morphological features based on Singh et al. (2005) revealed contrasting variations within and between the two geographical regions. Branching of thorns was observed in all the collections from Leh, whereas it appeared only in 26.8% of the Lahaul samples. Similarly, 92.3% of Leh collections showed excessive leafiness on thorns (more than 9 leaves); however, only 7.3% of Lahaul collections were found to have leafiness on the higher side (7 to 9 leaves); otherwise, leaf distribution on thorns remained sparse. The upper leaf surface of the samples collected from Leh was rough, contrary to the smooth surface observed in the Lahaul collections. Another contrasting feature was the occurrence of silvery scales on leaves, a completely absent feature in the Lahaul collections, in contrast to the presence of scales on the dorsal surface in 71.15% samples and on both the surface in 19.23% samples representing Leh. Silvery scales on stems were observed among 51.92% of Leh collections, whereas only 21.95% of Lahaul collections showed the presence of silver scales. All the above-listed features contributed markedly towards variation in morphological diversity prevailing in collections from Leh and Lahaul regions.

Some morphological characteristics like stem shape, stellate hairiness, young branches, number of leaves attached to a single point, stellate hairs beneath leaves, and dorsal leaf surface color did not reflect any significant variation among collections from Leh and Lahaul regions. The mature stem color in the majority of the collections from both the two major geographical regions was brown. In contrast, for Leh collections, the immature stem was silver, and for Lahaul collections, it was ashy, which could be attributed to the late accumulation of secondary metabolites in Lahaul collections, possibly a geographical area-specific characteristic. Different ordinal categories represented by a number were assigned to twenty morphological features and model values were calculated to assign the characteristic representative qualitative trait. Results obtained in the current study were generally in accordance with the previous studies conducted over collections representing different geographical regions (Singh 2002, 2014; Singh et al. 2005; Mathew et al. 2007). The other seven morphological features could be measured as metric traits, which showed contrasting variations among collections. The average plant height of the Leh population (range 15–180 cm, average 64.57 cm) was significantly shorter than in Lahaul collections (range 50–600 cm, average 216.83 cm). Values observed with the Lahaul *H. rhamnoides* population are in complete accordance with earlier studies conducted by Singh (2002, 2014), reporting

variations in plant height of ecotypes from Himachal Pradesh. A similar trend was observed for leaf length and leaf width. Characteristics like inter-branch distance, degree of thorniness, and leafiness were much higher for Leh collections in comparison to Lahaul collections.

A significant inverse relationship of altitude was observed with plant height and canopy width ($p > 0.001$). No relationship was observed between inter-branch distance and altitude. Leaf length and width showed a negative correlation ($p > 0.001$), whereas the degree of thorniness (number of thorns per 10 cm) and leafiness (number of leaves per 10 cm) were positively correlated with the altitude. Our results support some of the previous studies (Bartish et al. 2000; Korekar et al. 2011; Singh 2002, 2014). However, phenotypic variations existing in population are attributed to genetic background differences and variation in geographical conditions, including altitude, climate, and soil (Li et al. 2020).

Genetic Distance and UPGMA Cluster Analysis Based on Morphological Features

The pairwise genetic distance among pairs of ninety-three seabuckthorn collections was worked out using twenty-seven morphological traits. The average genetic distance among Lahaul collections was 0.57, which remained 0.43 for Leh collections. However, the overall average genetic distance among pairs of total collections was 0.61. The average genetic distance between the Leh and Lahaul collections was 0.75. Some of the samples collected from the same site did not show any genetic distance; a possible reason could be the clonal reproduction of one plant resulting in two distantly growing plants. Seabuckthorn root suckers are known to generate a large number of new plants covering large areas (Lian and Chen 2000). Among Lahaul collections, maximum genetic distance (0.81) was observed between Collection ID Lahaul 22 and 33. On the other hand, among Leh collections, maximum genetic distance (0.79) was observed between Collection ID Leh 22 and 12, and Leh 183 and 21. Overall, Leh 31, 41, and 42 displayed a maximum genetic distance of 0.91 in pair with Lahaul 43. Based on the genetic distance matrix of morphological characters, the ninety-three seabuckthorn collections were grouped into different clusters following UPGMA cluster analysis (Fig. 1). It was interesting to observe that collection from Leh (red; cluster I) and collection from Lahaul (blue; cluster II) formed completely distinct two major clusters reflecting a high level of genetic variations separating two geographical regions. Such categorical morphometric variations could be distinct environmental conditions of the two major geographical regions. It has already been witnessed that morphological variations sometimes are the result of $G \times E$ (Assogbadjo et al. 2005).

Fig. 1 Dendrogram showing relationships among *Hippophae rhamnoides* collections from Leh (red) and Lahaul (blue) derived using hierarchical unweighted cluster analysis of morphological characters. The collections from two geographical regions are clustered in separate groups

Microsatellite Marker Validation and Assessment

Implementing different strategies to isolate microsatellite markers in *H. rhamnoides*, amplifications of 45 microsatellite loci from microsatellite-enriched libraries, 30 from EST database, and 60 from whole transcriptome (randomly chosen) were facilitated using primer pairs complementary to flanking regions of microsatellite sequences. Among anonymous markers from the genomic library, 91.11% of primer pairs showed successful amplification at the respective annealing temperatures. However, while assessing these markers on the natural population collected from Leh and Lahaul, only four microsatellite markers were found polymorphic (Supplementary Fig. 1a, b; Table 2). Only 11.4% of the anonymous markers developed from the

microsatellite-enriched genomic library were polymorphic. Such a lesser frequency of polymorphic markers has been reported in some other plant species also (Crozier et al. 2007; Ma et al. 2007). Considering the outcrossing nature of the *Hippophae* species, the presence of a low number of polymorphic genomic markers remains a point for further investigation.

Sequence resources available in the public domain, especially the genic sequences, offer a cost-effective approach to microsatellite marker development for a variety of applications (Varshney et al. 2005). Our in-house established seabuckthorn EST database (Ghangal et al. 2012) and whole transcriptome assembly (Ghangal et al. 2013) were exploited for the presence of microsatellites yielding 9 and 19 markers, respectively, some of which have been reported earlier

Table 2 Data on genetic diversity parameters of Leh and Lahaul collections of seabuckthorn (*H. rhamnoides*) based on microsatellite markers

Primer ID	Leh collections						Lahaul collections					
	PIC	n	H _o	H _e	I	F _{IS}	PIC	n	H _o	H _e	I	F _{IS}
<i>HrMS 3</i>	0.466	3	0.3462	0.6067	1.0051	0.4294	0.464	3	0.293	0.6353	1.0481	0.5393
<i>HrMS 4</i>	0.422	2	0.3462	0.4815	0.6745	0.2811	0.439	2	0.3171	0.4893	0.6824	0.352
<i>HrMS 10</i>	0.424	3	0	0.6354	1.051	1	0.403	3	0	0.6044	1.011	1
<i>HrMS 12</i>	0.423	3	0.7308	0.6067	1.0051	−0.2045	Monomorphic marker					
<i>HrMS 14</i>	0.5	2	0.0192	0.4998	0.693	0.9615	0.464	3	0.439	0.6368	1.0522	0.3106
<i>HrMS 18</i>	0.486	2	0.1346	0.4954	0.6885	0.7283	0.484	2	0.195	0.4973	0.6905	0.6077
<i>HrMS 23</i>	0.417	3	0	0.6183	1.0263	1	0.401	4	0.122	0.735	1.3546	0.8341
<i>HrMS 25</i>	0.355	5	0.3281	0.7167	1.3739	0.5422	0.387	5	0.378	0.7731	1.5502	0.5113
<i>HrMS 26</i>	0.459	3	0.1154	0.6507	1.0752	0.8227	0.397	3	0.146	0.5473	0.927	0.7326
<i>USMM 1</i>	0.358	6	0.6731	0.7829	1.6517	0.1403	0.33	6	0.415	0.7784	1.6308	0.4673
<i>USMM 3</i>	0.311	6	0.2308	0.7807	1.6457	0.7044	0.326	6	0.4	0.7546	1.5792	0.4699
<i>USMM 5</i>	0.246	8	0.3077	0.8155	1.8621	0.6227	0.486	4	0.617	0.7417	1.3692	0.1681
<i>USMM 7</i>	0.453	5	0.7091	0.7821	1.5583	0.0934	0.381	5	0.273	0.7678	1.5534	0.6448
<i>USMM 12</i>	0.414	2	0.4038	0.4954	0.6885	0.1848	0.482	2	0.146	0.4926	0.6857	0.7029
<i>USMM 13</i>	0.496	2	0.0769	0.4993	0.6924	0.8459	0.482	2	0.122	0.4893	0.6824	0.7508
<i>USMM 16</i>	0.416	3	0.2097	0.5909	0.9872	0.6452	0.431	3	0.293	0.6139	1.0211	0.5233
<i>USMM 24</i>	0.339	6	0.4107	0.7878	1.6481	0.4786	0.383	3	0.562	0.6131	1.0065	0.0825
<i>USMM 25</i>	0.413	4	0.25	0.7132	1.3091	0.6495	0.396	4	0.171	0.7127	1.2946	0.7604
<i>USMM 26</i>	0.426	3	0.3594	0.6439	1.0618	0.4419	0.461	3	0.51	0.657	1.0836	0.2235
<i>USMM 30</i>	0.43	3	0.1346	0.613	1.0127	0.7804	0.482	3	0.341	0.6422	1.059	0.4683
<i>USMM 31</i>	0.355	4	0.0192	0.7228	1.332	0.9734	0.381	4	0.073	0.7207	1.319	0.8985
<i>USMM 34</i>	0.357	4	0.2308	0.6738	1.2484	0.6575	0.362	4	0.439	0.6344	1.1163	0.308
<i>USMM 66</i>	0.44	3	0.0962	0.6233	1.0364	0.8457	0.399	4	0.244	0.7032	1.2868	0.6531
<i>USMM 50</i>	0.412	3	0	0.6183	1.0263	1	0.422	4	0.386	0.7229	1.3268	0.4655
<i>USMMii_2</i>	0.414	3	0.2656	0.5851	0.976	0.546	0.271	3	0.605	0.5173	0.8362	−0.1688
<i>USMMii_7</i>	0.207	4	0.1731	0.4673	0.8795	0.6296	Monomorphic marker					
<i>USMMii_9</i>	0.416	3	0.4808	0.5516	0.9343	0.1284	0.442	3	0.195	0.6095	1.0024	0.6798
<i>USMMii_12</i>	0.37	4	0.1923	0.6428	1.1437	0.7008	0.42	3	0	0.6044	1.011	1
<i>GLC 12</i>	0.45	3	0.3167	0.6099	1.0009	0.4808	0.469	3	0.3542	0.6196	1.0182	0.4284
<i>GLC 2–5</i>	0.314	5	0	0.7862	1.573	1	0.428	3	0	0.6425	1.0612	1
<i>GLC 2–6</i>	0.317	6	0.25	0.7796	1.6245	0.6793	0.379	5	0.341	0.7686	1.516	0.5557
<i>GLC 2–14</i>	0.427	3	0	0.6398	1.0566	1	0.385	4	0.219	0.6874	1.2623	0.6807

by Jain et al. (2010, 2014) on limited genotypes. On screening microsatellite markers derived from the EST database on seabuckthorn collections, nine primer pairs were polymorphic in *H. rhamnoides* collections from Leh comparative to eight polymorphic primer pairs in Lahaul collections (Table 2). Similarly, nineteen microsatellite markers derived from unigenes of the whole transcriptome were polymorphic in Leh collections, and eighteen were polymorphic in Lahaul collections. Interestingly, two microsatellite markers, viz. HrMS12 and USMMii_7, monomorphic in Lahaul collections, were polymorphic in Leh collections. This observation could be attributed to the fixation of alleles as an adaptive characteristic for a particular environmental condition specific to a geographical area. Interestingly, microsatellite repeat in one of the markers was located in the CDS region. Thus, there exists a possibility of microsatellite loci being involved directly in imparting some functional role to cope up with specific environmental conditions.

In total, 35.44% of genic microsatellite markers were found polymorphic, pointing towards a better marker development efficiency in the present research compared to previous studies (Tang et al. 2009; Salem et al. 2010; Grover et al. 2012). Variation in allelic size in genic regions to sustain in different climatic conditions could be the possible reason behind the variations.

Like previous studies (Jain et al. 2010, 2014), markers from enriched genomic libraries showed cross-amplification and transferability in a limited collection of *H. salicifolia* (five) and *H. tibetana* (four) collected from DIHAR, Leh. All the primer pairs that produced amplification in *H. rhamnoides* cross amplified in *H. salicifolia* and *H. tibetana*. The cross transferability phenomenon observed in *Hippophae* species in our study demonstrates how to develop microsatellite markers for the species with no prior sequence information. Thus, these markers can be further explored for cross-species and cross-family transferability.

Diversity Analysis of Seabuckthorn Collections Using Microsatellite Markers

Occurrence of Microsatellite Alleles

Substantial variation in microsatellite allelic polymorphism was observed as the size of amplicons varied from 120 to 490 bp in the different collections. In total, 119 alleles, with an average of 3.71 alleles per locus from 32 microsatellite markers, were observed for Leh collections (Table 2). In comparison, 106 alleles, with an average of 3.53 alleles per locus, were recorded from 30 microsatellite markers in Lahaul collections (Table 2). Overall, the number of alleles varied from two to six in the current study. The number of alleles revealed in the present

study was relatively lower compared to another study conducted with microsatellite markers by Wang et al. (2008) in seabuckthorn (*H. rhamnoides*), where the number of alleles varied from 3 to 12 per locus. However, our results supported Li et al. (2017), who reported 2–5 alleles for RNA-Seq SSR markers. Such variations in allele frequency for microsatellite markers in different plant species have been attributed to the genetic differences and sampling strategies of the material under study.

Lacis and Dombrovskia (2014) showed the presence of 4 to 22 alleles averaging 10.25 alleles per locus on Latvian seabuckthorn applying microsatellite markers developed by Wang et al. (2008). However, on Indian seabuckthorn collections, these markers showed the presence of 2 to 4 alleles in the population averaging 2.83 alleles per locus (Srihari et al. 2013). A lower number of alleles in our study and in Srihari et al. (2013) could be a limitation of gel-based genotyping; higher resolution could be expected in genome analyzer-based studies like that of Wang et al. (2008). Similar results were obtained when some of these markers were assessed on seabuckthorn collection from the Karakoram region (Nawaz et al. 2018).

Polymorphism Analysis Using Microsatellite Markers

In Leh collections, polymorphism information content (PIC) values varied from 0.20 (USMMii_7) to 0.50 (HrMS14) with a mean of 0.39, whereas these values ranged from 0.27 (USMMii_2) to 0.48 (USMM5) with the mean of 0.41 in Lahaul collections (Table 2).

Srihari et al. (2013) reported PIC to vary from 0.23 to 0.73 with a mean value of 0.54 among Indian seabuckthorn. Based on PIC values, markers could be classified into three categories: highly informative (PIC > 0.5), moderately informative (0.25 < PIC < 0.5), and less informative (PIC < 0.25) (Yadav et al. 2011; Xu et al. 2012). Only a single microsatellite marker, USMMii_7, was found in the less informative category in the Leh population, and the rest of the markers exhibited moderate to high levels of PIC. PIC values are known to be impacted by sampling size and geographical location; thus, higher PIC values for markers investigated can be obtained if further applied over a larger and diverse collection. Sharma et al. (2014) suggested that differences in the range of PIC values observed across different populations could be because of the presence of certain unique alleles in certain individuals that differentiate them from the others. In comparison to most of the previous studies conducted on different marker systems such as RAPD and ISSR, the mean PIC value was almost similar to the study conducted by Li et al. (2020), possibly because of conserved sequences of these loci in seabuckthorn germplasm.

Assessment of Genetic Diversity Using Microsatellite Markers

The observed heterozygosity (H_o) for individual loci varied from 0.0000 to 0.73, with an average of 0.244 per locus, and the expected heterozygosities (H_e) or gene diversity (Nei's) ranged from 0.48 to 0.77 with an average of 0.641 per locus in Leh population (Table 2). In the case of Lahaul collection, H_o for individual locus varied from 0.0000 to 0.60 with an average of 0.286 per locus, and H_e ranged from 0.489 to 0.778 with an average of 0.647 per locus (Table 1). Like Wang et al. (2008), expected heterozygosity was found to exceed observed heterozygosity. Such H_e is attributed to heterozygote deficiency that could be because of factors like inbreeding pressure, small population size, presence of null alleles, or due to the Wahlund effect resulting in heterozygote deficiency resulted from subpopulation structure (Xuan et al. 2009). Wright's fixation index (F_{IS}), in the present study, ranged from (–) 0.20 to 1.00 with an average of 0.618 in Leh collection and 0.168 to 1.00 with an average of 0.555 in Lahaul collection (Table 2). The positive value of F_{IS} represents an excess of observed homozygotes, whereas the negative value demonstrates the presence of extra heterozygotes. The negative value of the fixation index could be attributed to the outcrossing nature of seabuckthorn. Thus, HrMS12 for Leh and USMMii_2 for the Lahaul collection showed a

negative value of the fixation index, which could be due to the existence of excessive heterozygotes for these particular loci. Similar excess of heterozygotes was observed in a previous study by Lacis and Dombrovskaya (2014) in seabuckthorn using microsatellite markers developed by Wang et al. (2008). Shannon's informative index (I) of loci varied from 0.693 to 1.86 with a mean value of 1.14 per locus in Leh collections, and it ranged from 0.682 to 1.63 with a mean value of 1.13 per locus in Lahaul collections (Table 2). The data on PIC values, observed heterozygosity (H_o), expected heterozygosity (H_e) or gene diversity (Nei's), fixation index (F_{IS}), and Shannon's informative index (I) of loci are presented in Table 2.

Genetic Distance and UPGMA Cluster Analysis Using Microsatellite Data

Among 52 Leh collections, maximum genetic distance (0.89) existed between collection ID Leh42 and Leh111, and minimum (0) between Leh11 and Leh12. Based on the UPGMA analysis, seabuckthorn collections from Leh were divided into two major groups (A and B) comprising seven clusters (Fig. 2a). The first major group B consisted of two subgroups (I and II) with 46 collections.

Subgroup I was further subdivided into Ia and Ib. Ia comprised three clusters, including the first cluster represented by nine collections, the second cluster included seven collections,

Fig. 2 a, b Dendrogram showing relationships among *Hippophae rhamnoides* collections from Leh (red) and Lahaul (blue), derived using hierarchical unweighted cluster analysis based on microsatellite marker data

Fig. 3 Dendrogram prepared using combined data on morphometric and microsatellite data on *Hippophae rhamnoides* collections from Leh (red) and Lahaul (blue), using hierarchical unweighted clus-

ter analysis. Regrouping of some collections from one geographical region in the other region is visible

and the third cluster was represented by 5 collections. Similarly, subgroup BIIb consisted of three clusters, including cluster IV comprising 7 collections, cluster V comprising a maximum of 10 collections, and cluster VI represented by eight collections.

On the other hand, major group A included six collections in cluster VII.

Further, genetic distance among 41 Lahaul collections was maximum (0.87) between collection ID Lahaul102

and Lahaul93 and minimum (0.06) between Lahaul41 and Lahaul42. The UPGMA clustering method classified Lahaul collections into two major groups (A and B) comprising nine clusters (Fig. 2b). Major group A consisted of 3 collections, whereas major group B consisted of two subgroups (I and II) accounting for 38 collections. Subgroup I was further subdivided into groups Ia and Ib. Major group A had one cluster, and group B consisted of eight clusters. Subgroup Ia accounted for the maximum number of clusters that could be further divided into two sections. Section 1 had four clusters, with the first one consisting of seven collections, and the second, consisting of six collections. Cluster III included six collections and cluster IV comprised three collections. The second section of subgroup Ia consisted of two clusters, including cluster V with 6 collections and cluster VI with 2 collections. Subgroup Ib had two collections in cluster VII. Subgroup II had cluster VIII comprising six collections. Major group A included only three collections in cluster IX. Collections from one sampling site, in most of the cases, got clustered together, depicting the similarity between the ecotypes from the same environmental conditions.

In order to see the placement of collections from two regions together, one combined dendrogram was constructed using a combined genetic distance matrix based on molecular data (Fig. 3). The average genetic distance observed within Leh collections was 0.71, which was found to be 0.68 in the case of Lahaul collections. However, the average genetic distance observed between the two populations was 0.75. It was really interesting to observe here that collections of cluster III of Leh collections got clustered within the Lahaul population, and cluster IX of Lahaul collection was found getting clustered around Leh collection but remained an outlier (Fig. 3). The only possible reason behind such observation could be possible human invasive activities, e.g., pollen transfer through some agents as collection sites were near the roads.

Comparison of Assessment of Diversity Using Microsatellite Markers and Morphological Traits

Information obtained in the present study using molecular markers was found to be highly useful in comparison to that generated from the study of morphological characteristics. For example, collection pairs showing no genetic distance such as in Lahaul51 and 53, Lahaul111 and 113, Leh132 and 133, Leh123 and 82, and Leh211 and 213 on the basis of morphological data were found to be diverse following molecular marker data. Similarly, collection Leh11 and 12, which showed a genetic distance of 0.59 as per morphological characteristics, were found to be similar on the application of microsatellite markers.

We used XLstat to calculate the correlation existing between morphological distance matrix and molecular

distance matrix to assess the relationship between the morphological and molecular data, and it was found to be non-significant (Mantel test, $r=0.203$). The Mantel Z test statistic was also not significant between the two matrices, indicating thereby a low correspondence between microsatellite markers and morphological traits.

Conclusion

Microsatellite-based markers have proved to be an efficient tool to characterize the genetic variability prevailing in seabuckthorn natural populations inhabiting the Leh and Lahaul regions of the Indian Himalayas in comparison to morphometric analysis. The sequence resources like EST database and transcriptome assembly developed in our laboratory have opened a wide opportunity for the development of microsatellite markers for diverse application in seabuckthorn conservation and breeding programs. Since a large number of markers are associated with the genic regions, there is a possibility of some functional role associated with these markers. Further experimental investigations will establish other applications of these markers for this non-model plant species with immense nutritional and medicinal value.

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Declarations

Conflict of Interest The authors declare no competing interests.

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