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# Microsatellite Polymorphism in Relation to Geographical Distribution and Adaptation of Seabuckthorn (*Hippophae rhamnoides* L.) in the Indian Himalayas

**Parneeta Chaudhary and Prakash Chand Sharma***

University School of Biotechnology, Guru Gobind Singh Indraprastha University, New Delhi, India
*Corresponding author: prof.pcsharma@gmail.com

**Abstract**

Microsatellites or Simple Sequence Repeats (SSRs) are the most popular molecular markers employed for the characterization of genetic diversity analysis, especially in non-model organisms. Seabuckthorn (*Hippophae rhamnoides* L.), a high altitude dioecious plant species, has attracted the attention of researchers and industrials as a future multipurpose crop, for its multifarious medicinal and nutritive properties. In the present study, we assessed the morphological and molecular diversity of *H. rhamnoides* representing diverse ecological sites in the different geographical areas of Union Territory of Ladakh, and Lahaul-Spiti region of Himachal Pradesh, India. Morphological diversity was assessed by screening for twenty-seven morphological characters. Molecular diversity employing fifteen microsatellite markers revealed 109 alleles, and 71.5% of the markers were polymorphic. The Polymorphic Information Content (PIC), Expected Heterozygosity ($H_e$), Nei's Diversity, Wright's fixation index ($F_{is}$), and Shannon's Informative Index (I) were also determined to study the phylogenetic relationships among collections representing different geographic regions. The molecular marker data matrix was used to prepare a UPGMA-based dendrogram that showed a clear demarcation between collections from different regions in the dendrogram, although a few collections clustered with collections from the other regions. Assessment and analysis of genetic diversity in seabuckthorn was more efficient and informative using microsatellite markers. The findings of this study will be useful in future breeding and conservation programs in *H. rhamnoides* and closely related species.

**Keywords**: Genetic diversity, Seabuckthorn, *Hippophae rhamnoides*, Microsatellite markers

## Introduction

The genus *Hippophae* L. (seabuckthorn, family Elaeagnaceae) represents multipurpose dioecious and deciduous nitrogen-fixing woody shrub species. The genus is represented by seven species and twelve sub-species that are distributed in diverse geographical regions ranging from the Atlantic coast of the Europe continent to temperate regions of Asia along seacoast, valleys, riversides, riverbeds, semi-arid deserts, alpine regions, and cold deserts. All seven species are diploid (2*n* = 24) and wind-pollinated (1, 2).

Seabuckthorn is an ecologically important species known for its immense use in the traditional medicine system for centuries in China, Russia, and Mangolia to treat a variety of ailments like gastric ulcers, influenza to skin infections, bowel irregularity to cardiovascular diseases with anti-inflammation, anti-microbial,

anti-atherogenic, and radio-protective effects. Seabuckthorn berry extracts also remain an important ingredient for the pharmacological and cosmetic industries for their high essential oil (linoleic and linolenic acids) content, carotene (lycopene, β-carotene and zeaxanthin), fatty acids (omega 3,6,9 palmitoleic acid, palmitic acid, and steric acid), flavonoids (quercetin, myricetin, kaempferol, isorhamnetin, etc.), vitamins (C, E, K), and many other bioactive compounds. The leaves of plant are rich in nutrient content like carotenoids, triterpenols, and sterols, free and esterified (3, 4).

The plant has remarkable extensive root system that shares a symbiotic relationship with an actinomycetes filamentous nitrogen-fixing bacteria *Frankia* (5) facilitating fixing of atmospheric nitrogen that improves soil fertility. The plants help in controlling desertification, stabilization of ecosystem, preventing soil erosion and therefore are used in land reclamation projects and wildlife habitat enhancement as effective ecosystem restorer to conserve soil erosion. Seabuckthorn is ecologically adapted and shows considerable tolerance towards abiotic stresses like temperature, drought, and salinity (6-8).

For the local population of Lahaul & Spiti Valley (Himachal Pradesh), and Leh, Nubra, Kargil (Union Territory of Ladakh), seabuckthorn is regarded as a very important economic resource as it provides employment to the people involved in cultivation, collection of berries, and pulp extraction for small scale cottage industries. Since the plant is of great importance for various purposes, it has been over-exploited causing great loss to its genetic diversity. This situation has become a major issue, which poses the threat of extinction of potential wild genotypes. So far, not much has been done to assess the genetic polymorphism of the genus *Hippophae* in different geographical regions, an activity that can help in the identification of useful genetic resources for more effective breeding, conservation and productivity of seabuckthorn (9).

Advances in marker technology are not much reflected in the progress of seabuckthorn improvement. The repertoire of marker resources available for the assessment of genetic diversity and gender specification is not sufficient in seabuckthorn (9). The available reports on molecular markers have primarily assessed the genetic diversity in the Chinese Seabuckthorn collections. Some reports suggest that Indian seabuckthorn could be more adaptive as well as diverse for its ability to grow in diverse climatic and geographical environments. Therefore, a detailed analysis of molecular diversity is required for formulating meaningful breeding and conservation programmes aiming towards seabuckthorn improvement. The principal aim of the present study was to study the genetic diversity among the seabuckthorn (*H. rhamnoides*) collections from the Indian Himalayas with respect to variation in geographical and ecological changes.

## Materials and Methods

***Collection of Plant Material:*** A total of 120 seabuckthorn (H. rhamnoides) leaf tissue samples were collected from forty-three diverse ecological locations in Union Territory of Union Territory of Ladakh, and Himachal Pradesh during the fruiting season in the month of September (2018-19). The locations for sample collection were selected such that they have certain variations in terms of altitude, water availability, exposure to sunlight, temperature and precipitation variations, and soil type. Every plant sample collected was geographically tagged using a GPS device i.e. Garmin, stored in ice-packs and transported to the laboratory and stored at -80°C until further use.

***Morphometric analysis:*** The phenotypic characters of each plant were recorded individually. Various plant characteristics were considered to discriminate sampled morphotypes. Characters for morphometric analysis included plant habitat, branching habit, phyllotaxy, leafiness and thorniness on the branches, number of leaves and thorns per

branch, color and venation pattern on the leaves, stem characteristics, color difference between the young and mature plant, attachment of leaf to the stem, and leaf tip shape. Other observations related to the habitat of collected samples collected included latitude, longitude, altitude, temperature, and soil type.

***Isolation of genomic DNA:*** Genomic DNA from young leaves was isolated following a CTAB-based protocol (10) with certain modifications. The use of PVPP 1.5% Polyvinylpolypyrrolidone (PVPP) and enhanced concentrations of β-mercaptoethanol (2%) was found suitable for ensuring good yield as well as high quality of genomic DNA (11). The DNA concentration was equated to 25 ng/µl for further use.

***Primer designing, validation of primers and detection of alleles:*** The microsatellite positive sequences were selected randomly from seabuckthorn transcriptome assembly developed in our laboratory (9) to design primers. Sixteen primer pairs were designed with the help of the software Primer3 (http://primer3.wi.mit.edu/ ) (12). Primer pairs designed considering the reference (9) from microsatellite positive sequences from genomic libraries, EST database were also screened in the present study. The parameters considered for primer designing were GC content of 45-55%, 18-22 bp length, and 50-60ºC annealing temperature. A PCR reaction was set up and amplifications were carried out in a Mastercycler (Eppendorf). The amplified PCR products were resolved by electrophoresis on 3% agarose in 1X TAE buffer solution. The amplicons were visualized and recorded using a gel documentation system (Alpha Imaging). For further analysis of the alleles obtained, in a binary data matrix in MS-Excel, the presence and absence of each allele was manually scored as 1 (presence) and 0 (absence).

***Statistical analysis of microsatellite markers:*** The binary data matrix recorded for the presence and absence of alleles for all individual microsatellite loci was analyzed using GenAlex software (13). Statistical tests, such as Analysis of molecular variance (AMOVA), $F_{ST}$ estimation, F-statistics, mean heterozygosity calculation, Nei's genetic similarity and differences estimation, and genetic distance were performed using the analysis tool PAST4 v4.07. A dendrogram was generated using Jaccard's coefficient and UPGMA cluster analysis in PAST4 v4.07 based on the binary data recorded for microsatellite markers in different *H. rhamnoides* collections. We also performed a cluster analysis based on Nei's genetic similarity matrix among the two populations of H. rhamnoides collection from different geographical regions following a published protocol (14). Further, POPGENEv1.32 (15) was used to evaluate other parameters such as PIC (Polymorphism Information Content), Nei's gene diversity ($H_e$), Shannon's information index (I), Wright's Fixation Index ($F_{is}$), the effective number of allele ($N_e$), and the observed number of allele ($N_a$).

## Results

***Morphometric analysis:*** It was observed that collections from Union Territory of Ladakh had branching of thorns, whereas this feature appeared only in 38.8% of samples from Lahaul-Spiti. Similarly, excessive leafiness on thorns (more than 9 leaves) was observed in 61.6% of Union Territory of Ladakh collections; however, 91.8% of collections from Lahaul-Spiti had leafiness on the higher side (7 to 9 leaves). The upper leaf surface of the samples collected from Union Territory of Ladakh was found to be rough contrary to the smooth surface observed in the Lahaul-Spiti collections. The occurrence of silver scales on leaves, a completely absent feature in Lahaul collections but scales existed on the dorsal surface of 78.5% of samples from Lahaul and while completely absent in Spiti was a contrasting trait observed. Incidentally, 19.23% of samples of Union Territory of Ladakh had scales on both leaf surfaces. Silvery scales on stems were observed in 85.72% Union Territory of Ladakh collections whereas only 14.28%

Lahaul collections showed their presence.

The stem shape, stellate hairiness on young branches, number of leaves attached to a single point, stellate hairs beneath leaves, and dorsal leaf surface colour did not show any significant variation between the collection sets from Ladakh, and Lahaul-Spiti and therefore were the same for both the populations (Table 1). Colour of midrib in 14.2% Lahaul collections and 96.15% Union Territory of Ladakh collections was found to be cream and remained green in the rest of the collections. Leaf attachment to stem was sessile in Ladakh collections, whereas 27.1% collection from Lahaul showed pedicellate attachment to stem. It was observed that in the majority of the collections from Ladakh, and Lahaul-Spiti, mature stem colour was observed to be brown, whereas immature stem colour in the Ladakh region was found silver compared to ashy in the case of Lahaul-Spiti collections.

Of the twenty-seven morphological characters studied, sixteen could be measured as metric traits and marked variations were observed among collections representing the two geographical areas (Table 2). Average canopy width in Ladakh collections (123±15 cm) and Lahaul-Spiti collections (203±56 cm) showed a marked difference. Plant height was also found varying significantly between the two collections sets such that collections from Ladakh were relatively shorter (range 28-670 cm, average 237.18 cm) comparative to those collected from Lahaul-Spiti (range 38-567 cm,

**Table 1**. Summary of morphological characteristics observed in H. rhamnoides collections from different geographical regions of Union Territory of Union Territory of Ladakh, and Lahaul-Spiti of Himachal Pradesh

| Character | Ladakh | Lahaul-Spiti |
|---|---|---|
| **Mature plant habit** | Intermediate | Erect |
| **Branching habit** | Dense | Dense |
| **Branching of thorns** | Branched | Unbranched |
| **Stem shape** | Round | Round |
| **Leafiness on thorns** | Abundant | Sparse |
| **Mature stem colour** | Brown | Brown |
| **Immature Stem colour** | Silver | Ashy |
| **Silver scales on stem** | Present | Absent |
| **Hardness of mature stem** | Medium | Hard |
| **Stellate hair on young branches** | Absent | Absent |
| **Number of leaves attached to single point** | Single | Single |
| **Doral leaf colour** | Green | Light green |
| **Leaf tip shape** | Very acute | Acute |
| **Upper leaf surface** | Rough | Smooth |
| **Silver scales on leaves** | Present on dorsal surface | Present in few collection of Spiti |
| **Stellate hair beneath leaves** | Absent | Absent |
| **Colour of mid-rib** | Cream | Off-white |
| **Leaf attached to stem** | Sessile | Sessile |
| **Phyllotaxy** | Zigzag | Zigzag |
| **Colour of leaf on ventral side** | Dark green | Green |

**Table 2.** Comparison of quantitative characteristics observed in H. rhamnoides collections from the two geographical regions

| Characters | Ladakh | | | Lahaul-Spiti | | |
|---|---|---|---|---|---|---|
| | Mean | Range | | Mean | Range | |
| | | Min. | Max. | | Min. | Max. |
| Canopy width (cm) | 123 ± 15 | 25.1 | 201 | 203 ±56 | 37 | 203 |
| Plant height (cm) | 237 ± 18 | 28.4 | 670.1 | 343 ±57 | 38 | 567 |
| Inter-branch Distance (cm) | 9 ± 2 | 2.9 | 16 | 5.8 ± 89 | 1.2 | 18.1 |
| Number of leaves (per 10 cm branch length ) | 122.9 ± 12 | 5.6 | 289 | 77.2 ± 7 | 9 | 240 |
| Number of thorns (per 10 cm branch length ) | 12.6 ± 0.78 | 2 | 25 | 7.9 ±0.8 | 2 | 21 |
| Leaf length (cm) | 2.6 ± 0.15 | 0.8 | 6 | 4.8±0.3 | 2 | 10.6 |
| Leaf width (cm) | 0.3 ± 0.10 | 0.1 | 29 | 0.61±0.03 | 0.2 | 1.3 |
| Thorn length (cm) | 1.25 ± 0.12 | 0.2 | 3 | 1.39 ±0.15 | 0.1 | 6.1 |
| Leaves on thorn (cm) | 6.6 ± 0.8 | 1 | 18 | 7.7 ± 2.02 | 0 | 67 |
| Number of berries ( per 10 cm branch length) | 51.36 ± 9.2 | 12 | 158 | 50.7 ± 6.6 | 15 | 255 |
| Berry's length (cm) | 0.69 ± 0.04 | 0.3 | 1 | 0.61 ± 0.03 | 0.3 | 0.9 |
| Berry's width (cm) | 0.14±28 | 0.3 | 0.7 | 1.42±19 | 0.4 | 0.8 |
| Weight of 20 berries (g) | 3.10 | - | - | 3.87 | - | - |

average 343.57 cm). However, characteristics like inter-branch distance, degree of thorniness, and leafiness were found significantly higher in the case of Ladakh collections in comparison to their Lahaul-Spiti counterparts (Table 2).

***Selection of seabuckthorn genic microsatellite markers:*** The screening of the whole transcriptome of seabuckthorn led to the identification of more than 8,000 microsatellite sequences occurring in 88,297 unigenes. The clustered ESTs were also screened for the presence of microsatellites (9) and primers designed from these were also screened in the present study. Sixteen primer pairs were considered to screen 120 collections of *H. rhamnoides* collections to assess the genetic diversity in relation to the geographical variability. A list of primer pairs has been presented in Table 3.

***Validation and assessment of microsatellite markers:*** The genetic diversity among hundred genotypes representing two geographically distinct populations was studied using sixteen microsatellite markers. The marker ESTSSR-25 was monomorphic in the Lahaul collection; however, the same marker showed

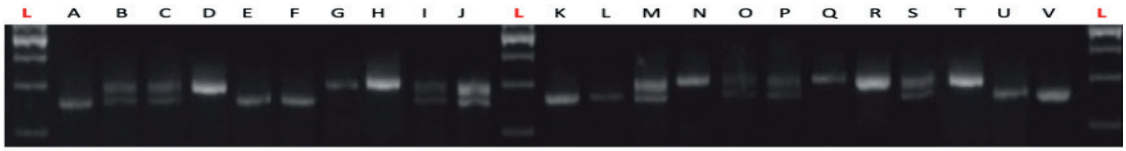


Fig. 1 Polymorphism displayed by microsatellite locus ESTSSR 25. L: Molecular size marker (Mix DNA ladder); A-J: Samples of *H. rhamnoides* representing different locations from Union Territory of Ladakh; K-V: Samples of *H. rhamnoides* representing different locations from Lahaul-Spiti in Himachal Pradesh

Fig. 1. Polymorphism displayed by microsatellite locus ESTSSR 25. L: Molecular size marker (Mix DNA Ladder); A-J: samples of *H. rhamnoides* representing different locations from Union Territory of Ladakh; K-V: samples of *H. rhamnoides* representing different locations from Lahaul-Spiti region in Himachal Pradesh

polymorphism in collections from Ladakh and Spiti region (Fig. 1). The varying environmental conditions and fixation of the allele to a particular area, prevailing at two geographical areas might have facilitated the natural selection of microsatellite alleles.

**Table 3.** Details of PCR primers of the microsatellite markers used in the present study

| Primer Id | Primer sequence 5'-3' | Microsatellite repeat | Tm (ºC) | Location | Size range (bp) |
|---|---|---|---|---|---|
| GLC 2-6 | F: GGT ATG TAG ATA AGG CCA CAA<br>R: CCA ATG ATA TAA ACC CTC CAC | $(CT)_7$ | 56.0 | Anonymous | 160-185 |
| GLC 2-5 | F: GTG ATG GCC CTA GTT ATA GA<br>R: CGT AAT ATC CAA ACA GAC CA | $(GA)_{13}$ | 53.0 | Anonymous | 360-380 |
| GLC 11 | F: ATT CGA AGA AGC AGG GAT A<br>R: GCA ATG AAG TCC TTG TTA TC | $(GA)_{12}$ | 54 | Anonymous | 320-350 |
| USMM | F: AAG GAT GTG GTC GAT CCA AG<br>R: GTT TGC AGG CAT TCC TTT GT | $(TTC)_{10}$ | 54.0 | CDS | 160-190 |
| USMM 1 | F: GGC GAA ACT TGA CTT GTT GC<br>R: ACC GAT CAA TAC CGT TCT GC | $(TAC)_{16}$ | 55.0 | 3'UTR | 180-220 |
| USMM 5 | F: TTC GAT CGG ATA AGG TCA TTG<br>R: GCA GTC GAG GAG GTT TGA AG | $(AC)_9(AT)_6$ | 56.0 | CDS | 190-240 |
| USMM 7 | F: TCG CCG TCT GTT TCA GAT AA<br>R: GCT GAT CCA ACG GTC TCA TT | $(AG)_{18}$ | 50.0 | 3'UTR | 180-210 |
| USMM 24 | F: TAG CAT TGC AGG CTC AGA GA<br>R: ATC CGT GGT TAA GGT TGC AC | $(AG)_{11}$ | 55.0 | 3'UTR | 240-270 |
| ESTSSR 25 | F: GTA CTG TGA CCA CGC TGC<br>R: GGG TTC AAA GTA ATG GCA AG | $(AG)_8$ | 53.0 | 3'UTR | 280-320 |
| BT-1 | F:TTC CCT GGT GAA CAA CCC CA<br>R:ACT GTT GGC CCA AGT AAG CCT | $(AAGA)_7$ | 48 | Anonymous | 150-180 |
| BT-2 | F: GGC GAA ACT TGA CTT GTT GCC CT<br>R: GGG TTC CAC CAC CAA AGC AAA CCA | $(TAC)_{16}$ | 54 | Anonymous | 120-150 |
| BT-3 | F:CAG AGT GGA GCG TCC GCG TG<br>R: GGC ACA GTA ACA CTA AGC ACC CCA | $(TAA)_{11}$ | 48 | Anonymous | 180-200 |
| BT-4 | F:AGG GGA CTT AAG AGC AGA GGC CA<br>R: CAT GCT CTG CAA CTT TTG GTT TCG T | $(TTTA)_5$ | 48.7 | Anonymous | 150-180 |
| BT-5 | F:TTG GTG CTC GAG CTG TTC GG<br>R: AGC ACG GAG GTG GAA AAG CC | $(CAA)_9$ | 52 | Anonymous | 140-190 |
| BT-6 | F:GGC CTA CTC CAG GTG TTG CAC<br>R:CCA ACC ATG GCC AGA AGG CA | $(TCA)_{10}$ | 48.3 | Anonymous | 120-150 |

GLC series marker: from microsatellite enriched genomic library; ESTSSR marker: from EST database screening; USSM and BT series markers from screening whole transcriptome; Tm (ºC): melting point; bp: Base pair

**Table 4**. Summary of genetic diversity parameters for H. rhamnoides collections from different regions using microsatellite markers

| Primer | PIC | Na | $H_o$ | He | I | Fis |
|---|---|---|---|---|---|---|
| **Union Territory of Ladakh** | | | | | | |
| ESTSSR 25 | 0.328 | 4 | 0.413 | 0.531 | 1.373 | 0.542 |
| USMM | 0.458 | 5 | 0.673 | 0. 782 | 1.651 | 0.140 |
| USMM 1 | 0.311 | 4 | 0.230 | 0.780 | 1.645 | 0.704 |
| USMM 5 | 0.546 | 6 | 0.307 | 0.815 | 1.862 | 0.622 |
| USMM 7 | 0.453 | 5 | 0.709 | 0.782 | 1.558 | 0.093 |
| USMM 24 | 0.339 | 4 | 0.310 | 0.585 | 1.648 | 0.378 |
| GLC 2-6 | 0.427 | 3 | 0 | 0.639 | 1.056 | 1 |
| GLC 2-5 | 0.417 | 6 | 0.259 | 0.786 | 1.624 | 0.679 |
| GLC 11 | 0.352 | 3 | 0.316 | 0.609 | 1.009 | 0.480 |
| BT-1 | 0.314 | 3 | 0.266 | 0.58 | 0.976 | 0.546 |
| BT-2 | 0.416 | 4 | 0.173 | 0.467 | 0.879 | 0.629 |
| BT-3 | 0.44 | 3 | 0.096 | 0.623 | 1.036 | 0.845 |
| BT-4 | 0.37 | 2 | 0.192 | 0.542 | 1.143 | 0.700 |
| BT-5 | 0.414 | 2 | 0.403 | 0.495 | 0.688 | 0.184 |
| BT-6 | 0.416 | 2 | 0.076 | 0.499 | 0.692 | 0.845 |
| **Lahaul** | | | | | | |
| ESTSSR 25 | 0.368 | 3 | 0.417 | 0.539 | 1.550 | 0.511 |
| USMM | 0.482 | 3 | 0.293 | 0.613 | 1.021 | 0.702 |
| USMM 1 | 0.431 | 4 | 0.415 | 0.778 | 1.630 | 0.467 |
| USMM 5 | 0.438 | 3 | 0.617 | 0.741 | 1.369 | 0.168 |
| USMM 7 | 0.383 | 3 | 0.562 | 0.613 | 1.006 | 0.082 |
| USMM 24 | 0.396 | 4 | 0.171 | 0.712 | 1.294 | 0.760 |
| GLC 2-6 | 0.469 | 3 | 0.354 | 0.619 | 1.018 | 0.428 |
| GLC 2-5 | 0.464 | 3 | 0.293 | 0.635 | 1.048 | 0.539 |
| GLC 11 | 0.337 | 2 | 0.122 | 0.489 | 0.682 | 0.702 |
| BT-1 | 0.371 | 3 | 0.605 | 0.517 | 0.836 | -0.168 |
| BT-2 | 0.442 | 2 | 0.146 | 0.492 | 0.685 | 0.702 |
| BT-4 | 0.42 | 3 | 0 | 0.604 | 1.011 | 1 |
| BT-5 | 0.396 | 2 | 0.403 | 0.495 | 0.688 | 0.184 |
| **Spiti** | | | | | | |
| ESTSSR 25 | 0.397 | 3 | 0.146 | 0.547 | 0.927 | 0.732 |
| USMM | 0.482 | 2 | 0.146 | 0.492 | 0.685 | 0.702 |
| USMM 1 | 0.431 | 3 | 0.293 | 0.613 | 1.021 | 0.523 |
| USMM 5 | 0.482 | 2 | 0.122 | 0.489 | 0.685 | 0.702 |
| USMM 7 | 0.383 | 3 | 0.293 | 0.613 | 1.021 | 0.523 |

| | | | | | | |
|---|---|---|---|---|---|---|
| USMM 24 | 0.383 | 2 | 0.562 | 0.613 | 1.006 | 0.082 |
| GLC 2-6 | 0.467 | 3 | 0.354 | 0.619 | 1.018 | 0.428 |
| GLC 2-5 | 0.428 | 2 | 0 | 0.642 | 1.061 | 1 |
| GLC 11 | 0.416 | 2 | 0.402 | 0.487 | 0.658 | 0.178 |
| BT-1 | 0.315 | 2 | 0.134 | 0.613 | 1.012 | 0.780 |
| BT-2 | 0.273 | 2 | 0.078 | 0.439 | 0.636 | 0.411 |
| BT-4 | 0.452 | 3 | 0.439 | 0.604 | 1.011 | 0.310 |

PIC: Polymorphic Information Content; Na: Number of alleles; $H_o$: Observed heterozygosity; He: Nei Expected heterozygosity; I: Shanon Information Index; Fis: Wright's Fixation Index

Only 15.03% of the markers developed by the screening of microsatellite enriched genomic library were polymorphic in the present study. On the other hand, 37.19% of markers developed as a result of screening genic resources were found polymorphic. The results suggest higher marker development efficiency in the present study compared to some of the previous studies (16, 17). All the primer pairs that produced amplification in *H. rhamnoides* were tested in the two other *Hippophae* sp. and these markers cross amplified in *H. salicifolia* and *H. tibetana* also. Interestingly, the markers polymorphic in *H. rhamnoides* were polymorphic in *H. salicifolia* but monomorphic in *H. tibetana*. The phenomenon of cross transferability of all microsatellite markers with expected product size in the above two close species of seabuckthorn that widely grow in the Indian Himalayas, suggests that these three species are genetically close to each other. The cross transferability phenomenon observed in *Hippophae* species in the present study also offers an opportunity to develop microsatellite markers for other closely related species in the genus *Hippophae*, and possibly in other related species in the family Elaeagnaceae, with no prior sequence information.

***Diversity analysis in H. rhamnoides collections using microsatellite markers:*** A set of sixteen microsatellite markers was employed to assess the genetic diversity prevailing among different *H. rhamnoides* collections representing diverse geographical regions, namely Leh, Nubra, and Kargil-Drass of Union Territory of Ladakh, and Lahaul and Spiti valley of Himachal Pradesh. A substantial variation was observed in microsatellite allele polymorphism (size range of amplicons: 120-450 bp) in different collections of seabuckthorn. By applying sixteen microsatellite markers on hundred collections, a total of 119 bands were generated for collections from Union Territory of Ladakh, with an average of 3.41 alleles per locus. The number of alleles ranged from 2 alleles (SBT6) to 6 alleles (USSM 5) per locus (Table 4). A total of 102 alleles for Lahaul and 98 alleles for Spiti collection were recorded with an average of 2.78 and 2.41 alleles per locus, respectively. Markers SBT3 and SBT6 were found monomorphic in all the collections of Lahaul and Spiti, and the number of alleles ranged from 2 alleles (SBT2 and SBT4) to 4 alleles (USSM 5, USSM 7, and USSM 24) per locus (Table 4).

***Polymorphism analysis and assessment of genetic diversity using microsatellite markers:*** The calculated PIC values varied from 0.546 (USSM 5) to 0.311 (USSM) for Ladakh collection (Table 4), 0.271(SBT1) to 0.469 (GLC 2-6) in Lahaul collection while PIC recorded for Spiti collection was 0.271 (SBT1) to 0.482 (USSM) (Table 4). Furthermore, we estimated and recorded the observed heterozygosity ($H_o$) that varied from zero to 0.605 with an average of 0.363 per locus. The expected heterozygosity ($H_e$) or gene diversity (Nei's) also ranged from 0.981 (Ladakh), 0.594 (Lahaul) and 0.517 (Spiti) with an average of 0.641 per locus (Table 4). Wright's Fixation Index ($F_{is}$) and Shannon's

**Table 5**. Hierarchical analysis of molecular variance in the two populations of *H. rhamnoides*

| Source | df | SS | MS | $E_{st}$ Var. | % Total Variance | $F_{ST}$ | $F_{IS}$ | $F_{IT}$ |
|---|---|---|---|---|---|---|---|---|
| **Among populations** | 1 | 519.43 | 193.23 | 16.31 | 81% | | | |
| **Among individual Population** | 86 | 1315.87 | 91.56 | 13.71 | 7% | | | |
| **Within Population** | 119 | 1502.71 | 81.24 | 69.98 | 12% | 0.271*** | 0.168*** | 0.312*** |
| **Total** | 206 | 3338.01 | 366.03 | 100.01 | 100% | | | |

df: Degrees of Freedom; SS- Standard Significance; MS- Mean Significance; $E_{st}$ Var.- Estimated Variation; $F_{ST,}$ $F_{IS}$ and $F_{IT}$-individual, inter-population and total fixation indexes, respectively; *** $p$<0.001.

informative index (I) ranged from 0.760 (Ladakh), 0.468 (Lahaul), and 0.411 (Spiti) with an average of 0.630, and 1.11 (Union Territory of Ladakh), 1.612 (Lahaul), and 1.492 (Spiti) (Table 4).

***Genetic distance and UPGMA cluster analysis using microsatellite data:*** Among 120 collections from distinct geographical regions, maximum genetic distance (6.75) existed between collections, namely Ladakh HRL26 and HRL19, and minimum (0) between HRL26 and HRL27, HRL6 and HRL28, HRL2 and HRL1, and HRLS1 and HRLS1 and HRLS3, HRLS48 and HRLS49 from Lahaul-Spiti region. Collection HRL30 and HRL31, HRL32 and HRL33 from (Leh) Ladakh and collection ID HRLS46 and HRLS61 (Spiti), HRLS43 and HRLS56 (Spiti), HRLS69 and HRLS73 (Lahaul) from Himachal Pradesh region also showed zero genetic distance.

Based on the UPGMA analysis, seabuckthorn collections from Union Territory of Ladakh and Himachal Pradesh were divided into two major groups i.e. A and B, comprising of five clusters (Fig. 2). Interestingly, group B consisted of twelve collections grouped together in cluster V (HRL16, HRL17, HRL21, HRL24, HRL25, HRL22, HRL23, HRL13, HRL15, HRL14, HRL20, and HRL19) from Nubra region of Union Territory of Ladakh that clustered together forming one group. The major group A consisted of a total of hundred and one (101) collections grouped in four clusters. Group A was further divided into sub-groups Ia and Ib. Subgroup Ia consisted of two clusters I and II comprising

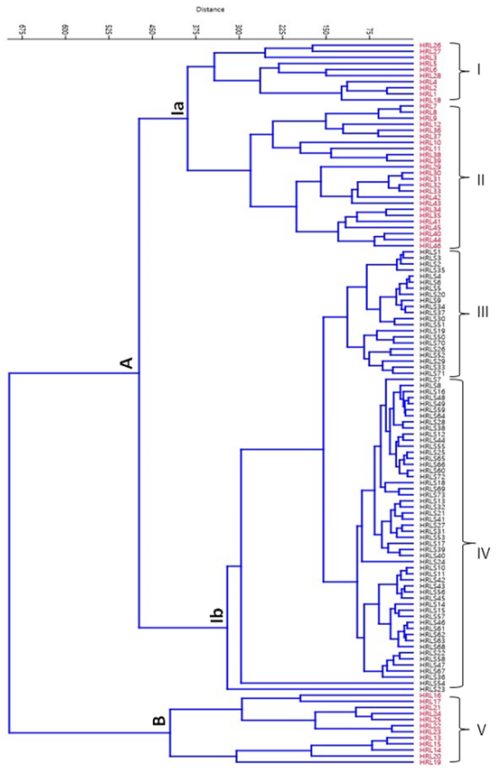


**Fig. 2.** Dendrogram generated using Jaccard's coefficient and UPGMA cluster analysis in PAST4 v4.07 software for *H. rhamnoides* collections analyzed using microsatellite-markers

of ten and twenty-four collections, respectively from Union Territory of Ladakh. The subgroup Ib comprised of seventy-four collections, including

**Table 6**. Nei's genetic similarity (above diagonal) and difference (below diagonal) in the two populations of H. rhamnoides

| Region | Population 1 | Population 2 |
|---|---|---|
| **Population 1** | - | 0.791 |
| **Population 2** | 0.389 | - |

Population 1: Union Territory of Ladakh; Population 2: Lahaul-Spiti

two clusters III (twenty collection IDs) and IV (fifty collection IDs) comprising of sample collections from Lahaul-Spiti region of Himachal Pradesh.

On performing various statistical tests and parameters like AMOVA, the results of the present study showed the highest genetic variation (81%) among the individuals across populations, 12% of the total genetic variation among the individuals within populations, and only 7% was among the populations (Table 5). To measure genetic differentiation of populations, $F_{ST}$ and $F_{IS}$ were also calculated and the values were low, (0.271 and 0.168), whereas $F_{IT}$ values were a little higher, however, still low (0.312) (Table 5). Nei's genetic similarity matrix was calculated following (14) from sixteen microsatellite markers using Unweighted Pair Group Method with Arithmetic mean (UPGMA) to study the overall genetic relationships (Table 6). The observations were based on the combined marker analysis. Overall, the two populations of *H. rhamnoides* from distinct geographical regions revealed a moderate level of genetic diversity among themselves. The possible reason behind such clustering could be habitat fragmentations due to vast geographical barriers. Similar observations of the clustering pattern of seabuckthorn collections according to their geographical affiliations have also been reported earlier (180.

## Discussion

The Indian Himalayas are home to three species of seabuckthorn, a medicinally and ecologically important plant, namely *H. rhamnoides*, *H. salicifolia*, and *H. tibetana*. In India, *H. rhamnoides* is primarily distributed in high terrains of Leh, Nubra, Kargil, Drass, and Zanskar valley of Union Territory of Ladakh, and Lahaul and Spiti in Himachal Pradesh. In the present study, a total of 120 leaf samples representing two populations of *H. rhamnoides* were collected from two distinct geographical regions of Ladakh and Lahaul-Spiti in Himachal Pradesh and analyzed for the presence of genetic diversity prevailing in these regions. Recent human invasive activities that have contributed to an adverse change in the environmental conditions might result in the genetic loss of important alleles of seabuckthorn in near future. Similar to other plant species, conservation and scientific exploration of genetic diversity is necessary for seabuckthorn improvement (19, 20).

In total, twenty-seven previously described morphological characteristics (21) were studied for discriminating seabuckthorn samples collected from two major geographical regions. The regions considered for exploration in the present study were significantly different in various environmental descriptors such as annual rainfall (Union Territory of Ladakh: 80 mm-200 mm; Lahaul: 120 mm-400 mm; Spiti: 150 mm-170 mm), temperature variation (Union Territory of Ladakh: -30ºC to 35ºC; Lahaul-Spiti: -18ºC to 38ºC), soil texture (Union Territory of Ladakh: loamy clay; Lahaul-Spiti: sandy loam), average soil pH (Union Territory of Ladakh: 7.3; Lahaul-Spiti: 6.7). Leaf samples were collected from hundred plants growing at forty-three different sites. Hence, morphological characteristics of 120 samples were considered for further analysis. Twenty morphological features between the two collections were classified into various ordinal categories represented by a number, and modal values were calculated to assign the characteristic representative qualitative trait (Table 1). The present study shares most of the observations

with the earlier studies conducted over collections representing different geographical regions of seabuckthorn growing areas (21-23).

Commonly available sequence resources have been exploited for their fast and cost-effective approach for the development of microsatellite markers (24). Nevertheless, the unavailability of genic sequences of seabuckthorn and other closely related species in the public databases prompted us to develop sequence resources in our laboratory. EST sequencing together with whole transcriptome sequencing generated a huge wealth of molecular resources and also offered an opportunity to isolate fast and cost-effective genic microsatellite markers (25).

The numbers of alleles determined in the present study were comparatively lower in comparison to the first study (26) in seabuckthorn (*H. rhamnoides*), where the number of alleles varied from 3 to 12 with an average of 6.66 alleles per locus. Microsatellite markers screened on Latvian seabuckthorn showed the presence of 4 to 22 alleles averaging to 10.25 alleles per locus (27), while application of the same set of markers on the Indian variety of seabuckthorn collected from diverse locations revealed the presence of 2-4 alleles in the population averaging 2.83 alleles per locus (18). The variation observed in the number of alleles is possibly due to the possible variation in the resolution of the traditional gel-based detection techniques. However, genotyping by sequencing has been performed in various other studies, by using a genetic analyzer allowing better resolution of small allelic variation among the genotypes. In the present study, all the markers exhibited moderate to high levels of PIC. Assessment of genetic diversity of medicinally and economically important species is associated with germplasm conservation and breeding, therefore, the application of molecular markers offers an effective solution to elucidate the genetic diversity among genotypes and can also prove helpful in solving taxonomic issues. A number of attempts have been made by researchers worldwide to assess the genetic diversity and phylogeny of *Hippophae* sp. (8, 18, 28-31).

Among 120 collections from distinct geographical regions of Leh, Nubra, Kargil, and Drass of Union Territory of Ladakh and Lahaul-Spiti region of Himachal Pradesh, a dendrogram was generated using Jaccard's coefficient and UPGMA cluster analysis in PAST software using sixteen microsatellite-markers. Moreover, the majority of the samples collected from one collection site got clustered together depicting the similarity between the collection ecotypes coming from one microenvironment. Overall, it was very interesting to note that the collections from different regions showed their respective region-specific genetic identity.

Utilizing these microsatellite markers, the presence of substantial genetic diversity was shown to exist in seabuckthorn collections. Therefore, on the basis of the prevalence of high genetic diversity in seabuckthorn, we suggest that there is ample scope of improvement in seabuckthorn cultivars through focused breeding and conservation programs.

## Conclusion

The present study on the assessment of genetic diversity in a large collection of *H. rhamnoides* from geographical regions of Leh, Nubra, Kargil, and Drass of Union Territory of Ladakh and Lahaul-Spiti region of Himachal Pradesh, generates important data for this unexplored species with significant economic and ecological value. The environmental variations prevailing in the areas covered in the present study have a determining effect on genetic diversity as revealed by the data by employing twenty-seven morphological characteristics and sixteen microsatellite markers. Overall, we observed that molecular markers are more efficient tools to assess the genetic variability existing in seabuckthorn populations in comparison to morphological characters. Our findings suggest that a significant level of diversity is available in seabuckthorn that offers

ample scope of improvement in seabuckthorn through focused breeding and conservation programs that can be facilitated by exploitation of the microsatellite marker-based resources.

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**References**

1. Bartish, I.V., Jeppsson, N., and Nybom, H. (1999). Population genetic structure in the dioecious pioneer plant species *Hippophae rhamnoides* investigated by random amplified polymorphic DNA (RAPD) markers. Mol Ecol 8: 791-802.
2. Chen, G., Wang, Y., Korpelsinen, H., and Li, C. (2008). Genetic diversity of *Hippophae rhamnoides* populations at varying altitude in Wolong natural reserve of China as revealed by ISSR markers. Silvae Genet 57:29-36.
3. Teng, B.S., Lu, Y.H., Wang, Z.T., Tao, X.Y., Wei, D.Z. (2006). In vitro anti-tumor activity of isorhamnetin isolated from *Hippophae rhamnoides* L. against BEL-7402 cells. Pharmacol Res 54: 186-194.
4. Suryakumar, G. and Gupta, A. (2011). Medicinal and therapeutic potential of seabuckthorn (*Hippophae rhamnoides* L.). J Ethanopharmacol 138: 268-278.
5. Gatner, E.M.S. and Gardner, I.C. (1970). Observation on the fine structures of root nodules endophyte of *Hippophae rhamnoides* L. Arch Mikrobial 70: 183:196.
6. Fuller, R.M. and L. A. Boorman (1977). The spread and development of Rhododendron ponticum L. on dunes at Winteron, Norflock, in comparison with invasion by *Hippophae rhamnoides* L. at Saltfleetby. Biol Conserv 12: 83-94.
7. Ruan, C., Qin, P., Zheng, J. and He, Z., (2004). Genetic Relationships among some cultivars of seabuckthorn from China, Russia and Mongolia based RAPD analysis. Sci Hortic 101: 417-426.
8. Sheng, H.M., An, L.Z., Chen, T., Xu, S.J., Liu, G.X., Zheng, X.L., Pu, L.L., Liu, Y.J. and Lian, Y.S. (2006). Analysis of the genetic diversity and relationships among and within the species of *Hippophae* (Elaeagnaceae) based on RAPD. Plant Syst Evol 260: 25-37.
9. Jain, A., Chaudhary, S. and Sharma, P.C. (2013). Application of DNA technologies for seabuckthorn improvement. In: Seabuckthorn- The Wonder Plant, Singh, V. (ed.), Daya Publishers, Delhi, pp. 167-178.
10. Doyle, J.J. and Doyle, J.L. (1990). Isolation of plant DNA from fresh tissue. Focus 12: 13-15.
11. Jain, A. (2015). Analysis of microsatellite polymorphism in relation to geographical distribution and adaptation of seabuckthorn in India, Ph. D. thesis, Guru Gobind Singh Indraprastha University, Delhi.
12. Rozen, S.G., Untergasser, A, Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C. and Remm, M. (2012). Primer3-new capabilities and interfaces. Nucl Acid Res 40: e115.
13. Peakall, R. and Smouse, P.E. (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 28: 2537-2539.
14. Nei, M. and Li, W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA, 76: 5269–5273.
15. Yeh, F.C. and Boyle, T.J.B. (1997). Population genetic analysis of co-dominant and dominant markers and quantitative traits. Belgian J Bot 129: 157-163.
16. Tang, S., Okashah, R.A., Cordonnier-Pratt, M., Pratt, L.H., Johnson, V.E., Taylor, C.A., Arnold, M.L. and Knapp, S.J. (2009). EST

and EST-SSR marker resources for Iris. BMC Plant Biol 9: 72.

17. Salem, K.F., Varshney, R.K., Röder, M. and Sand Börner, A. (2010). EST-SSR based estimates on functional genetic variation in a barley (*Hordeum vulgare* L.) Collection from Egypt. Genet Resour Crop Evol 57: 515-521.

18. Srihari, J.M., Verma, B., Kumar, N., Chahota, R.K., Singh, V., Rathour, R., Singh, S.K., Sharma, S.K. and T.R. Sharma (2013). Analysis of molecular genetic diversity and population structure in sea buckthorn (*Hippophae* ssp L.) from north-western Himalayan region of India. J Med Plant Research 7: 3183-3196.

19. Berding, N. and Roach, B.T. (1987). Germplasm collection, maintenance, and use. In: Heinz, D.J. (Ed.), Sugarcane Improvement Through Breeding. Elsevier, New York, pp. 143: 210.

20. Lian, Y.S. and Chen, X.L. (2000). The regular patterns of distribution on the natural components in plants of the genus *Hippophae* L. J. North Norm Univ 36: 113-128.

21. Singh, R., Dwivedi, S.K. and Mishra, S.N. (2005). Morphology and distribution of Seabuckthorn (*Hippophae rhamnoides* L.) population of cold arid Ladakh (India) using RAPD markers. Curr Sci 91: 1321-1322.

22. Singh, V. (2002). Studies on genetic diversity and improvement of seabuckthorn a multipurpose plant of cold desert Himalayas. Envis Bulletin 110: 8-9.

23. Singh, V. (2014). New findings on morphological variations and co-relationship of characteristics among various natural populations of seabuckthorn (*Hippophae* L.) growing in 5 districts of Himachal Pradesh, north-west Himalayas, India", In: Mörsel J.T, Zubrarev Y and Eagle D. (Eds) Seabuckthorn research for a promising crop: A look at recent developments in cultivation, breeding, technology, health and environment. Books on Demand, Norderstedt pp 139-162.

24. Varshney, R.K., Graner, A. and Sorrells, M.E. (2005). Genic microsatellite markers in plants: features and applications. Trends Biotechnol 23: 48-55.

25. Jain, A., Chaudhary, S. and Sharma, P.C. (2014). Mining of microsatellites using next generation sequencing of seabuckthorn (*Hippophae rhamnoides* L.) transcriptome. Physiol Mol Biol Plant 20: 115-123.

26. Wang, A., Zhang, Q., Wan, D., and Liu, A.T. (2008). Nine microsatellite DNA primers for *Hippophae rhamnoides* ssp. sinensis (Elaeagnaceae). Conserv Genet 9: 969-971.

27. Lacis, G. and Dombrovska, K.I. (2014). Assessment of genetic diversity of Latvian sea buckthorn (*Hippophae rhamnoides* L.) germplasm using molecular markers. Zemdirbyste-agric 101: 333-340.

28. Jepsson, N., Bartish, I.V. and Persson, H.A. (1999). DNA analysis as a tool in seabuckthorn breeding. ASHS Press, Alexandria, VA pp 338-341.

29. Tian, C.J., Lei, Y.D., Shi, S.H., Nan, P., Chen, J.K. and Zhong, Y. (2004). Genetic diversity of sea buckthorn (*Hippophae rhamnoides*) populations in northeastern and northwestern China as revealed by ISSR markers. New Forests 27:229-237.

30. Ruan, C. and Li, D. (2005). AFLP fingerprinting analysis of some cultivated varieties of sea buckthorn (*Hippophae rhamnoides*). J Genet 84: 311-316.

31. Raina, S.N., Jain, S., Sehgal, D., Kumar, A., Dar, T.H., Bhat, V., Pande, S., Vaishnavi, A., Bhargav, V., Singh, V., Rani, V., Tandon, R., Tewari, M. and Mahmoudi, A. (2011). Diversity and relationships of multipurpose seabuckthorn (*Hippophae* L.) germplasm from the Indian Himalayas as assessed by AFLP and SAMPL markers. Genet Resour Crop Evol 59:1033-1053.