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Analysis of molecular genetic diversity and population structure in sea buckthorn (*Hippophae spp* L.) from north-western Himalayan region of India

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Genetic diversity of 48 sea buckthorn genotypes collected from 16 locations in four diverse geographical regions of Himachal Pradesh and Jammu and Kashmir states of north-western Himalayan region of India, was analyzed using random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), simple sequence repeat (SSR) and MADS-box gene markers. While 21 decamer primers generated 155 loci, of which 148 (95.48%) were polymorphic with average of 7.3 loci per primer, ten ISSR primers generated 86 loci (8.6 loci per primer), out of which 83 (96.51%) were polymorphic. Six SSRs generated 18 loci (3 loci per primer), of which 17 (94.45%) were polymorphic, whereas 3 SSR primers resulted into monomorphic bands. Two MADS-box primers on the other hand generated 13 loci (6.5 loci per primers) with 100% polymorphism. RAPD, ISSR, SSR and MADS box gene-specific primers generated amplicons with polymorphism information content (PIC) values of 0.60 to 0.88, 0.60 to 0.91, 0.23 to 0.73 and 0.56 to 0.80, respectively. Cluster analysis of genotypes based on Jaccard's similarity coefficient using DARwin generated a dendrogram which revealed 11 groups that followed clustering pattern more or less according their geographical origin as the Mantel's test also indicated a good fit with r-value of 0.84. Internal transcribed spacer (ITS) sequence analysis revealed the distribution of *Hippophae salicifolia* in Sangla region of Kinnaur. Analysis of molecular variance (AMOVA) indicated sufficient genetic differentiation within populations and a low level of variation among populations. Clustering by DARWIN was supported by principal coordinate analysis (PCoA) and STRUCTURE. Some species specific diagnostic markers were also identified.

Key words: *Hippophae*, genetic diversity, simple sequence repeat (SSR), inter-simple sequence repeat (ISSR), Internal transcribed spacer (ITS), sequence analysis, analysis of molecular variance (AMOVA).

INTRODUCTION

Sea buckthorn (*Hippophae rhamnoides* L.) belonging to family Elaeagnaceae is a dioecious, deciduous and nitrogen fixing thorny plant. It grows widely in temperate Asia and Europe. Genus *Hippophae* has been divided

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into three species with nine subspecies (Rousi, 1971). Later, on the basis of extensive investigations and systemic studies, two sections were established and the number of taxa was increased to seven species and nine subspecies (Lian, 1988). According to the latest systematic treatments of the genus *Hippophae* L. (Bartish et al., 2001; Swenson and Bartish, 2002), the genus comprises seven species and the species *H. rhamnoides* circumscribes eight subspecies. All species are diploid (2n = 2x = 24) and wind pollinated. Several authors have designated *H. rhamnoides* in Indian Himalayas as *H. rhamnoides* sp. *Turkestanica* (Singh and Awasthi, 1995; Singh, 2003).

The taxonomic history of *Hippophae salicifolia* and *Hippophae tibetana* is extensive and contentious (Lian and Chen, 2000; Bartish et al., 2000; Sheng et al., 2006). The natural habitat of sea buckthorn extends widely in China, Mongolia, Sweden, Norway and India. After China and Commonwealth of Independent States (CIS), Indian Himalayan region is believed to possess world's second largest sea buckthorn resources (Singh et al., 1997). In India, sea buckthorn is naturally growing in cold desert regions and other high altitude Himalayan regions, comprising the states of Himachal Pradesh, Ladakh in Jammu-Kashmir, Uttrakhand, Sikkim and Arunachal Pradesh. The average rainfall in these regions fluctuates from 50 to 700 mm per year. The mean temperature shows considerable variation throughout the year (a maximum of 27°C during summers and minimum of -30°C in winters (Leh and Lahaul and Spiti regions). During winter months, the region is affected by a series of western disturbances which cause heavy snowfall (200 to 400 cm/year). *H. rhamnoides* sub species *turkestanica* is a predominant species that grows on riversides and ravines in these valleys that are characterized by harsh weather and tough terrain.

The berries of sea buckthorn are very nutritious and rich in vitamin C (400 mg/100 g) (Gutzeit et al., 2008). They also contain large amount of essential oil which is an important raw material for pharmaceutical and cosmetic industries (Rongsen, 1993). Sea buckthorn berries have considerable amount of proteins-globulin and albumin (average 15.0%). They also contain carotene, free fatty acids, flavonoids, vitamin E and many nutrients and bioactive substances. Sea buckthorn's nutritious products include tea, juice, puree, wine, jam, snacks etc. Sea buckthorn is well known for its strong antitumor activity (Teng et al., 2006). Today, a number of health products are being manufactured from sea buckthorn. It is beneficial for those suffering from cardiovascular diseases, cancer, skin problems, burns, digestive tract disorder, anti-senilism, anti-inflammation, anti-radiation effects etc. Sea buckthorn is a remarkably hardy bush that rapidly develops an extensive root system capable of fixing nitrogen. Thus, it is suitable for growth on marginal soils, eventually improving them to a level where they can support the growth of other plants. Hence, sea buckthorn has been used in checking soil erosion, land reclamation projects, wildlife habitat enhancement, farm stand protection and as ornamental bushes. The effective use of genetic resources in breeding programmes is based on the understanding of extent and distribution of genetic variation available in the species. Genetic diversity enables species to have a greater potential for the evolution of new combinations of genes and subsequently, a greater capacity for evolutionary adaptation to different environmental conditions. Hence, studies on molecular variability of sea buckthorn populations of north-western Himalayan region will help to characterize and understand the amount of genetic diversity available in the ecotypes.

Of the various molecular techniques available, random amplified polymorphic DNA (RAPD) is widely used for estimating genetic diversity and relationships in plant populations (Jiang and Liu 2011; Domingues et al., 2001; Mastan et al., 2011). New technological developments have expanded the range of DNA polymorphisms assays for genetic mapping, marker assisted breeding, genome finger printing and investigating genetic relatedness (Rafalski et al., 1996). In the case of sea buckthorn, it is also useful for clarification of taxonomy, sex determination and population genetic structure (Sheng et al., 2006; Chen et al., 2010). On the other hand, inter simple sequence repeat (ISSR) markers are abundant throughout the genome and show a higher level of polymorphism than any other genetic markers (Gutierrez et al., 2005). The sequences flanking specific micro-satellite loci in a genome are considered to be conserved within species, across species in a genus and perhaps even across the related genera (Varshney et al., 2002).

ISSRs have a number of advantages, for example, low quantities of template DNA are required, no sequence data are needed for primer construction, many informative bands are generated per reaction, and it is reliable and reproducible (Sharma et al., 2011). Consequently, ISSRs have been widely used in marker-assisted selection, genetic diversity analysis, DNA fingerprinting and evolution and molecular ecology (Li et al., 2009). Microsatellites or simple sequence repeats (SSRs) are tandemly repeated DNA regions with motif lengths of one to six base pairs. By virtue of their hypervariability, abundance, reproducibility, co-dominant nature and easy detection technique, SSRs are considered as one of the most efficient molecular markers which have a wide range of applications such as genetic mapping, gene tagging and studies of genetic diversity and evolution (Powell et al., 1996; Varshney et al., 2005). MADS-box genes encode a family of transcription factors which control diverse developmental processes

in flowering plants ranging from root development to flower and fruit development. Besides providing floral homeotic functions, MADS-box genes have many other roles within the gene networks that control reproductive development in angiosperms (Okada and Shimura, 1994). Nuclear DNA data provide valuable information in phylogenetic study of plants and the internal transcribed spacer (ITS) regions of the nuclear DNA have been shown to be a valuable source of evidence to resolve phylogenetic relationships at different taxonomic levels, in particular at intraspecific level because of relatively rapid evolutionary rates of the ITS fragment (Sun et al., 2002).

In this study, we used RAPD, ISSR, SSR and MAD-BOX gene primers to investigate the genetic diversity among 48 genotypes constituting 4 geographically distinct populations of *H. rhamnoides* collected from natural stands growing in northwestern Indian Himalayan region. The results would contribute to a better understanding of the population genetics and as a guide for strategies for conservation, selection and breeding of the species. We also analysed the nucleotide sequences of ITS region of the nuclear ribosomal DNA from out-group accessions to ascertain their species status. The main goal of the present study was to assess the degree of genetic diversity in natural sea buckthorn populations growing in the north-western trans Himalayan region of India using PCR-based molecular markers.

MATERIALS AND METHODS

Plant

A total of 48 genotypes from sixteen different sites comprising of three individual plant samples from each site were collected from four distinct geographic regions (considered as four populations) in the north western Himalayan region of India which include: Lahaul valley, Spiti region and Sangla-Kinnaur (Himachal Pradesh) and Ladakh region of Jammu and Kashmir (Table 1 and Figure 1). All these regions are characterized by arid temperate climate and remain snow covered during winters. Collections were made during the months of August to September, 2008.

Extraction of plant genomic DNA

Genomic DNA was isolated from young leaves (0.5 to 1 g) using cetyltrimethylammonium bromide (CTAB) method following Murray and Thompson (1980) with minor modifications. DNA concentration and quality was checked on 1.0% (w/v) agarose gels by electrophoresis using lambda DNA as a reference.

PCR amplification of DNA

One hundred and fifty random amplified polymorphic DNA (RAPD), 30 (3′ anchored) inter simple sequence repeats (ISSR) and 9 simple sequence repeats (SSR) primers of *Hippophae* (Wang et al., 2008) were used. Six MADS box gene primers including two corresponding to the candidate accessions (AB162020 and AB162019) were used. The sequence of candidate MADS box genes were downloaded from the gene bank (www.ncbi.nlm.nih.gov) and primers were designed using Primer 3.0 software (http://frodo.wi.mit.edu/primer3/) and 4 others were custom synthesized using the published information (Matsunaga et al., 2004; Nakayama et al., 2006). The polymerase chain reaction (PCR) conditions for RAPDs were optimized as initial cycle of 94°C for 5 min, 40 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 2 min with final extension at 72°C for 5 min, before cooling to 4°C. Amplification was carried out in 25 µl volumes containing 2.0 µl of dNTP mix (0.2 mM each of dATP, dGTP, dCTP and dTTP), 0.2 µl of *Taq* DNA polymerase (5 U/µl), 2.0 µl DNA template (20 ng/µl), 2 µl of primer, 2.5 µl of 10× PCR buffer, 1.5 µl of $MgCl_2$ (25 mM) and 14.8 µl of sterilized distilled water. The amplifications were carried out in a thermal cycler (Applied Biosystems, USA).

The PCR conditions for ISSRs were optimized as initial cycle of 94°C for 5 min, 35 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 2 min with final extension at 72°C for 7 min, before cooling to 4°C. Amplification was carried out in 25 µl volumes containing 2.0 µl of dNTP mix (0.2 mM each of dATP, dGTP, dCTP and dTTP), 0.2 µl of *Taq* DNA polymerase (5 U/µl), 2.0 µl DNA template (20 ng/µl), 2 µl of primer, 2.5 µl of 10× PCR buffer, 1.5 µl of $MgCl_2$ (25 mM) and 14.8 µl of sterilized distilled water. The amplifications were carried out in a Gen Amp PCR System® (Applied Biosystems, USA).

The PCR conditions for SSRs and MADS box genes primers were optimized as initial cycle of 94°C for 5 min, 39 cycles of 94°C for 40 s, 47 to 51°C for 40 s and 72°C for 45 s, with final extension at 72°C for 8 min, before cooling to 4°C. SSR marker amplification of DNA was carried out in 25 µl volume containing 2.0 µl of dNTP mix (0.2 mM each of dATP, dGTP, dCTP and dTTP), 0.2 µl of Taq DNA polymerase (5 U/µl), 2.0 µl DNA template (20 µg/µl), 1 µl each of primer, 2.5 µl of 10× PCR buffer, 1.5 µl of $MgCl_2$ (25 mM) and 14.8 µl of sterilized distilled water. The amplifications were carried out in a MyCycler™ (BioRAD, USA). Polymorphism survey was done on a panel of four genotypes representing diverse geographic locations.

Analysis of PCR products

Fifteen (15 µl) PCR products were mixed with 2 µl of gel loading dye (0.25% bromophenol blue and 40% sucrose) and electrophoresed at 100 volts for 90 min in 1.5% agarose gel in 1× Tris acetate-EDTA (TAE) buffer (40 mM Tris, 40 mM acetic acid Glacial, 1 mM EDTA, pH 8.0) for RAPDs and 1.8% gel for ISSR products. The amplification products of SSR and MADS box markers were analyzed in 3% agarose gel. After electrophoresis the gels were stained with ethidium bromide (0.5 µg/ml) for 10 min followed by de-staining in tap water for 30 min. The PCR products were visualized under ultraviolet transilluminator (Bio-Rad, USA) and photographed using the Gel-Documentation Unit (Bio-Rad, USA).

Analysis of RAPD, ISSR, SSR and MADS box data

The presence and absence of each band of a particular molecular weight in RAPD, ISSR, SSR and MADS box profiles of all the individuals was scored manually. For deducing relationship

Table 1. List of populations used in the present study.

Genotypes	Location	Region	Altitude	Latitude	Longitude
1 2	Madgran	LAHAUL	2695 m	32°44' N	76º37' E
3	-	-	-	-	-
4 5	Gemur	3259 m	32°36' N	77° 29' E	-
6	-	-	-	-	-
7 8	Jispa	3228 m	32° 38' N	77°11' E	
9	-	-	-	-	-
10 11	Goshal	2895m	32° 33’ N	76° 58’ E	-
12	-	-	-	-	-
13 14	Thirot	3120	32° 30’ N	77° 01’ E	
15	-	-	-	-	-
16 17	Phoora	2890	32° 37’ N	76° 52’ E	-
18	-	-	-	-	-
19 20	Kirting	2890	32° 36’ N	76° 54’ E	-
21	-	-	-	-	-
22 23	Poh	Spiti	3377 m	32° 02' N	78° 19' E
24	-	-	-	-	-
25 26	Chango	2995 m	31° 58' N	78° 35' E	-
27	-	-	-	-	-
28 29	Gulling	3360m	32° 02' N	78° 04' E	
30	-	-	-	-	-
31 32	Tabo	3285 m	32° 05' N	78° 23' E	-
33	-	-	-	-	-
34 35	Sangla	SANGLA	2586 m	31° 25' N	78° 15' E
36	-	-	-	-	-
37 38	Shey	LEH	3277 m	34° 04' N	78° 35' E
39	-	-	-	-	-
40 41	Budha garden Miru	3250 m	34° 13' N	77° 54' E	
42	-	-	-	-	-
43 44	Phyang	3535 m	34° 10' N	77° 29' E	-
45	-	-	-	-	-

Table 1. Contd.

46 47	Nang	3614 m	34° 02' N	77° 44' E	-
48	-	-	-	-	-

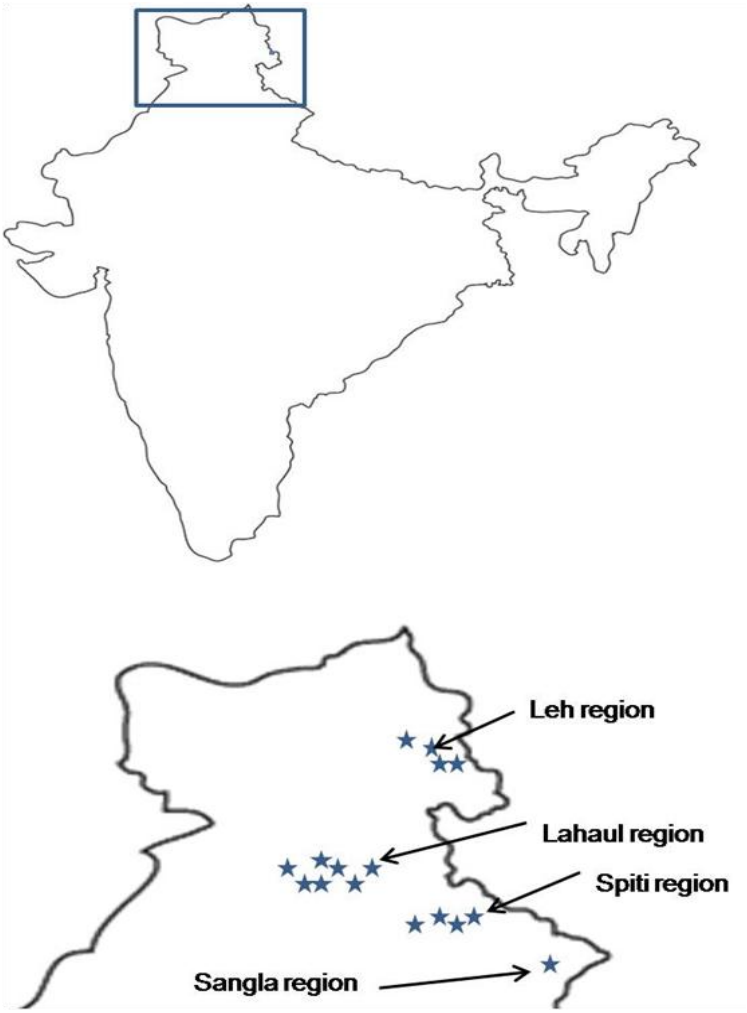

Figure 1. Map of India with north western Himalayas in box. At bottom the stars indicate 16 locations in four geographical regions used for collecting the DNA samples.

among individuals, each band of a specific molecular weight in the DNA profile of an individual was treated as locus/ marker. A binary data matrix with '1' indicating the presence and '0' the absence of a particular band was generated. The binary data matrix was prepared for each molecular marker separately and they were clubbed as combined data for overall analysis. The binary data for all individual marker types and the combined one were further analyzed with the help of GenAlex software (Peakall and Smouse, 2006) for analysis of molecular variance (AMOVA), principle coordinate analysis (PCoA), F_{st} estimation, mean heterozygosity calculation, Nei's genetic similarity and differences estimation, Mantel test of geographic and genetic distance etc.

To understand overall genetic relationships among *Hippophae* populations, a cluster analysis was carried out based on Nei's genetic similarity matrix following Nei and Li (1979) generated in GenAlex from 21 RAPDs, 10 ISSRs, 6 SSRs and 2 MADS box gene-specific primers using unweighted pair group method with arithmetic mean (UPGMA). This was further used for developing a dendrogram in SAHN

programme of NTSYS-pc 2.10 software (Rohlf, 2002). The combined binary data matrix was used for clustering of all the genotypes of *Hippophae* sp. by unpaired group mean arithmetic and Jaccard's similarity coefficients in DARWIN 5.0 software (Perrier et al., 2003) and NTSYSpc 2.10 (Rohlf, 2002). Popgene32 (Yea et al., 1999) was used to calculate polymorphic loci (%), Nei's gene diversity (h), Shannon's information index (I), effective number of alleles (ne), observed number of alleles (na), coefficient of population differentiation (Gst) and estimated gene flow (N_m). The program STRUCTURE version 2.3.3 (Pritchard and Donnelly , 2003) was used to test the hypotheses of K = 4 that is, 1 to 4 populations using an admixture model, with a burn-in phase of 1×10^4 and a sampling phase of 5×10^4 replicates.

Polymorphism information content (PIC) for each primer locus was calculated based on the number of bands/primer, as described by Weir, using the formula PIC = 1 - Pi^2, where *Pi* is the frequency of the *i*th band in the genotype examined (Weir, 1990). PIC compares the polymorphism levels across markers and is used to determine the usefulness of markers for specific studies.

Amplification and sequencing of ITS region

One genotype each from Lahaul (Madgran-2), Spiti (Poh-22), Leh (Nang-47) and Kinnaur (Sangla-34) regions was used for ITS sequence analysis. Double-stranded DNA of the complete ITS region (including ITS1, 5.8S and ITS2) was amplified with the primers P_1 (5′-AGAAGTCGTAACAAGGTTTCCGTAGG-3′) and P_4 (5′-TCCTCCGCTTATTGATATGC-3′). Amplifications were carried out following Sun et al. (2002) with minor modifications. Double stranded sequencing of the purified DNA was performed in an ABI PRISM® 377 DNA sequencing system with the ABI prism bigdye terminator cycle sequencing reading reaction kit. The two PCR primers were used as sequencing primers. Sequencing reactions were carried out as per Sun et al. (2002). DNA sequences were read for both strands. The sequence boundaries of two ITS regions and the coding region of nuclear rDNA were determined by comparison with the published sequences in the GenBank. The sequences were aligned manually with published sequences of *H. rhamnoides* and *H. salicifolia* using pairwise comparisons.

RESULTS

Marker polymorphism analysis

The genetic diversity among 48 genotypes representing 4 geographically distinct populations was assessed using 21 polymorphic RAPD primers which produced species specific amplicons and proved useful in diversity analysis. A total of 155 bands were generated of which 148 (95.48%) were polymorphic (Table 2). Amplicon number per primer ranged from 4 (OPN 20) to 11 (OPC 2 and OPH 15) with an average of 7.04 per primer. The PIC vales for RAPD varied from 0.60 (OPB 8 to 0.88 (OPC 2). RAPD primers OPG 10 (fragment 2), OPB 8 (fragment 1), OPB 8 (fragment 5), OPU 14 (fragment 9) OPAC 2 (fragment 1) were specific to *H. salicifolia* whereas, OPC 2 (fragment 10) and OPP 6 were specific *H. rhamnoides*. ISSRs on the other hand were more informative with 10 primers generating 83 fragments (8.3 bands per primer) with a high polymorphic percentage of 96.51. Number of polymorphic loci for ISSRs ranged from 4 (ISSR 01) to 15 (ISSR 26). PIC values were observed in the rage of 0.60 (ISSR 01) to 0.91 (ISSR 27) with an average value of 0.79. Some species (*H. salicifolia*) specific fragments were amplified for Sangla accessions by ISSR 11_{850}, ISSR 11_{600}. Similarly, ISSR 26_{750} and ISSR 17_{1500} were found *H. rhamnoides* specific. Some RAPD markers including: OPB8$_{1200}$, OPB8$_{620}$, OPU14$_{740}$ and OPAC2$_{1250}$ were amplified only in *H. salicifolia* genotypes from Sangla, whereas OPC2$_{350}$ was amplified only in *H. rhamnoides* accessions.

Six SSR primer pairs amplified a total of 17 polymorphic alleles ranging from 1 to 4 alleles per primer pair with a mean of 2.83 alleles per locus. PIC values for SSRs ranged from 0.23 (SSR 3) to 0.73 (SSR 5) with an average of 0.53. Only 2 MAD-Box gene primers were found polymorphic which amplified a total of 13 bands, all of which were polymorphic with average PIC value of 0.68. Overall, a total of 272 bands were amplified with 262 (96.32%) being polymorphic.

The percent polymorphic bands observed for population from Sangla region was 6.83, 15.12, 9.52, 23.08 and 10.32% for RAPD, ISSR, SSR, MADS-BOX and for combined data, respectively (Table 3). The observed (na) and effective number (ne) of alleles along with mean heterozygosity (Ht), Nei's genetic diversity (h) also followed the same trend that is, all these indices were observed to be the lowest for genotypes from Sangla region.

Genetic diversity and differentiation

The clustering of four populations based on Nei's genetic differences (Table 4) revealed that the Sangla population was out-grouped at 50% of genetic difference to all other populations which grouped at 9% of genetic differences. It was observed on the basis of individual as well as combined marker analysis. Cluster analysis of individuals based on Jaccard's similarity coefficient performed with UPGMA method using SAHN program of NTSYSpc version 2.10 generated a dendrogram delineating all the 48 sea buckthorn genotypes into two statistically robust clusters, A and B; encompassing 45 and 3 genotypes, respectively. Cluster A could be subdivided into six sub clusters denoted as A_1, A_2, A_3, A_4, A_5 and A_6, comprising 26, 5, 3, 5, 3 and 3 genotypes, respectively. Group A_1 further formed three sub-subgroups I, II, and III. Group A_1 exhibited 56.0% genetic similarity with

Table 2. Details of primers used for molecular analysis of 48 genotypes of *Hippophae* sp.

Primer	Sequence (5'-3')	GC%	TNLA	NPL	P (%)	PIC value
RAPD						
OPA -10	CAGGCCCTTC	70	6	5	83	0.74
OPAC-2	GTCGTCGTCT	60	8	8	100	0.78
OPB -7	GGTGACGCAG	70	6	4	66	0.78
OPB -8	GTCCACACGG	70	6	6	100	0.60
OPC-2	GTGAGGCGTC	70	11	11	100	0.88
OPC-6	GAACGGACTC	60	7	7	100	0.81
OPG -10	AGGGCCGTCT	70	7	6	86	0.72
OPG -17	ACGACCGACA	60	5	5	80	0.68
OPG -19	GTCAGGGCAA	60	8	8	100	0.85
OPG -9	CTGACGTCAC	60	8	8	100	0.81
OPH -15	AATGGCGCAG	60	11	11	100	0.84
OPH -2	TCGGACGTGA	60	10	10	100	0.83
OPH -4	GGAAGTCGCC	70	9	9	100	0.84
OPN -20	GGTGCTCCGT	70	4	4	100	0.80
OPP -16	CCAAGCTGCC	70	7	6	85	0.80
OPP -5	CCCCGGTAAC	70	7	7	100	0.86
OPP -6	GTGGGCTGAC	70	6	6	100	0.78
OPU – 14	TGGGTCCCTC	70	9	8	89	0.84
OPU- 2	CTGAGGTCTC	60	7	7	100	0.65
OPW- 3	GTCCGGAGTG	70	6	6	100	0.70
OPX -3	TGGCGCAGTG	70	7	6	86	0.75
Total			155	148	95.48	**0.77**
ISSR						
ISSR-01	CTCTCTCTCTCTCTCTCTCTG	52.4	4	4	100	0.60
ISSR-3	CTCTCTCTCTCTCTCTCTCTA	47.6	6	6	100	0.76
ISSR-10	AGAGAGAGAGAGAGAGAGAGG	52.4	6	6	100	0.81
ISSR-11	AGAGAGAGAGAGAGAGAGAGC	52.4	8	8	100	0.80
ISSR-12	AGAGAGAGAGAGAGAGAGAGT	47.6	11	11	100	0.87
ISSR-16	CTCCTCCTCCTCCTCCTCG	68.4	9	9	100	0.82
ISSR-17	CTCCTCCTCCTCCTCCTCT	63.2	4	4	100	0.66
ISSR-18	CTCCTCCTCCTCCTCCTCA	63.2	8	7	87	0.82
ISSR-26	ACCACCACCACCACCACCT	63.2	16	15	94	0.90
ISSR-27	ACCACCACCACCACCACCC	68.4	14	13	93	0.91
Total			86	83	96.51	**0.79**
SSR						
SSR-01	F: GGGATCGCCAAATAAACT R: TGCGTTGGTGCTTCGTTT	44.4 50	4	4	100	0.64
SSR-2	F:CGTCTTTCAATCGACACTTATTC R:GCTAGAAGAGTCTTTTCGTTGA	39.1 43.5	2	2	100	0.58
SSR-3	F: TCAATCCAAACTTGTTCGCC R: TATCGATTTCTCCCCAACTG	58.4 58.4	1	1	100	0.23
SSR-5	F: CTTGCCGCCGTGAGCTCTAG	65	5	4	80	0.73

Table 2. Contd.

	R:GCAATCATCGTCTCTTCTTCTCC	50				
SSR-6	F:CAACAAAATACAATTCGGAAAC R: AATAGGAGACACAGAGGCTTC	31.8 47.6	2	2	100	0.39
SSR-7	F: AGAATCACAAGGCTTCACCAC R: TAGTCCCCTTTGAGGTTGTAG	47.6 47.6	4	4	100	0.64
Total			18	17	94.45	0.535
MADS BOX gene primers						
MADS BOX-2	F:5' AAGCGCATTTTCTTGTTGAAA 3' R:5' AAAGGCAAAAACACGAGCAT 3'	33.34 40	8	8	100	**0.56**
MADS BOX-5	F:5'AAGACCCAGTCGATGCTCGATCAC 3' R:5'ACTTGAGCATGAGATGTCCCAGCA3'	52 52	5	5	100	**0.80**
Total			13	13	100	0.68

TNLA-Total number of loci amplified, NPL-Number of polymorphic loci, P-percent polymorphic loci

Table 3. Percentage of polymorphic loci observed and expected number of loci Nei's genetic distance and mean heterozygosity for each population of *Hippophae* sp.

Region	PPL (%)	na	ne	H	Ht
LAHAUL	66.90	1.69±0.46	1.34±0.35	0.20±0.18	0.20±0.01
SPITI	65.48	1.65±0.47	1.37±0.37	0.21±0.19	0.22±0.01
SANGLA	10.32	1.10±0.30	1.06±0.20	0.03±0.11	0.04±0.007
LEH	64.06	1.64±0.48	1.33±0.35	0.19±0.18	0.20±0.01
Overall	51.69	1.95±0.21	1.41±0.31	0.26±0.15	-

*PPL= Percentage of polymorphic Loci, na = observed number of alleles, ne = effective number of alleles, h = Nei's gene diversity, Ht = mean heterozygosity.

Table 4. Nei's genetic similarity (above diagonal) and differences (below Diagonal) of the four populations of *Hippophae* sp.

Region	Lahaul	Spiti	Sangla	LEH
Lahaul	-	0.946	0.596	0.919
Spiti	0.056	-	0.589	0.906
Sangla	0.518	0.530	-	0.644
Leh	0.084	0.099	0.439	-

group A_2, 53.0% with group A_3, 49.0% with group A_4, 47.0% with group A_5 and 42.0% with group A_6. Major clusters A and B exhibited least (26.0%) genetic similarity with each other.

Genotypes grouped in cluster B (Sangla) and A_6 (Poh) showed maximum divergence from other groups with bootstrap value of 100%. Genotypes clustered into group A_1 exhibited a similarity coefficient of 0.64, although they belonged to different geographic regions. We also performed cluster analysis of the 48 genotypes based on Jaccard's similarity coefficient with UPGMA method using DARWIN 5.0 (Sneath and Sokal, 1973) for generating a dendrogram (Figure 2). The dendrogram delineated all the 48 sea buckthorn genotypes into 11 clusters, A, B, C, D, E, F, G, H, I, J and K. The genotypes more or less clustered according to their geographical affiliations. Clusters I, J and K with 3 genotypes each from Poh (Spiti), Goshal (Lahaul) and Sangla (Kinnaur) were distinctively separated. Genotypes grouped in these clusters showed maximum divergence robustness from other groups with high bootstrap value of 100, 96 and 100%, respectively.

Genotypes clustered in group A exhibited close association, although they belonged to different

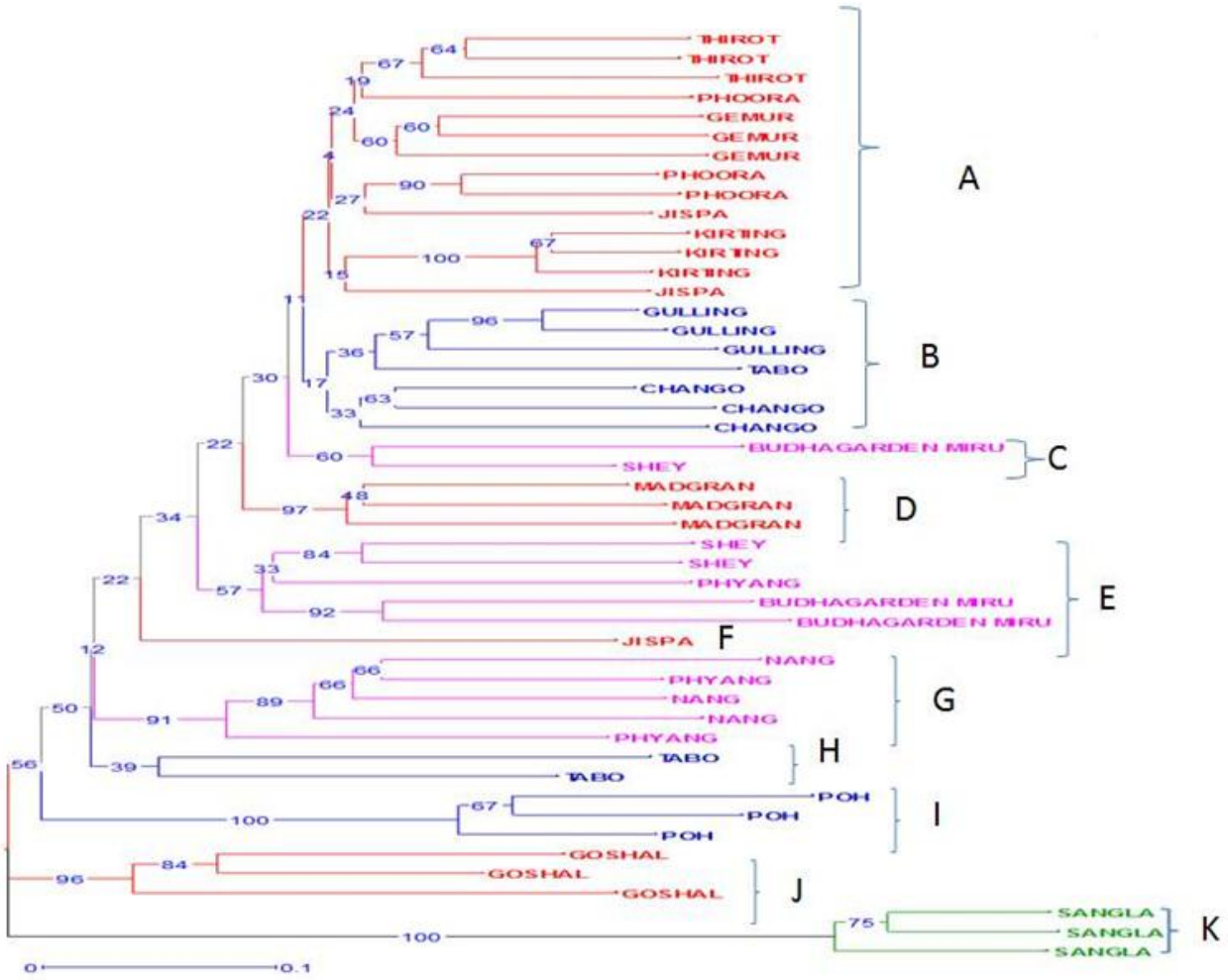

Figure 2. Dendrogram generated using Jaccard's cofficient and UPGMA cluster analysis in DARwin for 48 *Hippophae* L. genotypes analysed by 262 PCR-based markers.

geographic regions. Inconsistencies in the clustering pattern was evident from the mantel test with RAPD, SSR and MAD-Box gene primers exhibiting poor fit (r = 0.26 for RAPD, r = 0.62 for SSR and r = 0.006 for MAD-Box gene primers). ISSR and combined data showed good fit with r values of 0.77 and 0.84, respectively. Based on a set of 48 genotypes and data on 262 polymorphic markers, four populations were classified into small subgroups by STRUCTURE (Figure 3) and the pattern delineated was very similar to the clustering produced by DARWIN and NTSYSpc.

Analysis of molecular variance (AMOVA) based on individual marker data and combined markers data showed that differences among *H. rhamnoides* populations were significant at $p < 0.001$, having greater variation (72.0%) within population than among populations (28.0%) (Table 5).

Principal coordinate analysis

Principal coordinate analysis was performed to provide spatial representation of the relative genetic distances among individual and to determine the consistency of differentiation among populations defined by the cluster analysis. The first three principal coordinate axes obtained in the analysis of mixed populations accounted for 36.81, 20.40 and 16.22% of the total variation, respectively (cumulative value = 73.43%), while RAPD, ISSR, SSR and MADS-box marker accounted for 72.33, 76.90, 74.06 and 71.31% of the total variation for first three principal coordinates. Populations from Sangla valley expressed the highest level of genetic differentiation in relation to other populations, whereas the least population specific identity has been observed in populations

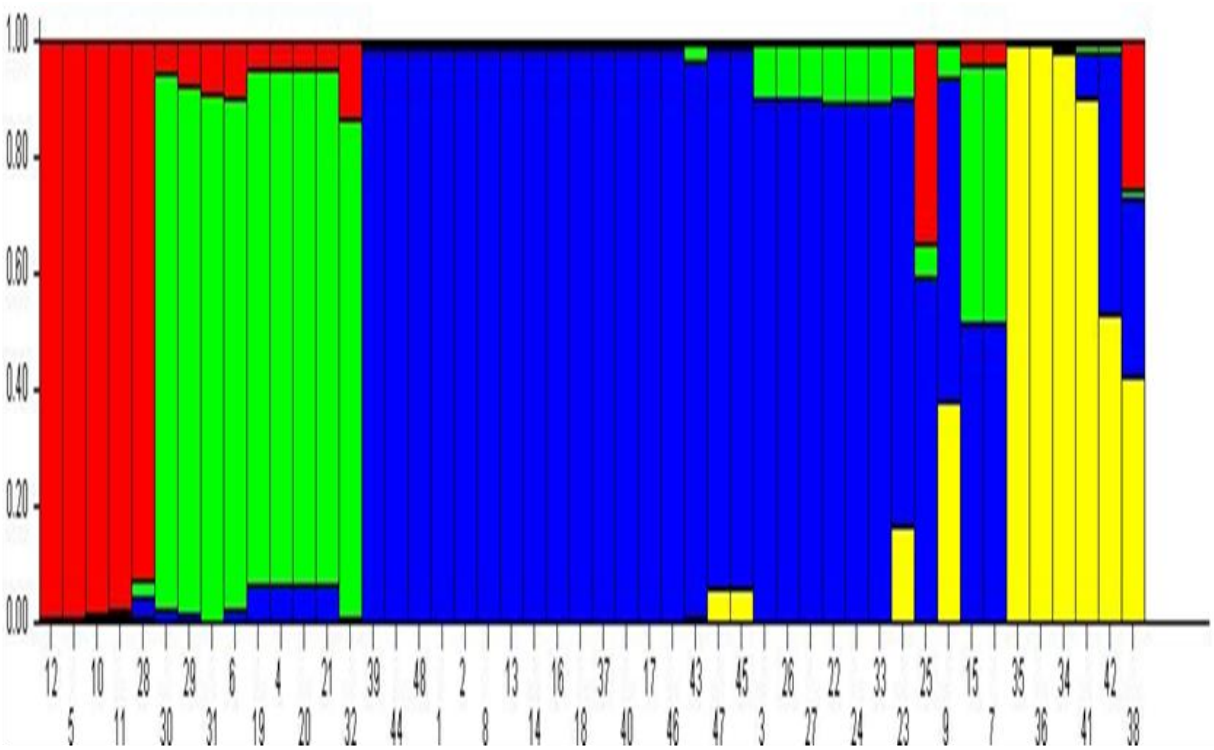

Figure 3. Structure bar plot of 48 genotypes of sea buckthorn from 16 locations showing the pattern of assignment into 4 broad clusters.

Table 5. Hierarchial analysis of molecular variance in four *Hippophe* sp. populations.

Source	df	SS	MS	Est. Var.	% Total variance	F_{st} value	Prob
Among pops	3	491.646	163.882	12.227	28%		
Within pops	44	1360.333	30.917	30.917	72%	0.283	0.001
Total	47	1851.979	194.799	43.143			

Pops = populations, ss = standard significance, MS = mean significance, Est var. = estimated variation, Prob = probability.

from Leh region. The populations from Lahaul valley were observed to be associated with that from Spiti valley, as both showed moderate population specific identity and both of which formed two looser sub-clusters of individuals. It was also indicated by Nei's genetic similarity values for nearly all individual markers (except MAD-Box gene markers) and combined marker analysis that population from Lahaul valley had maximum proximity with that from Spiti followed by population from Leh region. Population from Sangla-Kinnaur was most divergent which is congruent with Mantel test (Table 4).

The different pairwise population indices like phi-statistics, population differentiation, Shannon's information index and estimate of gene flow calculated for all the molecular markers separately and for combined data also revealed that the genotypes from Sangla region were genetically different as compared to other genotypes (Table 6). Populations from Lahaul and Spiti regions divulged maximum similarity.

ITS sequence analysis

Complete sequences of the ITS regions were generated from the accessions forming distinct groups. The boundaries of ITS1 and ITS2 and adjacent coding regions were determined by comparing the published sequences from Genebank. The ITS sequences varied in length from 617 to 638 bp. On alignment,

Table 6. Coefficient of pairwise population differentiation G_{st}, Phi statistics (F_{st}), Shannon's information index and estimate of gene flow N_m for all the Makers and for combined data among four *Hippophae* sp populations.

1st Population	2nd Population	F_{st}	G_{st}	N_m	I
LAHAUL	SPITI	0.111	0.07	6.31	0.35±0.24
LAHAUL	SANGLA	0.622	0.54	0.42	0.39±0.22
SPITI	SANGLA	0.578	0.55	0.41	0.43±0.22
LAHAUL	LEH	0.186	0.11	3.83	0.36±0.24
SPITI	LEH	0.176	0.124	3.52	0.37±0.23
SANGLA	LEH	0.561	0.539	0.428	0.39±0.24
OVERALL	-	-	0.17	2.37	0.41±0.20

accessions with known taxonomic status showed maximum identity with *H. rhamnoides* subspecies *turkestanica*. However, accessions from Sangla (Kinnaur) expressed maximum identity with *H. salicifolia* (99.0%). Populations from Poh (Spiti valley) and forming a distinct had, however, maximum identity with *H. rhamnoides* subspecies *turkestanica*.

DISCUSSION

Genetic diversity analysis

Assessment of genetic diversity within economically important plant species has implications in germplasm conservation and breeding, and molecular markers offer an effective solution to unravel the genetic diversity among genotypes due to their presence throughout the genome. Over the past 15 years, numerous attempts have been made to examine the genetic diversity and phylogeny of *Hippophae* (Jepsson et al., 1999; Tian et al., 2004, Ruan and Li, 2005; Sun et al., 2006; Chen et al., 2008; Raina et al., 2011). In the present study, four marker approaches, RAPD, ISSR, SSR and MADS box gene markers we applied to assess the extent and pattern of genetic diversity on a panel of 48 sea buckthorn genotypes representing 4 geographically distinct regions and 16 locations in the north western Himalayan region of India. We obtained a total of 272 bands with 262 (96.32%) markers being polymorphic. The populations were clustered into 11 distinct groups on the basis of Nei's genetic differences with population from Sangla forming an out-group. This grouping revealed that Sangla population was very divergent and we resorted to ITS sequence analysis to ascertain its species status. There is a general lack of authentic information on distribution of species in the Himalayan region of India and molecular markers can prove very useful in solving these taxonomic issues.

Genetic differentiation

Sea buckthorn in Indian Himalayas only thrives in mountain slopes and river valleys; therefore its distribution is restricted in limited geographical niches. The species with restricted eco-geographical distribution tend to have limited genetic differentiation within populations (Hamrick and Godt, 1990). Some studies have reported that geographic isolation significantly affects the differentiation of populations and that genetic distances are correlated with geographic distances (Schnabel and Hamrick, 1990; Alpert et al., 1993). Other studies yet suggested that geographic distances do not necessarily cause genetic differentiation and that the genetic distances among the populations have no significant correlation with the geographic distances (Li et al., 1995; Bartish et al., 1999; Wang et al., 2003).

In this study, we attempted to measure the correlation between geographic and genetic distances of the populations and it showed a significant ($p < 0.040$) and strong positive correlation (0.92) for combined marker data. The only molecular marker system which showed negative correlation was MADS-BOX gene primers which obviously may be due to very limited number of alleles amplified and only two primers used. Other molecular markers showed the same trend as observed for combined data. The Mantel test revealed that with the increase of geographical distance there is an increase in genetic distance too. It may be due to the dioecious nature of the species and the geographical boundaries which limit the scope of pollen flow to very long distance. Moreover, AMOVA indicated sufficient genetic differentiation within populations and very low level of variation among populations which is well supported by the Mantel test results. All four geographical regions are separated by high mountain passes and located in difficult and rugged terrain. Since these populations are surviving in harsh environment for a

long time, accumulation of genetic variation at species level is imminent for their adaptation to ambient pressures (Li and Midmore, 1999).

Sea buckthorn in western Indian Himalayas grows at a very high altitude (> 2600 m above mean sea level) with high ultra violet radiations and severely low temperatures (as low as 45°C during winters). Such extreme environmental factors not only affect morphological attributes but also the hereditary determinants (Chen et al., 2010). The *H. rhamnoides* populations from Goshal (Lahaul valley) and Poh (Spiti valley) showing divergence from other populations must have accumulated genetic mutations over time. The valley is characterized by arid cold weather devoid of vegetation and comprises of mountainous ravines having complex species composition. Such surroundings form distinct spatial heterogeneity and the resulting environmental selective pressures cause species plasticity that helps increase level of genetic diversity. Lack of genetic variation among the populations in a particular region could be ascribed to homogeneity of micro-environment and the individuals endure uniform selection pressure and tend to evolve in similar direction. Clonal reproduction behavour of sea buckthorn has also been ascribed as one of the possible reasons for low genetic diversity.

There are reports of negative correlation of genetic diversity with the altitude as a higher altitude sexual reproduction is adversely affected (Young et al., 2002). Chen et al. (2008) observed significantly positive correlation between genetic distance and altitude was detected while examining five populations and female sub-populations of *H. rhamnoides* (Chen et al., 2008). However, no such correlation was observed in this study.

Analysis of molecular variance (AMOVA) provided measures of genetic variation within and among populations. AMOVA based on combined marker data as well as on individual marker data showed greater variation within population than among populations. This observation correlates with the previous analysis in *Hippophae* based on RAPD and ISSR data (Bartish et al., 1999; Tian et al., 2004; Chen et al., 2008). The genetic structure of *H. rhamnoides* followed the general pattern detected in woody species with widespread distributions and out crossing mating systems. Such plants possess more genetic diversity within populations and less variation among populations than do species with other combinations of traits. The analysis showed that 72% of the variation of *Hippophae* was within the population and a meager 28% was found among the populations. The population genetic structure of the species is affected by a number of evolutionary factors, including mating system, gene flow, seed dispersal and mode of reproduction, as well as natural selection (Hamrick and Godt, 1990).

In *H. rhamnoides*, the sexual system is dioecious and the occasional hermaphrodite plants are highly self-incompatible. In order to assess whether the grouping of individuals based on marker data could be further resolved, principal coordinate analysis (PCoA) was used to examine the association among genotypes. The PCoA using separate and pooled markers data also placed the Sangla population separately to the other genotypes.

The pairwise population differentiation and F_{st} value for Sangla and other populations was observed to be very high along with high Shannon's information index and low level gene flow which clearly indicated that the Sangla genotypes are either a different strain or a close relative of *H. rhamnoides* ssp *rhamnoides*. Based on this assumption we sequenced the ITS region of one representative genotype from three populations. Interestingly, ITS sequence analysis revealed that *Hippophae* stands growing alongside Baspa river in Sangla valley (Kinnaur) are *H salicifolia* and not *H. rhamnoides* as was evident from the population analysis based on different molecular markers. This is new information and has implications in taxonomic classification and species conservation. The usefulness of molecular marker markers in genetic diversity studies, species identification, genetic mapping and marker-assisted breeding has been convincingly established.

Overall, the populations sampled revealed low levels of genetic diversity among themselves; however significant genetic differentiation existed at the population level. PCR-based molecular markers proved very effective in discerning genetic diversity and population structure. Complex and undulating topography and harsh environment throughout the sea buckthorn growing belts in the north-western Indian Himalayan region explain habitat fragmentation and restricted population size. Lack of genetic variation could also be due to high mountains that act as barrier to limit gene flow across *Hippophae* populations. ITS sequence data has proved very useful to assign a different species status to populations from Sangla valley in Kinnaur. Hence, molecular markers can be efficiently used to differentiate the genotypes from different species or strains.

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