EVALUATION AND UTILIZATION OF SSR MARKERS FOR GENETIC DIVERSITY CHARACTERIZATION IN SEABUCKTHORN

THESIS

By

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CERTIFICATE – I

This is to certify that the thesis entitled **"Evaluation and utilization of SSR markers for genetic diversity characterization in Seabuckthorn"** submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science (Agriculture)** in the discipline of **Agricultural Biotechnology** of CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Mr. Smitesh Sunil Dangat (Admission No. A-2017-30-004)** son of **Shri. Sunil Pandharinath Dangat** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

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CERTIFICATE- II

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TABLE OF CONTENTS

Chapter	Title	Page
1.	INTRODUCTION	1-3
2.	REVIEW OF LITERATURE	4-14
3.	MATERIALS AND METHODS	15-23
4.	RESULTS AND DISCUSSION	24-38
5.	SUMMARY AND CONCLUSIONS	39-40
	LITERATURE CITED	41-58
	APPENDICES	59-61
	BRIEF BIODATA OF THE STUDENT	

LIST OF ABBREVIATIONS USED

Sr. No.	Abbreviation	Meaning
1	%	Percent
2	µg	Microgram
3	µl	Microlitre
4	bp	base pair
5	N-J	Neighbor Joining
6	UPGMA	Unweighted pair group method of arithmetic mean
7	GPS	Global Positioning System
8	CTAB	CetylTrimethyl Ammonium Bromide
9	dATP	Deoxyadenosine triphosphate
10	dCTP	Deoxycytidine triphosphate
11	dGTP	Deoxyguanosine triphosphate
12	DNA	Deoxyribonucleic Acid
13	dNTP	Deoxynucleotide triphosphate
14	dTTP	Deoxythymidine triphosphate
15	EDTA	Ethylenediamine Tetra Acetic Acid
16	HCL	Hydrochloric Acid
17	$MgCl_2$	Magnesium Chloride
18	RNase	Ribonuclease
19	Tris	Tris (hydroxy methyl) amino methane
20	N	Nitrogen
21	TE	Tris EDTA buffer
22	AgNO3	Silver Nitrate
23	NaOH	Sodium Hydroxide
24	*Taq* polymerase	*Thermus aquaticus* DNA polymerase
25	et al	And Coworkers
26	Fig	Figure(s)
27	G	Gram
28	Kg	Kilogram
29	Mg	Milligram
30	ng	Nanogram
31	Hr	Hour
32	Sec	Second(s)
33	T_m	Melting Temperature
34	T_a	Annealing Temperature
35	^oC	Degree Celsius

Sr. No.	Abbreviation	Meaning
36	Gb	Giga byte
37	Kb	Kilo byte
38	Mbp	Million base pairs
39	Avg	Average
40	PCoA	Principal coordinate analysis
41	MCMC	Markov Chain Monte Carlo
42	Ne	Effective number of Allele
43	I	Shannon’s Information Index
44	Na	Observed number of Allele
45	Ho	Observed Heterozygosity
46	He	Expected Heterozygosity
47	Min	Minute(s)
48	Sec	Second(s)
49	L	Litre
50	Ml	Millilitre
51	M	Molar
52	mM	Millimolar
53	cm	Centimeter
54	m	Meter
55	Ha	Hectare
56	Ft	Feet
57	amsl	Above mean sea level
58	DNR	Di-nucleotide repeat
59	TNR	Tri-nucleotide repeat
60	TtNR	Tetra- nucleotide repeat
61	PNR	Penta-nucleotide repeat
62	HNR	Hexa-nucleotide repeat
63	P	Probability
64	PCR	Polymerase Chain Reaction
65	pH	Puissance de hydrogen (ion conc.)
66	PVP	Polyvinyl pyrrolidone
67	QTL	Quantitative Trait Loci
68	PAGE	Polyacrylamide gel electrophoresis
69	RAPD	Random Amplified Polymorphic DNA
70	RFLP	Restriction Fragment Length Polymorphism
71	AFLP	Amplified Fragment Length Polymorphism
72	EST-SSR	Expressed Sequence Tagged-Simple Sequence Repeats

Sr. No.	Abbreviation	Meaning
73	SNPs	Single Nucleotide Polymorphisms
74	SRAP	Sequence Related Amplified Polymorphism
75	SSR	Simple Sequence Repeat(s)
76	ISSR	Inter Simple Sequence Repeats
77	SAMPL	Selective amplification of microsatellite polymorphic loci
78	NGS	Next Generation Sequencing
79	V	Volts
80	W	watts
81	Rpm	Revolutions per minute
82	U	Units
83	UV	Ultraviolet
84	λ	Lambda
85	SBTMS	SeaBuckThorn MicroSatellite
86	DEB	DNA Extraction Buffer

LIST OF TABLES

Table No.	Title	Page
3.1	Location of *H. rhamnoides* leaf collection sites in trans-Himalayan Ladakh and Lahaul-Spiti region	15
4.1	Mining of SSRs from transcriptome data	25
4.2	List of seabuckthorn microsatellite primers, repeat type and annealing temperature used for PCR amplification.	29
4.3	Estimates of genetic diversity in seabuckthorn (*H. rhamnoides*)	31
4.4	Estimates of genetic differentiation and gene flow (Nm) in *H. rhamnoides* population	33

LIST OF FIGURES

Fig. No.	Title	Page
2.1	(A) Male (B) Female seabuckthorn plants	5
3.1	Map showing location of collected populations of *H. rhamnoides* (source: Google Maps)	16
3.2	Schematic representation of CTAB method for DNA isolation	19
3.3	Schematic representation of strategies used for the mining of microsatellites.	20
4.1	Quantification of purified genomic DNA isolated from seabuckthorn genotypes: Lane 1, Lane 49, Lane 51 and Lane 98 = 25 ng/µl λ uncut; 1-96 different seabuckthorn genotypes.	24
4.2	Distribution of nucleotide repeats in seabuckthorn transcriptome.	26
4.3	Localization of SSR repeats.	26
4.4	Experimental validation for polymorphic potential of SSR markers; Lane 1-6 random genotypes of seabuckthorn.	27
4.5	Amplification profile of 245 seabuckthorn genotypes with primer SBTMS-7 and L= 50 bp DNA ladder.	30
4.6	Total genetic variation among and within population of *H. rhamnoides* populations	34
4.7	Bayesian model population genetic structure harvester generated graph representing deltaK max	35
4.8	Neighbour joining dendrogram based on Jaccard's dissimilarity coefficient along with bootstrap values.	35
4.9	Bayesian model based Population genetic structure	36
4.10	Population structure of 245 seabuckthorn genotypes showing two genetic groups.	37
4.11	Principal coordinates analysis representing two populations of 245 Seabuckthorn accessions.	38

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Abstract

Seabuckthorn (*Hippophae rhamnoides,* diploid 2n=24) is a dioceous, wind pollinated, thorny shrub belongs to family Elaeagnaceae. In India, it grows naturally in high altitude and cold-arid regions of Ladakh, Sikkim, Jammu & Kashmir, Himachal Pradesh, and Uttrakhand. It is economically and ecologically important medicinal plant due to its high immense medicinal properties but there is unavailability of superior variety/ selection available for large scale commercial cultivation in India. Furthermore, validated microsatellite marker resources are also limited in this plant species. Therefore, a total of 882 novel SSR markers were mined from in-house *H. rhamnoides* transcriptome data. Among all, abundance of tri-repeats (49%) followed by di-repeats (46%) was observed. Further, evaluation and utilization of polymorphic SSR makers for genetic diversity assessment of 245 accessions of Seabuckthorn (*H. rhamnoides*) detected high average allele frequency (5.45 alleles/ primer) and high heterozygosity (Ho: 0.77; He: 0.75). Neighbor-Joining dendrogram, principal coordinate analysis (PCoA) and Bayesian model based population structure analysis grouped all the 245 individuals into two major groups with high within population gene flow suggesting that there are two genetic pools operating in the natural seabuckthorn populations of trans-Himalayan Leh and Keylong regions of India. The results of this study will be useful for identification and selection of potential genotypes to expedite the conservation and genetic improvement programme in seabuckthorn. Genome-wide microsatellite marker resource created during this study can be validated and utilized for futuristic evolutionary and various genotypic studies in seabuckthorn.

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1. INTRODUCTION

Seabuckthorn (*Hippophae rhamnoides,* diploid 2n=24), a widespread economically important dioceous thorny shrub belongs to family Elaeagnaceae (Elena et al. 2011). It is native to Eurasia and also widely distributed in cold regions of China and Russia at a high altitude range of 2500-4300 m (Bartish et al. 2002). In India, it naturally grows at high altitude and cold-arid regions of Ladakh, Himachal Pradesh, Sikkim, Jammu & Kashmir, and Uttarakhand. Seabuckthorn is one of the leading plants in Leh-Ladakh region where it is mainly distributed in Nubra, Indus and Changthang valleys covering approximately an area of 11,500 hectare of Leh district growing at altitudes of 8000-14000 ft. (Stobdan et al. 2008). *Hippophae rhamnoides* (L.) is a predominant species in the Trans-Himalayan region of India. Seabuckthorn has been found to grow in sundry areas with harsh environmental conditions; it has also been well documented for its ability to withstand drought and extreme temperate range between -45 to +43 °C (Ruan et al. 2007). In Ladakh, seabuckthorn is well known by its common name “Tsermang” or “Leh berry” (Roy et al. 2001; Singh et al. 2006).

All parts of seabuckthorn are considered to be great reservoir of important health-promoting substances. Leaves are rich in secondary metabolites like phenolics, flavonoids, carotenoids, free and esterified sterols, terpenols and isoprenols, lipids and volatile compounds (Goncharova and Glushenkova 1996; Beveridge et al. 1999; Tian et al. 2004; Suryakumar and Gupta 2011; Ahmad et al. 2012; Kashif and Ullah 2013). Leaves of seabuckthorn also contain a significant amount of proteins, folic acid, and catechin. Yasukawa et al. (2009) successfully isolated and determined three phenolic compounds, catechin, gallocatechin, and epigallocatechin and a triterpenoid, ursolic acid from the active fraction of the 70% ethanol extract of seabuckthorn leaves which are known for antitumor activity. The catechin content of seabuckthorn leaves is used to enrich the quality of tea.

Seabuckthorn berries are rich source of vitamins (C, E, and K), minerals, amino acids, fatty acids, carotenoids, flavonoids and other secondary metabolites (Zeb

2004; Suryakumar and Gupta 2011; Fatima et al. 2012). These bioactive compounds are highly valued for their antioxidant, hepatoprotective, anti-carcinogenic, immune-modulatory and cytoprotective effects (Michel et al. 2012). Quercetin is one of the main components that induce apoptosis in cancer cell treatment of patients having colorectal cancer, leukemia, and prostate cancer (Patel et al. 2012). Apart from its nutritional and medicinal properties, it also plays an important role in preventing soil erosion and improving soil fertility by forming a symbiotic association with *Frankia*, which is found to inhabit root nodules of plants. High-quality biomass produced by Seabuckthorn can be used as a potential source of green energy (Chaurasia and Singh 1996).

Seabuckthorn is considered as the “next major health food” due to medicinal and nutritional properties of its consumable berries and leaves. Pulp of the berries is used to make jams, marmalade and jelly while its unique aroma is used for flavoring dairy products (Tittinen et al. 2005). Likewise, leaves (either fresh or dried) used to prepare a nutritional tea, and leaves, young branches and fruit pulp are also used as animal fodder. The rural inhabitants of the upper Himalayan region have very limited opportunities for growing cash crops to earn income because of adverse climatic conditions (Dhyani et al. 2010). Therefore, progressive efforts should be taken to develop Seabuckthorn not only as functional food, but also to benefit the local villagers involved in small scale ventures including farming.

Variations observed at morphological, biochemical and physiological levels represent the manifestation of genetic variability required for imparting adaptations to sustain in diverse demographic challenging environmental conditions (Yao 1994). Plant height, leaf size, degree of thorniness, fruit shape and branching habits are some of the morphological characters showing vast variation in Seabuckthorn population worldwide (Yao 1994; Small et al. 2002). Additionally, Seabuckthorn in India faces great challenges of non-availability of superior varieties/ selection/ cultivars for large scale commercial cultivations. Till date very limited efforts have been made to assess these variations at the genetic level, wherein, anthropogenic activities may cause loss of genetic diversity of potential genotypes of Seabuckthorn.

The emergence of molecular marker techniques has provided the scientific tools for efficient genetic analysis of genomes without sequencing. Among the various molecular marker types, microsatellites (2-6 bp tandemly repeated DNA sequences) are advantageous and widely used in genetic diversity analysis, gene mapping, and marker-assisted selection because of their locus specificity, co-dominant inheritance, and even distribution throughout the genome (Zorrilla-Fontanesi et al. 2011; Akkaya et al. 1992; Huang et al. 2002; Zang et al. 2010; Hao et al. 2011). Additionally, high rate of cross-species transferability of microsatellites among closely related species or genera provides an important tool for ecological and evolutionary studies, population differentiation and speciation (Barbara et al. 2007; Rousseau-Gueutin et al. 2008).

Thus, in view of the above mentioned medicinal and economic importance, and various limitations of Seabuckthorn as a future horticultural crop, efforts were made for identification of polymorphic and reproducible SSR markers from in-house transcriptome data. Informative polymorphic SSR markers were utilized successfully for genetic diversity characterization and identification of core genotypes or populations to expedite conservation and genetic improvement programme in Seabuckthorn. The current master's degree dissertation was undertaken with the following objectives;

1. Evaluation of polymorphic potential of SSR markers
2. Utilization of SSR markers for genetic diversity characterization

2. REVIEW OF LITERATURE

The literature pertaining to different aspects of the present investigation has been reviewed under the following headings:

2.1 Morphology

Hippophae rhamnoides is a deciduous and hardy shrub grows between 2.0 m to 5.0 m height at a medium rate and rarely reaching to 10 meters in some parts of central Asia (Swenson and Bartish 2003). Male and female plants of seabuckthorn were depicted in fig. 2.1. The leaves are alternate, narrow and lanceolate. The branches are grey and spiny with silvery white leaves.

Seabuckthorn remains in the dormant stage during winters so it has adequate storage of carbohydrates that will helpful for launching their spring growth so that flowers bloom before the emerge of leaves in April, while seeds ripen from September to October. It is a dioceous plant having, male and female flowers produced on different plants, therefore, plant is cross-fertile with wind pollination resulted into berries in the female plants through-out the winter (Stobdan et al. 2008). The male flowers emerge in a condensed axillary form on the stem and are terminal with short pedicels. The male flowers are small (3.4 ± 0.1 mm in length) and each flower having four stamens surrounded with two perianth lobes. The pollen grains are small (26.47 ±0.37 μm), dry with starch grains as a prime reserve material (Mangla et al. 2013). The female flower represents a gynoecium which covered with two partially fused perianth lobes. Each flower produces only one ovary and one ovule. Stigma is nonpapillate, scrobiculate and dry type. At the time of anthesis, the ventral receptive side of the stigma faces the air and dorsal side towards the subtending bract (Mangla et al. 2013). Berries of Sea buckthorn are the oval shape and vary in color ranging from yellow to orange. Root system of *Hippophae rhamnoides* is extensive and horizontal with the maximum root spread of about 202.3 cm and the depth up to 24 to 72.6 cm.

The genus *Hippophae* comprises seven different species namely; *Hippophae salicifolia*, *Hippophae rhamnoides*, *Hippophae tibetana*, *Hippophae goniocarpa*, *Hippophae gyantsensis*, *Hippophae litangensis*, and *Hippophae neurocarpa,* out of that two are of the hybrid origin, native over a wide range of area from Europe and Asia (Bartish et al. 2002).

Fig. 2.1 (A) Male (B) Female seabuckthorn plants.

2.2 Distribution of Seabuckthorn

The natural habitat of seabuckthorn expands widely across colder region of Europe, Asian countries like India, China, Mongolia, Russia, and North America in Sweden, Finland and Norway (Rousi 1971; Lian 1988; Chen 1996; Lu et al. 1997; Lian and Chen 2000; Dwivedi et al. 2009). Among all, *Hippophae rhamnoides* is the most common and widely distributed species ranging from north-western Europe and spreads throughout central Asia to the Altai Mountain ranges, the northern part of Indian Himalaya, and the western and northern part of China.

In India, a joint national initiative launched by the Ministry of Environment & Forests and Defence Research and Development Organization for promoting seabuckthorn cultivation at high altitude, cold desert ecosystems. Defence Institute of High Altitude Research (DIHAR) and Defence Research and Development Organization (DRDO) has developed technologies for the preparation of food beverage from highly acidic berries. Nutritious and healthy 'Multivitamin Herbal Beverage' manufactured from seabuckthorn berries was developed and patented successfully in 2001 by the Defence Institute of High Altitude Research (DIHAR), Leh (Ballabh et al. 2007).

2.3 Pharmacological uses:

Medicinal and nutritional value of seabuckthorn is largely due to the presence of various bioactive compounds in different parts of plant wherein, berries, seeds, bark, leaves and roots are considered to be a good source of various compounds like carbohydrates, proteins, amino acids, vitamins, organic acids, polyphenolic acids, dietary minerals and β-sitosterol, etc. (Yang and Kallio 2002b; Pintea et al. 2005; Bal et al. 2011; Suryakumar and Gupta 2011). Seabuckthorn berries are recommended in Tibetan and Mongolian traditional medicinal literature as an effective remedy for patients suffering from cough and to improve blood circulation and functioning of the digestive system (Singh and Moersel 2005). It has been noted that the tribal communities in the Lahaul-Spiti region utilize seabuckthorn as a dry powder or in the form of juice to regulate menstruation cycle, to cure infecundity, liver disease and jaundice (Singh 2005). Extracts from seabuckthorn berries are commonly used for the treatment of dermatitis, asthma, jaundice, and gastro-intestinal disorders. Berries of

seabuckthorn and its extract are used as a laxative and for the treatment of rheumatism in Himalayan and some of the Russian tribes (Singh and Moersel 2005).

Antioxidant and immunomodulatory

Seabuckthorn leaf extracts show immunomodulatory activity that specifically activates the cell-mediated immune response is proved from in-vitro studies carried out using rat lymphocytes (Geetha et al. 2005). In oriental traditional medicines, seabuckthorn has been used to cure various inflammatory disorders. Seabuckthorn leaf extract has been found to have cytoprotective and antioxidant activity on glial cells across hypoxia-induced oxidative stress (Narayanan et al. 2005). The radial-scavenging activity has been enhanced by triterpenoids identified in seabuckthorn that significantly inhibits the production of nitric oxide (Yang et al. 2007). Further, clinically it has been ascertained that seabuckthorn extracts reduce tumor incidence of skin and fore stomach papilloma genesis (Suryakumar and Gupta 2011).

Anti-cancer activity

Extracts from seabuckthorn berries have efficient anticarcinogenic activity (Michel et al. 2012). Yang and Kallio (2002b) suggested that seabuckthorn oil is effective in palliation of haematological damage caused by chemotherapy, such as part of the treatment of leukemia. Therapeutic effects are attributed to substances such as catechin, gallocatechin, and epigallocatechin (Khan et al. 2010).

Hepatoprotective activity

Diseases like hepatitis or liver cirrhosis are caused by various environmental pollutants and drugs that weaken the liver (Zimmerman and Ishak 1994). Seabuckthorn extracts have the potential to protect the liver and cure liver diseases (Barkat et al. 2010).

Anti-stress and adaptogenic activity

The herbal formulations from seabuckthorn leaf extracts were used to study anti-stress and adaptogenic activity in rats using a cold (5 ^{0}C)–hypoxia (428 mmHg)–restraint (C–H–R) animal model (Saggu et al. 2007). Saggu and Kumar (2008) suggested that aqueous leaf extracts of seabuckthorn can effectively shift anaerobic

metabolism to aerobic during multiple levels of stress C–H–R exposure and post-stress recovery.

Modulation of hypoxia-induced transvascular leakage

The alcoholic extracts of Seabuckthorn leaf and seed oil are found to be effective against hypobaric hypoxia-induced trans-vascular fluid leakage in the lungs and brain of rats (Suryakumar and Gupta 2011).

Cardioprotective and anti-atherogenic effects

Different flavonoid compounds (mainly isorhamnetin and quercetin) in seabuckthorn berries and leaves improve the functioning of the cardiovascular system by providing protective effects on atherosclerotic heart disease and reperfusion, tumors, oxidative injury and aging (Eccleston et al. 2002).

Anti-bacterial and anti-viral effects

Discovery of a new phytochemical drug, 'Hiporamin' (purified fraction of polyphenols and containing a cocktail of monomeric hydrolyzable galloellagi-tannins), possessing a wide spectrum of anti-viral and antimicrobial activities from the seabuckthorn leaves (Suryakumar and Gupta 2011). It was also found to have very strong activity against Influenza and Herpes viruses (Shipulina et al. 2005) along with inhibitory effect against HIV infection in the cell culture and antimicrobial activity (Suryakumar and Gupta 2011). The seabuckthorn leaf extract also found to have significant activity against dengue (Jain et al. 2008) along with antibacterial activities against *Listeria monocytogenes* and *Yersinia enterocolitica* (Chauhan et al. 2007). Aqueous and hydro-alcoholic leaf extracts of seabuckthorn inhibit the growth of various bacteria like *Bacillus cereus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis* (Upadhyay et al. 2010).

Anti-radiation effects

Extract of the fresh berries of seabuckthorn has been reported to impart the protection to the whole mice along with various tissues, cells and cell organelles against harmful irradiation exposure and the functional integrity of mitochondria from radiation-induced oxidative stress (Goel et al. 2005; Chawla et al. 2007).

Healing effect on acute and chronic wounds

Seabuckthorn leaf extracts also provide effective protection against wounds, burns, scalds, ulcers and mucosal injuries (Gupta and Upadhyay 2011). Traditional use of seabuckthorn oil to promote recovery of skin injuries, healing of wounds, and skin diseases have also reported (Li and Schroeder 1996; Beveridge et al. 1999).

2.3 Ecological and Agronomical importance:

Apart from medicinal value, seabuckthorn is well known for its ecological and agronomical importance. Actinomycete, *Frankia* has an ability to fix atmospheric nitrogen that forms the symbiotic relationship with root nodules of seabuckthorn to enrich the soil nutrient (Gatner and Gardner 1970). Seabuckthorn root nodules can fix 180 Kg N/ha/year (Jike and Xiaoming 1992). A strong correlation exists between the seabuckthorn stand and concentration of different soil nutrients (Acharya et al. 2010). Seabuckthorn has an ability to develop an extensive root system in very short period of time helps in the soil nutrient improvement which makes it ideal for planting in degraded soil and in the barren lands (Stewart and Pearson 1967; Akkermans et al. 1983; Trajkovski and Jeppsson 1999).

Countries, like Germany and China, seabuckthorn plantation has been specially carried out for the ecological rehabilitation of degraded and barren lands particularly in afforestation of industrial and coal mining dumps in order to check soil erosion, conservation of soil nutrients, and ecological maintenance of ecosystem (Singh and Moersel 2005; Wei et al. 2007; Chen et al. 2008; Meng et al. 2008). Ledwood and Shimwell (1971) have been recognized as a key plant for stabilization of mobile sand dunes. Seabuckthorn has also been well known to grow efficiently in wastelands of oil shale, open mine areas and acts as an ideal pollution reducer and initial colonizer (Jalakas et al. 2003). Seabuckthorn is planted in some parts of North America as vegetation covers along highways where de-icing salt prevents the growth of other plants.

The many sharp and stout thorns present on the branches of seabuckthorn make it as an ideal plant for hedges around the houses and fields for protection against stray animals and acts as a barrier to pedestrian traffic (Pearson and Rogers 1962). Seabuckthorn has the quick re-growing ability as the propagation can be easily done

by seeds, cuttings or suckers so that it is considered as a good source of firewood (Li and Schroeder 1999). In arid zones of China, *Hippophae rhamnoides* is grown to overcome shortage of fuel forests (Gao and Xu 1991). Additionally, high-quality biomass produced by seabuckthorn can be used as a potential source of green energy (Chaurasia and Singh 1996).

2.4 Development of molecular markers:

The success of any genetic improvement and conservation program is dependent on the extent and distribution of genetic variation present in the population (Ellstrand and Elam 1993). Additionally, the availability of genomic resources provides better opportunities for future characterization, utilization, and bioprospecting of core genotypes.

Molecular markers are widely used for assessment of genetic diversity, construction of genetic linkage maps, identification of QTLs, molecular taxonomy, genome evolution, genome fingerprinting, gene localization, identification of core genotypes to expedite conservation and genetic improvement programme (Henry 1997; Jahufer et al. 2003; Weising et al. 2005; Koebner et al. 2001; Gupta and Rustgi 2004; Varshney et al. 2005; Agarwal et al. 2008; Grover and Sharma 2014). DNA markers are particularly useful if they can uniquely distinguish the closely related individuals within the same or different species. Such markers are called “polymorphic markers”, whereas markers that failed to distinguish between different genotypes are classified as “monomorphic markers”. DNA markers, which can identify variable sites in DNA, are most widely accepted marker types predominantly due to their abundance, accuracy, and reproducibility disregarding of changing the environment and the development stage of plant (Jones et al. 1997). These variations at the DNA level arise from different types of mutations, which include point mutations, insertions or deletions and errors in the replications of tandemly repeated DNA regions or called DNA slippage event (Paterson 1996). Molecular markers were preferred over morphological and biochemical markers in the recent past (Winter and Kahl 1995). The advantage of this technique is that genetic variations can be recorded without prior knowledge of the primer sequences in the target species. In the recent past there are different types of DNA based markers which were utilized for genetic

diversity, comparative, evolutionary and genome mapping studies (Bhandawat et al. 2019; Nag et al. 2015).

2.4.1 Simple sequence repeats (SSRs) or Microsatellites

Among the various methods available at present, detection of simple sequence repeats (SSRs) or microsatellites is one of the important methods emerge in the last decade of the 20th century due to its ability to target known sites in the plant genome (Tautz and Renz 1984). Microsatellites are tandemly repeated sequences of 2-6 base pairs of DNA that appears throughout the genome. Primers designed flanking to tandemly repeated regions represent one of the best co-dominant marker systems and are exploited in genome diversity, genome mapping and conservation studies in plant species. Microsatellite mutates much more rapidly than most other types of sequences and the high mutation rates of microsatellites allow a more detailed analysis of mutation patterns (Winter and Kahl 1995; Jones et al. 1997; Joshi et al. 1999; Kump et al. 2011; Kilian and Garner 2012). During the last two decades, microsatellites have become the most important and versatile source of highly informative and polymorphic genetic markers for the population and conservation genetics, management of biological resources and other related fields (Sunnucks 2000; Weising et al. 2005).

A traditional method for development of microsatellite markers was a cumbersome job due to some laborious and expensive protocols (Wright and Bentzen 1995; Gardner et al. 1999). Due to advent of high- throughput Next Generation Sequencing (NGS) platforms, the development of these markers has become rapid and cost effective. Additionally, mining of such markers from public data is even cheaper and less time consuming to expedite various molecular markers for genetic diversity, evolutionary and genome mapping studies. Mining of SSR markers from transcriptome/ Expressed Sequenced Tag (EST), is an excellent resource for rapid generation of functionally relevant microsatellite markers (Bouck and Vision 2007; Unamba et al. 2015). Furthermore, huge EST sequences are rich resources for gene annotation (Emrich et al. 2007), comparative genomics, development of molecular markers (Novaes et al. 2008) and population genomics studies associated with adaptive traits (Namroud et al. 2008). Recent years have witnessed a large number of

studies including marker development through high-throughput next-generation sequencing for model and non-model organisms (Bhandawat et al. 2019; Nag et al. 2015; Bhardwaj et al. 2013).

2.4.2 Molecular markers applications in Seabuckthorn

Random amplified polymorphic DNA (RAPD)

In the beginning, utilization of molecular markers for diversity characterization in seabuckthorn is restricted to arbitrary markers. RAPD markers revealed a considerably high level of species-level genetic diversity (Bartish et al. 1999). Five subsets of *Hippophae rhamnoides* L. and intraspecific hybrids between different species collected from *Hippophae* gene bank at Balsgard detected moderate to high level of genetic diversity with RAPD analysis (Bartish et al. 2000). Assessment of genetic diversity of seabuckthorn (*H. rhamnoides)* populations using RAPD markers from China revealed a high level of diversity without any significant correlation between genetic and geographic distances (Sun et al. 2006). However, utilization of RAPD markers by Sheng et al. (2006) indicated a significantly high level of genetic diversity among and within species of the genus *Hippophae* with a significant correlation between genetic and altitude distance.

Assessment of genetic diversity of Latvian seabuckthorn was done by using eight microsatellites and sixteen RAPD markers and confirmed that seabuckthorn (*Hippophae rhamnoides* L.) plant material grown in Latvia has high genetic diversity. RAPD markers are successfully able to distinguish the seabuckthorn populations in Latvia. (Lacis and Kota-Dombrovska 2014). Likewise, natural and cultivated populations of *Hippophae rhamnoides* subsp. *carpatica* collected from Romania recorded high genetic diversity (Alexandra et al. 2012). Sex-specific SCAR markers derived RAPD have successfully discriminated male and female genotypes (Korekar et al. 2012).

Amplified fragment length polymorphism (AFLP)

Among the dominant markers, AFLP markers were advantageous for detecting large number of polymorphic loci in a single reaction (Vos et al. 1995). 645 polymorphic bands using eight primer combinations have successfully revealed

genetic relationships among 15 cultivars of seabuckthorn collected from the different countries like China, Russia, and Mongolia (Ruan 2006). Cluster analysis revealed *H. rhamnoides* ssp. *sinensis* from China as the most distantly related to the 14 other varieties provides the basis for identification of superior genotypes and development of crossing strategies in a breeding program. Similarly, 25 ecotypes of *H. rhamnoides* from northern Pakistan not only revealed high genetic variability but also able to establish phylogenetic relationship. However, a close relationship exists between few ecotypes irrespective of their geographic distances and morphological characteristics (Shah et al. 2009). Selective amplification of microsatellite polymorphic loci (SAMPL) a variant of AFLP markers are dominant in nature but less employed in the assessment of genetic diversity (Morgante and Vogel 1994). Combined SAMPL and AFLP markers detected higher intrapopulation diversity than interpopulation of *H. rhamnoides* ssp. *turkestanica* (Raina et al. 2012).

Inter simple sequence repeats (ISSR)

ISSRs, a semi-arbitrary dominant marker system that allows amplification of region(s) between two inversely oriented microsatellite repeats are 2nd widely used marker type in seabuckthorn. Genetic variability in 11 natural populations from north eastern and north western China detected no significant correlation between genetic and geographic distances of the populations (Tian et al. 2004a). Contrarily, genetic variation and differentiation among five natural populations of Wolong Natural Reserve, China (an altitudinal range varies from 1,800 to 3,400 m above sea level) revealed significant positive correlation between genetic and altitudinal variations (Chen et al. 2008). Furthermore, 300 individuals of fifteen natural populations of Seabuckthorn (*Hippophae rhamnoides* ssp. *yunnanensis,* ssp. *sinensis,* and ssp. *gyantsensis*) from China detected subspecies level population differentiation in seabuckthorn (Tian et al. 2004b).

SSR or Microsatellite markers

A significant level of genetic diversity with expected heterozygosity ranged from 0.2659 to 0.4767 using SSR marker was reported in *Hippophae rhamnoides* ssp. *sinensis* of arid regions of northwest China (Wang et al. 2008). Further, Yanlin (2007) reported successful cross-amplification of 46 of 107 grapes microsatellite markers in Seabuckthorn. Additionally, genetic diversity along with dendrometric, fruit and leaf

morphometric traits were explored in seabuckthorn *(Hippophae rhamnoides L.)* populations sampled from different regions of Eurasia including the Karakoram Mountains of Pakistan (Gilgit-Baltistan territory) recorded the population level high diversity with high allelic richness using EST- SSR markers (Nawaz et al. 2018). Further, EST derived SSR marker-based characterization revealed a high genetic diversity in seabuckthorn populations existing in Leh region. Due to their derivation from functional genome, these markers were also recorded high level of cross-species transferability in related species of *Hippophae* (Jain et al. 2010).

Recently, NGS assisted de novo short read transcriptome sequencing produced 86,253,874 high-quality filtered short reads yielded 53 Mb non-redundant (NR) data in seabuckthorn. Utilization of NR transcriptome data produced 10,980 (12.4%) transcripts containing 13,299 SSRs with abundance of mononucleotides (56.4%) followed by dinucleotide (21.5%) and trinucleotide repeats (18.9%). Further, putative function assignment identified 7,421 transcription factor genes representing 80 different transcription factor families (Ghangal et al. 2013). Further, cold tolerance abiotic stress-responsive genes were identified through transcriptome analysis (Ghangal et al. 2012).

Current status on seabuckthorn diversity characterization and sequence based marker resources creation suggested that there are limitations of understanding large scale natural populations for selection and development of superior cultivars. Limitation of validated microsatellite markers in the existing resources provides the opportunity to undertake work for identification of additional polymorphic markers which will not only enhance the available marker resources but will also give valuable insight into the population structure and genetic diversity existing in Indian populations of seabuckthorn which in turn may assist to expedite effective breeding and conservation programmes.

3. MATERIALS AND METHODS

The present investigation was carried out in the Molecular Genetics and Genomics laboratory, CSIR- Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh. The material used and the methodologies adopted to achieve the objectives of the investigation are given here under:

3.1 Plant materials

Previously collected 245 genotypes of *Hippophae rhamnoides* from eight different locations of trans-Himalayan Ladakh and Lahaul-Spiti region were taken for this study. Geographical location and altitude of sampling sites are shown in Table 3.1 and Fig. 3.1 as established using GPS (Google maps). The fresh leaves were collected and preserved with silica gel in an air-tight ziplock bag to prevent DNA degradation.

Table 3.1 Location of *H. rhamnoides* leaf collection sites in trans-Himalayan Ladakh and Lahaul-Spiti region.

Geographical region	No. of individuals	Location details
Ladakh region	125	Chuchot, Shey Sakti, Stakna, Matho, Forest park, Choglamsar
Hippophae Resource Centre, CeHAB (Center for High Altitude Biology), Ribling, Lahaul and Spiti	120	Random genotypes collected

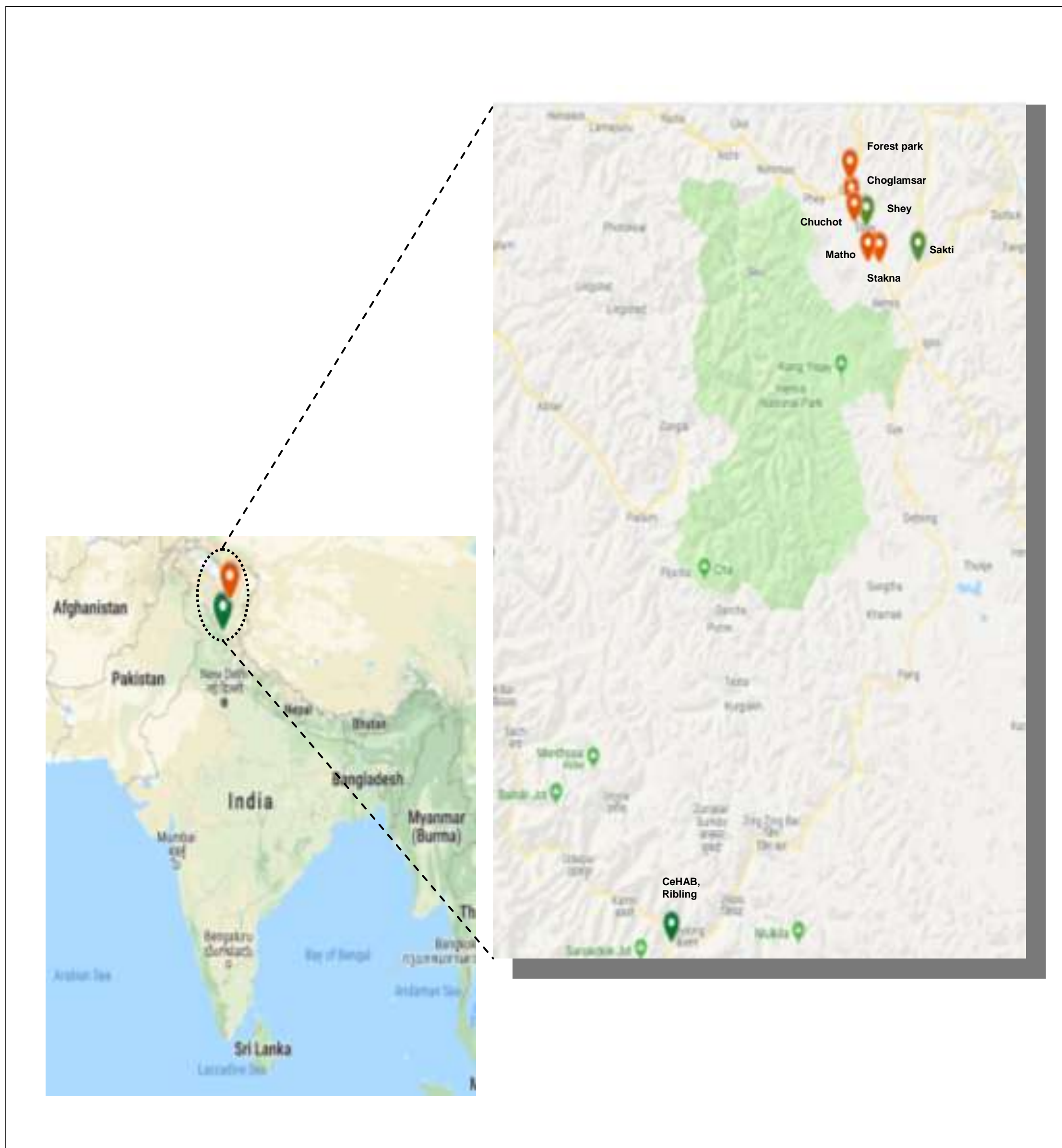


Fig.3.1 Map showing the location of collected populations of *H. rhamnoides* (source: Google Maps)

3.2 Methodology

3.2.1 Genomic DNA Isolation and quantification

DNA was extracted from leaf tissue of all the samples using the CTAB protocol (Doyle and Doyle 1990).

- The leaf tissue was rinsed in deionized water, dried on tissue paper discs and ground to a fine powder in liquid nitrogen using autoclaved pre-cooled pastels and mortars.
- The ground tissue was transferred to a separate 2 ml centrifuge tube containing 800 µl of extraction buffer (2 percent CTAB, 100mM Tris, 20mM EDTA, 1.4 mM NaCl and 1 per cent PVP, pH 8.0) pre-warmed at 65 ^{0}C in a water bath and mixed vigorously.
- The mixture was incubated at 65 ^{0}C for 1 hr with intermittent mixing after 10 min. An equal volume of Chloroform: Isoamyl alcohol (24:1) was added to the tubes followed by gentle mixing.
- The mixture was centrifuged at 10,000 rpm for 10 min at 4 ^{0}C. The aqueous phase was transferred to a 1.5 ml centrifuge tube, followed by addition of a 2/3rd volume of chilled-isopropanol. The contents of tubes were mixed gently and the mixture was incubated at -20 ^{0}C for 1 hr. DNA was precipitated by centrifugation at 10,000 rpm for 10 min at 4 ^{0}C using centrifuge (SIGMA, Laborzentrifugen, Germany).
- The supernatant was drained and the resulting pellet was washed twice with 1 ml of 70 per cent ethanol for 3 min. The pellet was air dried for 3-4 hr and then dissolved in 200 µl TE buffer (10 mMTris-HCL, 0.1 mM EDTA, pH 8.0).
- The dissolved DNA was treated with RNase (10 mg/ml) to rid the sample of RNA contamination.
- After 1 hr, 200 µl of Phenol: Chloroform: iso-amyl alcohol (25:24:1) was added, mixed well by inverting the tubes and centrifuged at 10,000 rpm for 5 min at the room temperature. The upper aqueous phase was transferred to fresh centrifuge tubes.

- The aqueous phase was further purified by adding 200 µl of Chloroform: Isoamyl alcohol (24:1) and centrifuged at 10,000 rpm for 5 min.

- The upper aqueous phase was transferred into a fresh centrifuge tube. 20 µl of 3M sodium acetate and 400 µl of chilled isopropanol were added to precipitate DNA. Tubes were kept at -20 ^{0}C for 30 min and DNA was pelleted by centrifugation at 13,000 rpm for 3 min. The supernatant was discarded and the pellet was retained.

- The pellet was washed with 70 per cent alcohol twice at 10,000 for 3 min, air dried and dissolved in 200 µl TE buffer.

- DNA was quantified using Nanodrop (Thermo Fisher Scientific, USA) to determine the concentration of DNA present in the extracted samples. Three parameters: DNA concentration, absorbance at wavelengths 260/280 ($A_{280/260}$), and 260/230 ($A_{260/230}$) were noted down.
- The samples were then run on a 0.8 percent agarose gel to check their quality. An uncut Lambda marker (25 ng/µl) was also used to quantify the DNA. After that, DNA dilutions of 25 ng/µl were made as required for PCR amplification.

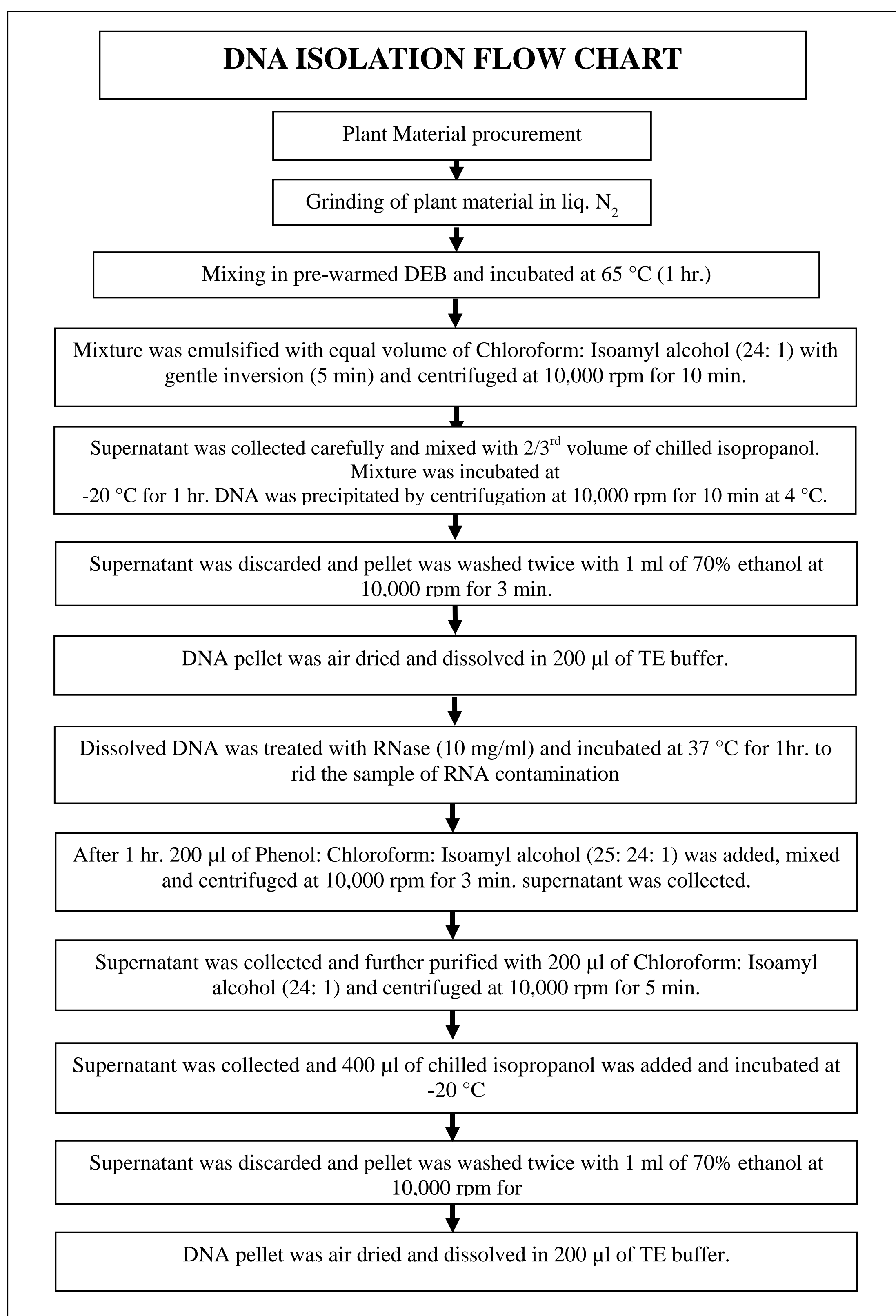


Fig.3.2 Schematic representation of CTAB method for DNA isolation

3.2.2 SSR mining and primer designing

In-house transcriptomic sequence reads were utilized for mining of SSRs with di-nucleotide repeats (DNR), tri-nucleotide repeats (TNR), tetra-nucleotide repeats (TtNR), penta-nucleotide repeats (PNR) and hexa-nucleotide repeats (HNR). The pearl script MISA (http://pgrc.ipk-gatersleben.de/misa/) was used for identification of microsatellites. The criteria for identifying SSRs for all possible combinations of sequences were 6 (DNR), 5 (TNR), 5 (TtNR), 5 (PNR) and 5 (HNR) with 100 bp maximum difference between two SSRs. The software Batch Primer3 (http://probes.pw.usda.gov/batchprimer3) was used to design the primers considering flanking regions of the targeted SSR, based on the following parameters: (1) primer length ranging from 18-22 bp with an optimum 20 bp, (2) optimum product size range of 100 – 300 bp, (3) annealing temperature ranging from 45-60 ^{0}C, (4) GC content ranging from 40-60 per cent with an optimum of 50 per cent.

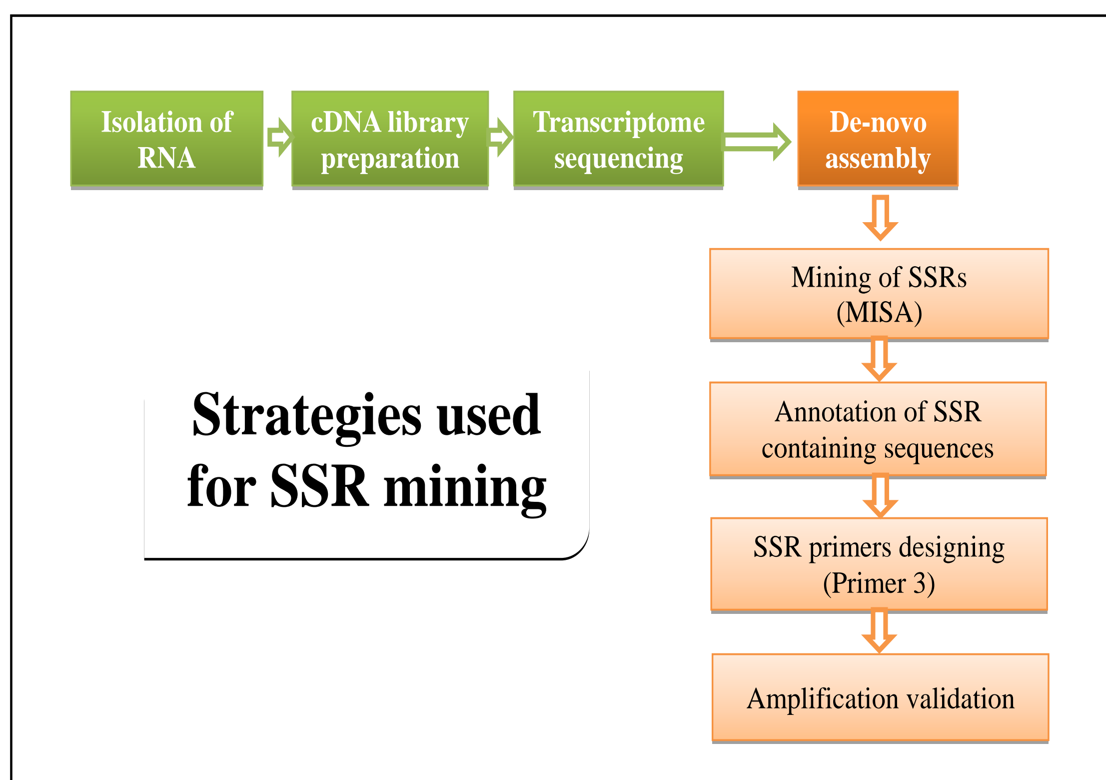


Fig.3.3 Schematic representation of strategies used for the mining of microsatellites.

3.2.3 Primer synthesis, PCR amplification and genotyping

PCR amplification of all the primers were performed in 10µl reaction volume consisting of 1.5 µl of 10× PCR buffer (10 mM Tris pH 9.0, 1.5 mM $MgCl_2$), 0.1 µl of 200 µM of each dNTPs, 0.5 µl each of forward and reverse primers, 0.1 µl Taq DNA polymerase (3U/µl)(NEW ENGLAND BioLabs) and 25-30 ng of template DNA. The PCR protocol consisted of one denaturation cycle at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, annealing at 55 ^{0}C (Ta) for 1 min, and extension at 72 °C for 1 min. The final extension cycle was carried out at 72 °C for 7 min. All the PCR reactions were carried in ABI Thermal cycler (Applied Biosciences, USA).

Amplification was checked on a 1.2 per cent agarose gel. A 50 bp ladder was used to check the size of the bands observed. Afterward, PCR products were run on a denaturing polyacrylamide gel (PAGE) containing 6 per cent polyacrylamide prepared with the fast silver staining protocol (Huang and Deng 2018).

- Pre-washed and air dried base and notch plate were wiped with isopropanol and ethanol alternatively twice using tissue paper.
- The adhesive was prepared (1 ml ethanol, 4 µl acetic acid, and 6.6 µl bind silane in 1.5 ml tube) and mixed by inverting two to three times and gently applied on the wiped side of the base plate with tissue paper and kept plate 15 min for heat drying.
- 200 µl of siliconizing reagent was poured and spread gently on the wiped side of notch plate with tissue paper and kept for drying for 15 min. The glass plates were assembled with a spacer to make a sandwich.
- 100 ml of 6 per cent non-denaturing polyacrylamide gel solution (Acrylamide 5.8 g, Bisacrylamide 0.2 g, 44 µl of tetramethylethylenediamine (TEMED)) was prepared and freshly prepared 1 mg of ammonium persulfate in 1 ml of distilled water was added and mixed gently.
- Gel solution was poured immediately in between the plate and the comb was inserted in an inverted position to make a cavity for loading of samples.
- After the gel was fully polymerized plate set was washed with tap water with the removal of comb to remove impurities in the cavity.

- Plate set was positioned in the vertical electrophoresis unit. 60 wells comb was inserted into the pre-cleaned cavity and the gel was pre-run at 1200 V, 45 W, 45 mA for 10-15 minutes.
- PCR product was denatured in Thermal cycler at 94 ^{0}C for 5 minutes and immediately transferred on ice.
- About 2 µl of PCR product was loaded into each well of the polyacrylamide gel with 1.5 µl 50 bp ladder in the first and last wells and assembly was run with previously decided configuration.
- After electrophoresis, glass plate along with gel was carefully removed and rinsed with 1 L of distilled water to remove the electrophoresis buffer for 5 min then the plate was submerged in 1 L of impregnation solution (1.5 g of AgNO3 in 1 L of distilled water) in the tray and kept on a Rocker (Tarsons, India) for 5 minutes.
- Residual impregnation solution was rinsed off from the surface of the plate and the gel using ample distilled water for 3-5 sec.
- The plate was submerged in 1 L of freshly prepared development solution (10 g of NaOH in 900 ml of distilled water, 1 ml of 37 per cent formaldehyde, and adjust to a final volume of 1 L using distilled water and shake gently at 50 rpm for ~5 min or until bands were clearly appeared. Plate along with gel was rinsed with ample distilled water in the tray, and air dried.
- Developed gel plate was scanned using scanner (EPSON, Japan) at a resolution of 400 dpi gives a clear visualization of DNA fragments and scored manually based on the DNA fragment size.

3.2.4 SSR Data Analysis

All SSR fragments were scored manually in a co-dominant manner and converted into a binary data matrix, i.e., 1 for the presence and 0 for the absence of the band. This binary matrix was used for estimation of Number of alleles obtained (Na), Number of effective alleles (Ne), Shannon's Information Index (I), Observed heterozygosity (Ho), Expected heterozygosity (He), and Hardy Weinberg Equilibrium analysis (HW) calculated using Popgene version 1.32 (Yeh And Boyle 1997). Polymorphism Information Content (PIC) was estimated using the software

PowerMarker (Liu and Muse 2005). Neighbor joining (NJ) dendrogram based on the unweighted pair group method of arithmetic mean (UPGMA) was constructed using Jaccard's dissimilarity coefficient with the help of DARwin6 (Perrier and Jacquemoud-Collet 2006). Analysis of Molecular Variance (AMOVA) and Principal coordinate analysis (PCoA) performed using GenAlEx 6.502 (Peakall and Smouse 2012). Genetic differentiation (F_{ST}) was calculated by using Popgene version 1.32 (Yeh And Boyle 1997) and gene flow (Nm) was calculated using Wright's equation ($Nm = [(1 / Fst) - 1] / 4$).

3.2.5 Population structure analysis

STRUCTURE version 2.3.4 (Pritchard et al. 2000), was used for Bayesian model-based clustering to estimate the genetic structure of the considered populations. The number of clusters of the population (K) was analyzed with a length of burn-in period 50,000; a number of Markov Chain Monte Carlo (MCMC) repeats after burn in 100,000 and K value ranging from 1 to 10 run with 5 iterations for each. The maximal value of LnP(D), the posterior probability of data as per (Evanno et al. 2005), was obtained using STRUCTURE HARVESTER (Earl and vonHoldt 2012).

4. RESULTS AND DISCUSSION

To strategize the commercial cultivation, efforts were made for identification of informative polymorphic microsatellite/ SSR markers. SSR markers with high level of polymorphic potential were successfully utilized for the genetic diversity assessment of 245 genotypes representing geographically separated natural populations of Indian Trans-Himalayan range.

The results obtained on different aspects of the present study have been presented and discussed below:

4.1 Evaluation of polymorphic potential of SSR markers

4.1.1 Genomic DNA Quantity and Quality

All the 245 genotypes were successfully processed for isolation of high quality genomic DNA. Quantitative and qualitative DNA estimation ranged from 200 ng/µl to 1200 ng/µl. (Fig.4.1).

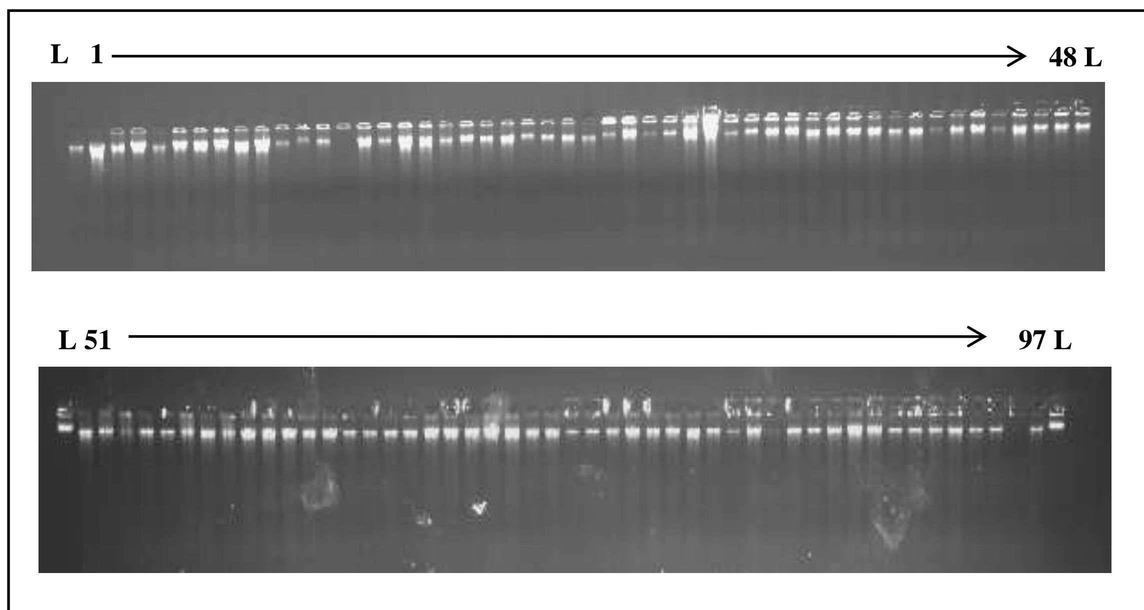


Fig.4.1 Quantification of purified genomic DNA isolated from seabuckthorn genotypes: Lane 1, Lane 49, Lane 51 and Lane 98 = 25 ng/µl λ uncut; 1-96 different seabuckthorn genotypes.

4.1.2 Mining and characterization of microsatellites

In-house generated non-redundant (NR) transcriptomic data (~ 9.8 GB; unpublished) was used for the mining of microsatellites. A total of 1229 microsatellite motifs were identified in 1113 transcripts. Among them, 97 transcripts were found to contain more than one SSR (Table 4.1).

Table 4.1 Mining of SSRs from transcriptome data.

Characteristics	Numbers
Total number of identified SSRs	1229
Number of SSR containing sequences	1113
Number of sequences containing more than one SSR	97
Number of SSRs present in compound formation	44
Total number of primers designed	882

Among all repeats, tri-repeats (49%) were the most abundant followed by di-repeats (46%), while penta-repeats (1.5%) and hexa-repeats were least common motifs (0.4%) (Fig.4.2). Abundance of di-nucleotide and tri-nucleotide repeats is also reported in many other plants, including cotton (Zang et al. 2014), pummelo (Liang et al. 2015), soybean (Cardle et al. 2000) and bamboo (Bhandawat et al. 2019) and in many other plant species (Tang et al. 2009; Roorkiwal and Sharma 2011). Trinucleotide repeats are the most abundant SSR motifs in the coding region of the genome as they are less prone to any kind of frameshift mutation in the gene product (Metzgar et al. 2000). The frequency of microsatellite was found to be one per 14 Kb of NR transcripts of Seabuckthorn data. Further, 1113 SSR motifs were successfully localized in the coding sequence (CDS), 5'-untranslated region (5'UTR) and 3'-untranslated region (3'UTR) with the abundance of maximum 54% in CDS region followed 31% and 15% in 5' and 3'-UTR , respectively (Fig.4.3). In total, 882 unique primer pairs flanking specific SSR motifs were designed in NR transcripts of *H. rhamnoides*.

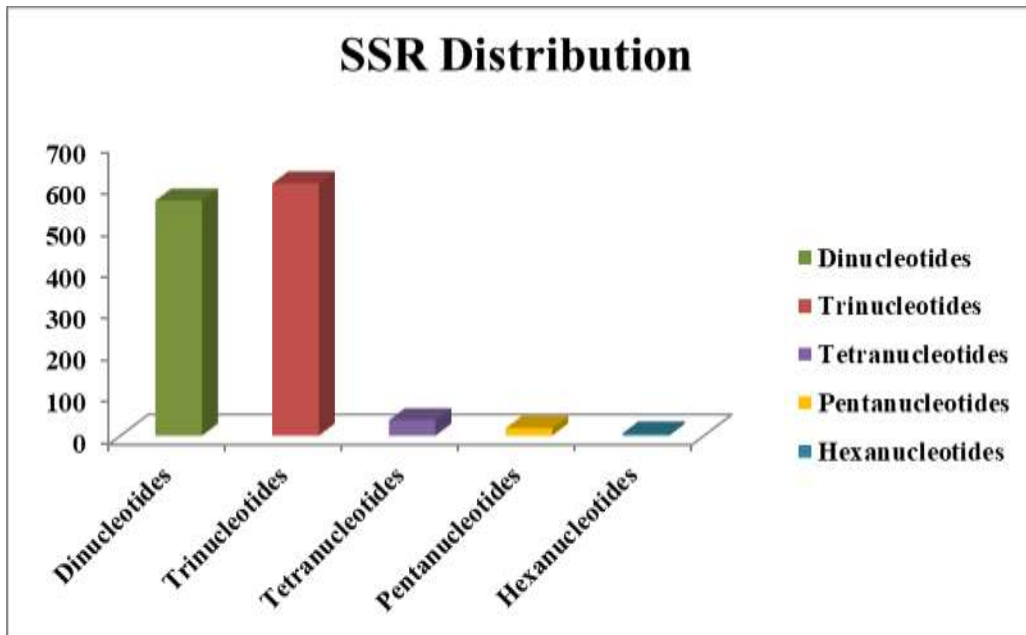


Fig.4.2 Distribution of nucleotide repeats in seabuckthorn transcriptome.

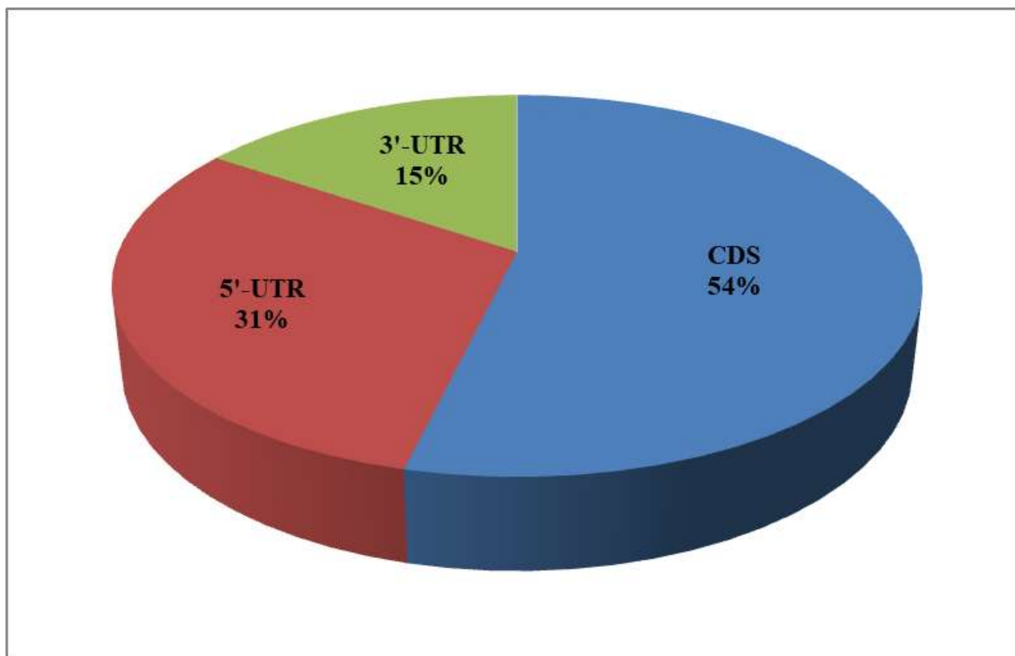


Fig. 4.3 Localization of SSR repeats.

4.1.3 Validation of SSRs

For this study, a total of 96 primer pairs representing transcription factors, pathway genes and some randomly distributed SSRs designated as "SeaBuckThorn MicroSatellite (SBTMS)" were selected and synthesized. All these markers were tested in 6 random genotypes (3 from Ladakh and Lahaul and Spiti region each) representing six geographically diverse populations. Of these, 87 SSR markers revealed desired amplification in three random repeated experiments with identification of 57 polymorphic SSR markers. Representative SBTMS markers were depicted in Fig.4.4.

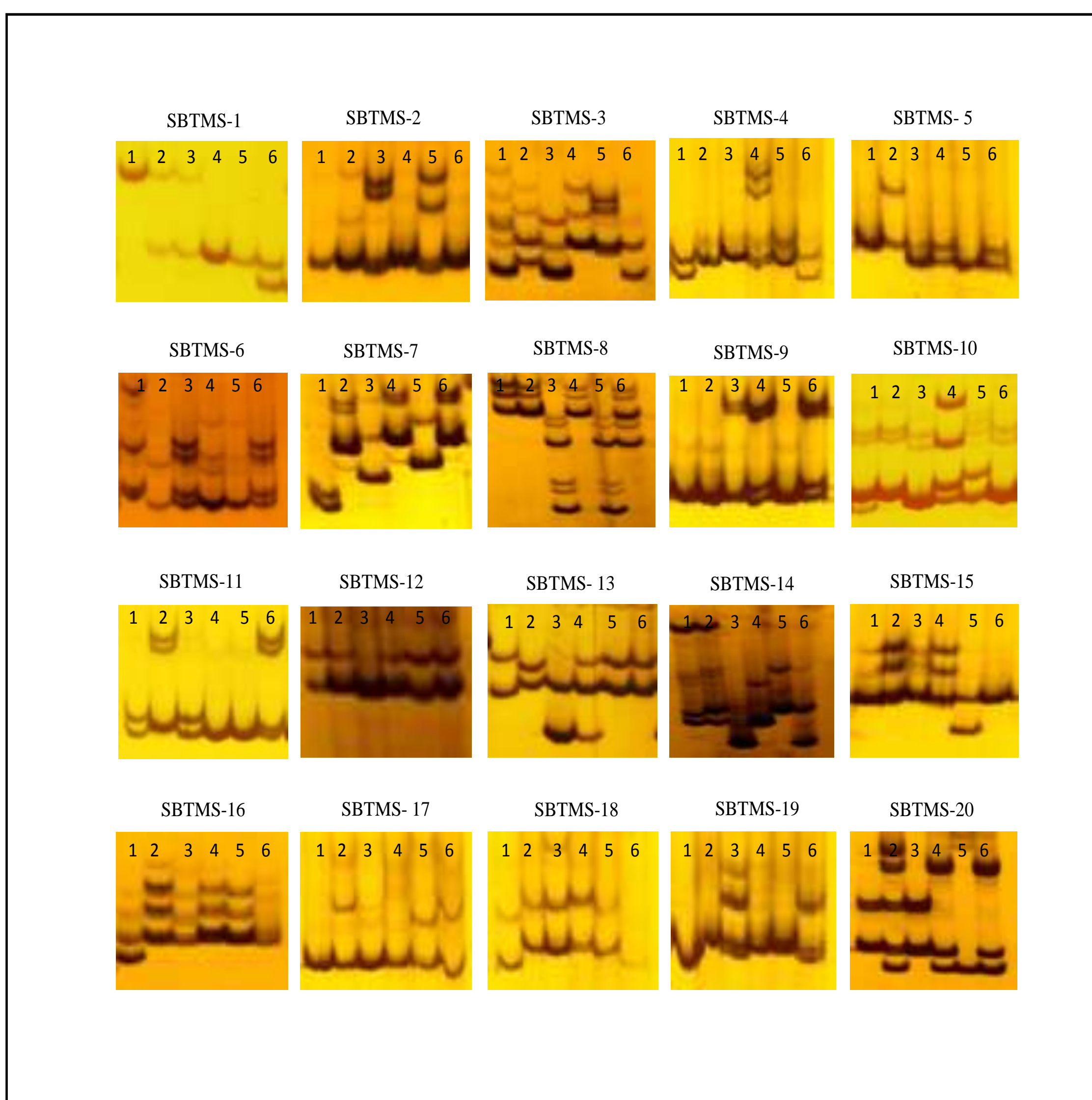


Fig.4.4 Experimental validation for polymorphic potential of SSR markers; Lane 1-6 random genotypes of seabuckthorn.

4.2 Utilization of SSR markers for genetic diversity characterization

4.2.1 Genetic diversity analysis in Seabuckthorn

Considering amplification and polymorphic potential, highly informative 20 SBTMS markers (Table 4.2) were selected for genotyping of 245 genotypes representing 8 geographically distinct populations and random collections for genetic diversity and population structure evaluation of *H. rhamnoides*. A total of 109 alleles were amplified with an average of 5.45 alleles per locus across 245 individuals. Allele size varied from 130-200 bp with the number of alleles ranged from a minimum of 2 to a maximum of 9 and mean effective allele (Ne) was recorded to be 4.70 per locus. A representative amplification profile of primer SBTMS-7 is depicted in Fig.4.5.

The polymorphic Information Content (PIC) value ranged from 0.05 to 0.61 with mean PIC value 0.32. The mean Shannon's information index (I) was 1.58. Observed heterozygosity (H_o) of each microsatellite loci ranged from 0.47 to 0.96 with an average of 0.77. While, expected heterozygosity (H_e) of each microsatellite loci varied from 0.49 to 0.90 (average H_e; 0.75) (Table 4.3). The mean observed heterozygosity was higher than expected heterozygosity as expected for outcrossed, plant species indicating high level of genetic diversity (Bartish et al. 2000). The mean fixation index (inbreeding coefficient) for the 20 primers was negative, suggesting an excess of heterozygotes present in seabuckthorn population. Low probability of inbreeding depression (low F_{IS} value) is correlated with outbreeding behaviour (wind pollination) and indicating sufficient pollen exchange within different populations of seabuckthorn.

Previously, SSR markers based study detected low to moderate genetic diversity with expected heterozygosity (H_e) ranging from 0.299 to 0.476 in *H. rhamnoides* ssp. *sinensis* (Wang et al. 2008). Further, 127 alleles with an average of 6.05 alleles and mean PIC value 0.359 per locus were successfully recorded in 15 tea accessions. High mean heterozygosity (Ho=0.775 and He=0.847) revealed that high level of genetic diversity exists in tea accessions (Bhardwaj et al. 2013). Similar observations were also recorded in other outcross plant species, wherein, high genetic diversity (H_e= 0.7566) with low genetic differentiation (F_{ST}: 0.086) and high level of within population molecular variation (94%) was observed in dioecious shrub *Salix viminalis* (Zhai et al. 2016). Likewise, SSR markers based genetic diversity evaluation

of *Ziziphus jujube* also recorded high average expected/ observed heterozygosity (H_e: 0.621; H_o: 0.678) and within population variations (Wang et al. 2014).

Table 4.2 List of seabuckthorn microsatellite primers, repeat type used for PCR amplification.

Sr No.	Primers	SSR type
1	SBTMS-1	Tri
2	SBTMS-2	Di
3	SBTMS-3	Di
4	SBTMS-4	Tri
5	SBTMS-5	Tetra
6	SBTMS-6	Di
7	SBTMS-7	Tri
8	SBTMS-8	Di
9	SBTMS-9	Tri
10	SBTMS-10	Di
11	SBTMS-11	Tri
12	SBTMS-12	Tri
13	SBTMS-13	Tri
14	SBTMS-14	Tri
15	SBTMS-15	Tri
16	SBTMS-16	Tri
17	SBTMS-17	Di
18	SBTMS-18	Tri
19	SBTMS-19	Tri
20	SBTMS-20	Di

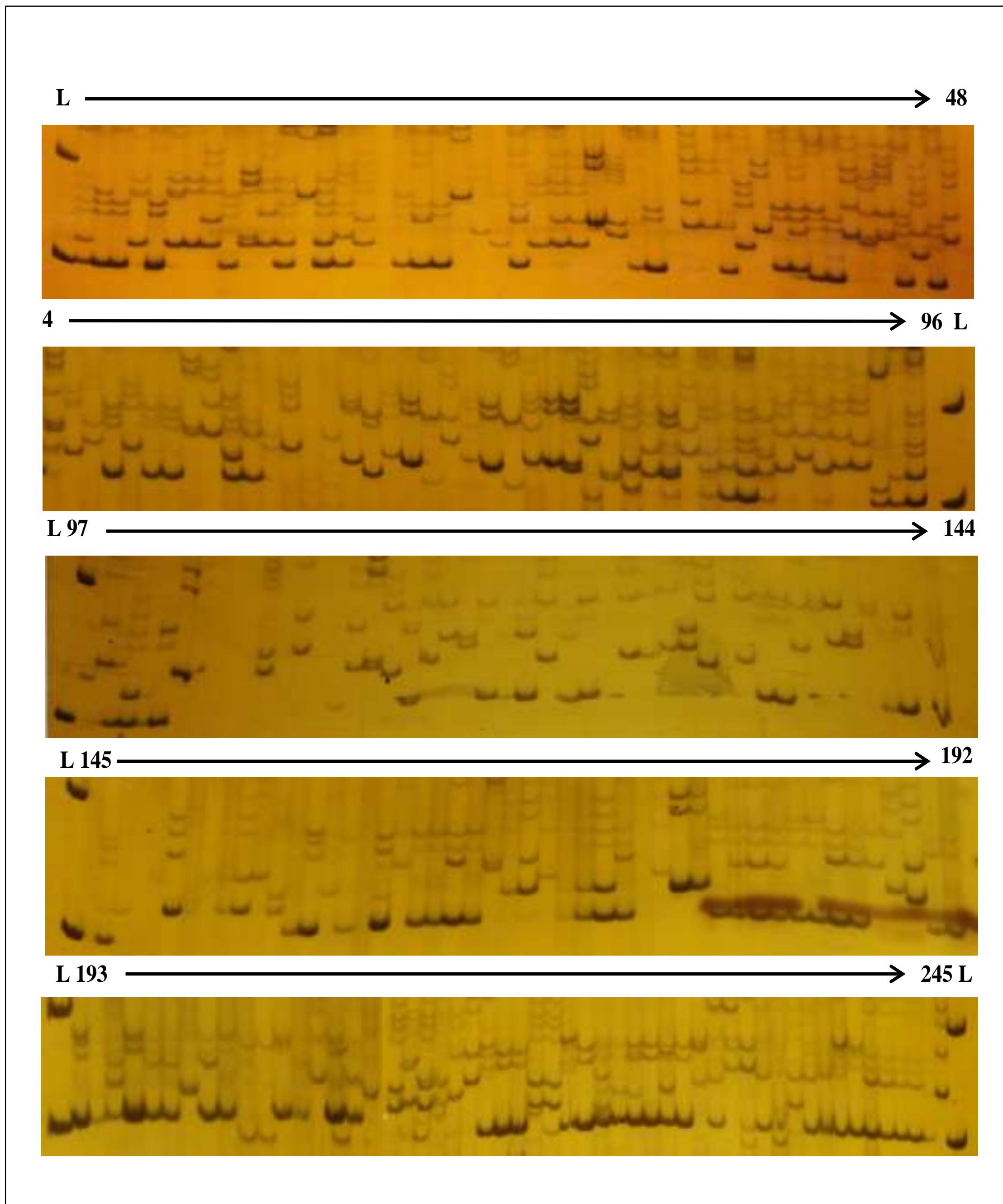


Fig. 4.5 Amplification profile of 245 Seabuckthorn genotypes with primer SBTMS-7 and L= 50 bp DNA ladder.

Table 4.3 Estimates of genetic diversity in seabuckthorn (*H. rhamnoides*).

Locus	Na	Ne	I	H_o	H_e	PIC	Allele Size range (bp)
SBTMS-1	5	4.72	1.66	0.74	0.79	0.391	140-200
SBTMS-2	6	3.53	1.50	0.75	0.72	0.212	135-200
SBTMS-3	7	6.45	1.95	0.86	0.85	0.453	150-200
SBTMS-4	5	3.63	1.47	0.80	0.73	0.155	150-180
SBTMS-5	4	3.68	1.46	0.89	0.73	0.019	150-200
SBTMS-6	8	8.13	2.14	0.94	0.88	0.522	150-200
SBTMS-7	9	9.45	2.27	0.89	0.90	0.615	150-200
SBTMS-8	8	7.21	2.07	0.74	0.86	0.614	130-180
SBTMS-9	5	4.67	1.62	0.92	0.79	0.186	140-200
SBTMS-10	9	7.51	2.14	0.96	0.87	0.480	140-200
SBTMS-11	7	3.60	1.56	0.67	0.72	0.317	150-200
SBTMS-12	2	1.97	0.71	0.47	0.49	0.052	150-175
SBTMS-13	4	4.05	1.44	0.60	0.75	0.355	150-180
SBTMS-14	4	4.34	1.52	0.73	0.77	0.247	150-200
SBTMS-15	3	2.81	1.11	0.91	0.65	0.289	150-180
SBTMS-16	5	4.50	1.57	0.66	0.78	0.370	150-200
SBTMS-17	5	2.43	1.11	0.79	0.59	0.174	150-200
SBTMS-18	3	3.15	1.25	0.52	0.68	0.329	150-200
SBTMS-19	4	2.84	1.19	0.96	0.65	0.253	150-200
SBTMS-20	5	4.70	1.61	0.89	0.79	0.283	150-200
Avg	5.45	4.67	1.58	0.77	0.75	0.3156	

bp= base pair; Na = Observed number of alleles; Ne = Effective number of alleles; I = Shannon's Information index; H_e = Expected heterozygosity; H_o = observed heterozygosity; PIC = Polymorphism Information Content

4.2.2 Genetic Differentiation, Gene flow, and AMOVA.

The overall genetic differentiation (F_{st} value) was low, i.e. 0.059. This observation was complimented with the results of AMOVA analysis (between population variation 6%). High gene flow (Nm 3.972) indicated high rate of migration of species (Table 4.4). Relatively low genetic differentiation with high gene flow was also observed in a dioceous, perennial, and wind pollinated out crossing plant species (Hamrick and Godt 1996). Despite, high altitude physical barriers in the Karakorum mountain regions, sufficient historic gene flow with relatively low F_{ST} values was observed earlier in *H. rhamnoides* populations (Nawaz et al. 2018). Inter-regional gene flow may be either contributed by long-distance dispersal of pollens by wind pollination or seed dispersal by birds (Mangla et al. 2015; Fan et al. 2007). Similarly, high gene flow (Nm = 3.648) with low genetic differentiation coefficient (F_{ST}= 0.063) was also reported in perennial, woody, outcrossing tree species *Quercus variabilis* (Shi et al. 2017).

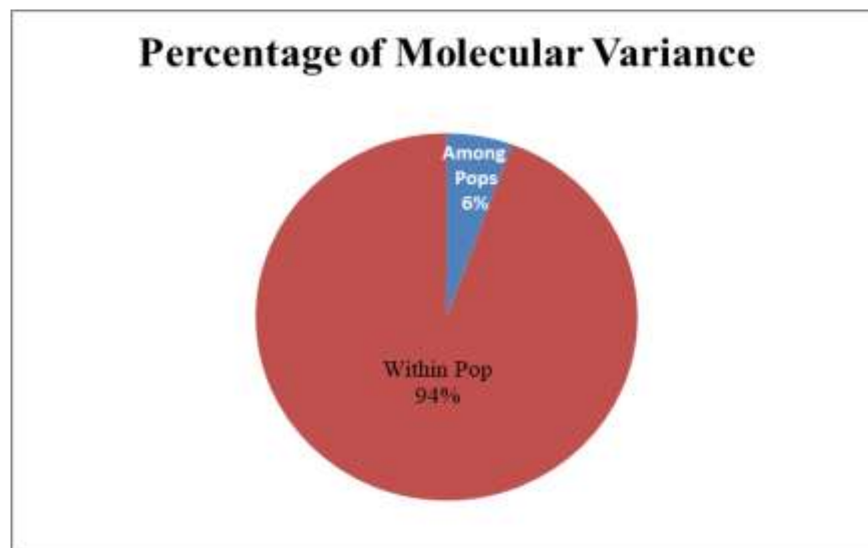
Analysis of Molecular Variance (AMOVA) was performed (Fig. 4.6) to record the genetic variation among the population and revealed that maximum (94%) variation was observed within a population, while 6% variation was recorded among populations ($P< 0.001$). Different factors such as mating system, population size and selection are the major factors contributing towards high within the population gene flow. *H. rhamnoides* resembled the general pattern of genetic structure detected in woody species with wide-spread distributions and outcrossing mating systems. Such plants possess more genetic variation within a population and less genetic differentiation among populations (Last et al. 2014). Utilization of AFLP markers in the dioceous plant, *Uapaca kirkiana* revealed low genetic differentiation (G_{ST}: 0.079) with higher genetic variation (92%) within a population than among population (7.8%) (Mwase et al. 2006).

Table 4.4 Estimates of genetic differentiation and gene flow (Nm) in *H. rhamnoides* population.

Locus	**Fis**	**Fit**	**Fst**	**Nm**
SBTMS-1	0.075	0.118	0.046	5.157
SBTMS-2	-0.100	-0.064	0.033	7.434
SBTMS-3	-0.090	-0.038	0.048	4.986
SBTMS-4	-0.198	-0.119	0.066	3.533
SBTMS-5	-0.269	-0.206	0.049	4.810
SBTMS-6	-0.085	-0.065	0.018	13.595
SBTMS-7	-0.056	-0.020	0.034	7.104
SBTMS-8	0.121	0.230	0.124	1.764
SBTMS-9	-0.215	-0.189	0.022	11.203
SBTMS-10	-0.122	-0.096	0.023	10.449
SBTMS-11	-0.038	0.015	0.050	4.712
SBTMS-12	-0.007	0.134	0.140	1.531
SBTMS-13	0.058	0.139	0.085	2.678
SBTMS-14	-0.079	-0.020	0.055	4.325
SBTMS-15	-0.409	-0.382	0.019	12.842
SBTMS-16	-0.068	0.078	0.136	1.583
SBTMS-17	-0.266	-0.154	0.089	2.570
SBTMS-18	0.086	0.139	0.058	4.049
SBTMS-19	-0.497	-0.338	0.106	2.107
SBTMS-20	-0.141	-0.112	0.026	9.509
Avg	-0.110	-0.044	0.059	3.972

Nm = Gene flow estimated from Fst = 0.25(1 - Fst)/Fst.

Fig.4.6 Total genetic variation among and within population of *H. rhamnoides* populations.



Source	df	%	P
Among Populations	10	6%	< 0.001
Within Population	234	94%	< 0.001
Total	244	100%	

D*f* degree of freedom, P probability

4.2.3 Population Structure

Genetic relationship among the eight populations (245 individuals) was evaluated by using three different approaches (Unweighted NJ phylogenetic analysis, Bayesian population structure model, and principal coordinate analysis). Out of 245 genotypes, 150 genotypes (the majority of Sakti, Shey, and CeHAB Ribling) were grouped into 1 cluster and 53 genotypes (majority from Stakna, Matho, Forest Park, Choglamsar, and Chuchot) were grouped into cluster 2 with some extent of admixtures interspersed within both the clusters (Fig.4.8). Bayesian model population structure analysis (Pritchard et al. 2000) of 245 genotypes grouped all the 245 genotypes into two major clusters (K= 2). Current analysis indicates that two distinct genetic pools existed in *H. rhamnoides* populations from Ladakh and Lahaul-Spiti region (Figs. 4.7; 4.9 and 4.10).

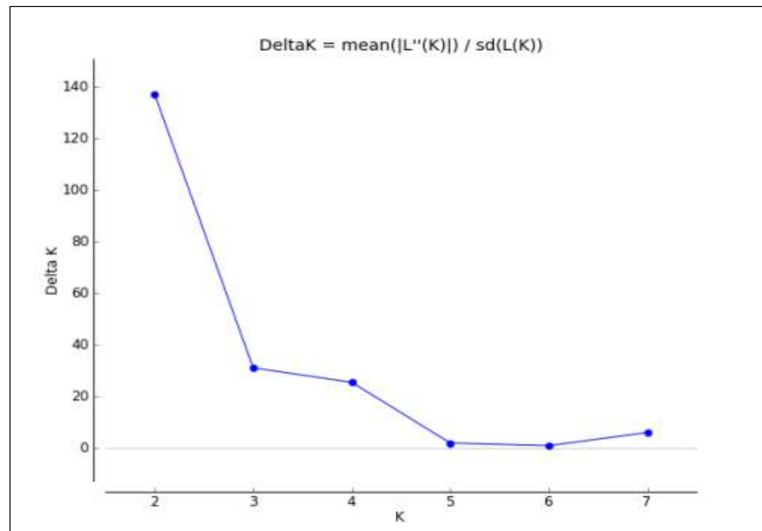


Fig.4.7 Bayesian model population genetic structure harvester generated graph representing delta K max.

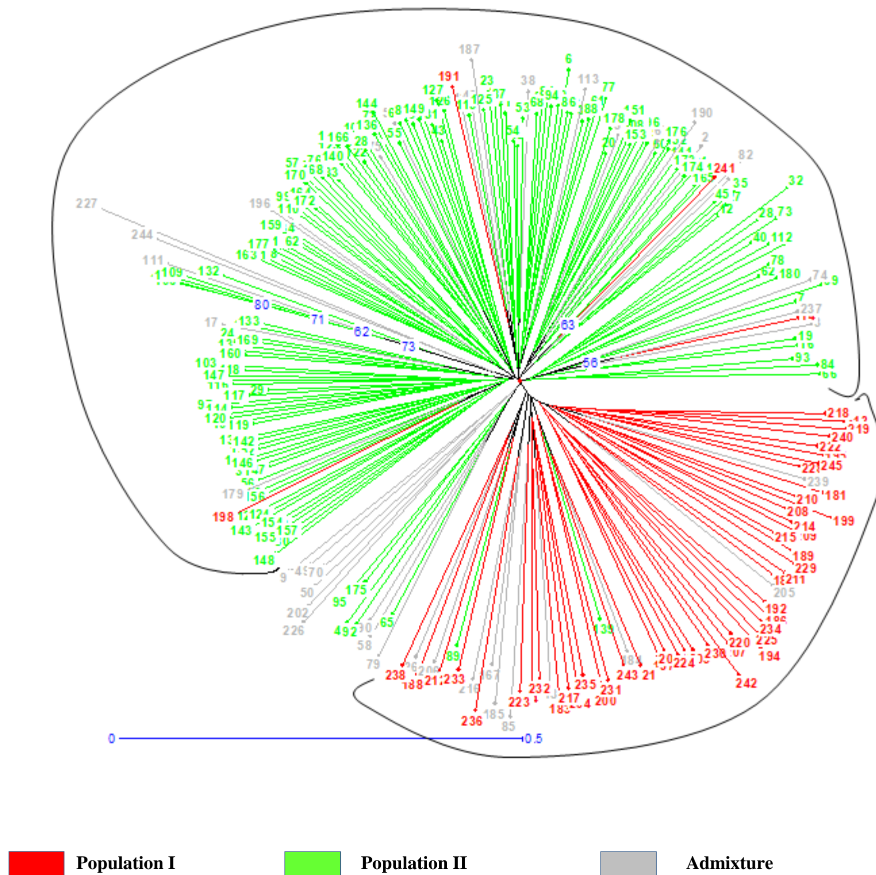


Fig.4.8 Neighbor-joining dendrogram based on Jaccard's dissimilarity coefficient along with bootstrap values.

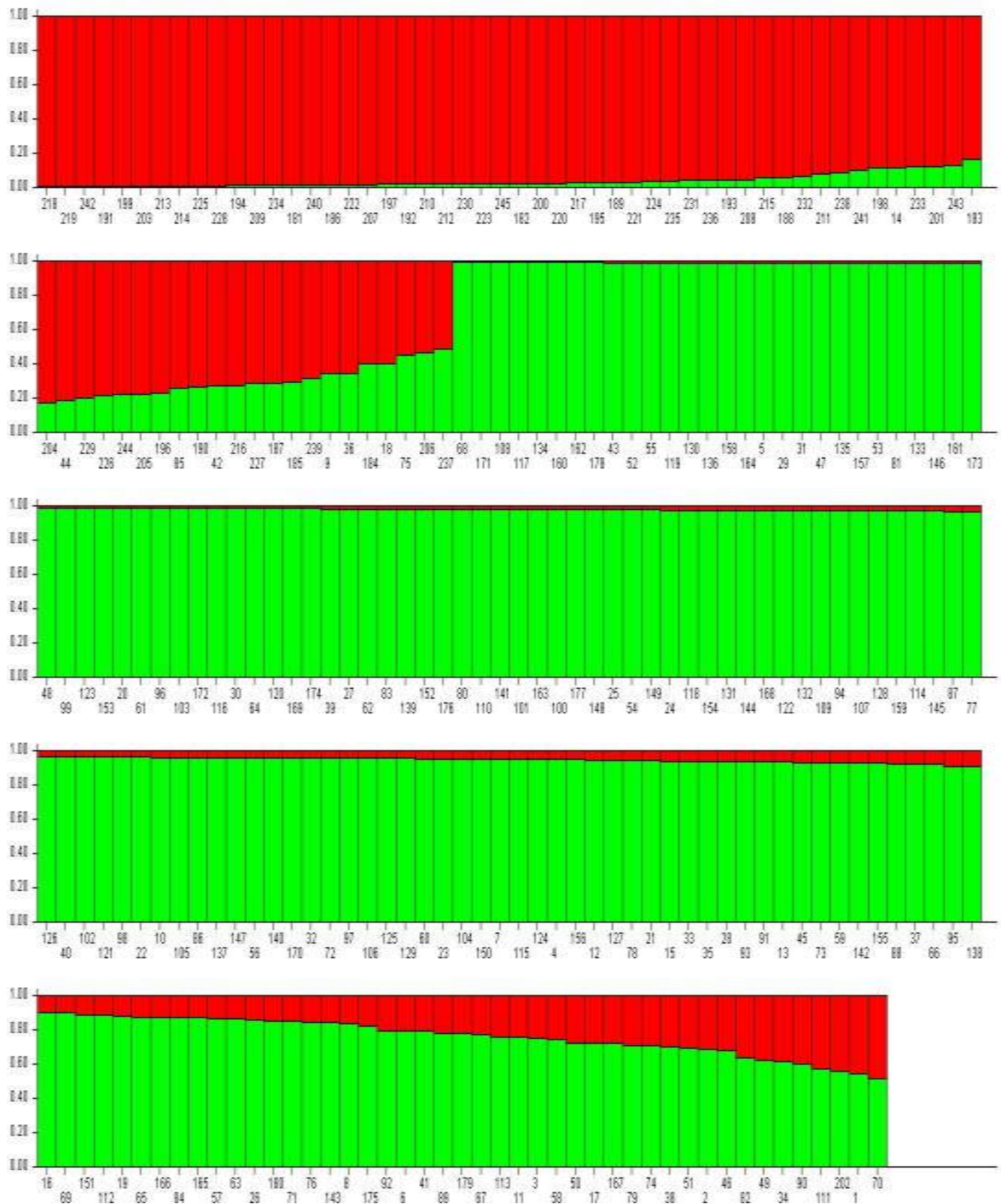


Fig.4.9 Population genetic structure showing 245 genotypes of *H. rhamnoides* grouped into 2 clusters based on largest LnP(D)-derived delta K (log probability of data derived delta K) value by STRUCTURE version 2.3.4. The single vertical line represents an individual genotype and segment of each vertical line show the extent of admixture in genotype.

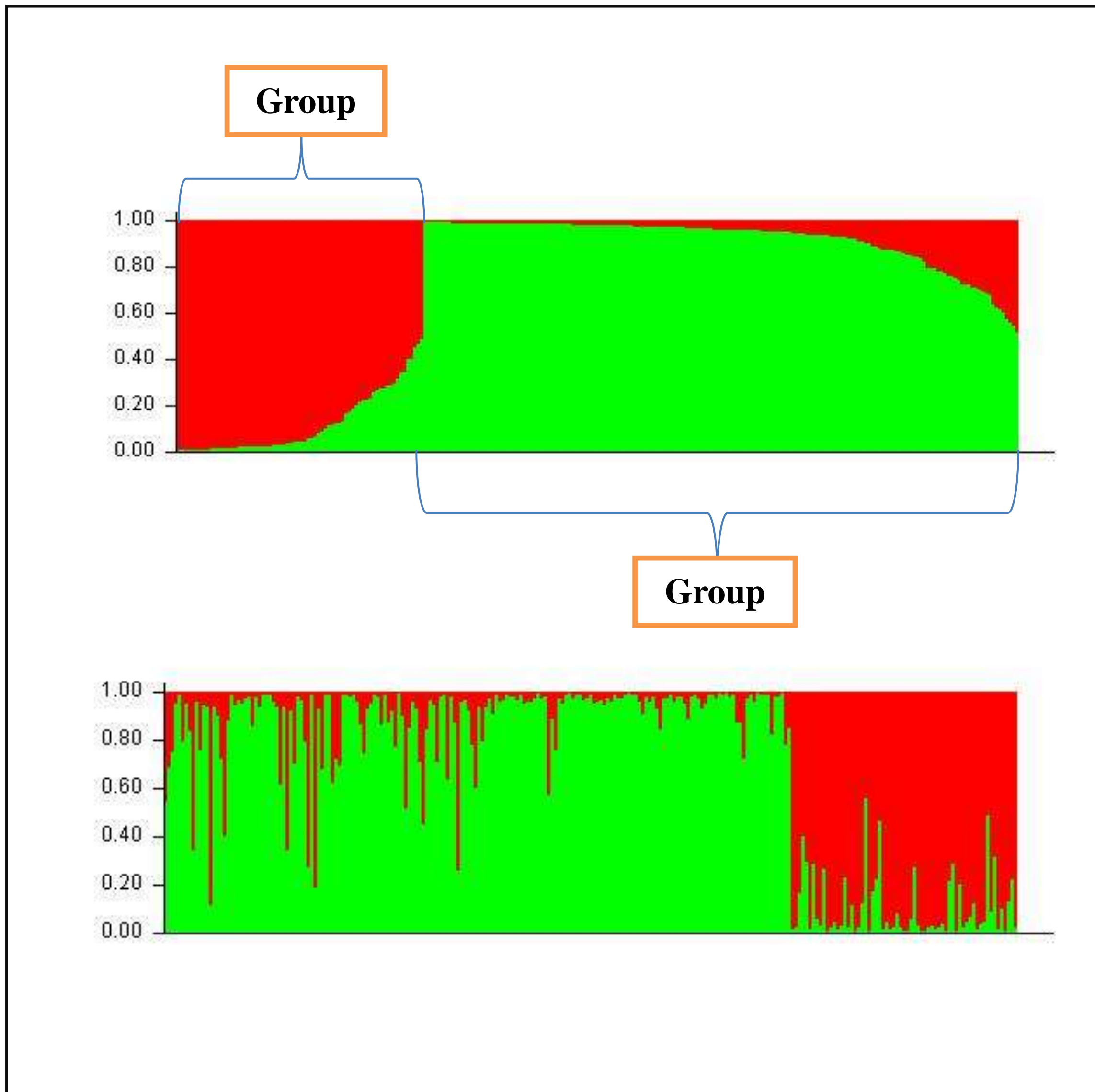


Fig.4.10 Population structure of 245 seabuckthorn genotypes showing two genetic groups.

Further, two-dimensional graphical views of genetic diversity in 245 genotypes of *H. rhamnoides* represented in the principal coordinate analysis (PCoA) clearly differentiate the eight populations into two groups with the distribution of admixture among both the groups and thus complementing the results of STRUCTURE and neighbor joining phylogenetic analysis (Fig. 4.11).

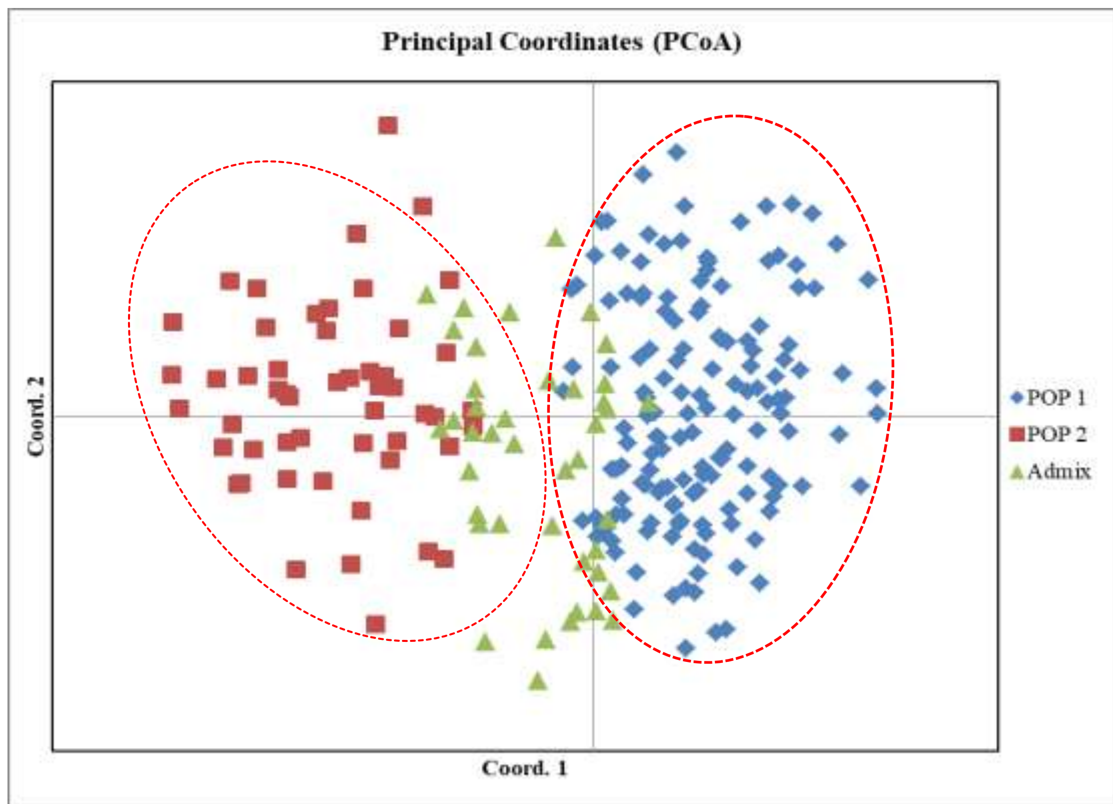


Fig.4.11 Principal coordinates analysis representing two populations of 245 seabuckthorn genotypes.

Mating system, gene flow, seed dispersal and mode of reproduction, as well as natural selection, affect the population genetic structure of plant species (Hamrick and Godt 1990). The mixing of populations could be due to many reasons; seeds of seabuckthorn can easily be dispersed to far-off regions by birds, grazing by domestic animals or anthropogenic intervention that leads to intermixing of populations. Besides, the local dwellers use seabuckthorn as bio fencing due to its thorny nature, thus anthropogenic causes further aid in intermixing of the populations.

Genetic diversity and population structure provide a basic genetic profile for implementation of *ex situ* conservation of genetic resources for appropriate management and selection of potential genotypes for implementation of genetic improvement programme in seabuckthorn. High level of genetic diversity detected in SSR analysis in the current dissertation suggests that there is huge potential of selecting potential cultivars for the commercial cultivation.

5. SUMMARY AND CONCLUSIONS

Current dissertation on "Evaluation and utilization of SSR markers for genetic diversity characterization in Seabuckthorn" was undertaken to identify informative novel microsatellite markers and their utilization for genetic diversity assessment of geographically distant population comprising 245 genotypes of seabuckthorn.

Microsatellite/ SSR markers being advantageous due to various desirable attributes such as fast, robust, reproducible and highly polymorphic with the codominant mode of inheritance have been widely accepted markers of choice for genetic diversity, evolutionary and genome mapping studies. In the present study, 882 SSR markers were identified from in-house generated transcriptomic sequence data (un-published). Out of 882 SSR primer pairs, 96 primer pairs were custom synthesized and experimentally validated in randomly selected six genotypes which detected 57 polymorphic markers. Further, utilization of 20 functionally relevant polymorphic SSR markers for genotyping of 245 genotypes identified 109 alleles with an average of 5.45 alleles per locus. The polymorphic Information Content (PIC) value ranged from 0.05 to 0.61 with an average PIC value of 0.32, suggesting that SSR markers used in this study are suitable for the large-scale characterization of seabuckthorn populations. The moderate to high level of genetic diversity, Shannon's information index (I) (1.58) high heterozygosity ($H_{o:}$ 0.77/ $H_{e:}$ 0.75) and high gene flow (Nm=3.97) suggesting that their no bottleneck observed in the survival of the seabuckthorn. Furthermore, inferences derived from cluster analysis, PCoA, and structure analysis suggests that there are two genetic pools operating in the population of Trans-Himalayan Ladakh and Keylong regions.

Outcome of the current study can be futuristically utilized for the development of appropriate strategies for conservation of natural populations and selection of potential individuals for commercial cultivation of seabuckthorn.

Conclusion

The functionally relevant novel 882 SSR markers identified in this study will enrich the existing microsatellite resources in seabuckthorn. Further, diversity inferences generated in this study can be utilized for identification of core genotypes/ populations for expediting the conservation plan. Further, chemical characterizations of these core populations can also help for implementation of genome-wide association studies for its genetic improvement. Current study possibly helps to strategize the commercial cultivation and selection of elite individual/ genotype in its major distribution site in India.

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APPENDICES

Table: List of SSR primers synthesized for this study

Sr. No.	Locus	Tm	Product Size	Repeat motif	Repeat type	PM/MM
1	SBTMS-1	56	150	TAC	Tri	
2	SBTMS-2	55	149	CT	Di	
3	SBTMS-3	55	154	AG	Di	
4	SBTMS-4	55	150	TCA	Tri	
5	SBTMS-5	55	151	AAAC	Tetra	
6	SBTMS-6	55	147	AT	Di	
7	SBTMS-7	55	145	TAC	Tri	
8	SBTMS-8	55	144	TG	Di	
9	SBTMS-9	55	148	GAA	Tri	
10	SBTMS-10	55	151	CA	Di	
11	SBTMS-11	55	154	ATC	Tri	
12	SBTMS-12	55	156	AGA	Tri	
13	SBTMS-13	55	163	AT	Di	
14	SBTMS-14	55	156	ATC	Tri	
15	SBTMS-15	55	137	AAT	Tri	
16	SBTMS-16	55	163	TCT	Tri	
17	SBTMS-17	55	185	TA	Di	
18	SBTMS-18	54	161	GTG	Tri	
19	SBTMS-19	55	140	GCT	Tri	
20	SBTMS-20	55	148	TCC	Tri	
21	SBTMS-21	55	147	CCA	Tri	
22	SBTMS-22	55	163	AG	Di	
23	SBTMS-23	55	151	CT	Di	
24	SBTMS-24	56	160	TG	Di	
25	SBTMS-25	55	145	AAC	Tri	
26	SBTMS-26	55	168	TC	Di	
27	SBTMS-27	55	148	AGT	Tri	
28	SBTMS-28	55	147	GA	Di	
29	SBTMS-29	55	140	TCA	Tri	
30	SBTMS-30	56	144	GA	Di	
31	SBTMS-31	55	162	TCT	Tri	

Sr. No.	Locus	Tm	Product Size	Repeat motif	Repeat type	PM/MM
32	SBTMS-32	55	146	TGG	Tri	
33	SBTMS-33	54	163	AAG	Tri	
34	SBTMS-34	54	170	TC	Di	
35	SBTMS-35	54	149	AAG	Tri	
36	SBTMS-36	55	150	GA	Di	
37	SBTMS-37	55	154	CT	Di	
38	SBTMS-38	54	143	ATT	Tri	
39	SBTMS-39	54	157	AT	Di	
40	SBTMS-40	55	149	TCC	Tri	
41	SBTMS-41	55	155	TC	Di	
42	SBTMS-42	55	215	TGG	Tri	
43	SBTMS-43	55	166	AGC	Tri	
44	SBTMS-44	55	150	TTC	Tri	
45	SBTMS-45	55	194	AAC	Tri	
46	SBTMS-46	51	142	TCC	Tri	
47	SBTMS-47	56	150	AGC	Tri	
48	SBTMS-48	56	181	CTC	Tri	
49	SBTMS-49	55	150	CAA	Tri	
50	SBTMS-50	55	158	TA	Di	
51	SBTMS-51	56	148	GTT	Tri	
52	SBTMS-52	55	148	TA	Di	
53	SBTMS-53	55	180	GTG	Tri	
54	SBTMS-54	55	162	CAG	Tri	
55	SBTMS-55	55	153	TAT	Tri	
56	SBTMS-56	55	145	TTC	Tri	
57	SBTMS-57	55	150	GGA	Tri	
58	SBTMS-58	55	156	GGT	Tri	
59	SBTMS-59	55	154	AC	Di	
60	SBTMS-60	55	148	TCT	Tri	
61	SBTMS-61	55	144	AG	Di	
62	SBTMS-62	55	153	CTT	Tri	
63	SBTMS-63	55	155	GAC	Tri	
64	SBTMS-64	55	165	GA	Di	
65	SBTMS-65	55	166	ATC	Tri	
66	SBTMS-66	55	164	AT	Di	
67	SBTMS-67	56	150	CT	Di	

Sr. No.	Locus	Tm	Product Size	Repeat motif	Repeat type	PM/MM
68	SBTMS-68	55	149	TA	Di	
69	SBTMS-69	56	174	AT	Di	
70	SBTMS-70	55	152	TGG	Tri	
71	SBTMS-71	55	150	AAG	Tri	
72	SBTMS-72	55	198	GA	Di	
73	SBTMS-73	55	146	GA	Di	
74	SBTMS-74	56	161	TC	Di	
75	SBTMS-75	55	158	TC	Di	
76	SBTMS-76	56	154	CT	Di	
77	SBTMS-77	55	149	AGC	Tri	
78	SBTMS-78	55	138	TC	Di	
79	SBTMS-79	56	153	GA	Di	
80	SBTMS-80	55	140	GGT	Tri	
81	SBTMS-81	55	152	GTT	Tri	
82	SBTMS-82	55	168	TCT	Tri	
83	SBTMS-83	55	144	TGA	Tri	
84	SBTMS-84	56	153	GGT	Tri	
85	SBTMS-85	55	158	GAT	Tri	
86	SBTMS-86	55	150	GGT	Tri	
87	SBTMS-87	54	155	TCT	Tri	
88	SBTMS-88	55	152	AT	Di	
89	SBTMS-89	55	169	GA	Di	
90	SBTMS-90	55	154	TAA	Tri	
91	SBTMS-91	55	163	AG	Di	
92	SBTMS-92	55	132	TAA	Tri	
93	SBTMS-93	55	138	AG	Di	
94	SBTMS-94	56	142	AG	Di	
95	SBTMS-95	54	152	CAC	Tri	
96	SBTMS-96	55	161	CA	Di	

Red colour, green colour and yellow colour representing monomorphic marker, polymorphic marker and markers that not amplified respectively.

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Higher Secondary	Apr., 2013	HSC Mumbai Board (MH)	64.67	1st
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M. Sc. (Agri. Biotechnology)	Jan., 2019	CSK HPKV, Palampur (H.P.)	78.50	1st

Title of M.Sc. Thesis:

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