GENETIC DIVERSITY OF SEABUCKTHORN (*HIPPOPHAE RHAMNOIDES* L.) IN TRANS-HIMALAYAN LADAKH REGION, INDIA

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IN

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By

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CERTIFICATE

This is to certify that the thesis entitled, **"Genetic diversity of Seabuckthorn (*Hippophae rhamnoides* L.) in trans-Himalayan Ladakh region, India"** which is being submitted by **Girish Korekar** in fulfillment for the award of degree of Doctor of Philosophy in Biotechnology by the **Jaypee University of Information Technology**, is the record of candidate's own work carried out by him under our supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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(Girish Korekar)

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Introduction

1.1 Seabuckthorn

Seabuckthorn (SBT) (*Hippophae* spp. L.) is an ecologically and economically important plant that belongs to the family Elaegnaceae. The name is from its habit of growing near the sea, and from the possession of many spines or thorns that are reminiscent of some buckthorn species (of the genus *Rhamnus*). It has silvery deciduous leaves and colourful red, orange or yellow berries that remain on the shrub throughout the winter. The plant is hardy and it can withstand extreme temperatures from -43°C to 40°C [Lu, 1992]. It is considered to be drought tolerant which is reflected from form and structure of leaves. SBT leaves are small, cuticle is thicker, crib-texture is well developed, leaf-back densely covered with scales and star-hair to cover stoma. The shrub develops extensive root system having ability to fix atmospheric nitrogen. It is therefore an ideal plant for soil erosion control, land reclamation, wildlife habitat enhancement, and farm stand protection in temperate region.

SBT is mentioned in the writings of ancient Greek scholars such as Theophrastus and Dioscorides. The plant is known as a remedy for horses. Leaves and branches were added to fodder to induce rapid weight gain and shiny coat, and in fact, the generic name *Hippophae* is classical Latin for 'shinning horse' [Lu, 1992]. The medicinal value of SBT was recorded as early as the 8th century in the Tibetan medicinal classic *rGyud Bzi*. Inspired by the ancient literatures, scientists in the former Soviet Union carried out research on SBT from the 1930s onward. In 1940s, especially after the Second World War, nutritionists and pharmacologists analyzed the vitamin components and found that SBT could be used not only as a food but also as a medicine [Lu, 1990]. Several countries including the Russia, Mongolia, Pakistan, Poland, Germany, Finland, India, Italy, Norway, Hungary, Canada and USA have been studying this amazing plant. SBT is known in different languages as *Shaji* in Chinese; *Duindoorn* in Dutch; *Sanddorn* in German; *Olivello Spinoso* in Italian; *Oblepicha* in Russian; *Tyrni* in Finnish; *Espino de Mar*, *Falso Espino*, *Espino Amarillo* in Spanish; *Havtorn* in Swedish

[www.seabuckthornresearch.com], *Yalanci igde*, *Karga dikeni* in Turkish [Baytop, 1999; Cakir, 2004], *sTar-Bu* in Tibetan, *tSer-Mang*, *tSer-Ta-Lu-Lu*, *Shib-Shu-Lu-Lu* in Ladakhi (India).

Every part of the plant *viz*. fruit, leaf, twig, root and thorn has been traditionally used as medicine, nutritional supplement, fuel and fence, and therefore, SBT is popularly known as 'Wonder Plant', 'Golden Bush' or 'Gold Mine'. Recent research has supported and extended the traditional uses of the plant and several products are being produced for nutraceutical and medicinal values. SBT has attracted international attention as a new crop opportunity. It is said to have momentous economic potential and is predicted by some as the next major health food fad. In view of the unique and valuable characteristics of SBT, the shrub serves as a storehouse for researchers in the field of biotechnology, nutraceutical, pharmaceutical, cosmetic, environmental and other disciplines [Stobdan et al., 2008].

Figure 1: Male (a) and female (b) SBT plant

SBT is dioecious and wind pollinated. The female plant bears red, orange or yellow berries on two-year-old thorny twigs. SBT berries are among the most nutritious of all fruits and have immense medicinal properties. Concentrations of vitamins B_2, B_3, B_5, B_6, B_{12}, C and E are much higher than other fruits, such as apricot, banana, mango, orange and peach [Stobdan et al, 2010]. SBT has been extensively studied for treatment of stomach malfunctioning, thrombosis, hepatic injury, tendon and ligament injuries and neoplasia. Clinical trials on patients with ischemic heart disease have shown that total flavonoids of SBT reduce cholesterol level and improve cardiac function. It is also established that antioxidant rich SBT juice reduces the risk factor for coronary heart diseases in human. Studies also demonstrated that SBT oil is effective in cancer therapy. Leaf extracts of SBT have been reported to have marked antibacterial, anti-viral and anti-tumour activities. SBT leaves have also been reported to have significant immunomodulatory and anti-inflammatory activities [Geetha & Gupta, 2011; Kanayama et al., 2012]. SBT extracts possess antibacterial activities and have shown protective effect against the toxic effect of mustard gas, a chemical warfare agent [Arora et al., 2012; Vijayraghavan et al, 2006]. Many of the claims associated with SBT are related to high nutritive value in terms of vitamins, organic acids, flavonoids, macro- and micronutrient elements. SBT pulps, seeds, leaves and stem bark contain high levels of total poylphenol content and antioxidants [Korekar et al., 2011]. The shrub serves as a storehouse for researchers in the field of biotechnology, nutraceutical, pharmaceutical, cosmetic and environmental sciences [Stobdan et al., 2008].

Believe it or Not

- There is enough vitamin C in seabuckthorn berries around the world to meet dietary requirement of the entire human population [Small et al., 2002].
- Seabuckthorn drink was the official beverage for Chinese athletes at the Seoul Olympic games in 1988 [Small et al., 2002].
- Russian cosmonauts were supplied with seabuckthorn beverage to enhance their health and resistance to stress. It was the first fruit juice in space [Small et al., 2002].

- Seabuckthorn oil was used by Russian cosmonauts for protection against harmful radiation.
- The medicinal value of seabuckthorn was recorded as early as the 8th century in the Tibetan medicinal classic *rGyud Bzi* [Lu, 1992].
- Seabuckthorn oil is approved for clinical use in China where it was formally listed in the Pharmacopoeia in 1977 [Xu, 1994].
- Seabuckthorn is believed to be the favored food of Pegasus, the winged flying horse of Greek mythology [Small et al., 2002].
- Seabuckthorn is credited as one of the factors that contributed to the Mongolian Genghis Khan's conquest of a large part of the world during the 12th and 13th century [Letchamo et al., 2007].
- The development of superior Seabuckthorn cultivars was a closely guarded secret under the Russian space program during the space race of the 1960s and 1970s [Letchamo et al., 2007].
- Commercial cultivation of Seabuckthorn started in Russia during the 1920s with development of cultivars for large commercial farms [Letchamo et al., 2007].

1.2 Taxonomy

All the species of the genus *Hippophae* are called SBT. SBT belongs to the family Elaegnaceae, which is in the major group *Angiosperms* (flowering plants). Genera in Elaegnaceae include *Elaegnus*, *Hippophae*, *Lepargyrea* and *Shepherdia*. Number of species under *Hippophae* is still unclear. The classification of genus *Hippophae* has been modified over the years. Originally it consisted of only one species, *H. rhamnoides*, with three subspecies, *rhamnoides, salicifolia* and *tibetana.* However, according to the latest systematic classification of the genus *Hippophae* L., the genus comprises of seven species, and the species *H. rhamnoides* circumscribes eight subspecies *viz. sinensis* Rousi, *yunnanensis* Rousi, *turkestanica* Rousi, *mongolica* Rousi, *caucasia* Rousi, *carpatica* Rousi, *rhamnoides* Rousi and *fluviatilis* van Soest. The precise classification of the genus is still debatable due to the variations found in the Himalayas and the adjacent areas of Central Asia. All species are diploid (2n = 24), wind pollinated, and

dioecious, and are restricted to the Qinghai Plateau and adjacent areas, with the exception of the species *H. rhamnoides* L. that occurs widely but sporadically in Asia and Europe. It is considered that the Hindu Kush-Himalayas, and in particular the Qinghai-Tibetan Plateau, are the main areas of distribution and origin of this genus [Lu, 1990]. The seven recognized *Hippophae* species are:

1. *H. rhamnoides* Linn.
2. *H. tibetana* Schltdl
3. *H. salicifolia* D. Don
4. *H. goniocarpa* Y.S. Lian, et al.
5. *H. gyantsensis* (Rousi) Y.S. Lian
6. *H. neurocarpa* S.W. Liu & T.N. He
7. *H. litangensis* Y.S. Lian & X.L. Chen ex Swenson & Bartish

1.3 Seabuckthorn in India

In India, SBT has received increasing attention after Defence Institute of High Altitude Research (DIHAR), formerly Field Research Laboratory (FRL), a constituent institute of Defence Research & Development Organisation (DRDO) has developed the technology for preparing beverage from its highly acidic fruit. The technology is well received by industrialists and ready-to-serve beverage is currently available in Indian market under brand name of 'Leh Berry', 'Ladakh Berry', 'Power Berry' etc. Interest in SBT fruit can be judged from the fact that the price of fruit in Ladakh region has increased from Rs 8/kg in 2001 to Rs 25/kg in 2012.

Potential of the lesser-known shrub has been recognized by several R&D organizations. DRDO has pioneered the SBT research in India and started several R&D projects since early nineties. Recently, biotechnological potential has been recognized by Department of Biotechnology (DBT), Govt. of India, which has initiated a project exclusively on SBT involving several R&D institutes and universities of India. Similarly, in the year 2008, Indian Council of Agriculture

Research (ICAR) has approved a mega programme 'A Value Chain on SBT (*Hippophae* L.)' in which research institutes of ICAR, DRDO, ICMR and NGOs are collaborative partners. In the year 2010, Ministry of Environment and Forest, Govt. of India has initiated a mega programme 'National Mission on SBT' to increase the area under SBT plantation in Jammu & Kashmir, Himachal Pradesh, Uttarakhand, Sikkim and Arunachal Pradesh. DIHAR (DRDO) is the nodal R&D institute for the 'National Mission on SBT'.

Hippophae rhamnoides, *H. salicifolia* and *H. tibetana* are the predominant SBT species in India. Of these, *H. rhamnoides* is widely distributed in the country. *H. rhamnoides* is naturally distributed in six valleys namely Leh, Nubra, Changthang, Suru, Lahaul and Spiti in Trans-Himalayan cold deserts of India. It is also found in few pockets of Nathula in Sikkim. Remote sensing and GIS have been used to map SBT in Lahaul and Spiti in Himachal Pradesh and Ladakh region in Jammu and Kashmir. Studies on satellite images by DIHAR and Defence Electronic Application Laboratory, DRDO, have shown that 11,500 ha of land is under pure natural SBT plantation in Ladakh. The shrub is mainly distributed in Nubra, Indus and Suru valleys. It is also found in Changthang valley at an altitude of 15,400 ft above MSL. Plantation is done around agricultural fields to serve as wind break and biofence.

SBT is a cross-pollinated crop and hence there exists high level of genetic diversity. Initial studies conducted by DIHAR (DRDO) on 17 morphotypes of SBT collected from Nubra valley revealed large genetic diversity. RAPD markers show five major groups in the natural population. None of the morphotypes shares a similarity more than 25 percent indicating high level of genetic diversity between the morphotypes [Singh et al., 2006]. However, a detail survey of SBT in Ladakh has not been conducted. Proximate composition of SBT berry has been extensively studied [Stobdan et al., 2010; Korekar et al., 2011; Chen et al., 1988; Tong et al., 1989; Zhang, et al., 1989; Kallio et al., 2002; Dhyani et al., 2007]. However, quantification of the health-promoting compounds has been studied either in elite selections [Tang, 2001] or within limited number of samples. TPC and TAC

depend on specific plant genotype and interaction of cultivation condition [Scalzo et al., 2005]. Although the importance of genotype in determining TAC in selected fruit has been demonstrated [Scalzo et al., 2005; Yildirim et al., 2010; Connor, 2005; Connor et al., 2002; Panico et al., 2009; Oomah et al., 79; Leccese et al., 2012]. Effect of genotype as health promoting compounds in SBT has not been deeply investigated. Ercisli et al., [2007] reported genotypic effect on chemical composition and antioxidant activity of SBT berry based on 10 wild genotypes from a single location in Turkey. Another aspect is antioxidant activity of leaf, fruit, seed and stem bark. To our knowledge, no information is available on the antioxidant properties of SBT stem bark. Extraction of phenolics and determination of antioxidant capacity of different parts of SBT using various solvent extracts have also not been investigated. In recent years, efforts have been made in developing molecular markers for sex determination in SBT [Person and Nybom 1998; Sharma et al., 2010]. However, the studies are confined to development of RAPD markers in F_1 progenies derived from crosses of selected varieties [Person and Nybom 1998] or within a small wild population comprising of few male and female plants only [Sharma et al., 2010]. There is urgent need to develop the sex specific SCAR marker in SBT. Another aspect is the storage of seed for short and medium term under naturally prevailing condition. Though few studies have been conducted on different aspects on seed germination of *H. rhamnoides* [Slabaugh 1974; Smirnova and Tichomirova 1980; Olmez 2011], no information is available on effect of ageing on seed moisture, germination percentage, mean germination time, germination index, seed vigour index and synchronisation index. In view of the rich genetic resources of SBT in cold desert Ladakh region, and considering the above mention problems, the present study has been conducted with the following objectives:

1. Role of geographical barrier in shaping population genetic structure of *Hippophae rhamnoides* L. in trans-Himalaya
2. Genotypic effect on antioxidant activity, total polyphenolic, carotenoids and ascorbic acid content in natural population of seabuckthorn

3. Phenolic content and antioxidant capacity of various solvent extracts from seabuckthorn fruit pulp, seed, leaf and stem bark
4. Identification and validation of sex-linked SCAR markers in *Hippophae rhamnoides* L.
5. Germination studies of seabuckthorn seed after 10 years of storage at ambient condition in Ladakh region

Each of the five pieces of work has distinct characteristics and are related to one another. To clearly and coherently demonstrate the goal, results and conclusion of each piece of work has been arranged chapter wise in a publishing format.

Chapter II

Role of geographical barrier in shaping population genetic structure of *Hippophae rhamnoides* L. in trans-Himalaya

Abstract

The role of geographical barrier in shaping the regional population genetic and morphometric structure within natural population and geographic distinctness of Seabuckthorn (SBT) (*Hippophae rhamnoides* L.) in trans-Himalaya was studied. Seventeen populations from two valleys, which are geographically separated by high mountains were collected. Genetic and morphometric diversity of *H. rhamnoides* L. was moderate to high with significantly low (r^2=0.1012) or no altitudinal variation among the populations. Closely related populations in the valleys were geographically restricted with significantly low genetic differentiation. A significant considerable isolation-by-distance pattern has been observed. Model-based Bayesian clustering, principal coordinate analysis and neighbor-joining analysis highlighted the role of high mountain Ladakh range (6500m amsl) as an important geographic barrier for this species in the studied site. Two main gene pools, one in Leh valley and other in Nubra valley have been observed. Moderate Nei's gene diversity (0.1559) was recorded with high gene flow (2.4215). A parallel pattern of morphometric and genetic diversity was found. The Shannon's diversity index for 20 quantitative morphometric characters was 0.859, which indicated high morphological diversity. Correlation studies between morphological, altitudinal and Nei's gene diversity suggested a strong correlation between characters such as fruit weight, fruit length, number of leafs/10cm^2, number of berries/10cm^2, fruit color in terms of lightness (L^*) and blueness (b). With altitudinal gradient, leaf density decreased while thorn density increases as a morphological adoption to sustain in harsh environment.

2.1 Introduction

Genetic diversity of germplasm determines the classification of collected accessions and identification of subsets of core accessions with possible utility for specific breeding purpose. Knowledge about germplasm diversity and genetic relationship among breeding materials or core collection could be an invaluable aid in crop improvement strategies [Prassanna & Mahammadi 2003]. Knowledge of the genetic structure of plants provides data on the levels and distribution of genetic variation within/among populations and the clues of long distance dispersal *via* seeds or pollen flow, thus providing fundamental information for conservation biology [Hamrick & Godt 1996; Zheng et al., 2008]. Alpine landscapes are characterized by pronounced environmental gradient and heterogeneous topography [Körner 2003]. High mountain ridges differentiate plants habitats and might consequently limit gene flow among plant population and colonization opportunities of new sites [Cain et al., 2000]. Limited gene flow due to habitat isolation could lead to a strong genetic differentiation among population compared with plants found in less isolated habitats and/or lower altitude [Till-Bottrand & Gaudeul 2002]. The reproductive system mostly affects the genetic diversity in plants population. Out crossing species usually have a high within population diversity and low genetic differentiation, while selfing plant populations has low within population diversity and high genetic differentiation among population [Hamrick & Godt 1996; Nybom 2004].

To explore the effect of natural isolation of alpine habitats on gene flow within and among plant population, the genetic variability and population structure of 17 populations of alpine plant *H. rhamnoides* L. was studied. Seed dispersal capacity of this species is limited as it lacks morphological adaptation for dispersal mechanism. Seeds disperse when birds and wild animals eats and disperse it through their waste. The genetic structure of *Hippophae* sp. in Trans-Himalayan region remains poorly documented. Except, few studies carried to estimate genetic diversity elsewhere in sub species *sinensis* and other related taxa using biochemical

and molecular markers such as isozyme and RAPD [Yao & Tigerstedt 1993; Bartish et al., 1999; Tian et al., 2004; Sun et al., 2006; Sheng et al., 2006] ISSR [Bartish et al., 2000] and most recently AFLP and SAMPL [Raina et al., 2011]. A consolidated status about the genetic differentiation, genetic and morphometric diversity of *Hippophae* sp. in Trans-Himalayan is non-existing.

In the present study, for the first time a high population density area was covered and combinations of molecular and morphological characters were used. Furthermore, a model based clustering method was used to determine the optimal number of genetic cluster in the population. A multivariate approach was used to differentiate plants population based on morphological characters and also determined correlation between molecular and morphological data with Mantel test.

The objectives of the study were as follows: (i) examine effect of geographical barrier on shaping regional population genetic structure (ii) assess the genetic and morphometric diversity within natural population of *H. rhamnoides* L. in Trans-Himalaya and (iii) to determine the degree of genetic differentiation among natural population.

2.2 Materials and methods

2.2.1 Population sampling and DNA extraction

Seventeen natural populations of *H. rhamnoides* L. (*N*=187 individuals) were sampled across the major distribution site of the species from Leh and Nubra valley in Trans-Himalaya. The altitude of collection site ranged from 2765.1 m to 3336.1 m amsl covering the densely distributed range of the species. Permission for survey and sample collection of *H. rhamnoides* L. natural population was obtained from Divisional Forest Officer, Leh Division, Office of Forest Department, Government of Jammu and Kashmir. All sampled individuals were collected based on visual observation of plant characteristics with at least 3-5 meters isolation distance (Table 2.1 and Fig 2.1). Leaf material and whole fruiting twig of plant were collected in zip log plastic bags and store at -20°C until DNA extraction and further morphological measurements. Disease free young leaves were treated with 2% (w/v) sodium hypochlorite for 20 min and total DNA was extracted using CTAB method as described by Doyle & Doyle [1990] with some minor modifications.

Table 2.1. Locations of 17 populations of *H. rhamnoides* L.

Valley	Sampling Localities	Population ID	Latitude (N)	Longitude (E)	Altitude (m)	Sample size
Leh	Choglamsar	CHO	34°06'.731"	77°35'.014"	3213.8	14
	Chuchot	CHU	34°05'.348"	77°35'.562"	3219.9	12
	Shey	SHY	34°04'.077"	77°37'.545"	3238.8	07
	Shey Forest	SHF	34°05'.241"	77°36'.227"	3222.6	10
	Phey	PHY	34°08'.207"	77°29'.044"	3185.5	17
	Shey Picnic	SHP	34°03'.557"	77°37'.505"	3239.4	35
Nubra	Skuru	SKR	34°40'.082"	77°17'.539"	3125.4	07
	Tyaxi	TYX	34°53'.219"	76°48'.313"	2765.1	07
	Turtuk	TRK	34°50'.491"	76°49'.478"	2869.4	17
	Bogdang	BGD	34°48'.112"	77°2'.443"	2987.0	03
	Changlung	CHG	34°55'.430"	77°28'.160"	3303.7	06
	Panamik	PNK	34°47'.392"	77°31'.504"	3196.1	09
	Sumur	SMR	34°37'.056"	77°36'.344"	3108.0	04
	Skuru Forest	SKF	34°41'.102"	77°16'.064"	3044.6	14
	Hunder	HUN	34°35'.009"	77°29'.494"	3089.7	08
	Thirth	TRT	34°32'.236"	77°39'.205"	3212.9	14
	Khalsar	KHA	34°29'.155"	77°42'.063"	3336.6	03

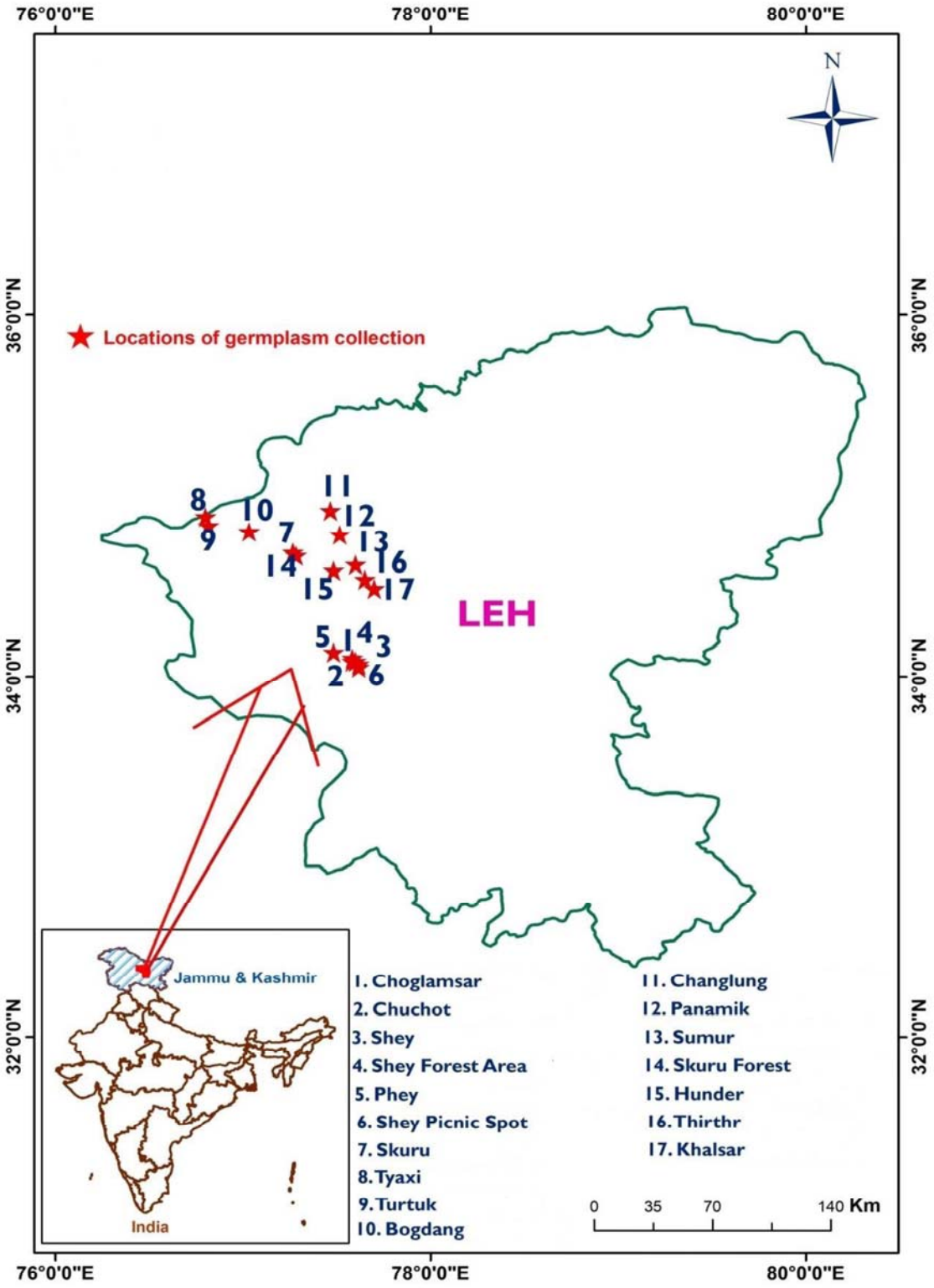

Figure 2.1: Geographical locations of *H. rhamnoides* L. collected from Trans-Himalaya.

Table 2.2: Mean morphological characters in 17 populations of *H. rhamnoides* L.

Populations	CHO	CHU	SHY	SHF	PHY	SHP	SKR	TYX	TRK	BGD	CHG	PNK	SMR	SKF	HUN	TRT	KHS
F Weight (mg)	163.52 ± 44.29^{a}	158.01 ± 35.27^{a}	171.61 ± 49.63^{a}	172.31 ± 42.09^{a}	140.45 ± 25.10^{a}	155.11 ± 37.31^{a}	164.98 ± 50.23^{a}	143.03 ± 48.18^{a}	156.01 ± 40.31^{a}	117.72 ± 27.40^{a}	141.47 ± 30.72^{a}	135.15 ± 44.54^{a}	148.97 ± 62.66^{a}	172.22 ± 34.32^{a}	161.77 ± 60.41^{a}	164.37 ± 41.80^{a}	168.28 ± 31.67^{a}
F Length (mm)	7.21 ± 0.98^{a}	7.34 ± 0.70^{a}	7.69 ± 1.49^{a}	7.63 ± 0.61^{a}	7.15 ± 0.41^{a}	7.33 ± 0.69^{a}	7.18 ± 0.91^{a}	6.87 ± 1.39^{a}	7.26 ± 0.94^{a}	6.24 ± 0.75^{a}	7.02 ± 0.39^{a}	6.82 ± 0.69^{a}	6.68 ± 0.73^{a}	7.10 ± 0.51^{a}	7.50 ± 1.27^{a}	7.37 ± 0.58^{a}	6.88 ± 0.70^{a}
F Width (mm)	6.46 ± 0.49^{ab}	6.54 ± 0.56^{ab}	6.44 ± 0.32^{ab}	6.48 ± 0.58^{ab}	6.02 ± 0.44^{ab}	6.22 ± 0.50^{ab}	6.38 ± 0.65^{ab}	5.57 ± 0.79^{a}	6.22 ± 0.55^{ab}	5.93 ± 0.37^{ab}	6.21 ± 0.60^{ab}	6.03 ± 0.68^{ab}	6.24 ± 0.95^{ab}	6.58 ± 0.58^{ab}	6.10 ± 0.64^{ab}	6.35 ± 0.70^{ab}	6.69 ± 0.72^{b}
No Berries/10cm^{2}	106.07 ± 26.35^{a}	120.50 ± 55.93^{a}	89.29 ± 35.25^{a}	103.60 ± 68.07^{a}	113.06 ± 48.33^{a}	114.09 ± 59.66^{a}	94.71 ± 18.65^{a}	120.57 ± 35.76^{a}	101.41 ± 30.24^{a}	102.33 ± 22.68^{a}	107.17 ± 20.46^{a}	82.56 ± 16.77^{a}	81.75 ± 10.84^{a}	95.29 ± 24.84^{a}	93.50 ± 16.00^{a}	98.86 ± 26.09^{a}	82.67 ± 14.05^{a}
No Berries/bunch	4.57 ± 1.09^{ab}	4.83 ± 0.94^{ab}	3.86 ± 0.90^{ab}	4.40 ± 1.07^{ab}	4.53 ± 1.12^{ab}	4.86 ± 1.59^{ab}	3.57 ± 0.53^{a}	5.00 ± 1.63^{ab}	4.47 ± 0.94^{ab}	6.00 ± 2.00^{b}	5.67 ± 1.63^{ab}	4.56 ± 0.73^{ab}	4.00 ± 0.73^{ab}	4.14 ± 0.86^{ab}	4.38 ± 1.06^{ab}	4.14 ± 1.10^{ab}	4.00 ± 1.00^{ab}
F Index	1.12 ± 0.12^{a}	1.13 ± 0.11^{a}	1.19 ± 0.18^{a}	1.18 ± 0.09^{a}	1.19 ± 0.10^{a}	1.18 ± 0.10^{a}	1.13 ± 0.13^{a}	1.23 ± 0.10^{a}	1.17 ± 0.09^{a}	1.05 ± 0.06^{a}	1.14 ± 0.13^{a}	1.14 ± 0.09^{a}	1.08 ± 0.08^{a}	1.09 ± 0.10^{a}	1.23 ± 0.13^{a}	1.17 ± 0.09^{a}	1.04 ± 0.18^{a}
Pedicel length (mm)	1.68 ± 0.49^{a}	1.47 ± 0.24^{a}	1.37 ± 0.23^{a}	1.63 ± 0.35^{a}	1.55 ± 0.35^{a}	1.65 ± 0.47^{a}	1.45 ± 0.37^{a}	1.21 ± 0.31^{a}	1.49 ± 0.34^{a}	1.49 ± 0.32^{a}	1.30 ± 0.73^{a}	1.54 ± 0.27^{a}	1.64 ± 0.22^{a}	1.21 ± 0.25^{a}	1.36 ± 0.45^{a}	1.38 ± 0.28^{a}	1.11 ± 0.37^{a}
S Wt(mg)	10.58 ± 2.87^{abc}	11.63 ± 2.06^{abc}	11.89 ± 4.02^{abc}	9.96 ± 2.19^{abc}	9.55 ± 1.99^{abc}	11.20 ± 1.96^{abc}	10.15 ± 1.16^{abc}	7.83 ± 2.20^{a}	11.44 ± 3.30^{abc}	8.31 ± 0.41^{ab}	10.12 ± 2.84^{abc}	12.26 ± 2.58^{bc}	11.63 ± 2.92^{abc}	13.06 ± 2.35^{c}	10.78 ± 1.91^{abc}	12.12 ± 2.06^{abc}	13.36 ± 2.27^{c}
S Length(mm)	3.87 ± 0.47^{ab}	4.06 ± 0.59^{ab}	4.02 ± 0.63^{ab}	3.99 ± 0.35^{ab}	3.90 ± 0.39^{ab}	4.07 ± 0.45^{ab}	3.84 ± 0.20^{ab}	3.50 ± 0.49^{a}	3.74 ± 0.53^{ab}	3.39 ± 0.42^{a}	3.34 ± 0.53^{a}	3.99 ± 0.67^{ab}	4.04 ± 0.31^{ab}	3.84 ± 0.36^{ab}	3.97 ± 0.71^{ab}	4.03 ± 0.25^{ab}	4.46 ± 0.62^{b}
S Width(mm)	2.37 ± 0.32^{abcd}	2.38 ± 0.23^{abcd}	2.39 ± 0.25^{abcd}	2.30 ± 0.11^{abc}	2.32 ± 0.19^{abcd}	2.31 ± 0.18^{abc}	2.44 ± 0.15^{abcd}	2.07 ± 0.21^{a}	2.41 ± 0.31^{abcd}	2.19 ± 0.01^{ab}	2.38 ± 0.29^{abcd}	2.59 ± 0.26^{bcd}	2.75 ± 0.31^{abcd}	2.65 ± 0.32^{cd}	2.46 ± 0.14^{abcd}	2.51 ± 0.23^{bcd}	2.72 ± 0.06^{cd}
S Index	1.66 ± 0.14^{ab}	1.71 ± 0.14^{ab}	1.69 ± 0.08^{ab}	1.74 ± 0.17^{b}	1.69 ± 0.19^{ab}	1.77 ± 0.15^{b}	1.59 ± 0.10^{ab}	1.70 ± 0.19^{ab}	1.56 ± 0.11^{ab}	1.58 ± 0.22^{ab}	1.42 ± 0.29^{a}	1.55 ± 0.24^{ab}	1.48 ± 0.12^{ab}	1.47 ± 0.18^{ab}	1.61 ± 0.23^{ab}	1.62 ± 0.10^{ab}	1.65 ± 0.25^{ab}
Leaf Area (cm^{2})	1.22 ± 0.65^{bc}	0.97 ± 0.29^{abc}	1.10 ± 0.12^{abc}	1.34 ± 0.32^{c}	0.86 ± 0.18^{abc}	0.93 ± 0.33^{abc}	0.73 ± 0.16^{ab}	0.80 ± 0.27^{abc}	0.73 ± 0.29^{ab}	1.07 ± 0.53^{abc}	0.60 ± 0.13^{a}	0.57 ± 0.31^{a}	0.71 ± 0.18^{ab}	0.73 ± 0.22^{ab}	0.71 ± 0.16^{ab}	0.82 ± 0.23^{abc}	0.85 ± 0.41^{abc}
No leafs/10cm^{2}	49.50 ± 19.16^{abc}	47.42 ± 16.69^{abcd}	55.14 ± 8.11^{abcde}	52.20 ± 7.25^{abc}	41.59 ± 18.02^{a}	49.11 ± 12.88^{ab}	62.29 ± 8.22^{abcdef}	77.86 ± 9.48^{ef}	76.94 ± 13.89^{ef}	84.00 ± 10.58^{f}	79.17 ± 18.56^{f}	75.78 ± 15.71^{ef}	78.25 ± 5.19^{ef}	72.57 ± 9.55^{cdef}	73.88 ± 10.32^{def}	72.07 ± 10.59^{bcdef}	66.33 ± 3.79^{abcdef}
No Thorn/10cm^{2}	7.07 ± 1.54^{a}	9.50 ± 3.50^{a}	8.71 ± 2.29^{a}	8.30 ± 3.43^{a}	8.12 ± 3.95^{a}	6.71 ± 3.44^{a}	5.86 ± 1.07^{a}	6.71 ± 2.50^{a}	5.76 ± 2.84^{a}	7.33 ± 4.16^{a}	7.17 ± 3.06^{a}	9.00 ± 3.46^{a}	7.00 ± 3.56^{a}	8.21 ± 4.10^{a}	6.63 ± 3.16^{a}	7.86 ± 3.48^{a}	7.67 ± 4.62^{a}
Plant Height (m)	2.74 ± 0.90^{bc}	1.45 ± 0.41^{a}	1.33 ± 0.46^{a}	2.12 ± 0.51^{abc}	1.52 ± 0.60^{ab}	2.29 ± 0.76^{abc}	2.41 ± 0.66^{abc}	2.46 ± 0.44^{abc}	2.42 ± 1.02^{abc}	2.13 ± 0.65^{abc}	2.38 ± 0.37^{abc}	2.19 ± 0.64^{abc}	3.25 ± 0.29^{c}	2.39 ± 0.53^{abc}	3.29 ± 0.38^{c}	2.16 ± 0.65^{abc}	2.13 ± 0.85^{abc}
Canopy width (m)	2.80 ± 1.17^{e}	0.93 ± 0.45^{abc}	0.86 ± 0.28^{ab}	1.26 ± 0.51^{abc}	0.82 ± 0.38^{a}	1.13 ± 0.27^{abc}	1.47 ± 0.94^{abcd}	2.61 ± 0.56^{de}	2.09 ± 0.90^{cde}	1.10 ± 0.10^{abc}	1.95 ± 0.76^{abcde}	2.04 ± 1.14^{bcde}	1.88 ± 0.25^{abcde}	1.74 ± 0.54^{abcde}	1.89 ± 0.83^{abcde}	1.78 ± 0.47^{abcde}	1.23 ± 0.71^{abc}
PH/CW	1.27 ± 0.92^{a}	2.12 ± 1.52^{a}	1.76 ± 1.05^{a}	1.85 ± 0.50^{a}	2.23 ± 1.06^{a}	2.11 ± 0.77^{a}	2.28 ± 1.40^{a}	0.97 ± 0.25^{a}	1.49 ± 1.09^{a}	1.93 ± 0.55^{a}	1.44 ± 0.75^{a}	1.24 ± 0.49^{a}	1.75 ± 0.20^{a}	1.43 ± 0.30^{a}	2.09 ± 0.99^{a}	1.34 ± 0.63^{a}	1.86 ± 0.34^{a}
L*	30.11 ± 3.83^{abc}	28.66 ± 3.37^{a}	27.44 ± 2.54^{a}	28.17 ± 2.66^{a}	31.57 ± 3.21^{abc}	33.61 ± 3.99^{abc}	33.48 ± 4.35^{abc}	33.56 ± 3.68^{abc}	30.78 ± 6.68^{abc}	28.40 ± 5.80^{a}	31.13 ± 3.82^{abc}	31.94 ± 4.89^{abc}	29.42 ± 1.77^{b}	33.42 ± 4.49^{abc}	37.88 ± 5.84^{c}	34.47 ± 2.79^{abc}	36.79 ± 7.58^{bc}
a	19.09 ± 4.41^{a}	20.40 ± 3.25^{a}	17.31 ± 1.95^{a}	18.30 ± 5.90^{a}	19.47 ± 3.68^{a}	20.97 ± 4.86^{a}	20.62 ± 3.66^{a}	18.26 ± 4.94^{a}	18.02 ± 3.05^{a}	19.19 ± 3.41^{a}	18.21 ± 1.44^{a}	17.24 ± 5.58^{a}	16.43 ± 9.64^{a}	17.71 ± 5.65^{a}	18.52 ± 4.40^{a}	17.60 ± 6.50^{a}	18.11 ± 5.31^{a}
b	16.63 ± 4.98^{abc}	13.73 ± 6.61^{a}	13.97 ± 4.68^{a}	15.81 ± 2.76^{ab}	19.60 ± 4.07^{abc}	21.46 ± 3.74^{abc}	23.55 ± 2.44^{bc}	22.64 ± 3.83^{bc}	21.32 ± 6.33^{abc}	18.01 ± 5.48^{abc}	20.90 ± 4.39^{abc}	21.36 ± 4.60^{abc}	15.76 ± 2.03^{ab}	20.42 ± 4.32^{abc}	24.34 ± 6.35^{c}	23.29 ± 1.32^{bc}	22.68 ± 5.28^{bc}

Values are given ± SD; For each row, different lowercase letters significantly differ at $p \leq 0.05$ as measured by 2-sided Tukey's HSD between populations.

2.2.2 AFLP fingerprinting and morphological characterization

AFLP fingerprinting was performed according to the original protocol given by Vos et al., [1995], with minor modifications. Five primer pairs (combination of FAM-labeled *Eco*RI-AAG + *Mse*I-A, *Eco*RI-ACA + *Mse*I-A, *Eco*RI-ACA + *Mse*I-G, *Eco*RI- ACG + *Mse*I-A, *Eco*RI-ACG + *Mse*I-G) were used for selective amplification. The resulting products were separated using an ABI 3730 xl DNA analyzer (Applied Biosystems) with a GeneScanROX-500 internal size standard. GENMAPPER 3.7 (Applied Biosystems) was used to analyze electropherograms. The amplified fragments of 50-500 base pairs having present (1) and absent (0) peaks were extracted using software GENMAPPER 3.7 (Applied Biosystems).

For morphological characterization, total 20 quantitative morphological characters were analyzed in 187 individual (Table 1.2). Characters such as plant height and canopy width were measured in the field while others such as fruit, seed and leaf characters were evaluated in the laboratory.

2.2.3 Genetic and morphological data analysis

Molecular diversity was calculated among population with mean number of alleles per locus (Na), Nei's gene diversity (H) [Nei 1973], Shannon's Information index (I) [Lewontin 1972], number of polymorphic loci (NPL), percentage polymorphic loci (PPL), population differentiation (Gst) and estimated gene flow Nm (Nm = 0.5 (1-Gst/Gst) using POPGENE v. 1.32 [Yeh et al., 1997]. The partitioning of variation at different levels was calculated by Analysis of Molecular Variance (AMOVA) using 999 permutations in GenAlEx v.6.3 [Peakall & Smouse 2006]. One thousand bootstrapped Nei's genetic distance metrics [Lynch & Millgan 1994] was generated using AFLP-SURV [Vekemans et al., 2002] and the Unweighted Pair Group

Method with Averages (UPGMA) as well as Neighbour-Joining (NJ) dendrograms were constructed by using NEIGHBOUR and CONSENSE module in the PHYLIP v. 3.69 [Felsenstein 1993] and tree was visualized by TREE-VIEW. STRUCTURE v. 2.3 [Pritchard et al., 2000; Falush et al., 2003; 2007] was used to calculate number of clusters (K) with probability of individuals to each cluster with no prior population information. Admixture allele model and correlated allele frequencies applied with a burn-in period of 100,000 followed by 250,000 MCMC replicates. The numbers of cluster (K) were predefined from 1 to 8. Three runs were performed for each predefined K. The Ln P (D) can be used as an indication of most likely number of groups, and it is usually plateaus or increased slightly after right K is reached [Evanno et al., 2005]. The result from STRUCTURE was interpreted by HARVESTER [Earl Dent et al., 2011] which estimates the correct K [Evanno et al., 2005]. Permutations of the most likely results among various runs for each K were conducted in CLUMPP [Jakobsson & Rogenberg 2007] and output of CLUMPP was visualized in DISTRUCT [Rosenberg 2004]. Geographical distinctness among population was estimated by isolation-by-distance model [Wright 1943] with Mantel test using GenAlEx [Peakall & Smouse 2006]. The regression analysis was performed between Nei's gene diversity and altitude among the population by using SPSS 17.0 for Windows. In order to determine the geographical location of the main genetic discontinuous among populations, we used the Monmonier's maximum difference algorithm with BARRIER ver. 2.2 [Manni et al., 2004].

Twenty quantitative characters were used in the multivariate statistical analysis of morphological data. One-way ANOVA with 2-sided Tukey's HSD at $p \leq 0.05$ were used to determine the significant differences among the population and 2-tailed Pearson correlation was used to identify relation between quantitative characters using SPSS 17.0 for Windows. Multivariate approaches were used to classify the plant population based on quantitative morphological characters using Gower general

similarity coefficient [Gower, 1971] in MVSP 3.2 including Shannon's Diversity Index for 20 quantitative characters. Mantel test was used to evaluate correlation between morphological and molecular markers.

2.3 Results

Use of five AFLP primer combination to analyze 187 individuals genotypes from natural population resulted in 862 markers out of which 861 (99.88 %) were polymorphic (Table 2.3).

2.3.1 Population genetic structure and gene flow

The Mantel test showed a significant correlation (Rxy = 0.609, P = 0.01) between geographical distances and Nei's genetic distances (Fig 2.2), which indicate significant isolation-by-distance. In Hierarchical cluster analysis (UPGMA), (Fig 2.3a), populations from Leh and Nubra valley grouped separately with the exception that a single population, SHP, from Leh valley was out group with 100% bootstrapped value. The NJ dendrogram supported results obtained using UPGMA analysis (Fig 2.3b). The populations from Leh and Nubra valley were separated with 100% bootstrapped value, which indicated the role of geographical barrier in shaping population genetic structure. For more illustration, a model based clustering method was implemented in STRUCTURE 2.3 [Pritchard et al., 2000; Falush et al., 2003; 2007]. Bayesian model based clustering was done without prior population information with admixture model, structure calculated the most likelihood of the data [LnP (D)] was highest when K = 2 (Fig 2.4a). For K > 2, LnP (D) slightly increased with more or less plateau. The ΔK (Fig 2.4b) analysis proposed by Evanno's [2006] also revealed the similar results that there are two genetically distinctive populations. STRUCTURE grouped all population in two separate groups (Fig 2.4c); green color comprised population from Leh while red comprised population from Nubra valley.

AMOVA revealed that 73% variation of these population was contributed by within populations, while only 27% belonged to among populations with ΦPT = 0.233,

P = 0.010, (Table 2.4). Since we sampled closely distributed populations from Leh valley than in Nubra valley, we selected only two populations (PHY and SHP) that were separated from each other and repeated the analysis of AMOVA.

Table 2.3: Genetic diversity among 17 populations of *H. rhamnoides* L.

Population ID	Nei's gene diversity (H) (Mean)	Shanon's Index (I) (Mean)	Number of Polymorphic Loci	Percentage of Polymorphic Loci
CHO	0.1742	0.2711	542	62.88
CHU	0.1532	0.2379	467	54.18
SHY	0.1526	0.1844	421	48.84
SHF	0.1660	0.2587	514	59.63
PHY	0.1818	0.2828	584	67.75
SHP	0.1933	0.2996	647	75.06
SKR	0.0925	0.1491	312	36.19
TYX	0.0966	0.1490	273	31.67
TRK	0.0958	0.1518	339	39.33
BGD	0.0674	0.0992	148	17.17
CHG	0.0711	0.1084	189	21.93
PNK	0.0887	0.1395	282	32.71
SMR	0.0772	0.1174	196	22.74
SKF	0.0932	0.1512	350	40.60
HUN	0.0857	0.1387	298	34.57
TRT	0.0890	0.1427	322	37.35
KHA	0.0810	0.1237	204	23.67

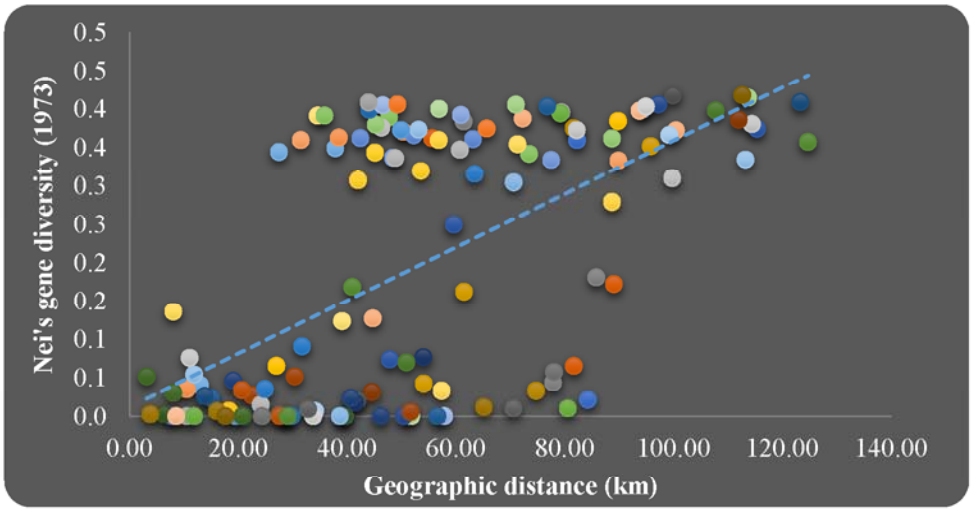

Figure 2.2: Relationship between Nei's gene diversity and geographical distances.

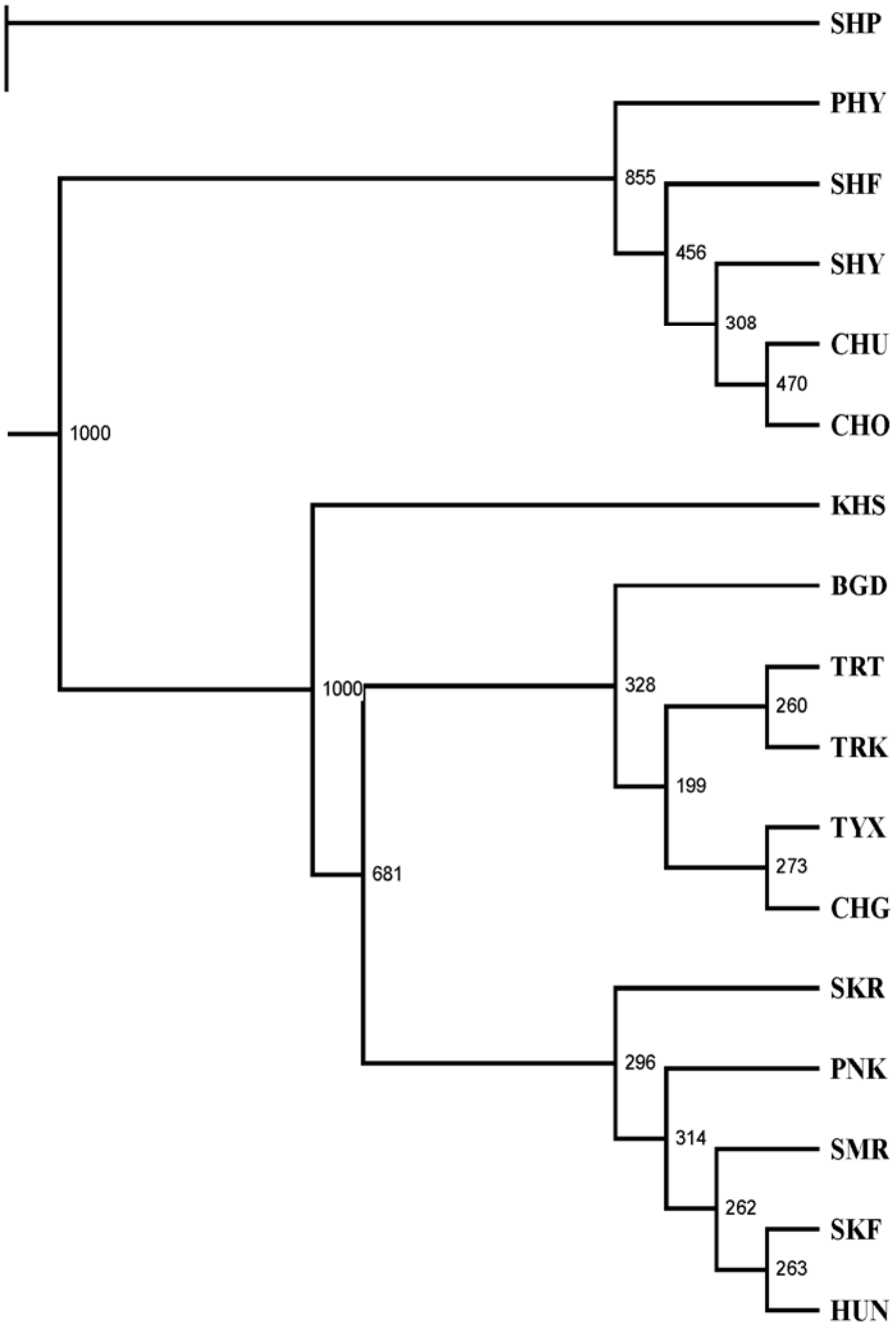

Figure 2.3a: UPGMA for populations from Nei's distance matrix.

We found a non-significant change in ΦPT = 0.231, P = 0.010 with 27% variation among populations while 73% within populations. This indicates high gene flow among populations.

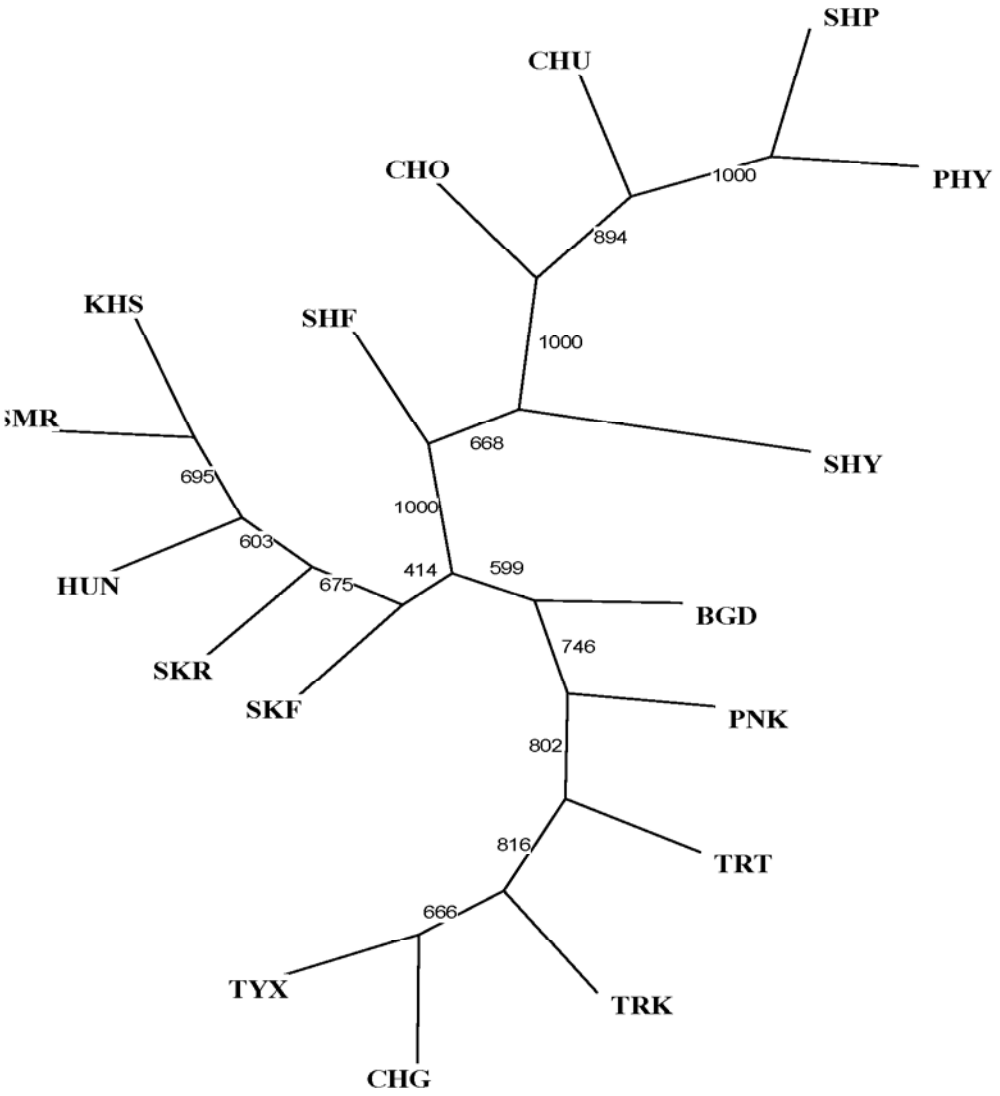

Figure 2.3b: Neighbor Joining tree for populations from Nei's distance matrix.

We concluded that there is considerable differentiation among valleys with high diversity within populations. The Monmonier's maximum difference algorithm, placed the genetic barrier between populations of Leh and Nubra valley (Fig. 2.6). This analysis was strongly congruent with the results from STRUCTURE, NJ and UPGMA based genetic distance analysis, which again highlighted the geography plays a significant role to shape the population genetic structure in the Himalayas.

The genetic differentiation in studied population was found to be low (ΦPT = 0.233, P = 0.010; Gst = 0.1711), thus suggested that only 17.11 % genetic differentiation exist between the populations. The estimated gene flow, Nm (Nm = 0.5(1- Gst/Gst), was 2.4215 high. The genetic differentiation in Leh valley was low (Gst = 0.0598) compare to that of Nubra valley (Gst = 0.1550) while gene flow was higher in Leh valley (Nm = 7.8638) than in Nubra valley (Nm = 2.7247) (Table 2.5).

Table 2.4: Results of AMOVA for *H. rhamnoides* L. based on AFLP markers.

Partitioning	*d.f.*	Sum of square	Mean Square	Variance components	Percentage of variation
Among Populations	16	5814.010	363.376	27.215	27%
Within Populations	170	12335.231	72.560	72.560	73%
Total	186	18149.241		99.775	100%

Table 2.5: Genetic variability among regions of *H. rhamnoides* L.

Regions	Total Nei's gene diversity (Ht)	Nei's gene diversity (Hs)	Shanon's Index (I)	Gene flow (Nm)	Number of Polymorphic Loci	Percentage of Polymorphic Loci	Genetic differentiation (Gst)
Leh	0.1810	0.1702	0.2956	7.8638	739	85.73	0.0598
Nubra	0.1009	0.0853	0.1637	2.7249	463	53.71	0.1550
Species level	0.1559	0.1292	0.2849	2.4215	861	99.88	0.1711

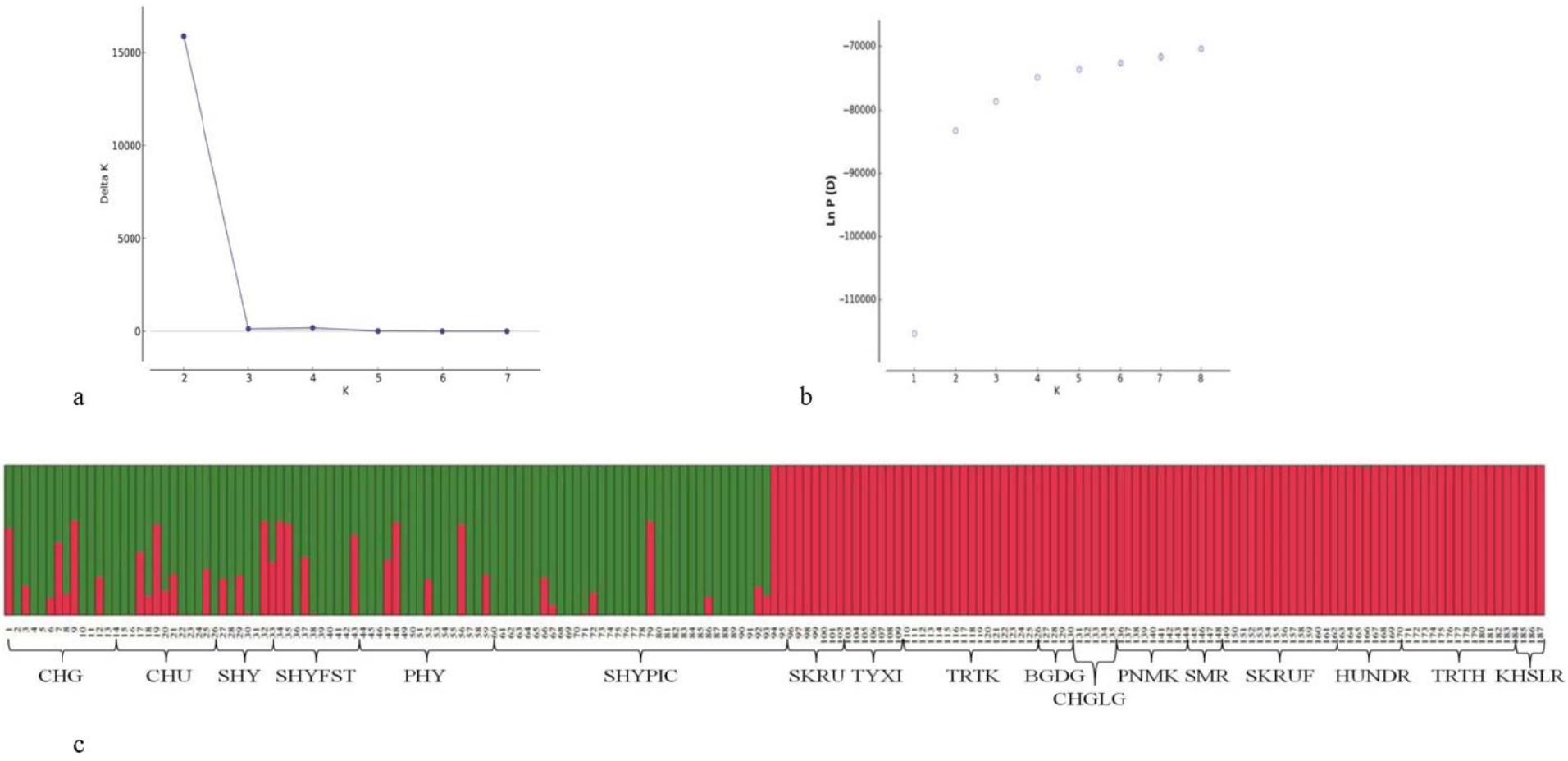

Figure 2.4: STRUCTURE analysis of *H. rhamnoides* L. populations. (a. relationship between K and ΔK; b.relationship between K and LnP(D); c. grouping when (K = 2).

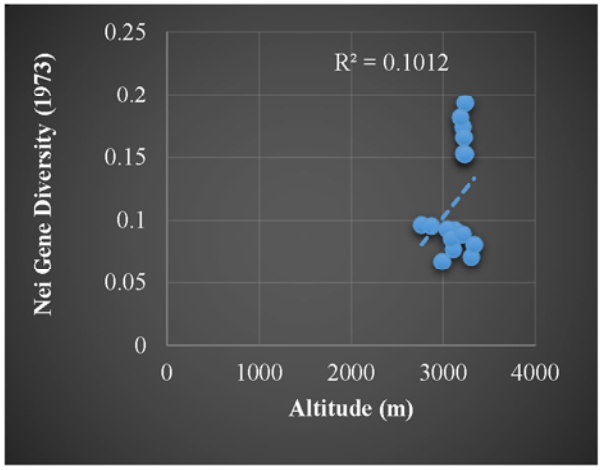

Figure 2.5: Relationship between Nei's gene diversity and altitude.

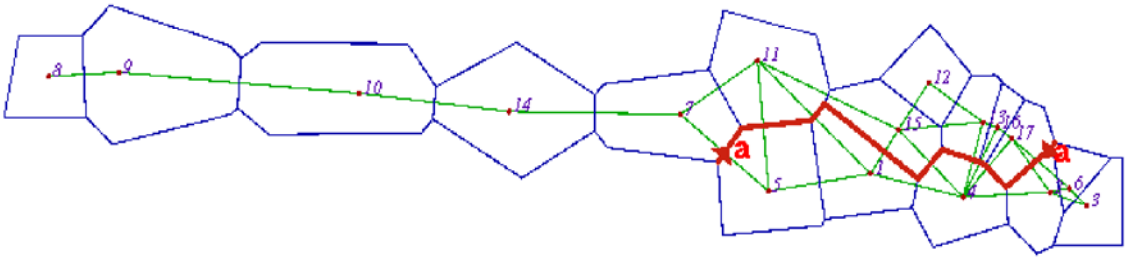

Figure 2.6: A barrier map showing the geographical barrier between two separate valleys

2.3.2 Population genetic diversity

The Nei's gene diversity (Table 2.3) of studied populations ranged from H=0.0674 (BGD) to H=0.1933 (SHP). Similar result was obtained with Shannon diversity index, which varied from I=0.0992 (BGD) to I=0.2996 (SHP). Within valley population from Leh valley have high average gene diversity (Hs = 0.1702) while that of Nubra was low (Hs = 0.0853). The total gene diversity of *H. rhamnoides* L. in Trans-Himalaya was Ht=0.1559 (Table 2.5), which was relatively high and therefore there exists moderate genetic diversity in studied populations. The total gene diversity of Leh valley was high (Ht=0.1812) than that of Nubra valley (Ht=0.1009) (Table 2.5). Relation between Nei's gene diversity and altitude using regression analysis, which revealed that there was no significant relation ($r^2 = 0.1012$) with altitude (Fig 2.5). This showed that there was no altitudinal effect on genetic diversity of SBT populations.

2.3.3 Morphological data in analyzed population

We carried out a comparative morphometric study of 17 populations (Table 2.2). The results revealed high morphological diversity in the species. Characters such as seed weight, seed width, seed index, leaf area, number of leafs/10cm^2, plant height, canopy width, fruit color in terms of lightness (L^*) and blueness (b) showed high variability among the populations (Table 2.2). Morphological data of the analyzed population varied significantly ($P \leq 0.05$). The mean value for each morphological data in the population is presented in Table 1.2. To differentiate the populations, we used multivariate approach. The first three Principle components (PC's) explained 96.57% variation of which PC1 explained 50.27% while PC2 explained 35.39% of total variation and the remaining 10.91% by PC3 (Table 2.6).

We also determine the characters that were responsible for variations (Table 2.7). The PC1 was positively correlated with fruit weight (0.804) and negatively to that of numbers of leafs/ $10cm^2$ (-0.590). The PC2 was negatively correlated with number of berries/$10cm^2$ and positively correlated with fruit weight (0.375) and number of leafs/cm^2 (0.463). PC3 have strong correlation, along with PC1 and PC2, with berries/$10cm^2$ (0.599), number of leafs/$10cm^2$ (0.627), and also with fruit weight (0.446), fruit color in terms of L^* (0.128) and b (0.165). The above results revealed that among the 20 analyzed traits, only five traits viz. fruit weight, number of berries/$10cm^2$, number of leafs/$10cm^2$, L^* and b (Table 2.7) were responsible for maximum variation.

Table 2.6: Eigenvalues and proportion of total variability among 17 populations as examined by first three principal components.

	PC 1	PC 2	PC 3
Eigenvalues	295.121	207.776	64.047
Percentage	50.269	35.391	10.909
Cum. Percentage	50.269	85.66	96.57

Table 2.7: Eigenvectors in first three principal components from PCA.

	PC 1	PC 2	PC 3
Fruit weight (mg)	**0.804**	**0.375**	**0.446**
Fruitlength (mm)	0.017	-0.001	0.007
Fruit width (mm)	0.012	0.007	-0.004
No. berries/10cm2	0.034	**-0.793**	**0.599**
No.berries/bunch	-0.023	-0.024	0.010
Fruitindex	0.001	-0.001	0.002
Pedicel length (mm)	0.001	-0.004	-0.011
Seedwt(mg)	0.036	0.064	-0.023
Seedlength(mm)	0.010	0.006	-0.012
Seedwidth(mm)	0.002	0.010	-0.005
Seedindex	0.003	-0.004	-0.002
Leaf area (cm^2)	0.006	-0.005	-0.005
No leafs/$10cm^2$	**-0.590**	**0.463**	**0.627**
No. thorn/$10cm^2$	0.016	-0.018	-0.051
Plant height (m)	-0.007	0.015	0.018
Canopy width (m)	-0.008	0.008	0.031
PH/CW	0.006	-0.005	-0.019
L*	0.007	0.065	**0.128**
a	0.016	-0.059	0.001
b	-0.046	0.058	**0.165**

We also used UPGMA method and PCA to determine the clusters in which the population they belongs by using Gower general similarity coefficient [Gower 1971]. The population from Leh and Nubra valley distinctly separated with the exception of two populations (BGD and TYX) from Nubra valley that formed a separate cluster (Fig 2.7).The PCA (Fig 2.8) also separated the populations from two the valleys supporting genetic clustering with Nei's genetic distances. We performed the Mantel test to estimate the relation between morphological and genetic data using software PAST [Hammer et al., 2001] and found the positive significant correlation (0.2385, $P \leq 0.00$) between morphological and genetic data.

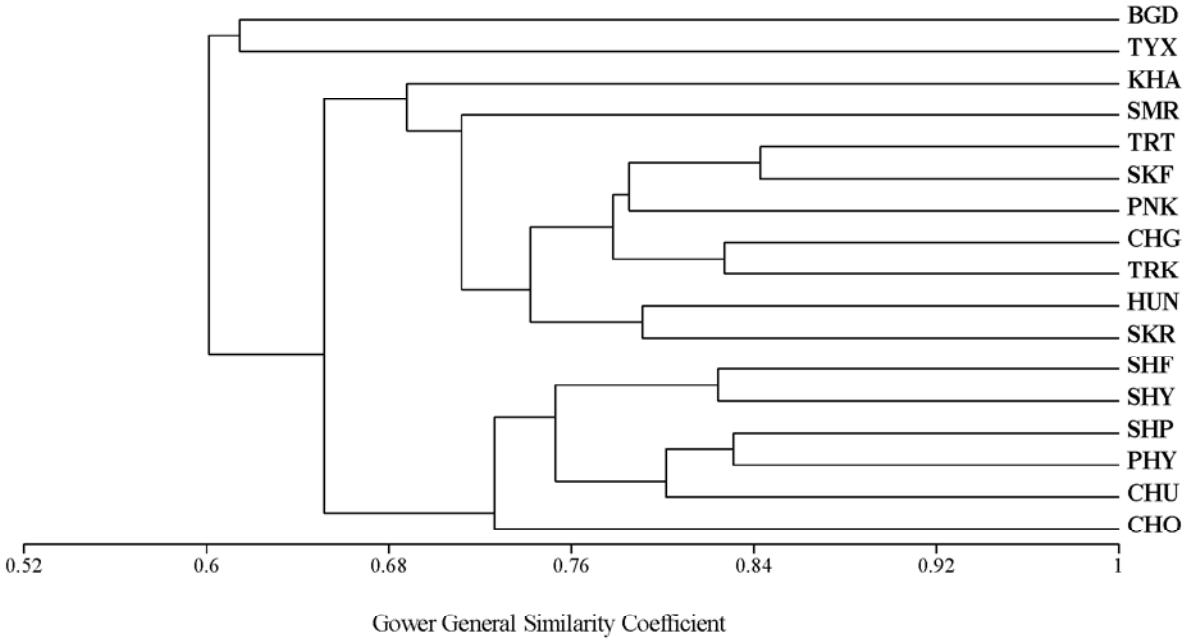

Figure 2.7: UPGMA cluster based on 20 quantitative characters by using Gower general similarity coefficient.

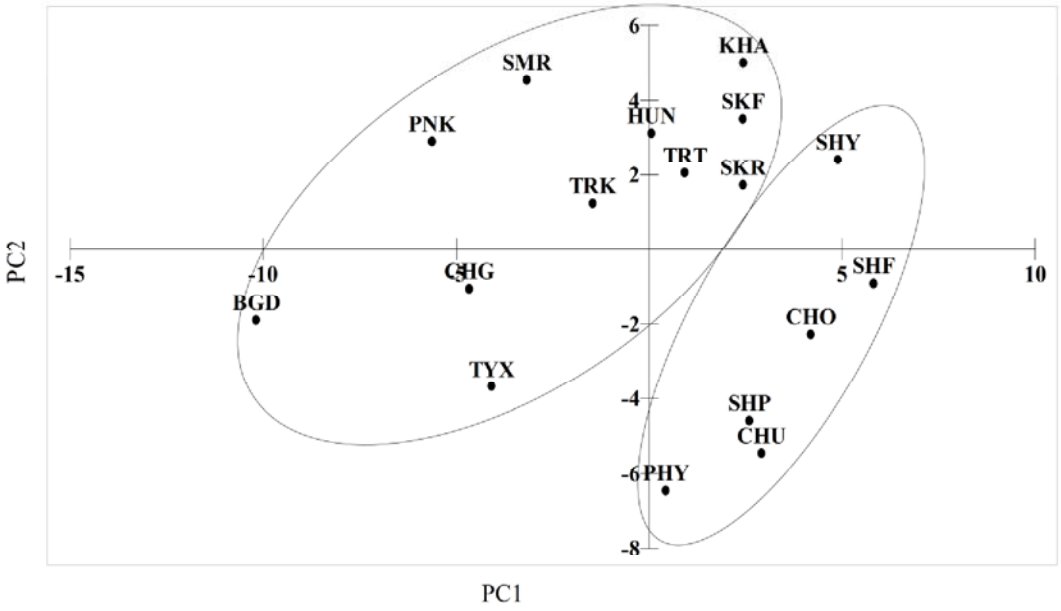

Figure 2.8: PCA based on 20 quantitative characters by using Gower general similarity coefficient.

2.3.4 Correlation

Significant correlation between fruit weight and fruit length (0.750, P= 0.01); between fruit weight and fruit width (0.750, P= 0.01); seed weight and seed length (0.721), seed weight and seed width 0.849 (P= 0.01); fruit color in terms of $L^*$and b (0.876, P= 0.01) was observed. Significant negative correlation between number of berries /10cm^2 (-0.788, leafs/10cm^{2}and seed index (-0.723, P= 0.01); number of berries / bunch and seed length (-0.699, P=0.01); number of P=0.01); plant canopy width and PH/CW ratio (-0.774, P=0.01). Similarly, significant negative correlation between Nei's gene diversity and number of leafs / 10cm^2 (-0.936, P=0.01) and a positive correlation with seed index (0.751, P=0.01) was observed. Besides, considerable correlation between Nei's gene diversity with fruit length (0.563, P=0.05); number of berries / 10 cm^2 (0.492, P=0.05); pedicel length (0.536, P=0.05) and leaf area (0.634, P=0.05) was observed. Positive correlation between altitude and characters such as fruit width (0.633, P=0.01); seed length (0.527, P=0.05); number of thorn/ 10cm^2 (0.528, P=0.05) and negative correlation with number of leafs / 10cm^2 (-0.494, P=0.05) were observed but values are not highly significant (Table 2.8).

Table 2.8: Correlation between the morphological characters, altitude and Nie's gene diversity.

	F Weight (mg)	F Length (mm)	F Width (mm)	No Berries/ 10cm2	No Berries/ bunch	F Index	Pedicel length (mm)	S Wt (mg)	S Length (mm)	S Width (mm)	S Index	Leaf Area (cm2)	No leafs/ 10cm2	No Thorn/ 10cm2	Plant Hight (m)	Canopy width (m)	PH/ CW	L*	a	b	Altitude (ft)	Nei Gene Diversity
F Weight (mg)	1	.760**	.750**	-0.192	-.749**	0.136	-0.171	.556*	.596*	0.356	0.236	0.26	-0.426	0.057	-0.008	-0.048	0.087	0.2	-0.046	-0.054	0.305	0.282
F Length (mm)		1	0.42	0.15	-0.471	.635**	0.089	0.26	0.378	-0.058	0.45	0.301	-.599*	0.203	-0.224	-0.164	0.183	0.03	0.13	-0.112	0.308	.563*
F Width (mm)			1	-0.318	-.511*	-0.428	-0.048	.725**	.610**	.575*	0.004	0.281	-0.397	0.272	-0.184	-0.3	0.254	-0.051	0.02	-0.299	.633**	0.194
No Berries/10cm2				1	.533*	0.416	0.128	-.583*	-0.42	-.788**	0.46	0.284	-0.355	0.102	-0.296	-0.02	0.029	-0.169	.624**	-0.102	-0.233	.492*
No Berries/bunch					1	-0.058	0.06	-.575*	-.699**	-.588*	-0.087	0.061	0.278	0.065	-0.059	0.061	-0.154	-0.25	0.218	-0.068	-0.181	-0.087
F Index						1	0.081	-0.322	-0.117	-.505*	0.422	0	-0.242	-0.032	-0.043	0.13	-0.07	0.136	0.091	0.193	-0.228	0.369
Pedicel length (mm)							1	-0.19	0.043	-0.149	0.241	0.382	-0.42	0.116	0.085	-0.036	0.238	-.515*	0.241	-0.439	0.129	.536*
S Wt(mg)								1	.721**	.849**	-0.201	-0.232	-0.053	0.351	-0.046	-0.121	-0.021	0.235	-0.331	-0.003	0.475	-0.067
S Length(mm)									1	.605*	0.387	0.131	-0.459	0.395	-0.113	-0.327	0.317	0.275	-0.05	-0.07	.527*	0.297
S Width(mm)										1	-.496*	-0.418	0.183	0.071	0.275	0.008	0.017	0.283	-0.478	0.078	0.39	-0.353
S Index											1	.656**	-.723**	0.36	-0.435	-0.352	0.303	-0.051	.498*	-0.193	0.11	.751**
Leaf Area (cm2)												1	-.600*	0.276	-0.336	-0.276	0.173	-.517*	0.243	-.621**	0.183	.634**
No leafs/10cm2													1	-0.452	485*	0.432	-0.455	0.21	-.527*	0.396	-.494*	-.936**
No Thorn/10cm2														1	-.624**	-.518*	0.104	-0.277	0.052	-0.468	.528*	0.477
Plant Hight (m)															1	.672**	-0.209	0.411	-0.245	0.385	-0.293	-0.439
Canopy width (m)																1	-.774**	0.226	-0.339	0.317	-0.418	-0.32
PH/CW																	1	0.005	.591*	-0.119	0.331	0.298
L*																		1	0.065	.876**	-0.019	-0.32
a																			1	0.073	0.104	0.482
b																				1	-0.217	-0.451
Altitude (ft)																					1	0.318
Nei Gene Diversity																						1

*Correlation significant at $p \leq 0.05$, **Correlation is significant at $p \leq 0.01$

2.4 Discussion

2.4.1 Population genetic structure and gene flow

Population differentiation estimation among the population is an essential component in conservation biology [Balloux & Lugon 2002]. Considerable significant correlation between genetic and geographic distances ($r = 0.601$, $P \leq 0.01$) revealed a pattern of isolation-by-distance. This pattern generally suggests that the gene flow most likely occur between neighboring populations [Hamrick and Godt 1990]. In contrast, high Nm (2.4215) values were unexpectedly detected in the studied populations, revealing an unusual migratory mode deviated from the regular stepping-stone, Wright's island, or isolation by distance models [Hamrick & Nason 1996]. The unusual phenomenon could be due to low pollen and seed flow across the long distance between the two geographical regions, which is over 150 km with 6500 m amsl Ladakh range as a geographical barrier. High Nm values estimated from genetic structure tend to represent historical genetic exchange, and are not indicative of current migration rates. For this species, high gene flow estimates are high since they may reflect previous intermingling of populations; and they should not be interpreted as indicating the present state of population isolation [Godt et al., 2005]. In this study, the high estimated Nm values between populations, plus moderate levels of genetic diversity in geographical region, suggested a likely migrant-pool migratory model [Wade and McCauley 1998], which describes a migratory pattern with colonists recruited from a random sample of previously existing populations. This model is usually associated with glaciation or vicariance events [Huang et al., 2001; Chiang and Schaal 2006]. According to the geological evidence, following the uplifting of the Himalaya, the climate in this region has remained relatively steady since the Tertiary. The refugia thereby provided shelters for *H. rhamnoides* during the Quaternary glaciations.

Subsequently, as Pleistocene glaciers retreated, plants from refugia expanded and colonized newly available habitats [Hewitt 2001].

The population genetic structure analysis (UPGMA, NJ and STRUCTURE) revealed that the populations are grouped together and are geographically restricted to each other. One exception to the differentiation was between CHG & PNK and TYX, TRK populations grouping together, despite its colonization to the geographically distinct sites. These results may reflect the colonization history and recent divergence of populations. Colonization may have occurred from CHG, PNK to TYX, TRK side, with sufficient time not having passed for complete divergence. AFLP analysis suggested low genetic differentiation and high gene flow among the populations (Gst=0.1711; Nm= 2.4215). Similar findings were found by Bartish et al., [1999], and Sun et al., [2006] with respect to genetic differentiation among groups of Chinese SBT using RAPD markers. The gene flow of outcross species is comparatively higher than average value (Nm=1.15) reported for out crossing species [Hamrick and Godt 1996]. The gene flow among natural populations strongly affects the distribution of genetic variation, as in the case of mating pattern of the species [Hamrick and Godt 1990]. The limited genetic differentiation among populations in ssp. *sinensis* may be due to the long distance seed dispersal [Sun et al., 2006; Lian et al., 2000]. We found contrasting result to that of previously studied population by AFLP (Gst=0.494, Nm=0.511) and SAMPL (Gst=0.607, Nm=0.322) markers [Raina et al,. 2011]. The current results based on hierarchical and model-based cluster analysis strongly suggest that the 17 populations of *H .rhamnoides* L. split into two clusters, one Leh and other Nubra. The populations of Nubra and Leh always grouped separately in all clustering analysis. This was supported by AMOVA analysis, which revealed the higher genetic variation within populations (73%) than among populations (23%). Topography of the region mostly affects the genetic structure of plant population in Trans-Himalaya, as revealed by the BARRIER analysis (Fig. 2.6). The strongest subdivision separated the populations of Nubra from Leh valley, the geographical location of the barrier coincided with the Ladakh mountain range, which reaches

6500 m amsl and probably act as a barrier to the movement of pollinators and seed dispersal agent.

2.4.2 Population genetic diversity

A moderate genetic diversity (Ht=0.1559) in *H. rhamnoides* L as revealed by AFLP markers in the present study was in accordance with previous studies by Bartish et al., [1999; 2000] and Sun et al., [2006]. The high genetic variability was found in Leh valley compared to Nubra valley with inverse relation to that of gene flow (Table 2.5). Indus River in the Leh valley could be a natural corridor for seed and pollen dispersal. Genetic variability within natural populations is directly linked to their mating system, pollen dispersion, seed propagation and their effective population size [Hamrick 1983]. High genetic diversity in the population is a reflection of high historic genetic variability, which is mostly common in recurrent plant species [Geert et al., 2008; Honnay et al., 2005]. In general, the out breeding plant species typically shows a higher genetic variability within populations [Aguillar et al., 2008], this is also supported by our results that AMOVA represents the 73% variance within populations. Stocklin et al., [2009] also found the similar results with alpine plant species.

The present study showed that there is no significant correlation between population genetic diversity and geographical altitude which is in agreement with previous reports [Stocklin et al., 2009; Thiel-Egenter et al., 2009]. The altitude does not affect the genetic diversity in *H. rhamnoides* L. in Trans-Himalaya. The species is found nearby river basin or glaciers water source, which leads to the similar adaptation to the altitudinal climatic conditions.

2.4.3 Morphological data in analyzed population

This is the first ever study on the morphometric data which covers the large SBT population size. The morphometric data (Table 2.2) shows significant differences among SBT populations. The Shannon's diversity index for 20 quantitative morphometric characters was 0.859, which indicated high morphological diversity in Trans-Himalaya. There are several reports on biotic variables being affected by the environmental conditions. A significant relationship between trait values for Shea tree [Soloviev et al., 2004], Savanna trees [Soloviev et al., 2004], and Agave [Silva-Montellano & Eguiarte 2003] have been reported. We conducted the PCA analysis to evaluate the exact relationship between variation in populations and morphometric characters. The first three PC's explain the 96.57% of total variation found in the populations. Five characters viz. fruit weight (mg), number of berries/10cm^2, number of leafs/10cm^2, fruit colour in terms of lightness (L^*) and blueness (b) showed discrimination ability in the populations. A parallel pattern of morphometric and genetic diversity in SBT populations was observed. Although it may well be recognized that the variations observed in morphometric characters are significantly correlated with abiotic factors of the environment [Assogbadjo et al., 2005], part of this variation within SBT population could be explain by genetic differentiation. In fact statistically significant correlation (0.2385, $P \leq 0.00$) was found between genetic and morphometric characters. These correlations point out towards some degree of genetic determinism for these morphological features. However, additional experiments like mapping studies are needed to identify specific genes that might be have a direct influence on the observed morphometric variations.

2.4.4 Correlation

Correlation studied between morphological, altitudinal and Nei's gene diversity (Table 2.8), suggested a strong correlation between characters such as fruit weight, fruit length, number of leafs /10cm^2, number of berries/10 cm^2, number of leafs /10 cm^2, fruit color in terms of lightness (L*) and blueness (b). There was a strong negative correlation between Nei's gene diversity and number of leafs/10cm^2, which suggested that with increasing population genetic diversity there is decrease in leaf density. A positive correlation between gene diversity and seed index was found, which suggested that seed shape increasing with increasing genetic diversity. Our result is agreement with that of Stocklin et al., [2009] that with altitudinal gradient fruit length and seed length increases significantly (Table 2.8). This could be due to the fact that with increasing altitude; plant life is challenged by low temperature, shorter vegetative period, more snow and harsh conditions. Phenotypic differences among population due to increasing altitude, as a result of increasing risk of not completing life cycle in time, and also the relative importance of sexual verses clonal reproduction might be changed [Stocklin et al., 2009]. Heavier and larger seeds are more likely to establish in more harsh conditions [Westboy et al., 1992]. The decreased leaf density and increased thorn density with increasing altitude might be due to the morphological adoption of the plant to sustain in the harsh environment in cold desert.

2.5 Conclusion

Genetic and morphometric diversity of *H. rhamnoides* L. was high and a significant isolation-by-distance pattern was found in the Trans-Himalaya. Significantly high gene flows with low genetic differentiation were observed in studied populations. Parallel pattern of morphometric and genetic diversity was found. Geographical barrier play a major role to shape genetic and morphometric

structure of population. We found that Ladakh mountain range (6500 m amsl) act as geographical barrier in the studied site, which separated the two major gene pools. With altitudinal gradient, significant effect on fruit and seed length was observed. Decrease in leaf density and increase in thorn intensity was observed with increasing altitude. Conservation and breeding strategies involving genetic studies are limited for Trans-Himalayan plants populations, and exploration of genetic diversity of this region is required. Intra-specific genetic diversity has become a fundamental parameter for the management of species with aim to maintain their evolutionary potential [Rajgopal et al., 2000]. Efficient resource management requires the identification of best focused conservation efforts. A representative sample of the natural populations of SBT could be then used to develop an in situ and ex situ conservation strategy for the species.

Chapter III

Genotypic effect on antioxidant activity, total polyphenolic, carotenoids and ascorbic acid content in natural population of Seabuckthorn

Abstract

Seventeen natural population of SBT, which comprised 187 plants from Trans-Himalaya, were studied to find out the variability and genotypic effect on total phenolic content (TPC), total antioxidant capacity (TAC), ascorbic acid and carotenoids content in fruit pulp. The fruits were found to be rich in TPC ranging from 963.8 to 10703.7 mg gallic acid equivalent /100 g. The free radical-scavenging activity in terms of inhibitory concentration (IC_{50}) ranged from 0.66 to 9.1 mg/ml and ferric reducing antioxidant potential (FRAP) from 179.8 to 1355.4 $FeSO_4.7H_2O$ µg/ml. The ascorbic acid and carotenoids content ranged from 55.6 to 3909.1 mg/100g and 0.07-14.4 mg/100 g, respectively. A variation of 1-11.1 fold in TPC, 1-13.7 folds in IC_{50} by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay and 1-7.5 fold in ferric reducing antioxidant potential, 1-70.3 fold in ascorbic acid content and 1-206.3 fold in carotenoid content among the examined fruit across 17 populations underlines the important role played by genetic background and the geographical location for determining the health promoting compounds of seabuckthorn fruit pulp. Significant correlation was observed between TPC, IC_{50}, FRAP, carotenoids, ascorbic acid, fruit lightness (L*) and plant height. Among the 20 morphological traits studied, fruit colour and plant height showed a positive correlation with the health promoting compounds.

2.1 Introduction

Natural products for food and nutritional supplements have gained increased attention in recent years. In this context, there is an increasing interest in the beneficial health effects of plant derived compounds. Epidemiological studies have demonstrated that there is a positive relation between intake of antioxidant rich diet and lower incidence of degenerative diseases including cancer, heart disease, inflammation, arthritis, immune system decline, brain dysfunction and cataracts [Ames et al., 1993; Gordon 1996; Halliwell 1996]. Along with other antioxidant components, polyphenols, carotenoids and ascorbic acid present in fruit and vegetable have been reported to play a major role in disease prevention. These results have stimulated research to characterize different types of plants with regards to their health promoting compounds.

Proximate composition of SBT berry has been extensively studied [Stobdan et al., 2010; Korekar et al., 2011; Chen 1988; Tong et al., 1989; Zhang et al., 1989; Kallio et al., 2002; Dhyani et al., 2007]. However, quantification of the health-promoting compounds has been studied either in elite selections [Tang & Tigerstedt 2001] or within limited number of samples. TPC and TAC depend on specific plant genotype and interaction of cultivation condition [Scalzo et al., 2005]. Although the importance of genotype in determining TAC in selected fruit has been demonstrated [Scalzo et al., 2005; Yildirim et al., 2010; Connor 2005; Connor et al., 2002; Panico et al., 2009; Leccese et al., 2012], the effect of genotype in SBT has not been deeply investigated. Ercisli et al. [2007] reported genotypic effect on chemical composition and antioxidant activity of SBT berry based on 10 wild genotypes from a single location in Turkey. However, studies involving large population have not been reported. Besides, limited studies have been conducted in fruit crops to study interrelationship between morphological and biochemical traits. To our knowledge extensive studies have not been conducted in SBT. Therefore, the objective of the present study was to investigate genotypic effect on TAC, TPC, ascorbic acid and total carotenoids content in SBT fruit from the 187 plants representing 17 natural

populations. Attempts have been made to find interrelationship between the health promoting compound content and 20 morphological traits in the plant.

2.2 Material and method

2.2.1 Sample collection

Seventeen natural populations of *Hippophae rhamnoides* L. comprising 187 female plants were sampled across the major distribution site from India Trans-Himalaya. The altitude of collection site ranged from 2765.1 m to 3336.1 m amsl (Table 2.1).

Table 3.1: Locations of 17 populations of *H. rhamnoides* L. from Indian trans-Himalaya.

Sampling Localities	Population ID	Latitude (N)	Longitude (E)	Altitude (m) AMSL	Sample size
Choglamsar	CHO	34°06'.731"	77°35'.014"	3213.8	14
Chuchot	CHU	34°05'.348"	77°35'.562"	3219.9	12
Shey	SHY	34°04'.077"	77°37'.545"	3238.8	07
Shey Forest	SHF	34°05'.241"	77°36'.227"	3222.6	10
Phey	PHY	34°08'.207"	77°29'.044"	3185.5	17
Shey Picnic	SHP	34°03'.557"	77°37'.505"	3239.4	35
Skuru	SKR	34°40'.082"	77°17'.539"	3125.4	07
Tyaxi	TYX	34°53'.219"	76°48'.313"	2765.1	07
Turtuk	TRK	34°50'.491"	76°49'.478"	2869.4	17
Bogdang	BGD	34°48'.112"	77°2'.443"	2987.0	03
Changlung	CHG	34°55'.430"	77°28'.160"	3303.7	06
Panamik	PNK	34°47'.392"	77°31'.504"	3196.1	09
Sumur	SMR	34°37'.056"	77°36'.344"	3108.0	04
Skuru Forest	SKF	34°41'.102"	77°16'.064"	3044.6	14
Hunder	HUN	34°35'.009"	77°29'.494"	3089.7	08
Thirth	TRT	34°32'.236"	77°39'.205"	3212.9	14
Khalsar	KHA	34°29'.155"	77°42'.063"	3336.6	03

The mean maximum and minimum temperature in the study site was 18.9±9.5ºC and -5.8±9.8ºC, respectively while the mean maximum and minimum relative humidity was 35.54±7.3 and 25.0±3.7, respectively during 2001-2011. Mean maximum temperature during cropping season (May-September) was 28.4ºC while the minimum was 4.5ºC during the last decade. Samples were collected with fruiting twigs and berries were removed manually in the Laboratory. Seed was separated and fruit pulp was lyophilized in a Laboratory freeze dryer (ALPHA 2-4 LD plus, Fisher Bioblock Scientific, France).

2.2.2 Morphological Characterization

Twenty morphological characters were analyzed in the 187 plants. Characters such as plant height and canopy width were measured in the field while fruit, seed and leaf characters were evaluated in the laboratory.

2.2.3 Preparation of the Extract

Freeze dried samples were defatted with hexane followed by two cycles of extraction with methanol. Each sample (20 to 40 mg) was extracted for 15 min with 1.5 ml methanol in a 2 ml micro centrifuge tube and vortexed at room temperature. The sample was centrifuged at 10,000 rpm for 10 min and the supernatant was recovered. The residue was mixed with 1.5 ml of water and the process was repeated as described above. TPC and TAC were measured directly in the methanolic and aqueous extracts and the values were combined mathematically.

2.2.4 Determination of Total Phenolic Content

The Folin-Ciocalteu reagent assay was used to determine the TPC [Singleton & Rossi 1965]. An aliquot of the samples (30 µl) was introduced into 96 well Elisa plate followed by 150 µl Folin-Ciocalteu reagent, which was previously diluted with distilled water (1:10) and 120 µl sodium carbonate (7.5%, w/v). The Elisa plate were vortexed, covered with parafilm and allowed to stand for 30 min in Elisa reader. Absorbance at 765 nm was recorded in an Elisa reader (SpectroMax M2 e, Molecular Devices, Sunnyvale, CA, United States). TPC was expressed in gallic acid equivalents (GAE mg per 100 g). The calibration equation for gallic acid was $y=0.0104x-0.0589$ ($R^2=0.9978$) where y is the absorbance at 765 nm and x is the concentration of gallic acid in mg/l.

2.2.5 Determination of Antioxidant Capacity

Ferric reducing antioxidant potential (FRAP) assay was conducted using the method previously described [Ikram et al., 2009] with minor modifications [Korekar et al., 2011]. The calibration equation for $FeSO_4.7H_2O$ was y=0.0018x-0.0292 (R^2=0.9911) where y is the absorbance at 593 nm and x is the concentration of $FeSO_4.7H_2O$ in µg/ml. Free radical scavenging method by DPPH developed by Brand-Williams et al. [Brand-Williams et al., 1995] was followed with minor modification [Korekar et al., 2011].

2.2.6 Determination of Ascorbic Acid

Ascorbic acid was determined following the method proposed by Rutkowski and Grzegorezyk [2007]. SBT pulp (1 ml) was mixed with Phosphotungstate reagent (PR) and left at room temperature for 30 min. The content was centrifuged at 7000X g for 10 min and the supernatant was used as test sample. Standard sample was prepared as above but without centrifugation. Absorbance of the test sample (A_X) and that of standard sample (A_S) was measured at 700 nm against PR: 50 mM solution of metaphosphoric acid = 1:1 (v/v) as a reference sample. Concentration of ascorbic acid (C_X) was calculated using the formula

$$Cx = \frac{Ax}{As}.Cs$$

Where, C_S is the concentration of the standard solution

2.2.7 Determination of Total Carotenoids

Carotenoids were analyzed using the method proposed by Ranjith et al., [2006]. Briefly, 1 ml of aliquot of oil in hexane (0.1g/ml) was added to 0.5 ml of 5 g/l NaCl, vortexed for 30s, and centrifuged at 1500g for 10 min. The supernatant was diluted, and the absorbance at 460 nm was measured. The amount of carotenoids

was calculated by plotting a calibration curve with standard β-carotene (1-10 ppm) and expressed as β-carotene.

2.2.8 Statistical Analysis

All the experiments were performed in triplicate. Influence of populations on studied parameters was performed in General Linear Model (GLM). One way ANOVA with 2-sided Tukey's HSD at $p \leq 0.05$ and 2-tailed Pearson correlation was done using SPSS 17.0 version for Windows.

2.3 Results

2.3.1 Total Phenolic Content and Antioxidant Capacity

TPC and TAC are presented in Table 2.2. SBT fruits were found to be rich in TPC ranging from 963.8 to 10703.7 mg GAE/100 g between individuals in the studied population. Therefore, a variation of 1-11.1 fold in TPC was observed. At population level, the highest TPC (6863.3 mg GAE/100g FW) was observed in HUN while the lowest in CHU population (2022.7 mg GAE/100g FW). The overall mean TPC was 2986.4 mg GAE/100g FW.

A large range of values was obtained for ferric reducing activity (Table 2.2). The ferric reducing activity ranged from 179.8 to 1355.4 mM $FeSO_4.7H_2O$. The difference in FRAP value between the genotypes showing the highest and lowest value was 1-7.5 folds. At population level, the highest value was 923.8 mM $FeSO_4.7H_2O$ (HUN) while the lowest was 358.26 mM $FeSO_4.7H_2O$ (TYX).

Table 2 showed the free radical-scavenging activity values measured using DPPH in terms of IC_{50} (mg/ml extract) of the examined SBT juice. A large range, 0.6 to 9.1 mg/ml, was observed. At population level the mean IC_{50} values was 4.16 mg ml^{-1} and the highest and lowest free radical scavenging activity was 1.79 mg ml^{-1} in HUN and 5.20 mg ml^{-1} in SHP, respectively (Table 2). The ascorbic acid content ranged from 55.6 to 3909.1 mg/100g and thus 1-70.3 fold variation was found. At population level the mean ascorbic acid value was 998 mg/100g FW. The highest content, 2318 mg/100g FW was in KHA and lowest value 621 mg/100g FW was observed in PHY. The carotenoids content ranged from 0.07-14.4 mg/100 g and thus 1-206.3 fold variation was observed between individual plants.

Table 3.2: Total phenolic content and antioxidant capacity within 17 populations and the effect of genetic background within a population

Population	TPC (mg GAE/100g FW)		IC_{50} (mg ml^{-1})		FRAP (µg/ml $FeSo_4$)	
	Range (Min - Max)	Mean	Range (Min - Max)	Mean	Range (Min - Max)	Mean
CHO	1464.84-6925.29*	3048.06 ± 1371^{ab}	1.21 – 7.35*	3.88 ± 1.79^{abc}	351.83-1069.06*	570.28 ± 193^{abc}
CHU	1157.29-3289.10**	2022.76 ± 607^{a}	3.02 – 8.62**	5.20 ± 1.88^{c}	272.83-666.72**	463.65 ± 127^{ab}
SHY	1742.31-4803.94**	2564.84 ± 1071^{ab}	2.98 – 6.73**	4.75 ± 1.52^{bc}	403.39-957.11**	564.66 ± 204^{abc}
SHF	1582.93-3558.63***	2397.66 ± 567^{a}	3.03 – 7.44***	4.49 ± 1.45^{abc}	363.06-808.72***	512.17 ± 146^{ab}
PHY	1109.70-6630.42***	2943.68 ± 1961^{ab}	1.29 – 8.05***	4.15 ± 1.93^{abc}	271.67-1258.17***	574.77 ± 293^{abc}
SHP	963.80-6965.26***	2133.86 ± 1074^{a}	1.30 – 9.08***	5.02 ± 1.73^{bc}	179.83-1037.89***	459.40 ± 196^{ab}
SKR	1244.97-3534.97***	2404.31 ± 789^{a}	2.31 – 7.20***	4.12 ± 1.54^{abc}	239.56-689.94***	413.59 ± 184^{ab}
TYX	1609.65-4715.38***	2264.11 ± 1055^{a}	2.40 – 6.57***	4.92 ± 1.44^{bc}	230.44-655.50***	358.46 ± 131^{a}
TRK	1272.10-4705.49***	2772.92 ± 968^{ab}	2.42 – 9.02***	4.23 ± 1.80^{abc}	271.72-655.67***	385.26 ± 129^{a}
BGD	1260.79-10703.71**	3617.42 ± 3085^{ab}	0.99 – 7.91**	4.06 ± 2.22^{abc}	286.56-889.61**	476.96 ± 203^{ab}
CHG	1645.52-7592.20***	2913.30 ± 1917^{ab}	1.12 – 7.02***	4.65 ± 1.67^{abc}	253.89-1138.06***	485.36 ± 271^{ab}
PNK	2060.37-5252.37***	3239.87 ± 1393^{ab}	2.21 – 4.28***	3.59 ± 0.94^{abc}	260.28-480.89***	413.20 ± 103^{ab}
SMR	2212.43-7039.87**	3790.39 ± 1445^{ab}	1.04 – 7.96**	3.30 ± 1.69^{abc}	247.94-757.94**	459.11 ± 175^{ab}
SKF	2286.73-7690.73***	5089.61 ± 2041^{bc}	0.68 – 4.06***	2.12 ± 1.20^{ab}	342.39-871.78***	557.99 ± 208^{abc}
HUN	4229.14-10691.49***	6863.26 ± 2439^{c}	0.66 – 2.89***	1.79 ± 0.92^{a}	592.06-1345.06***	923.81 ± 303^{c}
TRT	2002.32-7420.70***	3455.52 ± 1359^{ab}	0.88 – 5.67***	3.41 ± 1.33^{abc}	237.72-1355.44***	500.24 ± 317^{ab}
KHA	3569.44-4881.52***	4162.22 ± 665^{ab}	2.24 – 4.00***	3.12 ± 0.88^{abc}	585.89-1124.11	777.17 ± 301^{bc}
Avg		2986.44 ± 1714		4.16 ± 1.81		499.07 ± 228

*Significant difference between individual samples within a population at $p \leq 0.05$, ** $p \leq 0.0001$, *** $p \leq 0.000001$ level. Values represented as mean ± SD; for each column, different lowercase letters indicate significantly different at $p<0.05$, as measured by 2-sided Tukey's HSD between populations

However, at population level the carotenoids content do not vary significantly (Table 2), which ranges from 3.85 mg/100g FW in PNK to 7.77 mg/100g in KHA population.

Table 3.3: Ascorbic acid and corotenoids content within 17 populations and the effect of genetic background within a population.

Population	Ascorbic acid (mg/100g FW)		Carotene (mg/100g FW)	
	Range (Min – Max)	Mean	Range (Min – Max)	Mean
CHO	320.15 – 1991.16*	1142 ± 604^{ab}	2.49 – 8.57*	5.64 ± 1.55^{a}
CHU	61.69 – 1629.24**	709 ± 471^{a}	3.02 – 8.62**	6.03 ± 2.18^{a}
SHY	327.12 – 1789.58**	907 ± 512^{a}	2.98 – 6.73**	4.52 ± 1.55^{a}
SHF	179.92 – 1604.55***	713 ± 478^{a}	3.38 – 7.43***	5.15 ± 1.31^{a}
PHY	53.61 – 1849.58***	621 ± 542^{a}	1.54 – 8.94***	4.63 ± 2.29^{a}
SHP	79.99 – 2587.30***	910 ± 803^{a}	0.60 – 12.03***	4.68 ± 2.37^{a}
SKR	258.81 – 2773.16***	1293 ± 911^{ab}	4.49 – 8.69***	5.86 ± 1.34^{a}
TYX	114.37 – 1869.95***	1060 ± 749^{ab}	3.07 – 6.25***	5.26 ± 1.00^{a}
TRK	157.68 – 3909.13***	1207 ± 1064^{ab}	2.39 – 9.54***	5.78 ± 1.96^{a}
BGD	321.28 – 2432.06**	1144 ± 678^{ab}	1.31 – 11.22**	5.88 ± 3.33^{a}
CHG	300.78 – 2160.07***	952 ± 683^{ab}	0.07 – 14.44***	5.63 ± 4.51^{a}
PNK	852.44 – 1348.45***	1136 ± 220^{ab}	2.75 – 5.21***	3.85 ± 1.02^{a}
SMR	291.24 – 1704.51**	783 ± 381^{a}	2.75 – 9.78**	5.45 ± 1.99^{a}
SKF	537.48 – 2731.23***	1365 ± 716^{ab}	1.97 – 12.03***	7.07 ± 3.20^{a}
HUN	1267.12 – 1961.52***	1599 ± 301^{ab}	3.38 – 10.11***	5.97 ± 3.27^{a}
TRT	224.62 – 2198.82***	976 ± 593^{ab}	2.43 – 10.19***	4.93 ± 2.02^{a}
KHA	1847.21 – 2776.68***	2318 ± 465^{b}	4.25 – 11.51***	7.33 ± 3.75^{a}
Average		998 ± 711		5.33 ± 2.34

*Significant difference between individual samples within a population at $p \leq 0.05$, ** $p \leq 0.0001$, *** $p \leq 0.000001$ level.

2.3.2 Correlation Analysis

Table 2.4 displayed the correlation between TPC, TAC, ascorbic acid, carotenoid content and morphological traits that showed *r* value of more than 0.6 with one of the above character. TPC was significantly correlated with IC_{50} (-0.937, $p \leq 0.01$) and FRAP

Table 3.4: Pearson correlation to estimate the interrelationship between TPC, IC_{50}, FRAP, ascorbic acid, carotenoid and morphological characters

	TPC	IC_{50}	FRAP	Ascorbic acid	Carotenoids	L (lightness)	Plant Height (m)
TPC	1	-.937**	.770**	.558*	.439	.643**	.675**
IC_{50}		1	-.646**	-.573*	-.432	-.656**	-.684**
FRAP			1	.573*	.400	.508*	.301
Ascorbic acid				1	.657**	.633**	.452
Carotenoids					1	.609**	.303
L (lightness)						1	.546*
Plant Height (m)							1

*Correlation significant at $p \leq 0.05$, **Correlation is significant at $p \leq 0.01$

(0.770, $p \leq 0.01$).

2.3.3 Principal Component Analysis and Two-way ANOVA

Principal component analysis (PCA) was performed to understand how TPC, TAC, ascorbic acid, carotenoid content and the 20 morphological traits contribute to genotypic variability among the 187 genotypes. Eigenvectors resulting from PCAs show that 50% of the variation is explained by the first two principal components. PC1 represents mainly ascorbic acid (0.918) while PC2 was dominated by TPC, FRAP and IC_{50} (0.619, 0.451, -0.487, respectively).

We conducted a GLM model based analysis to determine the significant effect of populations on TPC, TAC, ascorbic acid content, carotenoid content and the 20 morphological trails. Populations significantly affect all the characters studied except for seven traits (Table 2.5).

Table 3.5: Eigenvector in the first two principal components from PCA and two-way ANOVA to estimate effect of populations on TPC, IC_{50}, FRAP, ascorbic acid, carotenoids and morphological characters.

Eigenvalues	Principal Components		Two-way ANOVA	
	PC1	PC2		
Eigenvalues	0.176	0.08		
% variation	34.3	15.6		
Cumulative %	34.3	49.9		
Variable			Source	$p<0.05$
TPC	0.253	**0.619**	Population	**.000**
FRAP	0.14	**0.451**		**.002**
Ascorbic acid	**0.918**	-0.363		**.008**
Carotene	0.122	0.154		.188
IC_{50}	-0.165	**-0.487**		**.000**
Fruit weight (mg)	0.015	0.021		.414
Fruit length (mm)	-0.008	0.008		.276
Fruit width (mm)	0.005	0.003		**.034**
No Berries/10cm^2	-0.025	-0.059		.706
No Berries/bunch	-0.01	-0.035		.074
Fruit index	-0.008	0.003		**.026**
Pedicel length (mm)	-0.003	-0.021		**.028**
Seed weight (mg)	0.008	0.009		**.000**
Seed length (mm)	-0.006	-0.003		**.015**
Seed width (mm)	0.006	0.009		**.000**
Seed index	-0.009	-0.01		**.000**
Leaf area (cm^2)	-0.027	0.001		**.000**
No leafs/10cm^2	0.105	0.038		**.000**
No Thorn/10cm^2	-0.048	-0.035		.284
Plant Height (PH) (m)	0.091	0.024		**.000**
Canopy width (CW) (m)	0.061	0.071		**.000**
PH/CW	0.018	-0.067		**.009**
Fruit colour				
L* (Lightness)	0.008	0.02		**.000**
a (Yellowness)	-0.004	-0.031		.467
b (Blueness)	0.057	0.008		**.000**

2.4 Discussion

2.4.1 Total Phenolic Content and Antioxidant Capacity

The order of population in terms of TAC by the two different antioxidant assays was different which could be due to presence of compounds having different affinity to react with DPPH and FRAP. Similar results have been reported earlier [Korekar et al., 2011]. The 1-13.7 fold variation in IC_{50} values highlighted unexploited variability among the SBT genotypes from trans-Himalayan region. Significant difference was observed between individual plants within a population in all studied 17 populations in terms of TPC, TAC, ascorbic acid and carotenoid content (Table 2.2 & 2.3). This underlines the importance of genetic background for determining health promoting compounds in SBT. Significant variation between plants within a population could be because SBT is a wind pollinated out-cross species and therefore high genetic variation is expected in natural population.

2.4.2 Correlation Analysis

Ascorbic acid content showed a positive correlation with TPC, FRAP and carotenoids, and a negative correlation with IC_{50}. This suggested that ascorbic acid contribute significantly towards TPC and TAC in SBT fruit. Among the 20 morphological traits studied, only fruit lightness (L*) and plant height showed significant correlation with health promoting compounds. L* showed positive correlation with TPC, TAC, ascorbic acid and carotenoid content. However, Capocasa et al., [2008] found a negative correlation between color (L*) and TPC in strawberry. The plant height was significantly correlated with TPC ($r = 0.675$) and IC_{50} ($r = -0.684$). Higher the plant height, higher is the TPC and TAC in SBT fruit suggesting interrelationships between morphological and biochemical traits. A negative correlation between seed weight and ascorbic acid was found in elite SBT selection [Tang & Tigerstedt 2001]. Similarly, a negative correlation between

strawberry fruit size and nutritional parameters (TPC, FRAP, TEAC) has been reported [Capocasa et al., 2008]. However, no significant correlation was found in SBT fruit.

2.4.3 Principal Component Analysis and Two-way ANOVA

The result demonstrated that genotypic effect is more important towards ascorbic acid, TPC and TAC content in SBT while contribution of morphological traits are not highly significant (Table 2.5). The result suggested that choice of population is an important criteria for selecting plant from natural population as a source of health promoting compounds. A large number of populations are needed to screen for their potential source as breeding stock to improve health promoting compounds.

2.5 Conclusion

The influence of plant genetic background on TPC, TAC, ascorbic acid and carotenoid content in SBT fruit pulp was demonstrated. Significant variation was found within and between the 17 natural populations, which underline the role of genetic background for determining the health promoting compounds in SBT. Many fold variation in TPC (1-11.1), ferric reducing activity (1-7.5), free radical scavenging activity (1-13.7), ascorbic acid content (1-70.3) and carotenoid content (1-206.3) was observed within the 187 studied female plants. Population has significant effect on the studied health promoting compounds. Plant height and fruit colour has significant positive correlation with TPC and TAC suggesting interrelationship between morphological and biochemical traits. Results obtained in this study can be considered for selection of genotype for breeding purpose to improve health promoting compounds in the berry.

Chapter IV

Phenolic content and antioxidant capacity of various solvent extracts from Seabuckthorn fruit pulp, seed, leaf and stem bark

Abstract

The outcome of various solvent extraction (water, methanol, acidic 50% methanol, 70% acetone, acidic 50% methanol followed by 70% acetone) on TPC and antioxidant capacity of fruit pulp, seeds, leaves and stem bark of SBT was investigated. The SBT extracts possess high phenolic content in terms of GAE/100g D.W. (1666-13769). The mean TPC was found highest in seeds (11148) followed by stem bark (10469), leaves (6330) and pulp (3579 mg GAE/100g D.W.). In general, the 70% acetone and acidic 50% methanol followed by 70% acetone extracts was found to contain significantly higher TPC than those obtained in other extracting solvents. Antioxidant capacity in terms of IC_{50} value of pulp (3.39 mg ml^{-1}) was up to 7.8 times higher than those reported for stem bark (0.43 mg ml^{-1}) and up to 2.4 times higher than those found in seeds (1.4 mg ml^{-1}). Further, antioxidant capacity by FRAP assay showed that the stem bark possess maximum antioxidant capacity (16.83) followed by seeds (15.26), leaves (12.73) and pulp (12.61), all as mM $FeSO_4$. Significant correlation was found between TPC and antioxidant capacity by DPPH and FRAP assays.

4.1 Introduction

Oxygen-centred free radicals and other reactive oxygen species (ROS), which are continuously produced *in vivo*, result in cell death and tissue damage. The role of oxygen radicals has been implicated in several diseases, ageing etc. [Capocasa et al., 2008]. Thus, it is considered important to increase the antioxidant intake in the human diet and one way of achieving this is by enriching food with antioxidants. As some synthetic antioxidants may require high manufacturing costs but show lower efficiency than natural antioxidants, there is a need to identify natural and possibly more economic and effective antioxidants with potential to be incorporated into foods. Several natural antioxidants have already been isolated from different kinds of plants. However, studies related to the antioxidant activities of various parts of SBT have been sparsely reported. Well-established dietary antioxidants are vitamin C, E, A, and carotenoids. Besides these antioxidants, other substances in plants such as polyphenols are an important class of defense antioxidants. Phenolics have received considerable attention because of their physiological functions, including antioxidants, antimutagenic and antitumour activities.

Proximate composition of SBT berry and oil composition has been well documented [Chen, 1988; Tong et al., 1989; Zhang et al., 1989; Kallio et al., 2002; Dhyani et al., 2007; Lu, 1992]. However, quantification of the health-promoting antioxidant component of the species especially the population growing in Trans-Himalaya has not been deeply investigated. To our knowledge, no information is available on the antioxidant properties of SBT stem bark. Extraction of phenolics and determination of antioxidant capacity of different parts of SBT using various solvent extracts have also not been investigated. Therefore, the objective of the present study was to investigate total phenolic content (TPC) and antioxidant capacity of various solvent extracts from SBT fruit pulp, seeds, leaves and stem bark for their potential as a natural source of antioxidant.

4.2 Materials and methods

4.2.1 Plant materials

SBT berries, leaves and stem bark were collected in 2009 harvest season from Nubra valley of cold desert Ladakh region, India. Upon arrival at the laboratory, the samples were freeze dried and stored at -20 °C until analysis. The collection site was at an altitude of 3269 meter above mean sea level (latitude N34°53.2′; longitude E77°28.9′) established using GARMIN *GPS 72*, USA.

4.2.2 Chemicals

Gallic acid and 2,2-diphenyl-2-picrylhydrazyl free radical (DPPH) were obtained from Sigma Chemicals Co. (St. Louis, USA). TPTZ were purchased from HiMedia (Mumbai, India). Folin-Ciocalteu phenol reagent was obtained from Merck (Darmstadt, Germany). All other chemicals used were of at least analytical grade.

4.2.3 Preparation of the extracts

Two cycle of extraction with water, methanol, acidic 50% methanol (50:50, v/v; pH 2; pH of solution was maintained by 1N HCl), 70% acetone (70:30, v/v), acidic 50% methanol followed by 70% acetone, were performed separately. Pulp, seeds, leaves and stem bark samples were lyophilized and finely grind in mortar and pestle. Lyophilized powder of each samples (0.5 g) were extracted separately with 20 ml solvent for 12 h in a capped bottle in an orbital shaker at 4 °C maintained at 180 r.p.m. The sample was centrifuged at 10,000 rpm for 10 min and the supernatant was recovered. The residue was mixed with 20 ml of the solvent and the process was repeated. TPC and antioxidant capacity were measured directly in the extract fractions.

4.2.4 Determination of total phenolic content

The Folin-Ciocalteu reagent assay was used to determine the TPC [Singleton and Rossi, 1965]. An aliquot of the samples (0.3 ml, triplicate) were introduced into test tubes followed by 1.5 ml of Folin-Ciocalteu reagent previously diluted with distilled water (1:10) and 1.2 ml of sodium carbonate (7.5% w/v). The tubes were vortexed, covered with parafilm and allowed to stand for 30 min. Absorbance at 765 nm was recorded in a PG Instruments Ltd, Germany, T80+ spectrophotometer. TPC was expressed in gallic acid equivalents (mg GAE per 100 g Dry Weight). The calibration equation for gallic acid was y=0.01x-0.066 (R^2=0.99) where y is the absorbance at 765 nm and x is the concentration of gallic acid in mg l^{-1}.

4.2.5 Determination of ferric reducing antioxidant potential

FRAP assay was conducted using method as described by Ikram et al., [2009]. A total of 75 µl of extract and 225 µl of distilled water were added to 2.25 ml of freshly prepared FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) solution and 1 part of 20 mM $FeCl_3.6H_2O$) and the reaction mixture was incubated in dark for 30 min. The increase in absorbance was measured at 593 nm. The antioxidant potential of the fruit extract was determined based on a calibration curve plotted using $FeSO_4.7H_2O$ at a concentration ranging between 5 and 100 mM. The calibration equation for $FeSO_4.7H_2O$ was y=4.758x+8.636 (R^2=0.98) where y is the absorbance at 593 nm and x is the concentration of $FeSO_4.7H_2O$ mM.

4.2.6 Determination of antioxidant activity by DPPH radical scavenging method

Method developed by Brand-Williams et al., [1995] was followed with minor modification [Zhang and Hamauzu, 2004]. 0.1 mM solution of DPPH in methanol was prepared and 4 ml of the solution was treated with 0.2 ml of the extracted sample. A control was treated with 0.2 ml of solvent instead of the extract. The mixture was left to stand at room temperature for 30 min before the decrease in absorbance at 517 nm was recorded. Antioxidant value was expressed as IC_{50}, the amount of sample extracted into 1 ml solution necessary to decrease by 50% the initial DPPH concentration. IC_{50} was derived from the % disappearance vs. concentration plot (concentration means mg D.W. of pulp, seeds, leaves and bark extracted into 1 ml solution).

4.2.7 Statistical analysis

All the experiments were performed in triplicate. Correlation analysis of TPC and antioxidant capacity by DPPH and FRAP assays was performed using one way ANOVA and Pearson correlation using SPSS for window 11.5 version.

4.3 Results

4.3.1 Total phenolic content

TPC and antioxidant capacity of various solvent extracts of pulp, seeds, leaves and stem bark are presented in Table 4.1. The TPC, expressed as mg gallic acid equivalent/100 g D.W. sample, ranged from 1666 to 15157 mg depending on the plant part and the extracting solvent. 70% acetone extracts and acidic methanol (50%) followed by 70% acetone extracts of leaves and stem bark present the highest TPC.

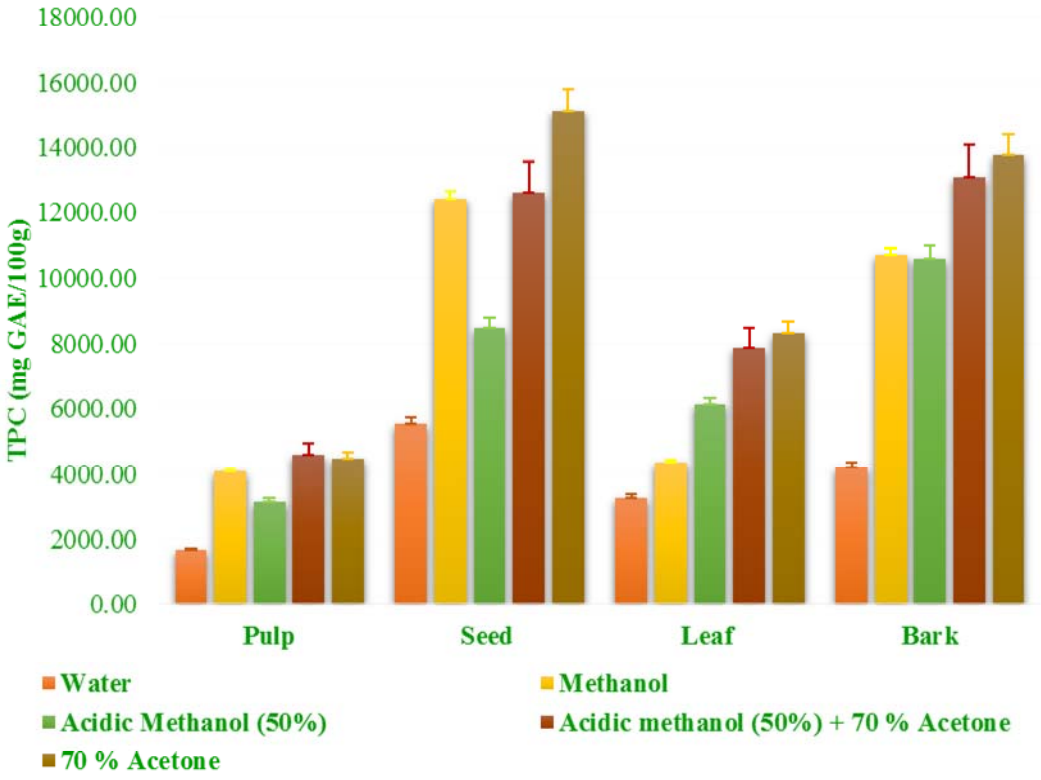

Figure 4.1: Total phenolic content (mg GAE/100g DW) in solvent extracts from seabucktheon pulp, seeds, leaves and bark

Table 4.1. Total phenolic content and antioxidant capacity of various solvent extracts from SBT fruit pulp, seeds, leaves and stem bark.

SBT part	Extraction solvents	Total phenolic content (mg GAE/100g DW)	Antioxidant capacity	
			IC_{50} (mg/ml)	FRAP (mM $FeSO_4$)
Fruit pulp	Water	1666 ±61.40[a]	6.90 ± 0.26[d]	7.50 ±0.49[a]
	Methanol	4056 ±70.12[c]	3.21 ± 0.06[c]	13.09 ±0.30[b]
	Acidic Methanol (50%)	3139 ±112.66[b]	3.09 ± 0.11[c]	13.73 ±0.63[b]
	Acidic methanol (50%) + 70 % Acetone	4578 ±343.68[d]	2.30 ± 0.18[b]	14.14 ±1.39[b]
	70 % Acetone	4455 ±196.57[d]	1.43 ±0.06[a]	14.57 ±0.83[b]
Seeds	Water	5503 ±202.81[a]	4.33 ±0.16[c]	9.20 ±0.53[a]
	Methanol	12440 ±215.02[c]	0.91 ±0.02[b]	16.01 ±0.28[b]
	Acidic Methanol (50%)	10010 ±303.77[b]	0.86 ±0.03[b]	16.74 ±0.60[b]
	Acidic methanol (50%) + 70 % Acetone	12633 ±948.36[c]	0.45 ±0.04[a]	16.87 ±1.29[b]
	70 % Acetone	15157 ±668.69[d]	0.44 ±0.02[a]	17.48 ±0.79[b]
Leaves	Water	3249 ±119.75[a]	5.20 ± 0.04[d]	8.00 ±0.45[a]
	Methanol	6100 ±75.31[b]	3.47 ±0.06[c]	11.18 ±0.20[b]
	Acidic Methanol (50%)	6107 ±219.18[b]	3.59 ±0.13[c]	13.53 ±0.48[c]
	Acidic methanol (50%) + 70 % Acetone	7888 ±592.20[c]	2.27 ±0.18[b]	15.00 ±1.39[d]
	70 % Acetone	8307 ±366.49[c]	1.55 ±0.07[a]	15.94 ±0.72[d]
Stem bark	Water	4188 ± 154.38[a]	1.2 ± 0.01[e]	12.17 ±0.41[a]
	Methanol	10701 ± 184.97[b]	0.21 ± 0.00[b]	17.43 ±0.28[b]
	Acidic Methanol (50%)	10595 ± 380.24[b]	0.32 ± 0.01[d]	18.36 ±0.62[b]
	Acidic methanol (50%) + 70 % Acetone	13090 ± 982.71[c]	0.28 ± 0.02[c]	17.75 ±1.28[b]
	70 % Acetone	13769 ± 607.46[c]	0.13 ± 0.01[a]	18.45 ±0.79[b]

Values represented as mean ± SD of three replication; for each column, values followed by the different letters are significantly differ at $P < 0.05$ for each plant parts as measured by the Duncan test by the Statistical software for windows SPSS 11.5 ver.

4.3.2 DPPH radical-scavenging activity

The mean IC_{50} value of different parts of SBT from five solvent extracts are in the following order from low to high: stem bark (0.43) < seeds (1.4) < leaves (3.22) < pulp (3.39), all in mg/ml. Higher IC_{50} value represents lower radical scavenging activity. The IC_{50} value of pulp (3.39 mg ml^{-1}) was up to 7.8 times higher than those reported for stem bark (0.43 mg ml^{-1}) and up to 2.4 times higher than those found in seed (1.4 mg ml^{-1}). The values suggested many fold higher antioxidant capacity of bark and seeds in comparison to pulp. In general, the antioxidant capacity by DPPH are significantly correlated with TPC for each parts examined (Table 4.2). The order of plant parts in terms of radical scavenging activities remains the same as that of TPC.

4.3.3 Ferric reducing activity based on FRAP assay

The values of total antioxidant activity expressed in terms of mM equivalent of $FeSO_4.7H_2O$ are shown in Table 4.1. The stem bark had the highest total mean FRAP value of 16.83 mM followed by seeds (15.26 mM), leaf (12.73 mM) and pulp (12.61 mM), all as mM $FeSO_4$. The order of plant parts in terms of FRAP value remain the same as that of TPC and antioxidant capacity by DPPH assay. Overall, stem bark and seeds showed a much higher antioxidant capacity and phenolic content than the leaves and pulp. Figure 4.3 shows the antioxidant capacity by FRAP assay of different extracting solvents from SBT pulp, seeds, leaves and bark.

Statistical correlations have been studied between TPC and antioxidant activity determined by different assays as shown in Table 4.2.

Table 4.2. Correlation coefficients, *r*, for relationships between total phenolic content and antioxidant capacity by DPPH and FRAP assays of various solvent extracts from SBT pulp, seeds, leaves and stem bark

Variables		TPC				IC_{50}				FRAP			
		Pulp	Seeds	Leaves	Bark	Pulp	Seeds	Leaves	Bark	Pulp	Seeds	Leaves	Bark
TPC	Pulp	1	.960(**)	.950(*)	.961(**)	-.937(*)	-.924(*)	-.924(*)	-.918(*)	.908(*)	.896(*)	.843	.851
	Seeds		1	.948(*)	.956(*)	-.958(*)	-.905(*)	-.952(*)	-.930(*)	.905(*)	.896(*)	.859	.863
	Leaves			1	.989(**)	-.976(**)	-.922(*)	-.988(**)	-.894(*)	.933(*)	.916(*)	.965(**)	.878(*)
	Bark				1	-.994(**)	-.968(**)	-.961(**)	-.949(*)	.974(**)	.963(**)	.951(*)	.935(*)
IC_{50}	Pulp					1	.973(**)	.950(*)	.964(**)	-.982(**)	-.976(**)	-.948(*)	-.956(*)
	Seeds						1	.864	.986(**)	-.996(**)	-.996(**)	-.888(*)	-.985(**)
	Leaves							1	.847	-.881(*)	-.861	-.950(*)	-.819
	Bark								1	-.979(**)	-.984(**)	-.839	-.976(**)
FRAP	Pulp									1	.999(**)	.917(*)	.990(**)
	Seeds										1	.900(*)	.995(**)
	Leaves											1	.877
	Bark												1

** Correlation is significant at the 0.01 level. * Correlation is significant at the 0.05 level.

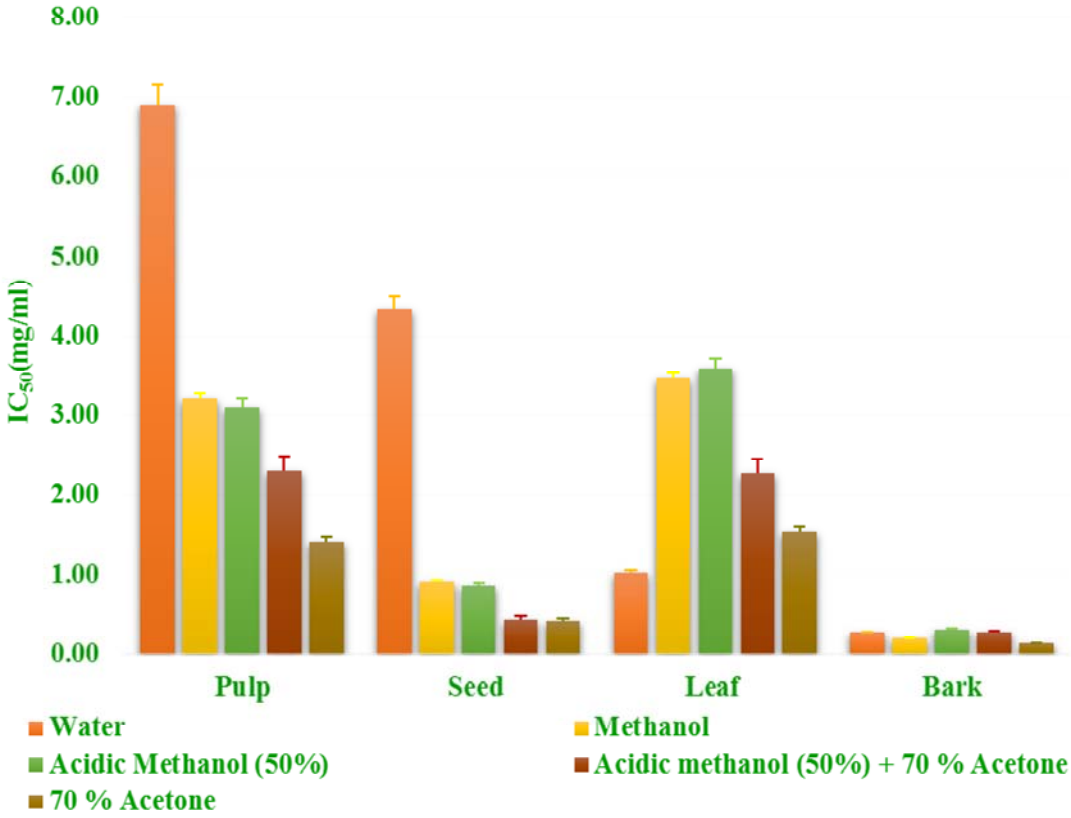

Figure 4.2: DPPH radical scavenging activity of solvent extracts from SBT pulp, seeds, leaves and bark

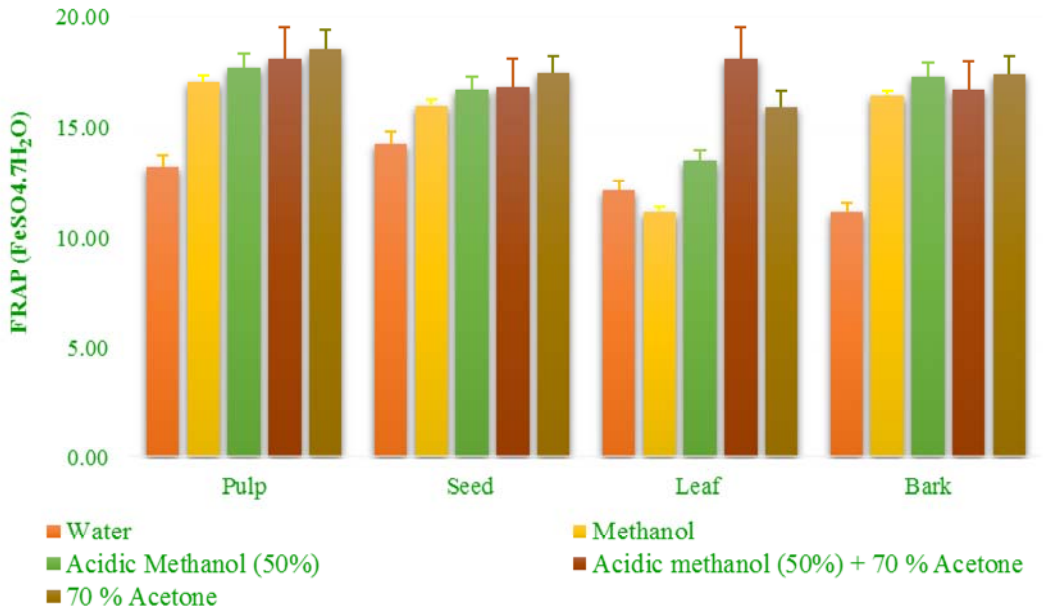

Figure 4.3: Ferric reducing antioxidant potential of solvent extracts from SBT pulp, seeds, leaves and bark

4.4 Discussion

4.4.1 Total phenolic content

On basis of total mean of five solvent extracts, TPC was found in the following order from high to low: seeds (11148) > stem bark (10469) > leaves (6330) > pulp (3579), all as mg GAE/100g D.W. Seed extracts contained 3 fold higher TPC than that of fruit pulp. The TPC in each parts examined was significantly correlated either at $P<0.01$ or at $P<0.05$ level (Table 4.2). The order of TPC in the examined SBT parts is in agreement with *Cassia fistula* L., which contains total phenolic in the following order: stem bark > leaves > pulp > flowers [Siddharaju et al., 2002]. However, Kubola and Siriamornpun [2008] reported TPC in Thia bitter gourd in the following order: leaves > green fruit > stem > ripe fruit.

Efficient extraction of total phenolics requires the use of solvents with different polarities and solubility. In the present study, the TPC in examined plant parts was found affected by the extracting solvent. TPC in SBT pulp in various extracting solvent was in the following order from high to low: acidic methanol (50%) followed by 70% acetone (4578) > 70% acetone (4455) > methanol (4056) > 50% acidic methanol (3139) > water (1666 mg GAE/100 g). TPC of acidic methanol (50%) followed by 70% acetone extraction was 2.7 fold that of aqueous extract. Similar results for TPC as a function of extracting solvent was observed for SBT seeds, leaves and stem bark (Table 4.1). Figure 4.1 presents a comparative TPC in various extracts from SBT parts. Variations in the TPC of the five extracts are attributed to the polarities of different solvents used in the experiment. The result indicated that the polarity of 70% acetone and acidic methanol (50%) followed by 70% acetone were more selective for phenolic compounds present in SBT than other three extracts. Significant differences in extraction of total phenolics in various extracting solvents from other plants have been previously reported [Jung et al., 2006; Pérez-Jiménez et al., 2008].

4.4.2 DPPH radical-scavenging activity

DPPH is a free radical compound and has been widely used to test the free radical-scavenging ability of various samples. It is a stable free radical with a characteristics absorbance at 517 nm. Antioxidants, on interaction with DPPH, either transfer an electron or hydrogen atom to DPPH, thus neutralizing its free radical character. The colour changes from purple to yellow and its absorbance at wavelength 517 nm decreases. To evaluate antioxidant capacity, the SBT fruit pulp, seeds, leaves and stem bark were freeze-dried to minimise losses of antioxidants. Freeze drying has been found to be superior drying method in preserving antioxidant potential in comparison to microwave, oven and sun drying methods [Chen et al., 2009].

The radical scavenging activities were determined in terms of inhibitory concentration (IC_{50}), the amount of sample extracted into 1 ml solution necessary to decrease by 50% the initial DPPH concentration. The result is in agreement with that of previous report by Siddharaju et al.,[2002] who found the antioxidant activity of *Cassia fistula* in the order of stem bark > leaves > flower > pulp. These results revealed that stem bark and seed extracts contain powerful inhibitor compounds, which may act as primary antioxidants that react with free radicals. However, Kubola & Siriamornpun [2008] found the radical-scavenging activities of different parts of Thai bitter gourd in the order leaf > green fruit > stem > ripe fruit.

Several procedures for extraction of antioxidants from plant materials have been described. Solvent extraction is the most common method used in sample preparations from plants. Efficient extraction of antioxidants requires the use of solvents with different polarities. No single solvent could extract all the antioxidants with different polarities and solubility. Water, ethanol, methanol and acetone are commonly used to extract plants. Use of acidified solvents has also

been used to improve efficiency of extraction of antioxidants [Pérez-Jiménez et al., 2008]. The radical scavenging activity in examined SBT parts was found affected by the extracting solvent. Radical scavenging activity in SBT bark in various extracting solvent was in the following order from high to low: 70% acetone (IC_{50} = 0.13 mg ml^{-1}) > methanol (IC_{50} = 0.21 mg ml^{-1}) > acidic methanol (50%) followed by 70% acetone (IC_{50} = 0.28 mg ml^{-1}) > 50% acidic methanol (IC_{50} = 0.32 mg ml^{-1}) > water (IC_{50} = 1.2 mg ml^{-1}). Up to 9.2 fold difference in IC_{50} value was observed for the stem bark in two different extracting solvents. Similar results were observed for pulp, seeds and leaves (Table 4.1). Figure 4.2 summarized the antioxidant capacity in terms of IC_{50} value of different extracting solvents of SBT pulp, seeds, leaves and bark. The result suggested that the choice of extracting solvent is an important factor for determining antioxidant capacity. This factor needs to be taken into consideration while comparing antioxidant capacity of various plant parts in the literature.

4.4.3 Ferric reducing activity based on FRAP assay

The ability of the fruits extracts to reduce ferric ions was determined using the FRAP assay. FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and producing a coloured ferrous tripyridyltriazine (Fe^{2+}-TPTZ), which absorb strongly at 593 nm [Benzie & Strain, 1996]. The result demonstrated that antioxidant capacity of a plant is influenced by the plant part and the choice of extracting solvents. Bark and seeds showed significantly higher antioxidant capacity than that leaves and fruit pulp.

4.4.4 Correlation

In general, a significant correlation between antioxidant capacity by DPPH in terms of IC_{50} and FRAP assay was found for all parts examined. However, the

exceptions were between IC_{50} value of leaves and FRAP value of seeds and bark; IC_{50} value of bark and FRAP value of leaves. Several studies focussed on the relationship between the antioxidant activities by different assay have showed similar results [Wong et al., 2006]. Significant correlation has also been found between ferric reducing power by FRAP assay and TPC of all examined SBT parts. This supports the results reported by Guo and co-workers, [2003] for different fractions of fruits.

This result was also in agreement with Benzie & Stezo [1999] and Kubola & Siriamornpun [2008] , who found a strong positive correlation between TPC and FRAP assay. Several studies focussed on the relationship between the antioxidant activity and total phenols suggested that polyphenols in the extract seemed to be an important factor partly responsible for antioxidant activities. A similar trend was reported by Wong et al., [2006] for 25 selected plants from Singapore wet-market. Similar results were reported in selected Czech honey [Lachman et al., 2010].

4.5 Conclusion

The present study demonstrated that extracts from SBT pulp, seeds, leaves and stem bark contain high levels of TPC and antioxidants. Among the parts examined, seeds and stem bark contains significantly higher antioxidant capacity that may be accounted for by the high phenolic content. The present investigation represents to our knowledge the first report of SBT stem bark as a rich source of natural antioxidant. A significant correlation was found between the antioxidant activity and phenolic content, indicating that phenolic compounds could be major contributor to antioxidant activity. The extracting solvent significantly affected the TPC and antioxidant activity of SBT extract. Seventy percent acetone and 50% acidic methanol followed by 70% acetone showed impressive phenolic content and antioxidant capacity as compare to use of water, methanol and 50% acidic methanol as extracting solvent. Collectively, our data suggest that, when a comparison is made among the data for phenolic content and antioxidant activity of

plant parts in the literature, the choice of extracting solvent should be known and considered. Isolation of bioactive components in the extracts would ascertain the individual potency of the compounds which could be further exploited in food and pharmaceutical industries.

Chapter V

Identification and validation of sex-linked SCAR markers in *Hippophae rhamnoides* L.

Abstract

SBT is a wind pollinated dioecious crop. Study was initiated to develop a robust PCR-based marker(s) which can distinguish male genotypes from female genotypes early in the vegetative growth phase. DNA bulk samples from 20 male and 20 female plants each were screened with sixty RAPD primers. Two primers, OPA-04 and OPT-06 consistently amplify a female specific polymorphic fragment of 1164 and 868 bp, respectively that were absent in the male samples. DNA sequence of the two markers did not exhibit significant similarity to previously characterized sequences. A sequence-characterized amplified region (SCAR) marker *HrX1* (JQ284019) and *HrX2* (JQ284020) designed for the two fragments, continued to amplify the female specific allele in 120 female plants but not in 100 male plants tested in the current study. These results strongly suggest that *HrX1* and *HrX2* are female-specific markers, which could provide an imperative tool for early determination of sex in SBT and expedite commercial cultivations with elite genotypes for industrial applications.

5.1 Introduction

Sex determination of dioecious plant is generally under the influence of an XY chromosome system, wherein males are heterogametic XY [Westergaard 1958; Liu et al., 2004] and females are homogametic XX [Ainsworth 2000; Matsunaga & Kawano 2001; Charlesworth 2002]. In flowering plants, however, sexual specialization of the sporophyte is rare, with only 6% of the 240000 species being dioecious [Renner & Ricklefs 1995]. Only a dozen species in five genera – *Cannabis*, *Humulus*, *Rumex*, *Silene* and *Coccinia* – are known to have morphologically distinct sex chromosomes [Ming et al., 2007]. Although there is no evidence that all organisms with separate sexes are destined to acquire morphologically distinct sex chromosomes [Lynch 2007]. Unravelling the sexual dimorphism in angiosperms has captivated researchers worldwide, and significant strides have been made in understanding the process of flower development in the model plant *Arabidopsis* [Huang et al., 1996; Honma & Gato 2001] and in *Antirrhinum majus* [Huijser et al., 1992; Tröbner et al., 1992].

Early sex determination of dioecious plants has commercial application. Financial resources and valuable time can be saved if undesired male/ female plants can be discarded at an early stage of research trials and commercial plantation. DNA based markers have been widely used in view of its advantages over morphological and biochemical markers for sex determination in plants: *Bryonia dioica* [Oyama et al., 1998]; *Carica papaya* L. [Deputy et al., 2002]; *Pandanus fascicularis* L. [Vinod et al., 2007]; *Salix viminalis* L. [Gunter et al., 2003]; *Asparagus officinalis* L. [Nakayama et al., 2006]; *Actinidia deliciosa* [Shirkot et al., 2002].

For commercial berry production, SBT female plant is critical whereas 10% male plants are needed in the field to produce large amount of fertile pollen. Unfortunately, gender of SBT seedlings cannot be determined until flowering, which usually takes place after 3-4 years in the field. This represents a serious

inconvenience to plant breeders who have to retain large numbers of superfluous males for several years. Studies devoted to early determination of sex in SBT, have been carried out in three basic directions, i.e., morphological, physiological and biochemical characteristics. However, these methods do not offer a reliable and simple method for early determination of sex at young stage [Lebeda 2003]. In recent years, efforts have been made in developing RAPD based marker for sex determination in SBT [Persson & Nybom 1998]. However, the studies are confined to F_1 progenies derived from crosses of selected varieties [Persson and Nybom 1998] or within a small wild population comprising of few male and female plants only [Sharma et al., 2010].

In this study, for the first time, sex-specific SCAR markers were developed for *H. rhamnoides* and further validated in a population consisting of 100 male and 120 female plants. The robust SCAR markers developed and validated in this study will provide early determination of sex in SBT and enhance its production for commercial and pharmaceutical exploitations.

5.2 Material and methods

5.2.1 DNA extraction

Disease free young leaves of *H. rhamnoides* subsp. *turkestanica* were collected from Leh (latitude 37°05.56′N- 37°08.44′N, longitude 77°29.07′E-77°35.90′E, altitude 3192.5-3233.9 m) and Nubra (latitude 34°29.53′N-34°52.68′N, longitude 77°02.60′E- 77°42.81′E, altitude 2801.1-3224.8 m) valley of Trans-Himalayan Ladakh region, India during 2009 to 2011. The leaves were treated with 2% sodium hypochlorite for 20 min and total DNA was extracted using CTAB method as described by Doyle and Doyle [1990]with some minor modifications. DNA concentration and quality was checked on 1.5% agarose gel by electrophoresis using Lambda DNA as a reference.

5.2.2 Identification of sex-linked RAPD marker

Bulk DNA samples from 20 male and 20 female plants each were screened with 60 RAPD primers. PCR amplification was done at following cycle conditions: initial denaturation at 94°C (5 min) followed by 45 cycles of 94°C (1 min), 37°C (1 min) and 72°C (2 min) and a final extension step at 72°C (7 min). The PCR products were run at 1.2% agarose gel. Primers that showed sex-specific amplification with the bulk DNA samples were used to screen individual 20 male and 20 female plants each.

5.2.3 RAPD fragment isolation, cloning and sequencing

RAPD markers that were female-linked were excised from three different plants from the gel and cloned independently into pGEMT-Easy vector (Promega, Madison, WI, USA). Atleast three clones for each band were sequenced using 3730XL DNA Analyzer (Applied Biosystems) in 20 µl of sequencing reaction to verify the correctness of the sequence.

5.2.4 SCAR marker development and testing

The sequences obtained from sex-specific RAPD markers were aligned using the MegAlign module of DNASTAR software and was used to design the sequence characterized amplified regions (SCAR) primers. Primer OPA04(Female specific, FS)F (5'-TATGAGCTCTCGACTGACAGCCA-3') and OPA04(FS)R (5'-CTGTTGTCCGAGATGACGCGT-3') for marker OPA04(FS) and primers OPT06(FS)F (5'-AAGTGTGGCCACCGTCGTAAGA-3') and OPT06(FS)R (5'-ACCGTGTCGATGCACTGTGTATAG-3') for marker OPA06(FS) were developed. PCR amplification was performed at following cycle conditions: initial denaturation at 94^{O}C (5 min) followed by 35 cycles of 94^{O}C (30 sec), 56^{O}C (30 sec) and 72^{O}C (1 min) and a final extension step at 72^{O}C (7 min). The PCR products were run at 1.2% agarose gel. Sex specificity of the SCAR primers was tested with genomic DNA of 100 male and 120 female plants of *H. rhamnoides*.

5.3 Results

5.3.1 Sex-linked RAPD marker

Of the 60 RAPD primers (decamer) screened in the current study, two primers viz. OPA-04 (5'-AATCGGGCTG-3') and OPT-06 (5'-CAAGGGCAGA-3') consistently amplified a female specific polymorphic fragment of 1164 and 868 bp, hereafter named as OPA-04(FS) and OPT-06(FS), respectively in the female bulk samples. The primers were then used to screen 40 genotypes containing 20 male and 20 female plants. The OPA-04(FS) and OPT-06(FS) specific amplifications were observed in all the female genotypes, while none of the male-plant yielded the said amplifications (Fig. 5.1&5.2), thus proving "female specificity' of the observed markers.

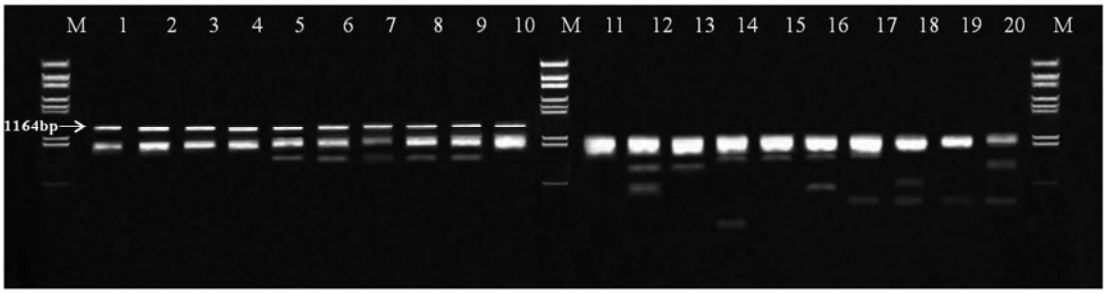

Figure 5.1: Amplification profile ofRAPD primer OPA-04 in 20 SBT genotypes. Arrow indicate 1164 bp. M, Lambda DNA/HindIII marker; lanes 1-10, female; lanes 11-20, male

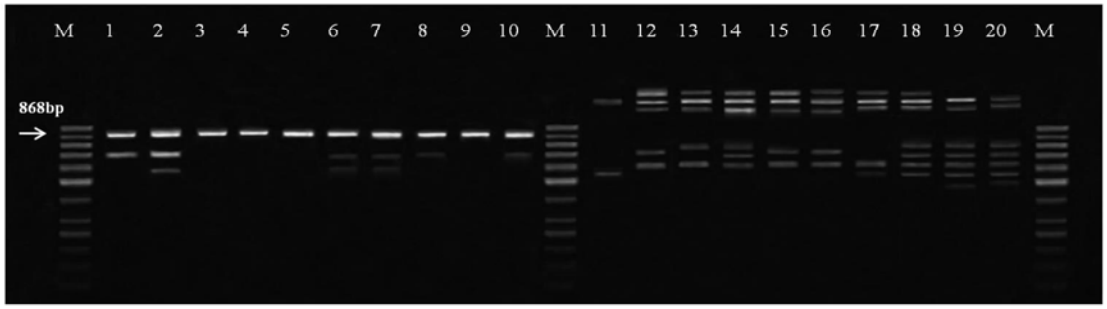

Figure 5.2: Amplification profile ofRAPD primer OPT-06 in 20 SBT genotypes. Arrow indicate 868 bp. M, 100 bp ladder marker; lanes 1-10, female; lanes 11-20, male

5.3.2 DNA sequence of female-specific fragments

The gene sequences obtained from cloning and sequencing OPA-04(FS) and OPT-06(FS) amplicons were deposited in NCBI GenBank under the accession number JQ284019 and JQ284020, respectively. The sequence homology search of the sequences was conducted in the publicly available NCBI database. Megablast analysis of OPA-04(FS) sequence showed a single hit with hypothetical oxidoreductase domain containing protein of *Pyrenophora tritici-repentis* (XM-001933228.1) while OPT-06(FS) showed no similarity. However, BLASTn analysis of OPA-04(FS) sequence showed a sequence identity (E value of 7e-151, 54% query coverage) with the oxidoreductase domain containing protein of *Pyrenophora tritici-repentis* Pt-1C-BFP while that of OPT-06(FS) sequence showed similarity with BNR/Asp-box repeat domain containing protein of the same fungus (E value of 2e-43, 54% query coverage). The two markers did not show any sequence homology between them and the reported plant sequences.

5.3.3 SCAR marker development and testing

Based on the DNA sequence obtained for the OPA04 (FS) marker, a SCAR marker (primer combination OPA04(FS)F and OPA04(FS)R) was developed which showed a 470 bp fragment in all the 120 female genotypes, while no amplification was observed in the 100 male genotypes, each obtained from different locations (Fig. 5.3). The SCAR-470 was named as *HrX1*. Similarly, the OPT06(FS) sequence was converted into a SCAR marker (primer combination OPT06(FS)F and OPT06(FS)R), providing a 386 bp amplification in all female genotypes, and not in the male genotypes tested (Fig. 5,4). The SCAR-386 was named *HrX2*.

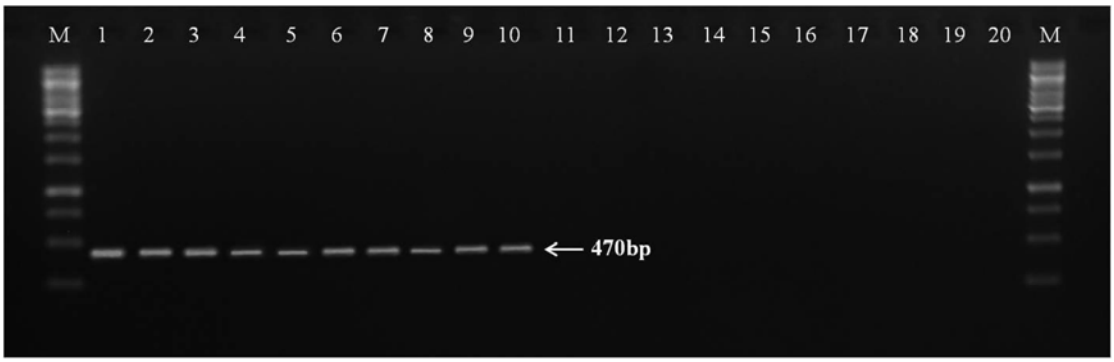

Figure 5.3: Amplification profile of the SCAR marker *HrX1* in SBT, showing the 470 bp fragment. M, 100 bp ladder marker; lanes 1-10, female; lanes 11-20, male

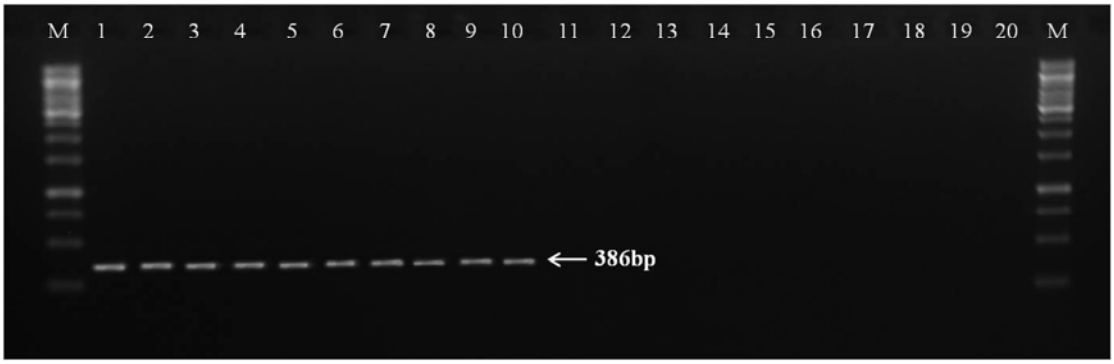

Figure 5.4: Amplification profile of the SCAR marker *HrX2* in SBT, showing the 386 bp fragment M, 100 bp ladder marker; lanes 1-10, female; lanes 11-20, male

5.4 Discussion

5.4.3 DNA sequence of female-specific fragments

SBT is a diploid species with a basic chromosome number n = 12. An X/Y gender determination system is indicated by the findings of a heteromorphic chromosome pairs in male plants [Schapov 1977; Elena et al., 2011]. The total chromosome length of Y-heterosome exceeds X-chromosome [Elena et al., 2011]. Presence of female-linked DNA based markers in large number of population, as shown in this study, confirms that gender in SBT is most likely genetically determined. Sequence homology search of the two marker sequences did not show any sequence homology between them and the reported plant sequences and in all probability represents independent gene loci which are linked to the female-specific loci (X-chromosome) belonging to the *H. rhamnoides* lineage. A BLASTn search showed that 54% sequence coverage of OPA-04(FS) and OPT-06(FS) markers have similarity with hypothetical oxidoreductase and BNR/Asp-box repeat domain containing protein of *Pyrenophora tritici-repentis* Pt-1C-BFP, respectively. However, the remaining 46% of the sequence did not show any significant sequence similarity. Vinod et al., [2007] also did not find significant sequence homology with sex-specific fragment of *Pandanus fascicularis* L. but showed 40-50 bp homology with genomic clones of grapevine, mouse, zebrafish, human etc.

5.4.2 SCAR marker development and testing

Previous attempt to identify sex-linked molecular marker in SBT have met with variable success. The first report on finding RAPD markers for gender determination has been reported by Persson and Nybom [1998]. Marker OPD15-600 obtain using RAPD primer OPD-15 (5'-CATCCGTGCT-3') was observed in the male parent and in all the 17 males of F_1 progenies derived from the crosses 'Leikora' × 'Pollmix I' commercial varieties of SBT. However, the same marker

OPD15-600 was observed in only one of the males of F_1 progenies derived from the cross 'BHi 10224' × '2-24'. The result from the two segregating population clearly suggested that the marker OPD15-600 cannot be designated as sex-specific marker in SBT. Second attempt to identify sex-specific marker in SBT was reported by Sharma et al., [2010] wherein a male-specific RAPD marker OPD-20_{911} was identified in SBT. However, the study is confined to five male and five female plants only. More importantly, both the above studies involve development of male specific RAPD markers. However, for commercial berry production and breeding program females are desirable since only 10% males are needed in the field to product large amount of fertile pollen. Therefore development of a female-specific marker will be of great utility for SBT breeding program.

Since RAPD based markers are sensitive to the subtle differences in the PCR reaction condition, they are found to lack the reproducibility of the desired marker from a pool of multiple fragments and thereby having variable success rate with the sex-determination in crops. The use of SCAR marker is therefore preferred over the RAPD marker [Jang et al., 2004]. A sex-linked SCAR marker developed in *Bryonia dioica* was found to be perfectly linked to male-sex in *B. dioica* from northern Europe [Oyama et al., 1998]. In yet another study, a SCAR marker developed in *Pandanus fascicularis* was found to be perfectly linked to the male plants [Vinod et al., 2007].

5.5 Conclusion

In this context, we for the first time report two robust sex-linked SCAR markers in SBT. Our newly developed sex-linked markers namely *HrX1* and *HrX2* have been successfully validated in larger sets of female (120) and male (100) genotyped and are proved to be perfectly linked to female sex in *H. rhamnoides* would revolutionized its future cultivations. Despite their great value in field studies including identification of female genotypes, these markers could also assist the future chromosome mapping of X-linked locus in *H. rhamnoides* L.

Chapter VI

Germination studies of Seabuckthorn seed after 10 years of storage at ambient condition in Ladakh region

Abstract

For the first time, two important aspects of germination of SBT seeds: (a) germination related studies of aged seed stored up to 10 years under ambient condition in cold arid condition and (b) the effect of seed pre-soaked treatment on germination related parameters of aged seeds have been addressed. Seed stored upto six years does not show any significant difference in germination percentage. However, seed aged 9 and 10 years showed significant reduction in germination percentage, being 65.3 and 65.67%, respectively, compared to 100 and 99% in one and two years old seeds, respectively. KNO_3 pre-soaking treatment showed negative effect on seed germination. Correlation studies showed that with advancement of age of SBT seed, the moisture content, germination percentage and seed vigour index decrease. It takes more time for seed to germination with ageing. Similarly, decrease in moisture content results in decrease germination percentage and seed vigour index. On account of our experimental observations, it could be concluded that short and medium term storage of seed could be achieved at ambient condition in cold arid region at lower cost without the limitation of space.

6.1 Introduction

Study of behaviour of seed germination and the factors controlling the process in an environment is an important aspect not only for physiologists and ecologists, but also for seed technologists. Seed moisture content and storage temperature are the most important factors affecting seed longevity and vigour during storage [Sun 1997]. Preferable conditions for long term seed storage is 3-7% moisture content and -18°C [FAO/IPGRI 1994]. However, its use in developing countries has been greatly limited because of the high cost of building and operation [Huang 2003]. Storage of seed in cold arid region may serve as an alternate cheaper means of seed storage for short and medium terms in view of naturally prevailing low temperature and relative humidity in the region.

Though few studies have been conducted on different aspects on seed germination of SBT [Slabaugh 1974; Smirnova & Tichomirova 1980], no information is available on effect of ageing on seed moisture, germination percentage, mean germination time, germination index, seed vigour index and synchronisation index. In this study, for the first time, two important aspects of SBT seeds: (1) germination related studies of aged seed stored up to 10 years under ambient condition in cold arid condition and (2) the effect of seed pre-soaked treatment on germination related parameters of aged seeds have been addressed. The results in this study provide new knowledge on germination and effect of pre-soaking treatment on aged SBT seeds. Besides, feasibility of cost effective seed storage for short and medium term in cold arid region have been addressed.

6.2 Material and method

Seeds of three healthy SBT plants were collected from Leh valley (3235 m amsl, N37°05.5, E077°35.8) of Trans-Himalayan Ladakh region, India during October 2001, 2002, 2005, 2009, 2010 and 2011. The seeds were stored in 3-ply aluminium laminated (10 μm) poaches in a room with natural air and temperature. The outside mean maximum and minimum temperature in the experimental locality was 18.9±9.5°C and -5.8±9.8°C, respectively while the mean maximum and minimum relative humidity was 35.54±7.3 and 25.0±3.7, respectively during the study period. The average annual precipitation was less than 200 mm of which more than 70% was in the form of snowfall.

6.2.1 Seed moisture

Seed moisture content was determined using the oven drying method [ISTA 1985] and expressed as percentage fresh weight.

6.2.2 Experimental design

Germination experiment was conducted in December 2011. Three sets of seeds from each plant was made and pre-treated with two cheap methods as follows: (a) submersion in distilled water for 48 hours; (b) submersion in 0.1% KNO_3 for 48 hours; (c) control without any treatment. Four replicates of 30 seeds each were placed on one sheet of filter paper (Whattman No. 1) in 50 mm diameter Petri dishes and germinated at predetermined optimal dark condition at 25°C. The filter paper was moistened as needed with distilled water and seedling counts were performed after every 24 hours. Final germination rate (measured as %) was recorded after 35 days.

6.2.3 Mean Germination Time (MGT)

Mean Germination Time (MGT) was determined using the formula [Labouriau 1983]:

$$MGT = \sum_{i=l}^{k} n_i t_i \,/ \sum_{i=l}^{k} n_i$$

where t_i is the day from the start of the experiment to the i^{th} observation; n_i is the number of seeds germinated in the day i; k is the last day of germination.

6.2.4 Germination Index (GI)

Germination Index (GI) was calculated using the formula [Melville et al., 1980]:

$$GI = \sum_{i=l}^{k} |(36 - D_i)G_i| \,/S$$

Where n is the number of germination counting (days); 36 is the total number of days spent in the germination test plus 1; D_i is the number of days until the i^{th} reading; G_i is the number of normal seeds germinated in the i^{th} day; and S is the total number of seeds used in the test.

6.2.5 Germination Synchrony (GS)

Germination Synchrony (GS) was calculated [Labouriau & Valadares 1976] as:

$$GS = -\sum_{i=l}^{k} f_i log_2 \, f_i$$

, being

$$f_i = n_i \sum_{i=l}^{k} n_i$$

where f_i is the relative frequency of germination; n_i is the number of seeds germinated on the day i; and k is the last day of observation.

6.2.6 Seed Vigour Index (SVI)

Seed Vigour Index (SVI) was calculated at the final count by measuring average seedling length of 20 seedlings [Abdul-Baki and Anderson 1972] as:

$$\text{SVI} = \frac{\text{germination (\%)} \times \text{seedling length (cm)}}{100}$$

6.2.7 Statistical analysis

All experiments were conducted in a completely randomized block design. Germination data were arcsine transformed before analysis of variance. One-way ANOVA was performed with the help of 2-sided Tukey's HSD at $p \leq 0.05$ and 2-tailed Pearson correlation using SPSS for window 17.0 version. Two-way ANOVA was used to test the effect of the main factors and their interactions (age and treatment) on seed germination %, mean time to germinate, germination index, seed vigour index and synchronization index.

6.3 Results

6.3.1 Germination percentage

No significant decline in germination percentage of seed stored up to six years was observed (Table 6.1). Therefore SBT seeds can be kept satisfactorily up to six years without significant loss of viability at room temperature in cold desert condition. However, seed aged 9 and 10 years showed significant reduction in germination percentage to 65.3 and 65.67%, respectively, compared to 100 and 99% in one and two years old seeds, respectively.

Two-way ANOVA for pre-soaking treatments, age of seed and their interaction showed that pre-soaking treatment has significant influence on seed germination percentage (Table 6.2). KNO_3 treatment has negative effect on germination of SBT seed of all ages.

6.3.2 Mean Germination Time (MGT)

Influence of age and pre-soaking treatment on seed mean time to germinate is shown in Table 6.1. No significant difference in mean germination time with respect to age of seed was observed. However, 9 and 10 years old seeds treated with water showed significant difference as compare to seed stored for six years or less. Two-way ANOVA for pre-soaking treatments, age of seed and their interaction showed that pre-soaking treatment as well as age of seed has significant influence on seed mean time to germinate (Table 6.2). The combined effect of seed pre-soaking treatment and age of seed is also significant. Significant difference in germination index was observed for seed stored for 9 and 10 years old as compare to others (Table 6.3). Two-way ANOVA for pre-soaking treatments, age of seed and their interaction showed that pre-soaking treatment as well as age of seed has significant influence on seed germination index (Table 6.4). However, the combined effect of seed pre-soaking treatment and age of seed is not significant.

Table 6.1: Moisture content and the effect of seed age and pre-soaking treatments on seed germination percentage and mean germination time

Age of seed (Year)	Moisture (%)	Germination (%)			Mean germination time (day)		
		Treatments		Control	Treatment		Control
		KNO_3	Water		KNO_3	Water	
0	8.25 ± 0.57^{b}	83.00 ± 5.57^{c}	98.67 ± 1.15^{b}	92.00 ± 13.86^{b}	9.97 ± 1.29^{a}	9.35 ± 1.60^{ab}	10.95 ± 2.13^{a}
1	8.01 ± 0.36^{b}	91.00 ± 11.27^{c}	98.33 ± 2.89^{b}	100.00 ± 0.00^{b}	9.39 ± 3.14^{a}	6.73 ± 1.33^{a}	9.79 ± 1.38^{a}
2	8.01 ± 0.62^{b}	77.67 ± 16.74^{c}	99.33 ± 1.15^{b}	99.00 ± 1.73^{b}	10.26 ± 1.16^{a}	6.90 ± 1.62^{a}	10.57 ± 1.58^{a}
6	5.78 ± 0.88^{a}	68.67 ± 1.53^{bc}	95.00 ± 5.00^{b}	94.33 ± 4.04^{b}	9.93 ± 0.61^{a}	8.28 ± 0.47^{a}	11.70 ± 1.40^{a}
9	5.56 ± 0.70^{a}	44.00 ± 2.00^{ab}	55.33 ± 3.06^{a}	65.33 ± 3.06^{a}	10.53 ± 0.26^{a}	12.24 ± 1.94^{b}	12.77 ± 0.61^{a}
10	4.09 ± 0.69^{a}	26.33 ± 15.37^{a}	44.00 ± 23.07^{a}	65.67 ± 10.69^{a}	7.68 ± 1.98^{a}	12.97 ± 0.65^{b}	11.96 ± 1.74^{a}

Values represented as mean ± SD; for each column, different lowercase letters indicate significantly different at $P < 0.05$, as measured by 2-sided Tuckey's HSD between seed age.

Table 6.2: Two-way ANOVA for pre-treatment, age of seed and their interactions on germination % and mean germination time

	Germination Percentage					Mean Germination Time				
Independent Variable	Sum of Squares	df	Mean Square	F	$p \leq 0.05$	Sum of Squares	df	Mean Square	F	$p \leq 0.05$
Treatment	0.441	2	0.220	24.901	0.000	38.010	2	19.005	7.978	0.001
Age of seed	2.138	5	0.428	48.305	0.000	58.779	5	11.756	4.935	0.002
Treatments x Age of seed	0.139	10	0.014	1.570	0.156	80.357	10	8.036	3.373	0.003

6.3.3 Seed Vigour Index (SVI)

Vigour index of untreated seed did not show any significant decline with respect to age of seed. However, loss of vigour was significantly high in KNO_3-pretreated seeds with storage period (Table 6.3). Two-way ANOVA for pre-soaking treatments, age of seed and their interaction showed that age of seed had significant influence on seed vigour index (Table 6.4). However, pre-soaking treatment and the combined effect of pre-soaking treatment and age of seed is not significant.

6.3.4 Germination Synchrony (GS)

Untreated seed did not show any significant difference in terms of synchronization index with respect to age of seed (Table 6.3). However, significant difference in synchronization index was observed for 10 years old seeds when pre-soaked treatment was given with water as well as KNO_3. Two-way ANOVA for pre-soaking treatments, age of seed and their interaction showed that pre-soaked treatment and the combined interaction of age of seed and pre-soaking treatment had significant influence on synchronization index (Table 6.5).

Table 6.6 displayed the correlation among seed age, moisture content, germination, vigour index and synchronization index. Correlation studies showed that with advancement of age of SBT seed, the moisture content, germination percentage and seed vigour index decrease. It takes more time for seed to germination with ageing. Similarly, decrease in moisture content results in decrease germination percentage and seed vigour index.

Table 6.3: Effect of seed age and pre-soaking treatments on germination index, vigour index and synchronization index

Age of seed (Year)	Germination index (seed day^{-1})			Seed vigour index			Synchronization index		
	Treatment		Control	Treatment		Control	Treatment		Control
	KNO_3	Water		KNO_3	Water		KNO_3	Water	
0	2.37 ± 0.16^{c}	2.82 ± 0.03^{b}	2.63 ± 0.40^{b}	2.12 ± 0.47^{bc}	2.44 ± 0.43^{b}	2.15 ± 0.19^{a}	0.190 ± 0.009^{a}	0.207 ± 0.003^{a}	0.248 ± 0.085^{a}
1	2.60 ± 0.32^{c}	2.81 ± 0.09^{b}	2.86 ± 0.00^{b}	2.55 ± 0.62^{c}	2.63 ± 0.43^{b}	2.30 ± 0.58^{a}	0.154 ± 0.015^{a}	0.221 ± 0.019^{ab}	0.256 ± 0.030^{a}
2	2.22 ± 0.48^{c}	2.90 ± 0.12^{b}	2.83 ± 0.05^{b}	2.08 ± 0.16^{bc}	2.15 ± 0.22^{ab}	2.10 ± 0.58^{a}	0.174 ± 0.023^{a}	0.220 ± 0.012^{ab}	0.191 ± 0.021^{a}
6	1.96 ± 0.05^{bc}	2.95 ± 0.15^{b}	2.88 ± 0.08^{b}	1.92 ± 0.30^{bc}	2.23 ± 0.73^{ab}	1.31 ± 0.89^{a}	0.186 ± 0.006^{a}	0.232 ± 0.000^{ab}	0.201 ± 0.008^{a}
9	1.24 ± 0.06^{ab}	1.74 ± 0.08^{a}	2.01 ± 0.09^{a}	1.13 ± 0.37^{ab}	1.53 ± 0.20^{ab}	1.02 ± 0.63^{a}	0.184 ± 0.009^{a}	0.209 ± 0.011^{ab}	0.244 ± 0.030^{a}
10	0.75 ± 0.44^{a}	1.26 ± 0.66^{a}	1.88 ± 0.31^{a}	0.56 ± 0.37^{a}	1.07 ± 0.46^{a}	1.41 ± 0.36^{a}	0.267 ± 0.037^{b}	0.257 ± 0.036^{b}	0.213 ± 0.011^{a}

Values represented as mean ± SD; for each column, different lowercase letters indicate significantly different at $P < 0.05$, as measured by 2-sided Tuckey's HSD between seed age.

Table 6.4: Two-way ANOVA for pre-treatment, age of seed and their interactions on germination index and seed vigour index

	Germination Index					Seed Vigour Index				
Independent Variable	Sum of Squares	df	Mean Square	F	$p \leq 0.05$	Sum of Squares	Df	Mean Square	F	$p \leq 0.05$
Treatment	4.521	2	2.260	30.932	0.000	990047.683	2	495023.841	2.385	0.106
Age of seed	17.244	5	3.449	47.194	0.000	1.546E+07	5	3.093E+06	14.902	0.000
Treatments x Age of seed	1.398	10	0.140	1.913	0.076	2.205E+06	10	220527.834	1.063	0.415

Table 6. 5: Two-way ANOVA for pre-treatment, age of seed and their interactions on synchronization index

	Synchronization Index				
Independent Variable	Sum of Squares	df	Mean Square	F	$p \leq 0.05$
Treatment	0.013	2	0.006	8.244	0.001
Age of seed	0.013	5	0.003	3.374	0.013
Treatments x Age of seed	0.026	10	0.003	3.341	0.004

Table 6.6: Pearson's correlation for seed age, moisture content, germination performance, vigour index and synchronization index

	Age	Moisture %	G%	MGT	GI	SVI	SI
Age	1	-0.970**	-0.870*	0.857*	-0.800	-0.912*	-0.247
Moisture %		1	0.817*	-0.791	0.757	0.853*	0.272
G %[1]			1	-0.847*	0.983**	0.765	-0.099
MGT[2]				1	-0.749	-0.950**	-0.135
GI[3]					1	0.639	-0.131
SVI[4]						1	0.209
SI[5]							1

*Correlation is significant at 0.05 level (2-tailed), **Correlation is significant at 0.01 level (2-tailed)

6.4 Discussion

6.4.1 Germination percentage

In contrast it has been reported that dry seeds can be keep satisfactorily for 1 to 2 years at room temperature [Slabaugh 1974] and 60% viability has been reported for seeds stored for 4 to 5 years [Smirnova and Tichomirova 1980]. Higher seed germination of aged seeds in our study could be due to lower temperature and relative humidity in the storage condition. Seed storage stability and the kinetics of seed viability loss are largely dependent upon seed water content and storage temperature [Sun 1997]. High temperature during storage enhances seed deterioration as does high seed moisture content. A drop of 5°C in storage temperature doubles seed longevity [Harrington 1972; Gómez-Campo, 1985]. Relative effects of seed moisture content and temperature on longevity differ with species, and the structural and biochemical composition of seeds. A complete pattern of loss in viability could be understood on the basis of seed moisture and storage temperature [Ellis et al., 1982]. In the present study, moisture content of seed stored at ambient condition ranges from 4.09- 8.25% and the outside mean maximum and minimum temperature in the experimental locality was 18.9±9.5°C and -5.8±9.8°C, respectively. The phenomenon may have ecological importance since SBT is a pioneer plant species and it is selectively advantageous to maintain high germination rate stretched over a period of time to offset unfavourable conditions for germination in adverse condition prevailing in cold arid environment.

KNO_3 is a growth regulating and germination stimulating substance that can either stimulate or inhibit seed germination depending on the plant species. Adverse effect of KNO_3 has also been observed in *Terminalia sericea* [Amri 2010]. However, significant reduction in seed germination was observed in 9 and 10 years old seed when treated with water. The aged seed have lower moisture content (4.09-5.56%) and significant reduction in germination could be due to susceptible

to imbibition injury during soaking. It is established that when very dry seed are tested for germination, the rapid uptake of water which ensues on contact with water can lead to imbibition injury and decreased germination [Ellis & Roberts 1982].

6.4 Conclusion

On account of our experimental observations, it could be concluded that short and medium term storage of SBT seed could be achieved at ambient condition in cold arid region. This will significantly reduce the cost of storage and will be of practical application. Long term storage in seed bank with controlled temperature and moisture is not always possible especially in developing countries due to high cost of building and operations. Besides, seed banks are public institutions whose management is influenced by political decisions, personnel shortage or changes and economic limitations [Pita et al., 1998]. Seeds developed by research institutions and seed companies in tropical and sub-tropical conditions can be stored in bulk for short and medium term in cold arid region for practical application at a lower cost without the limitation of space.

6.6 Tricotyledony in *Hippophae rhamnoides* L.

The phenomenon of typically dicotyledonous plants producing three cotyledons has been referred to as tricotyledony or tricotyly. More generally, the production of an abnormal number of cotyledons has been referred to as pleiocotyly. Molecular studies have shown that a few mutated genes could produce the tricotyledonous traits in the model plant *Arabidopsis* [Conway & Poething, 1997]. Tricotyledonous seedlings occur sporadically in nurseries of dicotyledonous plant species in over 15 families of plants [Vernon et al, 2001]. However, the phenomenon has not been reported in SBT (Elaeagnaceae).

During our study in 2009 to check seed viability of a 10 year old SBT seed stock, we observed few seedlings with three cotyledon leaves. To check the frequency of tricotyledony in SBT, seed from 30 different plants maintained in field gene bank (latitude 34°08.2′N, longitude 77°34.3′E, altitude 3340 m) at Defence Institute of High Altitude Research were collected in 2010. Seedlings were raised in pots and emergence of cotyledon number was checked on each plant approximately every 3 days. Plants were scored in three categories: two full cotyledons, three full cotyledon and greater than three cotyledons. Tricotyledonous seedlings were transplanted into greenhouse.

The observed tricotyledon frequencies among the 2798 germinated seedlings from 30 plants ranged from 0 to 6.4 %, with an average of 0.64%. A rare single tetracotyledon seedling was also observed. Low frequency of tricotyledony has also been reported in *Brassica oleracea* var. *capitata* (0.6%) [Gupta & Jain, 1980], *Crotolaria juncia* (1.05%) [Purkayastha, 1940], *Raphanus raphanistrum* (0.53%) [Conner and Agrawal, 2005].

None of the cotyledonary leaves showed any sign of external distortion or splitting and were arranged symmetrically in a whorl. Seedlings bearing three cotyledons also bear three true leaves at each internode of the first few internodes (Fig 6.1). Plants with three cotyledons is potentially useful for faster establishment

of seedlings after planting because of the larger leaf area in the early growing stages and may serve as a morphological characteristics for distinguishing cultivars [Hu et al, 2006].

Figure 6.1: SBT seedling with three cotyledons and three true leaves.

Conclusion

Role of geographical barrier in shaping population genetic structure of *Hippophae rhamnoides* L. in trans-Himalaya

Genetic and morphometric diversity of *H. rhamnoides* L. was high and a significant isolation-by-distance pattern was found in the Trans-Himalaya. Significantly high gene flows with low genetic differentiation were observed in studied populations. Parallel pattern of morphometric and genetic diversity was found. Geographical barrier play a major role to shape genetic and morphometric structure of population. We found that Ladakh mountain range (6500 m amsl) act as geographical barrier in the studied site, which separated the two major gene pools. With altitudinal gradient, significant effect on fruit and seed length was observed. Decrease in leaf density and increase in thorn intensity was observed with increasing altitude. Conservation and breeding strategies involving genetic studies are limited for Trans-Himalayan plants populations, and exploration of genetic diversity of this region is required. Intra-specific genetic diversity has become a fundamental parameter for the management of species with aim to maintain their evolutionary potential. Efficient resource management requires the identification of best focused conservation efforts. A representative sample of the natural populations of SBT could be then used to develop an in situ and ex situ conservation strategy for the species.

Genotypic effect on antioxidant activity, total polyphenolic, carotenoids and ascorbic acid content in natural population of Seabuckthorn

The influence of plant genetic background on TPC, TAC, ascorbic acid and carotenoid content in SBT fruit pulp was demonstrated. Significant variation was found within and between the 17 natural populations, which underline the role of genetic background for determining the health promoting compounds in SBT. Many fold variation in TPC (1-11.1), ferric reducing activity (1-7.5), free radical scavenging activity (1-13.7), ascorbic acid content (1-70.3) and carotenoid content

(1-206.3) was observed within the 187 studied female plants. Population has significant effect on the studied health promoting compounds. Plant height and fruit colour has significant positive correlation with TPC and TAC suggesting interrelationship between morphological and biochemical traits. Results obtained in this study can be considered for selection of genotype for breeding purpose to improve health promoting compounds in the berry.

Phenolic content and antioxidant capacity of various solvent extracts from Seabuckthorn fruit pulp, seed, leaf and stem bark

SBT extracts from pulp, seeds, leaves and stem bark contain high levels of TPC and antioxidants. Among the parts examined, seeds and stem bark contains significantly higher antioxidant capacity that may be accounted for by the high phenolic content. The present investigation represents to our knowledge the first report of SBT stem bark as a rich source of natural antioxidant. A significant correlation was found between the antioxidant activity and phenolic content, indicating that phenolic compounds could be major contributor to antioxidant activity. The extracting solvent significantly affected the TPC and antioxidant activity of SBT extract. Seventy percent acetone and 50% acidic methanol followed by 70% acetone showed impressive phenolic content and antioxidant capacity as compare to use of water, methanol and 50% acidic methanol as extracting solvent. Collectively, our data suggest that, when a comparison is made among the data for phenolic content and antioxidant activity of plant parts in the literature, the choice of extracting solvent should be known and considered. Isolation of bioactive components in the extracts would ascertain the individual potency of the compounds which could be further exploited in food and pharmaceutical industries.

Identification and validation of sex-linked SCAR markers in *Hippophae rhamnoides* L.

We for the first time report two robust sex-linked SCAR markers in SBT. Our newly developed sex-linked markers namely *HrX1* and *HrX2* have been successfully validated in larger sets of female (120) and male (100) genotyped and are proved to be perfectly linked to female sex in *H. rhamnoides* would revolutionized its future cultivations. Despite their great value in field studies including identification of female genotypes, these markers could also assist the future chromosome mapping of X-linked locus in *H. rhamnoides*.

Germination of seabuckthorn seed after 10 years of storage at ambient condition in Ladakh region

On account of our experimental observations, it could be concluded that short and medium term storage of SBT seed could be achieved at ambient condition in cold arid region. This will significantly reduce the cost of storage and will be of practical application. Long term storage in seed bank with controlled temperature and moisture is not always possible especially in developing countries due to high cost of building and operations. Besides, seed banks are public institutions whose management is influenced by political decisions, personnel shortage or changes and economic limitations. Seeds developed by research institutions and seed companies in tropical and sub-tropical conditions can be stored in bulk for short and medium term in cold arid region for practical application at a lower cost without the limitation of space.

Tricotyledony in *Hippophae rhamnoides* L.

Plants with three cotyledons has been reported for the first time in SBT, which is potentially useful for faster establishment of seedlings after planting

because of the larger leaf area in the early growing stages and may serve as a morphological characteristics for distinguishing cultivars.

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List of Publications

Publication in Peer-reviewed Journal

1. **Girish Korekar**, Ram Kumar Sharma, Rahul Kumar, Meenu, Naveen C. Bisht, Ravi B. Srivastava, Paramvir Singh Ahuja, Tsering Stobdan. (2012) Identification and validation of sex-linked SCAR markers in dioecious *Hippophae rhamnoides* L. (Elaeagnaceae). Biotechnology Letters 34(5): 973-978. (Impact Factor: 1.768)

2. **Girish Korekar**, Tsering Stobdan, Om P Chaurasia and Shashi B Singh. (2011) Phenolic content and antioxidant capacity of various solvent extracts from seabuckthorn (*Hippophae rhamnoides* L.) fruit pulp, seeds, leaves and stem bark. Acta Alimentaria 40(4): 449-458 (Impact factor: 0.379)

3. **Girish Korekar**, Harvinder Singh, Ravi B. Srivastava, Tsering Stobdan. Tricotyledony in *Hippophae rhamnoides* L. (Elaeagnaceae). (2012) Current Science 25(2): 159-160 (Impact factor: 0.897)

4. **Girish Korekar**, Harvinder Singh, Sanjai K Dwivedi, Ravi B. Srivastava, Tsering Stobdan. (2013) Germination of *Hippophae rhamnoides* L. seed after 10 years of storage at ambient condition in cold arid trans-Himalayan Ladakh region. Current Science (In Press)(Impact factor: 0.897)

5. **Girish Korekar**, Tsering Stobdan, Richa Arora, Ashish Yadav, Shashi Bala Singh (2011). Antioxidant capacity and phenolic content of apricot (*Prunus armeniaca* L.) kernel as a function of genotype. Plant Food for Human Nutrition 66: 376-383 (Impact factor: 2.463)

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