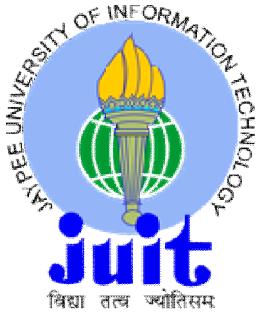
ADAPTATION, POPULATION GENETICS AND FRUIT QUALITY CHARACTERISTICS OF *MORUS ALBA* L. FROM TRANS-HIMALAYAN LADAKH REGION, INDIA



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JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT

By

PRABODH KUMAR BAJPAI

2014

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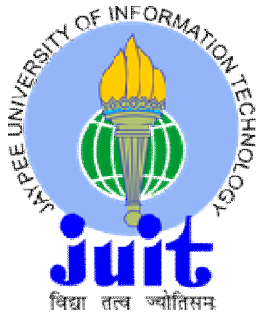
PRABODH KUMAR BAJPAI

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY



JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY
WAKNAGHAT

MAY, 2014

CERTIFICATE

This is to certify that the thesis entitled, **"Adaptation, population genetics and fruit quality characteristics *of Morus alba* L. from trans-Himalayan Ladakh region, India"** which is being submitted by **Pabodh Kumar Bajpai** 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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DECLARATION

I certify that

a. The work contained in this thesis is original and has been done by me under the guidance of my supervisor.
b. The work has not been submitted to any other organization for any degree or diploma.
c. Whenever, I have used materials (data, analysis, figures or text), I have given due credit by citing them in the text of the thesis.

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(Prabodh Kumar Bajpai)

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LIST OF ABBREVIATIONS

Abbreviations	Full form	Abbreviations	Full form
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)	DF	Dilution factor
AFLP	Amplified fragment length polymorphism	df	Degrees of freedom
AMOVA	Analysis of molecular variance	DIC	Deviance information criterion
ANCOVA	Analysis of covariance	DNA	Deoxyribose nucleic acid
ANOVA	Analysis of variance	dNTP's	di-nucleotide triphosphates
asl	Above sea level	DPPH	2,2-Diphenyl-2-picrylhydrazyl
b	Slope of the log linear distance between regression and kinship	DW	Dry weight
BHA	Butylated hydroxyanisole	EDTA	Ethylenediamine tetraacetic acid
BHT	Butylated hydroxytoluene	ESI-MS	Electron spray ionization-mass spectrometry
CE	Catechin equivalent	ε	Extinction coefficient
CIMMYT	International Maize and Wheat Improvement Center	*f*	Analogous to Wright's inbreeding coefficient (F_{IS})
CTAB	Cetyl tri-methyl ammonium bromide	FAO	Food and Agriculture Organization
CV	Coefficient of variation	FDR	False discovery rate
C3GE	Cyanidin-3-glucoside equivalent	$f_{ij(1)}$	Average kinship in the first distance interval
DAMD	Directed amplification of minisatellite-region DNA	FRAP	Ferric reducing antioxidant power
Dbar	Measure of model fit	F_{ST}	Wright's genetic differentiation coefficient

Abbreviations	Full form	Abbreviations	Full form
GAE	Gallic acid equivalent	MCMC	Monte carlo markov chain
G_{ST}	Nei's genetic differentiation coefficient	MAS	Marker assisted selection
GC-MS	Gas chromatography-mass spectroscopy	MW	Molecular weight
GPS	Global positioning system	Nm	Gene flow
H	Nei's genetic diversity	NO	Nitric oxide
HPLC	High performance liquid chromatography	NPL	Number of polymorphic loci
Hs	Average gene diversity of sub-populations	PBS	Phosphate buffer saline
HSD	Honestly significant difference	PCA	Principal component analysis
Ht	Gene diversity of total population	pD	Measure of model complexity
I	Shannon's information index	PPI	Phenotypic plasticity index
IC_{50}	Inhibitory concentration needed to scavenge 50% radical	PPL	Percentage polymorphic loci
ICP-OES	Inductively coupled plasma-optical emission spectrometry	PHYLIP	Phylogeny inference package
IBD	Isolation by distance	QE	Quercetin equivalent
ISSR	Inter simple sequence repeat	QTL	Quantitative trait loci
K	Number of clusters	RAPD	Random amplified polymorphic DNA
LnP(D)	Most likelihood	RH	Relative humidity
LPI	Lipid peroxide inhibition	rpm	Rotations per minute
		SCAR	Sequence characterized amplified region
LDL	Low density lipoprotein	SD	Standard deviation

Abbreviations	Full form	Abbreviations	Full form
SGS	Spatial genetic structure	TPAC	Total proanthocyanidin content
Sp	Descriptive measure for SGS intensity	TPC	Total polyphenol content
SPAR	Single primer amplification reaction	TPTZ	2,4,6-tripyridyl-*s*-triazine
SPSS	Statistical package for social sciences	TSS	Total soluble solids
SRAP	Sequence related amplified polymorphism	UPGMA	Unweighted pair group method with averages
SSR	Simple sequence repeat	USDA	United States Department of Agriculture
TAC	Total anthocyanin content	UTM	Universal transverse mercator
TBARS	Thiobarbituric acid reactive species	Φ_{PT}	Genetic differentiation coefficient equivalent of binary data
TFC	Total flavonoid content	θ^{B}	Analogous to Wright's F_{ST}
TP	α-tocopherol		

ABSTRACT

Ten quantitative morphological characters were studied in 56 *Morus alba* L. trees representing three natural populations from the trans-Himalayan Ladakh region. The altitude of collection sites ranged from 2815 to 3177 m asl. Coefficient of variation (CV) showed high phenotypic variation in *M. alba* populations from the trans-Himalaya. Phenotypic plasticity measured using phenotypic plasticity index revealed significant contribution of phenotypic plasticity towards phenotypic variation. Linear regression analysis revealed that size of leaf and fruit characters decreases with increase in altitude. High CV was observed for leaf length (26.65%), leaf width (26.21%), petiole length (32.21%), leaf area (50.34%), inter-nodal distance (38.11%), number of nodes (46.01%), bud length (23.58%), fruit length (13.14%), fruit width (21.52%) and fruit weight (44.14%). Similarly, high phenotypic plasticity was observed for bud length (0.32), leaf length (0.5), leaf width (0.5), petiole length (0.61), leaf area (0.73), and inter-nodal distance (0.51), number of nodes (0.6), fruit length (0.25), fruit width (0.31) and fruit weight (0.66). For every 100 m increase in altitude, leaf length, leaf width and leaf area decreased by 1 cm, 0.8 cm and 16.6 cm^2, respectively. Analysis of covariance showed a predominant altitudinal effect on the morphological characters in comparison to the population effect. A small change in altitude caused significant change in plant morphological characters. The present investigation represents to our knowledge the first study addressing phenotypic variation in mulberry along altitudinal gradient.

Sequence-related amplified polymorphism (SRAP) markers were used to assess the genetic structure in three natural populations of *Morus alba* from trans-Himalaya. Multi-location sampling was conducted across 14 collection sites. The overall genetic diversity estimates: percentage polymorphic loci (89.66%, Nei's gene diversity (0.2286) and Shannon's information index (0.2175) were high. At regional level, partitioning of variability assessed using analysis of molecular variance (AMOVA), revealed 80% variation within and 20% among collection sites. Pattern appeared in STRUCTURE, BARRIER and AMOVA results clearly demonstrated that there is gene flow between Indus and Suru population while there is geographical barrier between Indus-Suru and Nubra population which effectively hinders gene flow. The results showed significant genetic differentiation, population structure, high to restricted gene flow and high genetic diversity. Assumption that samples

collected from the three valleys represent three different populations does not hold true. The fragmentation present in trans-Himalaya was more natural and less anthropogenic.

Genome scan was done with 439 dominant marker loci to identify outlier loci in three populations of *Morus alba* from trans-Himalayan region. Employing two population genetics model viz. finite and hierarchical island model, we detected 30 (6.85%) loci under selection of which 3.64% was under directional selection and 3.19% under balancing selection. Hierarchical island model was efficient in detecting signatures of natural selection with low occurrence of false positives. Data showed significant spatial genetic structure (SGS), especially in population from Nubra valley with significantly high SGS. Bayesian approach revealed high genetic differentiation and inbreeding in Nubra population. Indus and Suru valleys populations showed comparatively low SGS, genetic differentiation and inbreeding. The study showed that *M. alba* falls in the range of mixed mating and outcrossing species. However, population from Nubra valley showed selfing with high SGS. Significant SGS strengthens the presence of natural selection in *M. alba* natural populations.

Mulberry is listed in modern Chinese Materia Medica for its medicinal uses. Our aim was to evaluate the antioxidant capacities and bioactive compounds of methanolic extract of *Morus alba* fruit from the trans-Himalaya. Thirty seven genotypes of *M. alba* with white, pink and black fruit were studied to find antioxidant capacities, total phenolic content (TPC), total flavonoid content (TFC), total proanthocyanidin content (TPAC) and total anthocyanin content (TAC) in fruit. We have identified and quantified the phyto-chemotypes present in the methanol extracts by gas chromatography/mass spectrometry (GC/MS) technique. We have also estimated mineral content of *M. alba* fruit by inductively coupled plasma/optical emission spectrometry (ICP/OES). The ferric reducing antioxidant potential (FRAP) ranged from 34.2 to 46.2 µM Fe(II)/g DW. A 1-25 fold variation in IC_{50} value was observed for DPPH radical scavenging capacity of extracts. However, the fold variation in IC_{50} by ABTS radical cation scavenging, lipid peroxide inhibition (LPI) and nitric oxide (NO) radical scavenging capacity of extracts was lower i.e 1–11.5, 1–5.6 and 1–3.4, respectively. The fruits were found to be rich in TPC ranging from 1.8 to 8.3 mg gallic acid equivalent (GAE)/g DW. The TFC and TPAC ranged from 0.4 to 0.9 mg quercetin equivalent/g DW and 0 to 0.02 mg catechin equivalent/g DW, respectively. Two way analysis of variance (ANOVA) revealed that fruit color has no significant relationship with TPC, TAC and TPAC. However, fruit color showed inconsistent effect on antioxidant capacities as determined by different methods. Sampling location has significant effect on antioxidant capacities, TPC,

TFC and TAC. Interaction between fruit color and sampling location showed significant effect on antioxidant capacities and TAC. The study therefore showed that source of genotypes from different sampling location is more important as compare to fruit color in determining antioxidant capacities, TPC and TAC in *M. alba* fruit from the trans-Himalaya. GC/MS analysis revealed the presence of healthy ω-6 fatty-acids. It appeared from the results that mulberry is a good source of macro and micro nutrients.

CHAPTER 1

INTRODUCTION

Mulberry (*Morus spp.*; Moraceae) is a fast-growing deciduous woody perennial, wind pollinated and outbreeding tree. Mulberry has tremendous economic importance in developing countries in Asia, where sericulture is an important industry. The silkworm, *Bombyx mori* L., exclusively feeds on the leaf of mulberry, and hence, sustainability of sericulture heavily depends on the productivity of high-quality mulberry leaf. Mulberry is also used as fodder because of its highly nutritious, palatable and digestible [Sanchez, 2000 and 2000a] leaves. Fruits of mulberry are used for human consumption either as fresh fruit or in the form of various confectionary products such as jam, marmalade, frozen desserts, pulp, juice, paste, ice cream and wine [Soufleros et al., 2004; Yua et al., 2008]. In spite of high commercial importance, very little effort has been made to harness the benefits of the recent technological advancements in plant genomics for mulberry genetic improvement.

1.1 Origin and distribution

The domestication of mulberry must have started several thousands of years ago as a requirement for silkworm rearing [FAO, 1990]. Considering that the silk trade has been going on for a long time throughout the Old World, and that this plant is also cultivated for its fruit and for landscaping, mulberry germplasm has been taken to a lot of countries, and it now has a very wide distribution range in Asia-Europe (from Korea to Spain, including China, India, Central Asia and Near East); in Africa (North and East Africa) and in America (from the U.S.A. to Argentina, including Mexico, Central America, Colombia and Brazil). The origins of most cultivated mulberry varieties are believed to be in the area of China-Japan and in the Himalaya foothills, and later it spread to other parts of world [Sanchez, 2000a]. Today, it is present in all regions between 50° N latitude and 10° S latitude [Yokoyama, 1962] from sea level to altitudes as high as 4,000 m asl [Machii et al., 1999; Tutin, 1996].

1.2 Taxonomy and botany

Taxonomy of *Morus* genus has been unstable, with great variation in the numbers of species recognized by different workers. The taxonomic difficulties in *Morus* genus may be due in part to its very wide distribution, overlapping ranges of distribution of some taxa, morphological plasticity and hybridization. For example, Linnaeus [1753] established the genus with seven species, Bureau [1873] recognized only five species, and Koidzumi [1917], who

provided the most recent genus-wide treatment, recognized 24 species. According to Koidzumi [1917], the genus *Morus* is divided into two sections, the Dolichostylae (long style) and the Macromorus (short style), and each section is again divided into two groups namely: Papillosae and Pubescentae, based on the nature of stigmatic hairs. Currently, however, more than 68 species are widely recognized [Datta, 2000]. Out of which, only a few species such as *M. alba*, *M. indica*, *M. bombycis*, *M. latifolia* and *M. multicaulis* are cultivated for leaves to feed the silkworm while *M. nigra*, is cultivated for its fruits. Among different species *M. alba* is the most important species from economic importance point of view. Classification of *M. alba* is given below.

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Rosales

Family: Moraceae

Genus: *Morus*

Species: *alba* L.

M. alba belongs to moraceae family, commonly known as white mulberry. *Morus* is old classic latin name of black mulberry; *alba* refers to the white fruits. *M. alba* grows as a dense, round-topped, perennial small to medium sized shrub or tree, reaching heights around 50 feet. The stem is slender, yellowish green to brownish gray, smooth, more or less shining, slightly sweetish, if chewed. The bark is light brown, thin, shallowly furrowed and has long, narrow ridges. The twigs are thin, light brown and glabrous. The sap is milky. The leaf is alternate, simple, stipulate and with degree of lobing, sometimes unlobed but often 3 or 5 lobed, coarsely serrate or dentate, ovate to broad ovate, 6-15 cm long and 50-10 cm wide, acute or short acuminate, rounded or cordate at base, long petioled, 12×8 cm on fruiting branch, up to 25×20 cm on vigorous non-fruiting branches. The bud are imbricate, terminal-absent, laterals-small 0.3-0.7 cm long, ovoid, 3-6 scales, appressed, sharp or blunt pointed, light brown to reddish

brown, often set oblique to leaf scar, margins of bud scales somewhat finally hairy. Inflorescence is catkin. The flowers are small, greenish yellow, in dense spikes to 2 cm long, sepals 4, stamens 4, staminate and pistillate on different tree. Pistils with two styles; staminate spike soon become decidous; pistillate spikes maturing into an aggregate fruit (syncarp) of drupelets. The fruits are small, 1-seeded, numerous, ovoid to oblong cylindric, crowd into cluster 10-20 mm long, white or purplish to nearly black, edible long before ripe, sweet but insipid. The ovoid nutlet has a thin, soft shell and the seed has a "hard bony coat". The fruit is botanically called sorosis, it is juicy and has a sweet taste with some sourness that is more prominent in the less mature fruits. Tree, inflorescence and fruiting shown in Figure 1.1.

Figure 1.1 *M. alba* tree: a – a wild tree, b – male inflorescence, c – female inflorescence and d – ripe fruits

1.3 Other important species

According to Nepal [2008] twelve important species of mulberry are

1. *Morus australis* Poiret.
2. *Morus cathayana* Hemsley
3. *Morus celtidifolia* Kunth
4. *Morus insignis* Bureau
5. *Morus macroura* Miquel
6. *Morus mesozygia* Stapf ex A. Chev.
7. *Morus microphylla* Buckley
8. *Morus mongolica* (Bureau) C. K. Schneider
9. *Morus nigra* L.

10. *Morus notabilis* C. K. Schneider
11. *Morus rubra* L.
12. *Morus serrata* Roxburgh

1.4 Mulberry in India

Mulberry is grown in India mainly for sericulture purposes, it is found wild in Himalayas. There are mainly four species of *mulberry* commonly found in India *viz. M. alba*, *M. indica*, *M. serrata* and *M. laevigata* (Table 1.1).

Table 1.1 Characteristic features of *Morus* species found in India

***Morus* species**	**Characteristic features**
M. indica	Teeth of leaves unequal, the lateral nerves running straight into the teeth or forked within the margin; leaves usually medium to long; male spike less than 2.5 cm long; style is long, hairy; fruit ovoid or cylindrical; unripe fruit colour red, becomes black when ripe. Bud colour dark brown, elongated, triangular in shape.
M. alba	Teeth of leaves uniform, usually blunt; segments of the perianth of female flowers in four numbers, the two outer keeled; style very short; fruit colour white, pink and black. Bud colour brown, oval round in shape.
M. laevigata	Teeth of leaves fine; the lateral nerves abruptly curved upwards within the margin; style is very short. Fruit is long greenish, white, dark purple. Bud colour brown, elongated, lengthy and elliptical in shape.
M. serrata	Teeth of leaves usually coarse, somewhat unequal and sharp; leaves velvety, coarse, full of minute hair on both the side; segments of the perianth of female flowers 2-4, usually 3, all similar, style is medium; fruit colour white, pink and mucilage fruit. Bud colour dark brown, round and bigger in size.

1.4.1 *Area under mulberry cultivation in India*:

The mulberry was first grown in the foothills of the Himalayas, but now, almost all Indian states cultivate mulberry. The primary grower of the mulberry is Karnataka, distantly come Andhra Pradesh and West Bengal, respectively. The total acreage of mulberry in India is around 282,244 ha. The details of area under mulberry cultivation in different states in India are shown in the Table 1.2 and Figure 1.2.

Table 1.2 Area under mulberry cultivation in different states [Datta, 2000]

Sr. No.	State	Area (ha)	Sr. No.	State	Area (ha)
1	Andhra Pradesh	38084	7	Manipur	25975
2	Assam	2813	8	Tamil Nadu	9491
3	Jammu & Kashmir	4717	9	Uttar Pradesh	5665
4	Karnataka	166000	10	West Bengal	21358
5	Kerala	1164	11	Other	4934
6	Madhya Pradesh	2043		Total	282244

Figure 1.2 Area under mulberry cultivation (ha) in India [Datta, 2000]

1.4.2 ***Mulberry production in India***

Unfortunately for fruit lovers, farmers care more about the taste buds of silk worms than humans when it comes to growing this crop. Because India is the country known for its beautiful, flowing saris, most of mulberry production is dedicated to harvesting leaves for silkworm food and later, silk extraction. So mulberry production in India can be assessed by raw mulberry silk production (Table 1.3). But there is no data available on mulberry fruit production.

Table 1.3 Mulberry and raw silk production in India (Central Silk Board, 2013)

Particulars	2007-08	2008-09	2009-10	2010-11	2011-12	2012-13$_{(P)}$
Mulberry plantation area (Lakh hactares)	1.85	1.78	1.84	1.7	1.81	1.92
Raw silk production (Metric tonnes)						
Bivoltine	1175	1250	1200	1400	1685	1984
Crossbreed	15070	14360	15122	14960	16587	16711
Sub total	16245	15610	16322	16360	18272	18755

P=provisional

1.5 Mulberry in Ladakh

M. alba is the dominant species in Ladakh. In the trans-Himalayan Ladakh region of India, *M. alba* is found at 2700-3300 m asl. A long spell of subzero temperatures forces *M. alba* trees to remain in dormant condition from October to February. The dormant buds get sprouted on the onset of spring during March-April along with floral buds. Fruiting occurs in the trans-Himalayan region in July and fruit color varies from white, purple, pink or red. Mulberry is the first fruit crop which comes early in the season in Ladakh region, so in historical time, it is common notion among local people that there must be a mulberry tree in every backyard. So mulberry is very important fruit crop of this region as there are few fruit crop available in this region. Traditionally, local people use mulberry as fresh or in dried form. A dish called “kroyesh” is prepared by mixing mulberry fruit with wheat grains and roasting them together.

1.6 Nutritional value of mulberry

According to the USDA nutrient database, 100g of fresh raw mulberry fruit of *M. nigra* species contains the following values (Table1.4)

Table 1.4 Nutritional compositions of mulberry fruit (*Morus nigra,* fresh raw)

Nutrient	Unit	Value/100 g	Nutrient	Unit	Value/100 g
Proximates			Vitamin A, IU	IU	25
Water	g	87.68	Vitamin E (α-tocopherol)	mg	0.87
Energy	Kcal	43	Vitamin D (D2 + D3)	µg	0
Protein	g	1.44	Vitamin D	IU	0
Total lipid (fat)	g	0.39	Vitamin K (phylloquinone)	µg	7.8
Carbohydrate, by difference	g	9.8	Minerals		
Fiber, total dietary	g	1.7	Calcium, Ca	mg	39
Sugars, total	g	8.1	Iron, Fe	mg	1.85
Vitamins			Magnesium, Mg	mg	18
Vitamin C, total ascorbic acid	Mg	36.4	Phosphorus, P	mg	38
Thiamin	Mg	0.029	Potassium, K	mg	194
Riboflavin	Mg	0.101	Sodium, Na	mg	10
Niacin	Mg	0.62	Zinc, Zn	mg	0.12
Vitamin B-6	Mg	0.05	Lipids		
Folate, DFE	µg	6	Fatty acids, total saturated	g	0.027
Vitamin B-12	µg	0	Fatty acids, total monounsaturated	g	0.041
Vitamin A, RAE	µg	1	Fatty acids, total polyunsaturated	g	0.207
			Cholesterol	mg	

1.7 Medicinal properties of mulberry

According to Hartwell [1967–1971], the fruit juice is used in folk remedies for tumors of the throat. Reported to be antidotal, antiphlogistic, antitussive, antivinous, astringent, bactericide, diaphoretic, ditiretic, emollient, escharotic, expectorant, fungicide, laxative, nervine, purgative, refrigerant, restorative, sedative, tonic, and vermifuge. White mulberry is a folk remedy for aphtha, armache, asthma, bronchitis, bugbite, cachexia, cold, constipation, cough, debility, diarrhea, dropsy, dyspepsia, edema, epilepsy, fever, headache, hyperglycemia, hypertension, inflammation, insomnia, melancholy, menorrhagia, snakebite, sorethroat, stomatitis, tumors, vertigo, and wounds [Duke and Wain, 1981]. Roots and bark are purgative, anthelmintic, and astringent. Leaves are considered disphoretic and emollient; a decoction of leaves being used as a gargle for inflammation of throat [Reed, 1976].

Mulberry fruit is classified in the modern Chinese Materia Medica as a blood tonic, and is listed along with herbs having similar traditional uses. Traditionally, mulberry fruit has been used as a medicinal agent to nourish the blood, benefit the kidneys, and treat weakness, fatigue, anemia, and premature graying of hair. It is also utilized to treat urinary incontinence, tinnitus, dizziness, and constipation in the elderly and the anemic.

Root is one of the constituents of drug named, "Glucosidase" which is used for treatment of high blood pressure. Root juice agglutinates the blood and is useful in killing the worms in digestive system [Shivkumar et al., 1995]. The stem bark is used as purgative and vermifuge [Singh and Ghosh, 1992]. The leaves are diaphoretic and emollient. Leaf juice keeps skin smooth, healthy and prevent throat infections, irritations and inflammations.

1.8 Review of Literature

1.8.1 *Phenotypic variation in genus Morus*

Banerjee et al. [2007] studied fourteen morphometric traits in 25 mulberry genotypes from varied agroclimatic conditions of India to examine the genetic divergence. Wide variation was observed for all the traits. The genotypes irrespective of their place of collection were grouped into 10 different clusters. Seven accessions, that is, Baragura-2, Gorabandha-2, Kalimpong, Herbertpur, Kollegal, Resham majri-7, and UP-14 were reported to be useful genetic resources. Correlation and path analysis suggest the direct selection of lamina length, fresh leaf weight, leaf area, and single leaf weight for mulberry leaf yield improvement.

Tikader and Kamble [2008a] studied performance of exotic mulberry germplasm on growth and yield traits in Indian condition. Exotic mulberry germplasm accessions were evaluated under tropical dry agro-climatic condition of India. Mulberry accessions showed great extent of variation in growth and yield traits. The analysis of variance revealed significant differences in all growth and yield traits. The interaction between accession x season was significant for all traits except number of branches per plant, total shoot length and internodal distance. The coefficient of variation was maximum in leaf yield per plant followed by total shoot length and minimum in leaf moisture content. The relationship among different growth traits indicated that leaf yield is dependent on number of branches per plant and total shoot length. The CIMMYT selection index was used to identify the best accessions studied in the experiment. The divergence analysis using R statistics grouped the accession into 7 clusters of which maximum numbers of accessions were in cluster

IV and minimum in cluster V. Inter-cluster distance was maximum in between cluster V and VII whereas minimum in cluster IV and I. The intra cluster distance was higher in cluster VI and minimum in cluster V. The authors suggested that diversity among the accessions measured by D^2 values may be used for selection of exotic mulberry accessions for crop improvement.

Özgen et al. [2009] studied morphological characters of several *Morus* species from Turkey. He studied *M. alba, M. nigra, M. rubra* and *M. laevigata*. More than 200 promising mulberry accessions were visited and preliminary tree and fruit characteristics were investigated. Based on the first year results, a total of 22 superior accessions from four different species were morphologically characterized in the second year. Considerable variations were observed for most of the traits. For example, fruit weight ranged from 3.3 to 8.2 g with an average of 4.9 g. *M. nigra* accessions had much smaller mean values when compared to those of *M. rubra* and *M. alba*. The juice yield ranged from 56.1 to 67.8% for *M. nigra* accessions, 20.0 to 31.1% for *M. rubra* and 25.6 to 35.0% for *M. alba.* The mean total acidity of *M. nigra* was 2.7%, 3.1-fold higher than those of *M. rubra* and *M. alba* accessions. Soluble solids ranged from 14.1 to 27.1% with an average of 21.8%. These fruit traits were subjected to principle component analysis and the results indicated that *M. nigra* accessions were grouped together, while *M. laevigata* accession was distinct from other genotypes. The accessions of *M. rubra* and *M. alba* separated from *M. nigra* and *M. laevigata* accessions, although the two groups did not separate from each other.

Few other authors also studied phenotypic variation in *Morus* species. Rao et al. [2005] conducted variability analysis for morphological characters of 14 mulberry collections from two districts of Ladakh. The collected germplasm exhibited wide variation in respect to morphological and reproductive characters. The trees were predominantely dioecious female and rarely monoecious in nature. The monoecious trees had all categories of inflorescence *viz.* pure male, pure female and bisexual in the same branches or different branches of the same tree with different degree of cleistogamous condition. Irrespective of the species, the female flowers had short styles with diveritcate and spreading stigma, which indicated the hybrid nature of the population between *M. indica* and *M. alba* species.

Chatterjee [2004] carried out variability analysis for 12 morpho-biochemical characters in *M. laevigata*. Pandit et al. [2006] evaluated 30 mulberry varieties by evaluation index based on

six morphological characters including primary and secondary branch lengths, nodes/meter primary and secondary branch, leaf index and leaf yield per plant. Koyuncu [2004] studied morphological and agronomical characters of native black mulberry (*M. nigra*) in Sutculer, Turkey. He studied the dates of bud burst, appearance of flowers, fruit coloring, beginning and end of harvest for each genotype. But there is no study which addresses about phenotypic variation in *M. alba* along altitudinal gradient.

1.7.2 ***Genetic diversity and genetic structure studies in genus Morus***

Owing to the great economic importance, large numbers of mulberry germplasm accessions have been maintained in several countries. For instance, China, India, Japan, Korea, and Bulgaria have, respectively, more than 1860, 1120, 1375, 615, and 140 germplasm accessions [FAO 2003; Machii et al., 1999; Pan, 2000; Tzenov, 2002; Tikader and Dandin, 2006; Tikader et al., 2009]. A better information on the genetic make-up of germplasm accessions enables better management and utilization of mulberry genetic resources.

Molecular markers have been extensively used to characterize *Morus* species. Bhattacharya and Ranade [2001] performed molecular distinction amongst varieties of mulberry using RAPD and DAMD profiles. Twenty three arbitrary sequence decamer primers for RAPD and three minisatellite core sequence primers for DAMD reactions were used. The triploid varieties were found to be most similar to each other using RAPD analysis, while the varieties S13 and S34 were more similar using DAMD analysis. Nearly 85% of the RAPD bands and 91% of the DAMD bands were polymorphic across the nine varieties. They concluded that the mulberry varieties can be distinguished with as many as five RAPD primers and one DAMD primer that generated profiles that can together differentiate all the nine varieties in terms of unique bands.

Vijayan et al. [2004a] investigated the mulberry genotypes using ISSR and RAPD markers to find out the possibility of using DNA markers to confirm the identity of genotypes in mulberry spp. Fifteen each of ISSR and RAPD primers generated 86% and 78% polymorphism and the polymorphism among the species varied from 50% to 57% in ISSR markers and 31% to 53% in RAPD markers. Based on different parameters and the result of cluster analysis it was concluded that *M. laevigata* can be considered as a separate species of mulberry, whereas the other four species (*M. alba* L., *M. latifolia* Poir, *M. bombycis* Koidz and *M. indica* L) may be grouped together and treated as subspecies.

Vijayan et al. [2004b] studied the genetic analysis of Indian mulberry varieties through molecular markers. Seventeen RAPD and eleven ISSR primers were used to analyze the genetic relationships among these varieties. The RAPD and ISSR primers revealed more than 75% polymorphism among the eleven varieties (Mysore local, Bomaypiasbari, Kanva-2, Bilidevalaya, Kajli, S1, BC259, C776, RFS-175, S-36 and Victory-1). The genetic similarity estimated from RAPD markers varied from 0.645 to 0.887 and of ISSR ranges from 0.6 to 0.873. These markers grouped the varieties into three major groups comprising the low yielding, medium yielding and high yielding.

Aggarwal et al. [2004] firstly reported the isolation and characterization of six novel microsatellite markers for mulberry (*M. indica*). Microsatellite markers were developed from an enriched genomic library of *M. indica*. These markers revealed a high degree of polymorphism (14–26 alleles per locus; polymorphic information content, 0.85–0.9) and a broad cross-species affinity when tested on a set of 43 elite genotypes including 13 related *Morus* species. The data demonstrated the utility of microsatellite markers as potentially efficient genetic markers for germplasm characterization, crop improvement and molecular systematics of mulberry.

Awasthi et al. [2004] employed RAPD and ISSR markers to study the genetic diversity and interrelationships among twelve domesticated and three wild mulberry species. Nineteen RAPD primers generated 128 discrete markers ranging from 500–3000 bp in size. One-hundred-nineteen of these were polymorphic (92%), with an average of 6.26 markers per primer. Among these were a few putative species-specific amplification products which could be useful for germplasm classification and introgression studies. The ISSR analysis employed six anchored primers, 4 of which generated 93 polymorphic markers with an average of 23.25 markers per primer. Two clusters were observed one comprising polyploid wild species and the other with domesticated (mostly diploid) species. They concluded that RAPD and ISSR markers are useful for mulberry genetic diversity analysis and germplasm characterization, and that putative species-specific markers may be obtained which can be converted to SCARs after further studies.

Bhattacharya et al. [2005] compared exotic and indigenous mulberry varieties. Twenty seven mulberry varieties (exotic as well as indigenous) were subjected to diversity analysis using four DAMD, three ISSR and twenty RAPD primers. All three methods revealed wide range of distances supporting a wide range of mulberry genetic diversity. When considered as two groups (10 exotic and 17 indigenous varieties), the mulberry varieties in the exotic group were found to

have slightly greater diversity than the indigenous ones. Their results supported the concept of naturalization of mulberry varieties at locations distant from their origins.

Studies on molecular characterization and identification of markers associated with yield traits in mulberry using ISSR markers was conducted by Vijayan et al. [2006]. They initiated to identify parents suitable for breeding to raise high-yielding varieties for the non-traditional areas of Kerala, India and attempted to identify markers associated with leaf yield attributing traits in 44 mulberry genotypes. Variability on morpho-biometric traits and molecular markers, generated with 12 selected ISSR primers, was estimated. The dendrogram generated with morpho-biometric characters clustered the genotypes into three distinct groups and one isolate, while the same using ISSR markers clustered the genotypes into five groups and six isolates. The greater resolving power of the ISSR markers was evident. Using step-wise multiple regression analysis, a number of markers associated with number of branches, total shoot length, leaf weight, internodal distance, leaf chlorophyll, protein, leaf moisture percentage were identified. These markers could be of much use in marker assisted selection breeding programs in mulberry, especially when no genetic information in terms of linkage maps and quantitative trait loci (QTLs) is available in a plant with high heterozygosity and a long juvenile period.

Kafkas et al. [2008] carried out molecular characterization of mulberry accessions in Turkey by AFLP markers. They attempted to characterize 43 *Morus* accessions originating from distinct regions of Turkey using fluorescent dye AFLP markers and capillary electrophoresis. The accessions belonged to *M. alba, M. nigra* and *M. rubra*. Eight primer combinations generated a total of 416 bands, 337 of which were polymorphic (80.5%). Resolving powers of the AFLP primers ranged from 0.41 to 0.942 making a total of 5.015, whereas the polymorphic information content ranged from 0.662 to 0.898 with an average of 0.812. Unweighted pair-group method of arithmetic mean (UPGMA) clustering of the accessions showed three major groups representing *M. nigra, M. rubra* and *M. alba* accessions. The *M. alba* group had two subgroups that were not correlated with fruit color. The UPGMA dendrogram of average taxonomic differences confirmed these results. The principle coordinate analysis demonstrated that *M. nigra* accessions had limited genetic variation. In conclusion, the study indicated that *M. nigra* and *M. rubra* are molecularly distinct from *M. alba*. The results also suggest that *M. nigra* accessions have a low level of morphological variation and similar at molecular level.

Tikader et al. [2009] reviewed on conservation and management of mulberry germplasm through biomolecular approaches. They concluded that RAPD markers are cost effective than other molecular markers but suffer from poor reproducibility. Other markers like ISSR, SSR and AFLP are found more suitable for the purpose. But till date studies related to assessment of population structure and genetic diversity of natural populations of *M. alba* from the trans-Himalayan region of Ladakh has not been attempted.

1.8.3 ***Natural selection studies in genus Morus***

Unleashing the genetic basis of adaptation to different environment is the central topic in evolutionary biology [Storz, 2005]. The detection of signatures of natural selection within the genome of organisms is key, since it may allow a greater understanding of what proportion of a genome or which genes are being shaped by ongoing natural selection [Joost et al., 2007]. But there is no study till date on natural selection in mulberry.

1.8.4 ***Bioactive content, mineral content and antioxidant activity in genus Morus***

Phytochemicals rich plants played a significant role in diet based therapies to cure various maladies [Butt et al., 2009; Ramaa et al., 2006]. Consumer's trend is being widened due to awareness spread and research interventions indicating potential health benefits associated with consumption of plants and their functional components [Butt and Sultan, 2009; Gosslau and Chen, 2004]. Detailed study of phytochemistry of fruits and vegetables provides insight about phenolic compounds [Balasundram et al., 2006; Randhir et al., 2004]. These phenolic compounds often exhibit wide range of physiological activities that include antioxidant, antimicrobial and anti-inflammatory properties [Halliwell, 2007; Manach et al., 2005].

Traditional medicines also show reliance on phytochemicals rich plants extracts to cure various maladies. Studies have been reported on the pharmacological activity and chemical composition of some mulberry species specially from China, India, Japan, South Korea, Pakistan and Turkey [Gerasopoulos and Stav-Roulakis 1997; Elmacı and Altuğ 2002; Darias-Martin et al., 2003; Arabshahi-Delouee and Urooj 2007; Ercisli and Orhan 2007; Gungor and Sengul 2008; Koca et al., 2008; Nikkah et al., 2009; Imran et al., 2010; Kutlu et al., 2011; Du et al., 2008 and Arfan et al., 2012]. Mineral content of 3 *Morus* species fruit were reported by Ercisli and Orhan [2007], Imran et al. [2010] and Karlidag et al. [2012]. Bioactive content, antioxidant activity,

chemical composition and mineral content studies carried out in *Morus* genus is presented in Table 1.5, 1.6 and 1.7.

Table 1.5 Bioactive content and antioxidant activity studies in mulberry

Year	Country	Research aspect	Reference
1999	China	Antioxidant activity of aqueous and ethanolic extract of mulberry leaf	Zhishen et al.
2006	Japan	Ethanolic extract of mulberry leaves to show LDL antioxidant activity	Katsube et al.
2007	India	Antioxidant activity of methanol, acetone and water extracts of *M. indica* L. leaves	Arabshahi-Delouee and Urooj
2007	South Korea	Antioxidant activities of ethanolic extract of five different mulberry cultivars.	Bae and Suh
2007	Turkey	Chemical composition of *M. alba* and *M. rubra*	Ercisli and Orhan
2008	Turkey	Antioxidant activity of methanolic extract of *M. nigra*.	Ercisli and Orhan
2008	Turkey	Antioxidant activity *M. alba* L. fruit.	Gungor and Sengul
2008	Turkey	Antioxidant activity of purple mulberry fruit.	Koca et al.
2008	Italy	Quali-quantitative analyses of flavonoids of *M. nigra* L. and *M. alba* L. fruits	Pawlowska et al.
2009	Iran	*in vitro* antioxidant activity of *M. alba* var. nigra berry	Nikkhah et al.
2010	Pakistan	Chemical composition and antioxidant activity of certain *Morus* species	Imran et al.
2011	Taiwan	Antioxidant and antityrosinase activity of *M. alba* L. twigs	Chang et al.
2011	Turkey	Physicochemical properties and fatty acid content	Gecgel et al.
2011	Pakistan	Antimicrobial activity, phytochemical profile and trace minerals	Khalid et al.
2011	Turkey	Antioxidant properties of different extracts of black mulberry	Kutlu et al.
2012	China	Composition of anthocyanins in mulberry and their	Du et al.

		antioxidant activity	
2012	China	*in vitro* antioxidant and antimicrobial activity of extracts from *M. alba* L. leaves, stems and fruits.	Wang et al.
2012	Serbia	Free radical scavenging activity, total phenolic and flavonoid contents of mulberry	Rodojokovic et al.
2012	Pakistan	Antioxidant activity of mulberry fruit extracts	Arfan et al.

Table 1.6 Chemical composition analysis studies carried out in mulberry

Year	Country	Bioactive Compound isolated/separated/quantified	Reference
1999	China	Quercetin and rutin separated using HPLC from mulberry leaves.	Zhishen et al.
2006	Japan	Three flavonol glycosides: quercetin [3-(6-malonylglucoside)], rutin (quercetin 3-rutinoside) and isoquercitrin (quercetin 3-glucoside)] were identified as the major LDL antioxidant compounds by LC-MS and NMR in *M. alba* leaves	Katsube et al.
2007	South Korea	Cyanidin 3-glucoside and Cyanidin rutinoside quantified in mulberry using HPLC.	Bae and Suh
2007	Turkey	Three fattyacids: linoleic acid (54.2%), palmitic acid (19.8%) and oleic acid (8.41%) identified in mulberry.	Ercisli and Orhan
2008	Turkey	Myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, nonadecenoic acid identified in *M. nigra* fruits using GC	Ercisli and Orhan
2008	Turkey	Cyanidin 3,5-diglucoside, Cyanidin 3-glucoside, Cyanidin 3-rutinoside, Pelargonidin 3-glucoside, Peonidin 3-glucoside quantified in *M. rubra* by HPLC	Koca et al.
2008	Italy	Quercetin 3-O-glucoside, quercetin 3-O-rutinoside, kaempferol 3-O-rutinoside, 5-O-caffeoylquinic acid and four anthocyanins (cyanidin 3-O-glucoside, cyanidin 3-O-rutinoside, pelargonidin 3-O-glucoside, and pelargonidin 3-O-rutinoside) quantified and characterized by Sephadex LH-20 column chromatography, HPLC/MS and NMR.	Pawlowska et al.
2011	Taiwan	Maclurin, rutin, isoquercitrin, resveratrol and morin quantified in extract of twigs *M. alba*.	Chang et al.
2011	Turkey	Fatty acid methyl esters, linoleic acid, palmitic acid, oleic acid and stearic acid quantified by GC. α-tocopherol ranged between 0.17 and 0.20 mg in 100 g seed oil. Sterols isolated β-sitosterol, Δ5-avenasterol,	Gecgel et al.

		Δ5, 23-stigmastadienol, clerosterol, sitosterol and Δ5, 24-stigmastadienol.	
2012	China	Cyanidin 3-O-(6”-O-α-rhamnopyranosyl-β-D-glucopyranoside), Cyanidin 3-O-(6-O-α-rhamnopyranosyl-β-D-galactopyranoside), Cyanidin 3-O-β-D-glucopyranoside, Cyanidin 3-O-β-D-galactopyranoside and Cyanidin 7-O-β-D-glucopyranoside separated and identified by high-speed counter-current chromatography (HSCCC), ESI–MS and one/two-dimensional NMR spectra.	Du et al.
2012	Serbia	Phenolics quantified using HPLC: Gallic acid, chlorogenic acid, ferulic acid, sinapic acid, rutin and quercetin	Radojokovic et al.
2012	Pakistan	Two phenolics: chlorgenic acid and rutin quantified in *M. alba* and *M. nigra*	Arfan et al.

Table 1.7 Mineral content reported in different studies (mg/100g dry weight)

Elements	Ercisli and Orhan [2007]	Imran et al. [2010]	Karlidag et al. [2012]
Nitrogen	0.75	-	3.19
Phosphorous	247	-	0.342
Potassium	1668	1731	1.96
Calcium	152	576	2.78
Magnesium	106	240	0.647
Sodium	60	280	65.8
Iron	4.2	73	122.4
Copper	0.5	-	14
Manganese	3.8	-	72.9
Zinc	2.8	50.2	31.3
Nickel	-	2.2	-

1.9 Research gap: *M. alba* in the trans-Himalaya

Mulberry is a promising plant for studying phenotypic variation. The genus is cosmopolitan in nature and easily adapted to different ecological conditions, and easily hybridized both naturally and artificially, which create a wide range of variability in the existing

gene pool [Banerjee et al., 2007]. Gray [1990] reported high phenotypic plasticity in leaf and fruit characters of mulberry. However, there is no study which shed light on the phenotypic variation of mulberry along altitudinal gradient particularly in harsh environmental conditions such as the trans-Himalaya. *M. alba* populations in the trans Himalaya is separated by geographical barriers but there is no report on genetic structure of *M. alba* populations. Gene flow also severely affected by geographical barriers but no information is available on levels of gene flow within and among populations. The trans-Himalayan region is characterized by prolonged sub zero temperatures, low precipitation, sandy soils with low water holding capacity, low relative humidity, intense solar radiation, low atmospheric pressure, high wind velocity and rugged terrain, due to such features mico-climatic conditions vary frequently in the trans-Himalya which imposes selective pressure on plants. But no attempt has been made to quantify the part of *M. alba* genome under selection. *M. alba* in the trans-Himalaya bears colored fruit ranging from white to black. Studies to related effect of color and sampling location on bioactive content and antioxidant activity of *M. alba* fruit has not been attempted. There is no previous report on volatile and aromatic compounds and mineral profiling of in *M. alba* from the trans-Himalaya.

1.10 Objectives of the research work

Keeping above research gap in mind we have devised following research objectives for the present study.

1. Phenotypic variation in *M. alba* L. along altitudinal gradient.
2. Structure and genetic diversity of natural populations of *M. alba* L.
3. Detecting molecular signatures of natural selection in *M. alba* L. populations.
4. Chemical composition and antioxidant activities of *M. alba* L. fruit.

Each of the four pieces of work has distinct characteristics and is 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 2

PHENOTYPIC VARIATION IN *MORUS ALBA* L. ALONG ALTITUDINAL GRADIENT

Abstract

Ten quantitative morphological characters were studied in 56 *Morus alba* L. trees representing three natural populations from the trans-Himalayan Ladakh region. The altitude of collection sites ranged from 2815 to 3177 m asl. Coefficient of variation (CV) showed high phenotypic variation in *M. alba* populations from the trans-Himalaya. Phenotypic plasticity measured using phenotypic plasticity index revealed significant contribution of phenotypic plasticity towards phenotypic variation. Linear regression analysis revealed that size of leaf and fruit characters decreases with increase in altitude. High CV was observed for leaf length (26.6%), leaf width (26.2%), petiole length (32.2%), leaf area (50.3%), inter-nodal distance (38.1%), number of nodes (46%), bud length (23.6%), fruit length (13.1%), fruit width (21.5%) and fruit weight (44.1%). Similarly, high phenotypic plasticity observed for bud length (0.3), leaf length (0.5), leaf width (0.5), petiole length (0.6), leaf area (0.7), and inter-nodal distance (0.5), number of nodes (0.6), fruit length (0.2), fruit width (0.3) and fruit weight (0.7). For every 100 m increase in altitude, leaf length, leaf width and leaf area decreased by 1 cm, 0.8 cm and 16.6 cm^2, respectively. Analysis of covariance showed a predominant altitudinal effect on the morphological characters in comparison to the population effect. A small change in altitude caused significant change in plant morphological characters. The present investigation represents to our knowledge the first study addressing phenotypic variation in mulberry along altitudinal gradient.

2.1 Introduction

An important consequence of the sessile lifestyle of plants is that they cannot escape from the environment in which they grow or from any environmental changes. To cope with this, many plants are able to alter one or more morphological characters in response to both abiotic (e.g., climate and weather) and biotic (e.g., grazing and competition) factors of the environment with a potential effect on resource acquisition. For example, leaf size and leaf area of many alpine plants are reported to change with altitude [Meinzer et al., 1985; Körner et al., 1989]. Some arctic plants produce more or larger leaves during warmer summers than during colder ones [Havström et al., 1995; Stenstrom et al., 2002]. This adjustment to environment is referred as phenotypic plasticity. Phenotypic plasticity plays an important role in resource acquisition by plants; variation of the size and placement of resource acquiring organs such as leaves are critical to a plant's adjustments to resource availability [Sattarian et al., 2011]. The current interest in plasticity results from an urgency to predict species responses to global climate change [Potvin and Tousignant, 1996; Rehfeldt et al., 2001] and from the emerging ideas on the importance of plasticity for understanding trait-mediated species interactions [Callaway et al., 2003; Valladares et al., 2006]. Study of phenotypic plasticity is more important in plants with long life span, such as trees, which may experience large changes in climatic conditions during their life time [Rehfeldt et al., 2001; Valladares et al., 2005].

Altitudinal gradients are among the most powerful 'natural experiments' for testing ecological and evolutionary responses of biota to geophysical influences, such as low temperature [Körner, 2007]. The four primary atmospheric changes associated with altitude are: (i) decreasing total atmospheric pressure and partial pressure of all atmospheric gases; (ii) reduction of atmospheric temperature, with implications for ambient humidity; (iii) increasing radiation under a cloudless sky, both as incoming solar radiation and outgoing night-time thermal radiation; and (iv) a higher fraction of UV-B radiation at any given total solar radiation [Körner, 2007].

Altitudinal gradient are particularly relevant in order to study plants phenological responses to temperature since they provide a wide temperature range over a very short distances. The distributions of species in mountain regions are typically restricted to relatively

narrow and well-delineated altitudinal bands in comparison with often broad and poorly defined latitudinal distributions in the lowlands [Jump et al., 2009]. The relationship between altitude and plant morphology is of great interest to plant physiologists, ecologists and palaeobotanists alike. High-altitude species tend to be morphologically and physiologically distinct from closely related species from lower altitudes. Altitude also has a major effect on leaf morphology and physiology within a species [Hovenden and Vander Schoor, 2003].

Mulberry is a promising plant for studying phenotypic plasticity. The genus is cosmopolitan in nature and easily adapted to different ecological conditions, and easily hybridized both naturally and artificially, which create a wide range of variability in the existing gene pool [Banerjee et al., 2007]. Gray [1990] reported high phenotypic plasticity in leaf and fruit characters of mulberry. However, there is no study which shed light on the phenotypic response of mulberry along altitudinal gradient particularly in harsh environmental conditions such as the trans-Himalaya. Basically there are three principle sources of phenotypic variation viz. genetic variation, phenotypic plasticity and developmental instability. However, in present investigation we try to reveal contribution of phenotypic plasticity along altitudinal gradient in the trans-Himalaya. Therefore, present investigation was undertaken to study the effect of altitudinal gradient on the phenotypic variation of *M. alba* in the trans-Himalayan Ladakh region. Hence, in present investigation our working hypothesis is: 'Is there a statistically-significant effect of altitude on phenotypic variation of *M. alba*?' The present investigation represents to our knowledge the first study addressing phenotypic plasticity in mulberry along altitudinal gradient.

2.2 Materials & methods

2.2.1 *Study site*

Representative samples from 56 sporadic wild *M. alba* trees, representing three natural populations, were collected from 14 different collection sites spread across Indus, Suru and Nubra valleys in the Indian trans- Himalaya during 2010 (Table 2.1 and Figure 2.1). The valley divisions were considered as separate populations and collection sites as sub-populations. The altitude of collection sites ranged from 2815 to 3177 m asl. Altitude and location of study sites was established using GARMIN GPS 72, Olathe, Kansas, USA. The outside mean maximum and minimum temperature during 2001-2011 recorded at 3235 m asl (N37º05.5, E077º35.8) in the

Table 2.1 Multi-location sampling of three *M. alba* L. population from trans-Himalaya Ladakh region

SN	Collection sites	Collection site code	Collection site id	Populations	No. of samples	Altitude (m asl)	Latitude	Longitude
1	Skurbuchan	1	SKB	Indus	20	2893.4664	34° 25'877''	76° 43' 106''
2		1				2920.2888	34° 25' 846''	76° 43' 084''
3		1				2951.0736	34° 25' 801''	76° 43' 092''
4		1				2970.0736	34° 25' 798''	76° 43' 050''
5	Domkhar	2	DMK			3050.6	34° 23' 333''	76° 45' 576''
6		2				2987.6496	34° 23' 330''	76° 45' 572''
7		2				3001.0608	34° 23' 321''	76° 45' 566''
8		2				3024.2256	34° 23' 323''	76° 45' 571''
9	Achinathang	3	ACH			2874.264	34° 30' 320''	76° 37' 562''
10		3				2916.936	34° 30' 322''	76° 37' 564''
11		3				2879.4456	34° 30' 379''	76° 37' 566''
12		3				2892.2472	34° 30' 377''	76° 37' 618''
13	Beama	4	BMA			2840.4312	34° 36' 014''	76° 30' 874''
14		4				2741 .676	34° 35' 955''	76° 31' 105''
15		4				2742.2856	34° 35' 916''	76° 31' 118''
16		4				2777.0328	34° 35' 915''	76° 31' 119''
17	Sanjok	5	SNJ			2928.2136	34° 34' 458''	76° 31' 584''
18		5				2870.2	34° 34' 500''	76° 31' 570''
19		5				2793.492	34° 34' 651''	76° 31' 588''
20		5				2795.6256	34° 34' 528''	76° 31' 555''
21	Batalik	6	BTK	Suru	16	2818.7904	34° 39' 348''	76° 20' 419''
22		6				2856.2808	34° 39' 316''	76° 20' 100''
23		6				2839.5168	34° 39' 366''	76° 20' 420''
24		6				2821.8384	34° 39' 327''	76° 20' 495''
25	Achkamal	7	AKM			2844.3936	34° 33' 424''	76° 09' 569''
26		7				2854.452	34° 33' 422''	76° 09' 556''
27		7				2901.0864	34° 33' 422''	76° 09' 554''
28		7				2876.04	34° 33' 425''	76° 09' 557''
29	Paschym	8	PSY			2877.6168	34° 31' 284''	76° 10' 945''
30		8				2888.5896	34° 31' 287''	76° 10' 941''
31		8				2872.74	34° 31' 277''	76° 10' 897''
32		8				2879.7504	34° 31' 228''	76° 10' 959''
33	Lochum	9	LCM			3034.24	34° 28' 019''	76° 15' 280''
34		9				3003.1944	34° 28' 034''	76° 15' 457''
35		9				3020.2632	34° 28' 053''	76° 15' 210''
36		9				3057.7536	34° 28' 064''	76° 15' 337''
37	Chalunka	10	CHK	Nubra	20	2959.9128	34° 49' 315''	76° 56' 293''
38		10				2962.656	34° 49' 308''	76° 56' 293''
39		10				2970.8856	34° 49' 341''	76° 56' 248''

40		10		2991.0024	34° 49’ 461’’	76° 56’ 148’’
41	Bokdang	11	BKD	2980.9256	34° 48’ 240’’	77° 02’ 460’’
42		11		2991.01	34° 48’ 242’’	77° 02’ 480’’
43		11		3000.1	34° 48’ 244’’	77° 02’ 547’’
44		11		3009.9	34° 48’ 257’’	77° 02’ 696’’
45	Turtuk	12	TRK	2876.7024	34° 50’ 823’’	76° 49’ 292’’
46		12		2911.4496	34° 50’ 823’’	76° 49’ 295’’
47		12		2864.5104	34° 50’ 818’’	76° 49’ 401’’
48		12		2873.0448	34° 50’ 790’’	76° 49’ 411’’
49	Tyakshi	13	TYK	2968.4472	34° 53’ 114’’	76 ° 48’ 390’’
50		13		2962.9608	34° 53’ 110’’	76° 48’ 406’’
51		13		2953.8168	34° 53’ 120’’	76° 48’ 275’’
52		13		3008.376	34° 53’ 122’’	76° 48’ 281’’
53	Hunder	14	HUN	3110.484	34° 35’ 087’’	77° 27’ 938’’
54		14		3176.9304	34° 35 143	77° 27’ 731’’
55		14		3202.8384	34° 35 298	77° 27’ 824’’
56		14		3163.5992	34° 35 298	77° 28’ 072’’

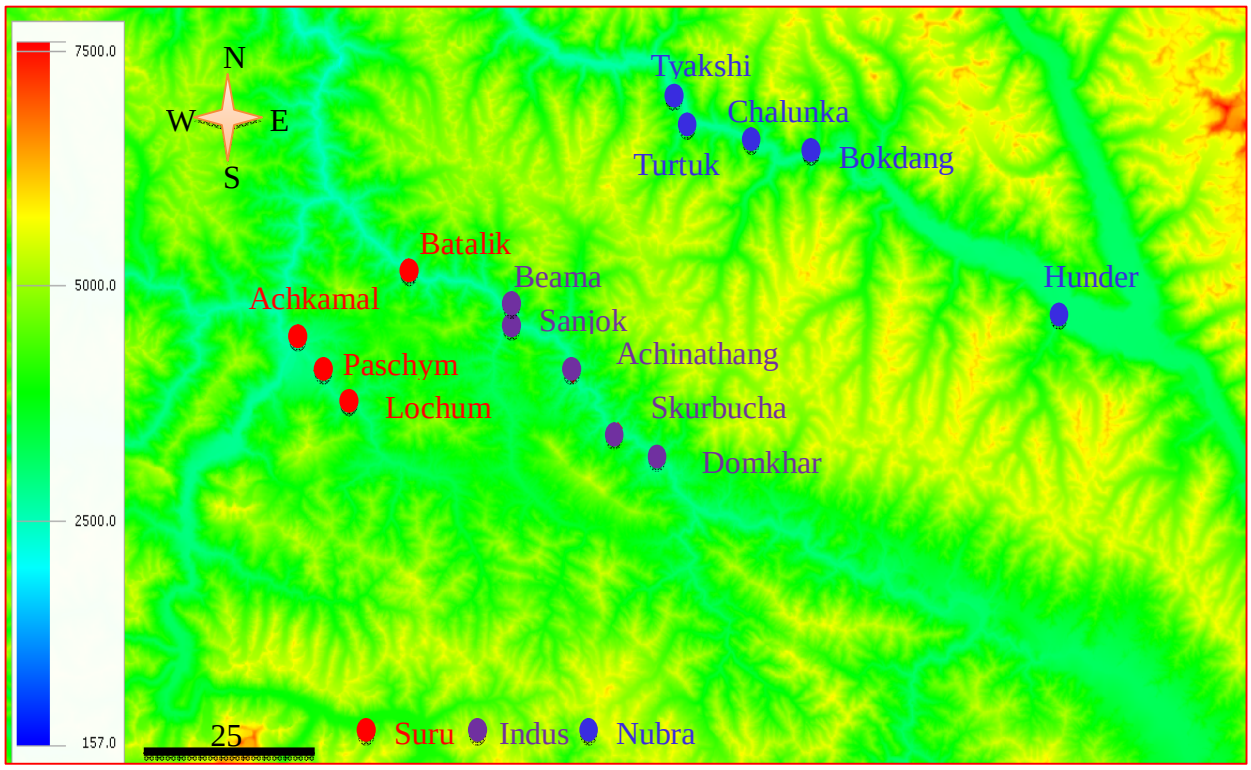

Figure 2.1 Sampling sites of *M. alba* L. populations from trans-Himalaya India

region was 18.9±9.5ºC and -5.8±9.8ºC, respectively, while the mean maximum and minimum relative humidity was 35.5±7.3 and 25.0±3.7%, respectively. The average annual precipitation was less than 200 mm of which more than 70% was in the form of snowfall [Korekar et al. 2013]. A herbarium of mulberry representative samples collected from the three valleys was prepared and voucher specimens submitted to Botanical Survey of India, Kolkata, India, to ascertain the *Morus* species status.

2.2.2 ***Quantitative characters studied***

Ten quantitative morphological characters were analyzed based on recommended plant descriptors for mulberry by Food and Agricultural Organization (FAO) [www.fao.org)]. The morphological measurements taken for each Operational Taxonomic Unit (OTU) include: (1) length of mature bud from the basal portion to the tip of the bud, (2) length of leaf blade from the leaf base at the juncture of the petiole attachment to the leaf tip leaving the extended portion of the tip, (3) width of the leaf from the widest point from both sides of the leaf margins, (4) petiole length by cutting petiole portion of the leaf from the base of the leaf blade, (5) inter-nodal distance of longest shoot, (6) number of nodes, (7) leaf area, (8) fruit length (9) fruit width and (10) fruit weight. We have sampled four trees per subpopulation for assessment of morphological variation. For the leaf morphological characters three leaves were used from upper, middle and lower portion of the branch. Fruit characters were measured from three ripen fruits from each individual. The number of nodes was counted from the same shoot of which inter-nodal distance was measured. Length and width measurements were done with a digital vernier caliper (MITUTOYO, Japan). Leaf area was measured with a portable leaf area meter (CI 202), CID Inc, Camas, WA, USA. The weight of ripen fruit was measure in an electronic balance to an accuracy of 0.001g.

2.2.3 ***Statistical analysis***

Minimum, first quartile, median, third quartile and maximum values were calculated for each data set. Turkey's honestly significant difference test was used to assume equal variances with $p<0.05$. Box plots were produced to show minimum, median and maximum values of each variable. Assumptions of normality were checked for all variables with Kolmogorov-Smirnov test and variables that significantly deviate from normality were log transformed. To test the

effect of population and altitude on the morphological characters, one way analysis of covariance (ANCOVA) was conducted with population as fixed factor and altitude as covariate. To examine the relationship between morphological characters and altitude, the data were with fitted simple linear model (y=a+bx). Principal component analysis (PCA), based on the correlation matrix was performed in order to see whether data reduction obtained through the new set of variables (PCs) revealed a pattern of variation that is consistent with the distribution rate of *M. alba* populations. Statistical analysis was carried out in MS excel 2007 and SPSS software package v.17.0 for Windows (SPSS Inc., 2008). Coefficient of variation (CV) for each trait as a complementary index to interpret intraspecific variation was computed using the formula: CV=standard deviation × 100/mean. Phenotypic plasticity index (PPI) was calculated by following formula: PPI=maximum mean - minimum mean/Maximum mean [Balaguer et al., 2001; Gratani et al., 2003; Valladares et al., 2006].

2.3 Results

2.3.1 *Variation in morphological characters*

Morphological characters showed high variation. CV was 23.6% for bud length, 26.6% for leaf length, 26.2% for leaf width, 32.2% for petiole length, 50.3% for leaf area, 38.1% for inter-nodal distance, 46% for number of nodes, 13.1% for fruit length, 21.5% for fruit width and 44.1% for fruit weight. Six morphological characters viz. leaf length, leaf width, leaf area, petiole length, fruit length and fruit weight showed significant variation among sub- populations. High variability is evident from Figure 2.2 and 2.3, which represent box plot showing minimum, first quartile, median, third quartile and maximum value of different morphological characters.

The PCA (Figure 2.4) based on correlation matrix reduced the 10 morphological characters into three principal components. The first three PCs explained 78% of the total variance (36, 25 and 17%, respectively). The highest loadings on the first PCA axis (PC1) correspond to leaf width (0.9), leaf area (0.9), leaf length (0.9), petiole length (0.8) and inter-nodal distance (0.6). The variables with highest loading on the second PCA axis (PC2) were fruit length (0.9), fruit weight (0.9) and fruit width (0.8).

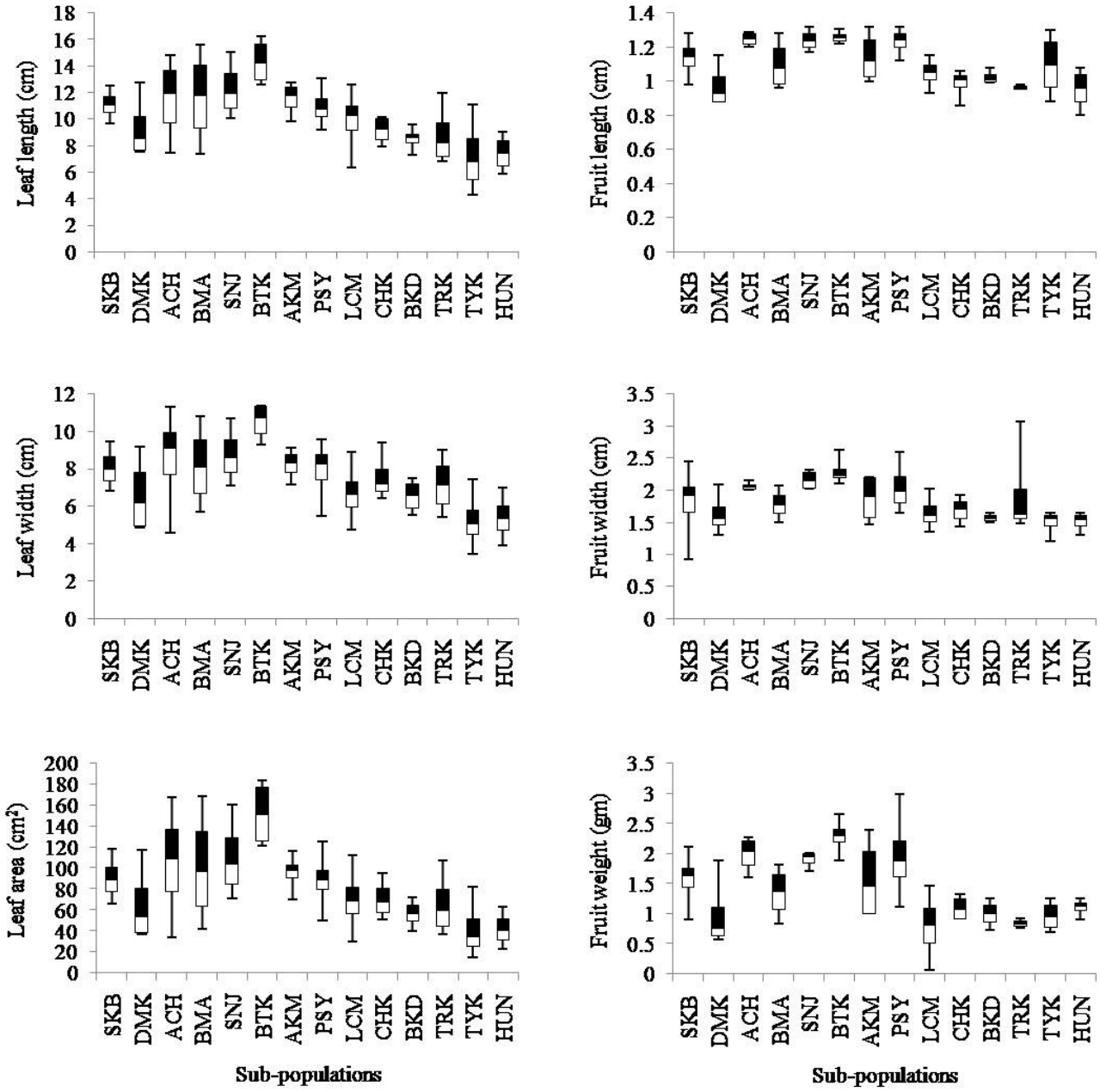

Figure 2.2 Box plot showing minimum, 1st quartile (blank box), median, 3rd quartile (black box) and maximum values of leaf and fruit characters in 14 sub-populations of *M. alba* L.

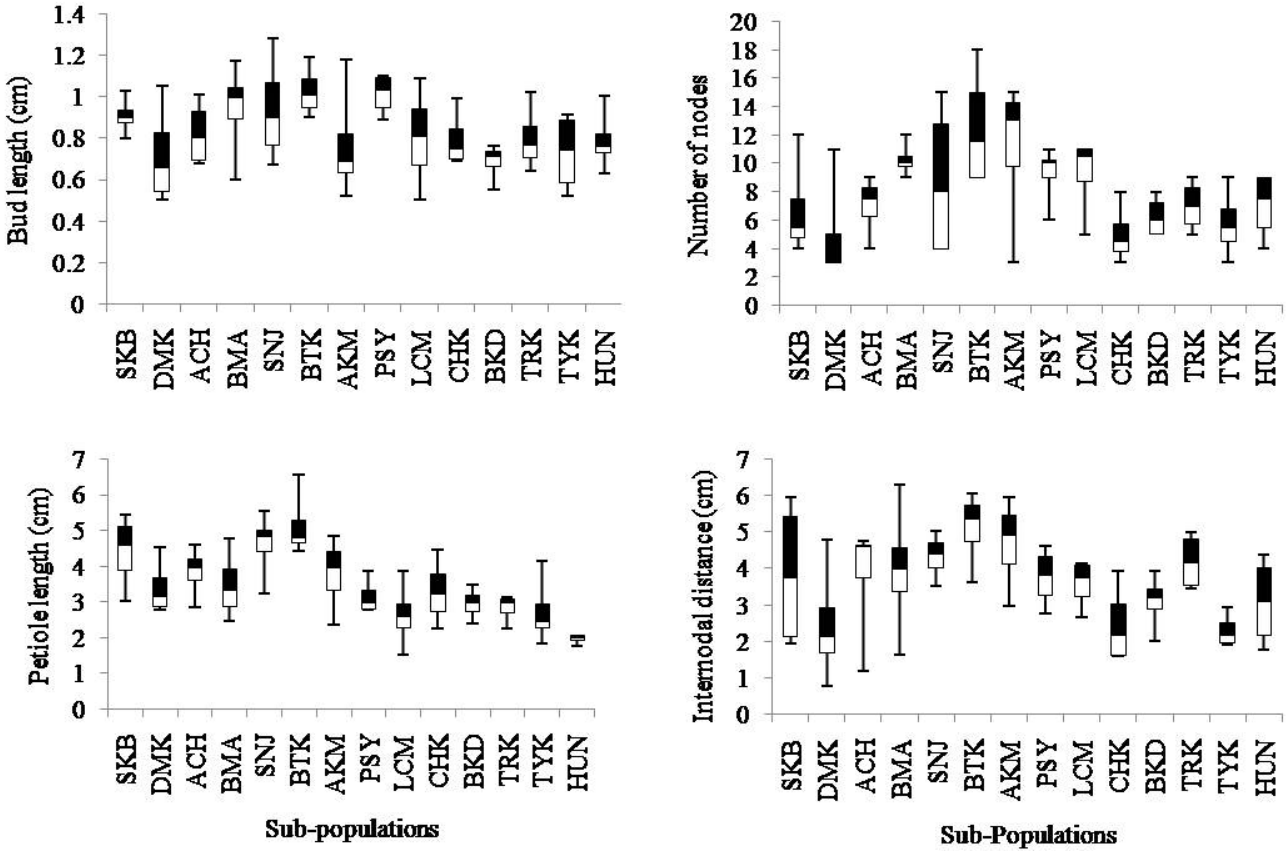

Figure 2.3 Box plot showing minimum, 1st quartile (blank box), median, 3rd quartile (black box) and maximum values of bud length, petiole length, number of nodes and inter-nodal distance in 14 sub-populations of *M. alba*.

Overall in PC1 the variability is explained mainly by leaf traits and in PC2 the variability is explained mainly by fruit traits (Figure 2.4). Phenotypic plasticity index for 10 morphological characters is presented in Figure 2.5. High phenotypic plasticity was observed for bud length (0.3), leaf length (0.5), leaf width (0.5), petiole length (0.6), leaf area (0.7), inter-nodal distance (0.5), number of nodes (0.6), fruit length (0.2), fruit width (0.3) and fruit weight (0.7).

2.3.2 ***Effect of altitudinal gradient on morphology***

ANCOVA was carried out to see the effect of population and altitude on morphological characters. ANCOVA clearly revealed predominant effect of altitude on morphology (F_1=4.93, $p\leq0.05$ for bud length; F_1=15.24, $p\leq0.001$ for petiole length; F_1=6.64, $p\leq0.01$ for inter-nodal distance; F_1=45.72, $p\leq0.01$ for leaf length; F_1=13.53, $p\leq0.001$ for leaf width; F_1=12.87, $p\leq0.001$

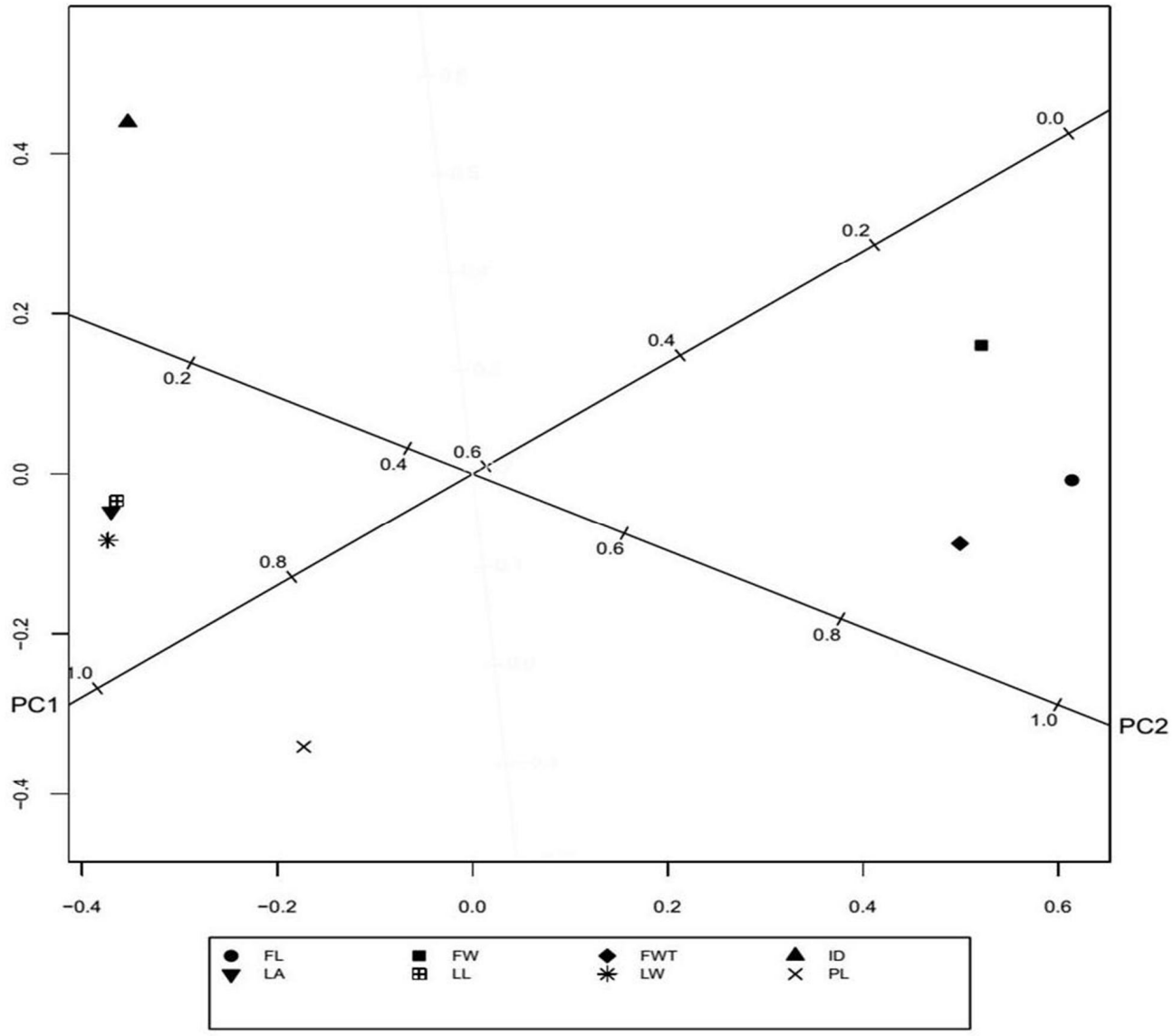

Figure 2.4 Biplot showing first two components of principal component analysis (LL=leaf length; LW=leaf width; PL=petiole length; LA=leaf area; ID=inter-nodal distance; FL=fruit length; FW=fruit width; FWT=fruit weight).

for leaf area; F_1=12.30, $p \leq 0.001$ for fruit length; F_1=9.72, $p \leq 0.01$ for fruit width and F_1=20.47, $p \leq 0.001$ for fruit weight). Population and interaction between population and altitude showed somewhat effect on petiole length (Table 2.2). Altitudinal variations presents in eight morphological characters viz. leaf length, leaf width, leaf area, petiole length, number of nodes

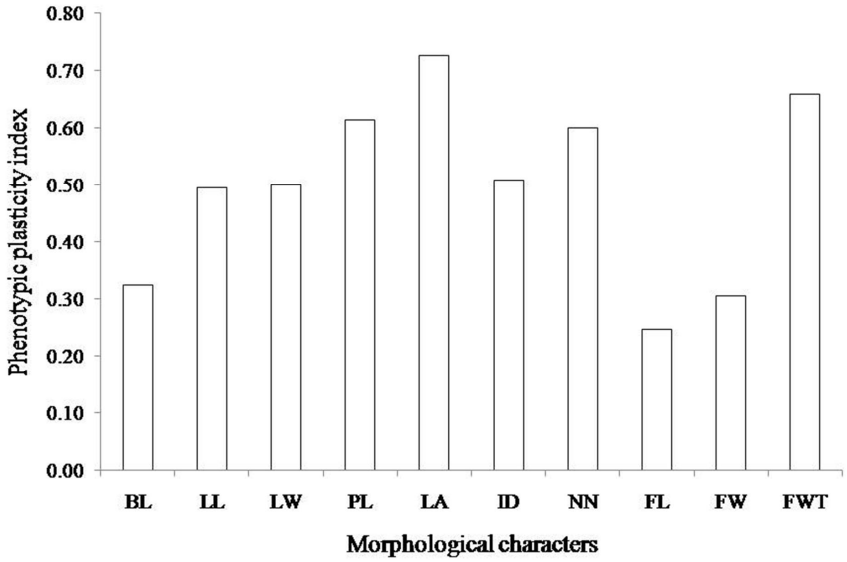

Figure 2.5 Phenotypic plasticity index of ten morphological characters (BL=bud length; LL=leaf length; LW=leaf width; PL=petiole length; LA=leaf area; NN=number of nodes; ID=inter-nodal distance; FL=fruit length; FW=fruit width; FWT=fruit weight)

Table 2.2 One way ANCOVA analysis for morphological characters with population as fixed factor and altitude as covariate

Source	d.f	*F*	*F*	*F*
		Bud length	Leaf length	Fruit width
Populations	2	1.41	1.79	1.17
Altitude	1	4.93*	9.89**	9.72**
Pop x Alt	2	1.31	7.23	1.13
		Petiole length	Leaf width	Fruit weight
Populations	2	3.36*	1.80	12.00***
Altitude	1	15.24***	13.53***	20.47***
Pop x Alt	2	3.35*	1.69	11.44***
		No. of nodes	Leaf area	
Populations	2	1.23	2.41	
Altitude	1	3.12	12.87***	
Pop x Alt	2	1.12	2.22	
		Inter-nodal distance	Fruit length	
Populations	2	0.37	3.29*	
Altitude	1	6.64**	12.30***	
Pop x Alt	2	0.31	2.92	

The F ratio (*F*) is presented for each factor; d.f: Degrees of freedom

*Significant at $p \leq 0.05$, **Significant at $p \leq 0.01$, ***Significant at $p \leq 0.001$

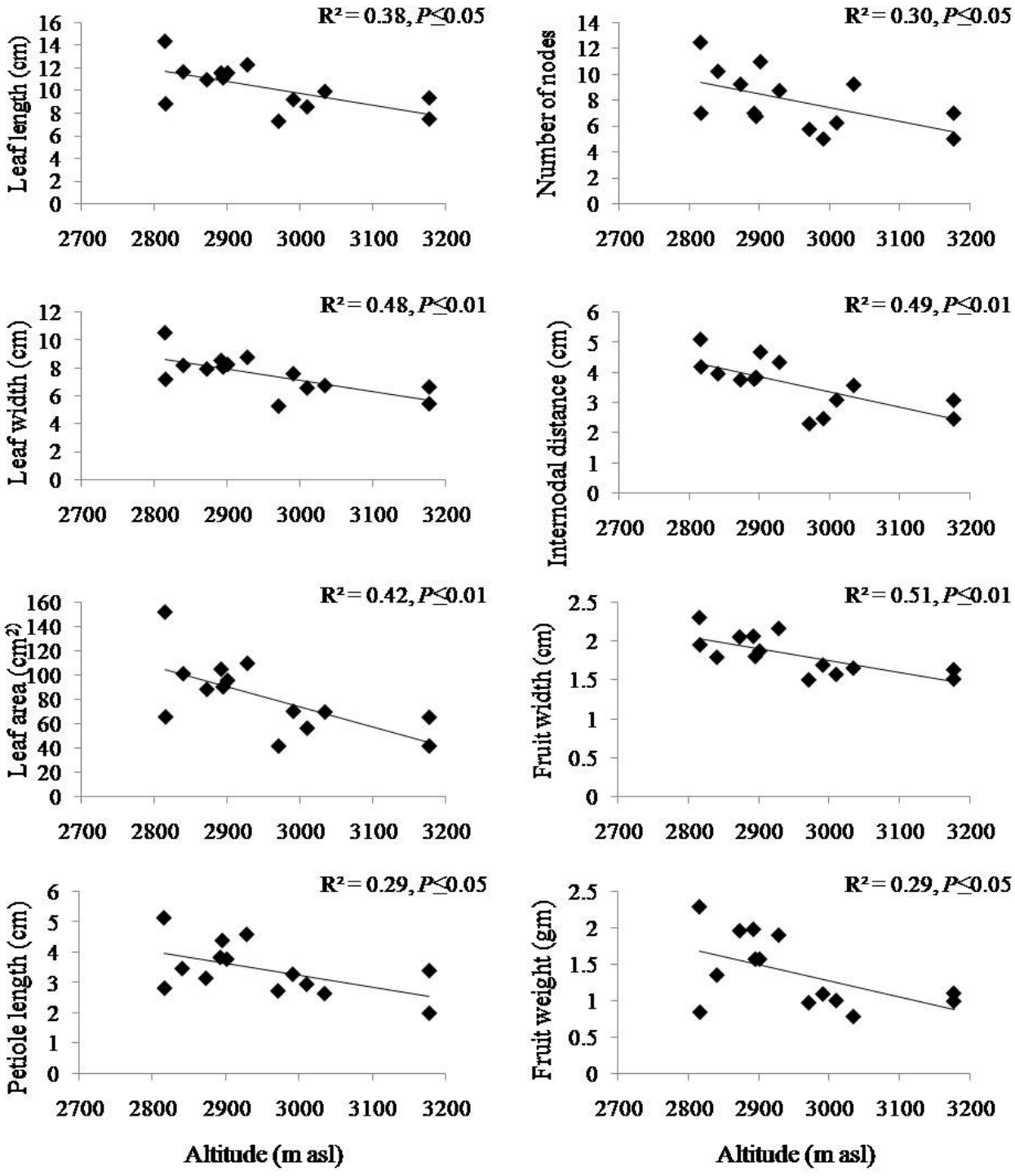

Figure 2.6 Altitudinal variations in morphological characters

inter-nodal distance, fruit width and fruit weight are presented in Figure 2.6. Leaf length (d.f=1, 12; b=-0.010; R^2=0.38; $p \leq 0.05$), leaf width (d.f=1, 12; b=-0.008; R^2=0.48; $p \leq 0.01$) and leaf area

(d.f=1, 12; b=-0.166; R^2=0.42; $p \leq 0.01$) showed 1 cm, 0.8 cm and 16.6 cm^2 decrease per 100 m increase in altitude, respectively. Petiole length (d.f=1, 12; b=-0.004; R^2=0.29; $p \leq 0.05$), inter-nodal distance (d.f=1, 12; b=-0.005; R^2=0.49; $p \leq 0.01$) and number of nodes (d.f=1, 12; b=-0.005; R^2=0.30; $p \leq 0.05$) displayed negative relationship with altitude. Fruit size decreased significantly with increase in altitude, especially fruit width (d.f=1, 12; b = -0.002; R^2=0.51; $p \leq 0.01$).

2.3.3 ***Correlation among morphological characters***

Pearson correlation among different morphological characters is presented in Table 2.3. Bud length, leaf size (lamina length, lamina width and leaf area), petiole length and inter-nodal distance showed significant correlation with fruit size (fruit length, width and weight).

Table 2.3 Pearson correlation among different morphological characters

Variables	BL	LL	LW	PL	LA	ID	NN	FL	FW	FWT
BL		0.76**	0.74**	0.6*	-0.03	0.64*	0.66*	0.7**	0.76**	0.77**
LL			0.96**	0.88**	-0.32	0.81**	0.76**	0.8**	0.86**	0.84**
LW				0.89**	-0.29	0.79**	0.67**	0.74**	0.90**	0.82**
PL					-0.2	0.62*	0.46	0.73**	0.76**	0.78**
LA						-0.31	-0.45	-0.15	-0.2	-0.03
ID							0.85**	0.61*	0.81**	0.65*
NN								0.59*	0.62*	0.58*
FL									0.78**	0.91**
FW										0.84**
FWT										

*Significant at $p \leq 0.05$, **Significant at $p \leq 0.01$

BL=bud length; LL=leaf length; LW=leaf width; PL=petiole length; LA=leaf area; NN=number of nodes; ID=inter-nodal distance; FL=fruit length; FW=fruit width; FWT=fruit weight

2.4 Discussion

The genus *Morus* is interesting for a systematic study because of its wide geographical distribution, morphological plasticity [Gray, 1990], hybridization [Burgess et al., 2005], long history of domestication and introduction. High phenotypic plasticity in leaf and fruit characters

of mulberry growing in northern America has been reported. However, the study did not provide empirical evidence that either links the variation to specific environmental conditions [Gray, 1990]. Besides, the study is based on four *M. alba* and six *M. rubra* trees. In the present study a high morphological diversity was observed in *M. alba* of trans-Himalayan origin in terms of leaf length, leaf width, leaf area, petiole length and fruit characteristics. The effect of altitudinal gradient on morphological characters of *M. alba* was observed.

Coefficient of variation is a simple and common measure of phenotypic variation used in various studies. *M. alba* from the trans-Himalayan region collected from an altitudinal range of 2815-3177 m asl showed high phenotypic variation. We observed high CV for leaf length (26.65%), leaf width (26.21%), petiole length (32.21%), leaf area (50.34%), internodal distance (38.11%), number of nodes (46.01%), bud length (23.58%), fruit length (13.14%), fruit width (21.52%) and fruit weight (44.14%) in a relatively short altitudinal gradient of 362 m. In comparison, Ligaretto et al. [2011] reported 17.37%, 20.58%, 18.02%, 17.19% of CV in leaf length, leaf width, fruit length and fruit width, respectively, in wild populations of *Vaccninium meridionale* Swartz. at an altitudinal gradient of 2357-3168 m asl with an altitudinal difference of 811 m. In another study in Iran on *Populas euphratica* Oliv.at a lower altitude (50-1820 m), with an altitudinal difference of 1770 m, Calagari et al. [2006] found 29.80%, 27.90% and 44.60% CV in leaf length, petiole length and leaf area, respectively. Chalcoff et al. [2008] reported 37.7%, 53.3%, 48.1% CV in leaf length, leaf width and petiole length, respectively in *Emborthrium coccineum* J. R. Forster and G. Forst in southern Andean forest at an altitudinal gradient of 10-1100 m.

We analyzed phenotypic plasticity in terms phenotypic plasticity index (PPI) [Balaguer et al., 2001; Gratani et al., 2003]. PPI is a simple and robust plasticity index widely used in previous studies [Valladares et al., 2006]. High plasticity was observed in all the morphological characters specially the leaf area and fruit weight. Plant response to environmental variation includes passive limitation of growth under low resource conditions as well as active developmental plasticity that enhances resource acquisition in each resource environment [Sultan and Bazzaz, 1993; Sultan, 1995]. Therefore with change in altitude the climatic conditions changes, which leads to variation in resource acquisition pattern, and this change of pattern is manifests in phenotype.

The findings shed light on the fact that a small change in altitude in the trans-Himalaya cause large change in phenotype. Several studies have indicated that morphological variation is apparently the result of an adaptive response to the environment; for example, variation in growth traits and phenological traits is associated with a latitudinal and altitudinal range [Kleinschmit, 1993; Beaulieu et al., 2004]. ANCOVA showed that morphology in *M. alba* is significantly affected by altitude in the trans-Himalayan region, a trend similar to that of many other species [Halloy and Mark, 1996]. Different altitudinal gradient represents varying abiotic and microclimatic conditions. Plants respond to such varying habitat conditions by modifying its phenotypic features. *M. alba* adjusts itself to varying habitat conditions by modifying leaf size, petiole length, number of nodes, inter-nodal distance. Phenotypic plasticity in foliar characters has been reported in several studies [Meinzer et al., 1985; Körner, 1989; Havström et al., 1995]. Reduction in leaf size (lamina length, lamina width and leaf area), petiole length and inter-nodal distance along an altitudinal gradient has been reported earlier in *Metrosideros polymorpha* Gaud. [Cordell et al., 1998]. Reduction in size is an important strategy employed by plants at high altitude to withstand decrease in temperature and reduced nutrient availability. At high altitude plant increase supercooling capacity by decreasing cell size and intercellular spaces [Goldstein et al., 1985], which eventually leads to decrease in overall plant size. The effect of low temperature is more pronounced in trees as compared to low stature vegetation [Körner, 2007]. Water stress is also a crucial factor in this rain shadow trans-Himalayan region. Plants decrease size of its parts to reduce water loss through transpiration. Other possibility is that colder soils reduce the water uptake of the root system and induce water stress [Magnani and Borghetti, 1995]. Fruit morphological characters such as length, width and weight also decreased significantly with increase in altitude. Fruit morphological characters were significantly correlated with leaf size (leaf length, width and area), petiole length and inter-nodal distance. It is generally assumed that the size of the photosynthetically active leaf area supplying resources to individual fruit is a main factor in determining its size. The total active leaf area per tree is therefore, one of the factors determining fruit size [Fishler et al., 1983]. Therefore, reduction in leaf size ultimately leads to overall decrease in fruit size. The trans-Himalayan high altitude region is characterized by high UV-B radiation influx. The increase in UV-B radiation results in decrease in chlorophyll content of plants [Roblek et al., 2008], which further leads to overall reduction in photosynthetic output. Therefore, the adverse climatic conditions change the

resource allocation pattern of plants and invest more of its resources in fighting stress. So changes in allocation strategy, enhances plants fitness at the expense of reduction in size of different plant parts [Weiner, 2004].

Mulberry leaf is a major economic component in sericulture since the quality and quantity of leaf produced per unit area has a direct bearing on cocoon harvest. Improved mulberry varieties with higher leaf productivity are essential for enhancing and sustaining profitability in sericulture. Morphometric characterization can be used as a tool to analyze the genetic relationship among different genotypes of mulberry and the information is utilized in mulberry improvement program [Tikader, 1997; Masilmani and Camle, 1998; Tikader and Roy, 1999; Masilamani et al., 2000]. High variation in mulberry leaf characters observed in the present study revealed unexplored potential of the mulberry genetic resources from the trans-Himalaya for sericulture industry.

2.5 Conclusion

The present study revealed significant variation in morphological characters among *M. alba* populations in the trans-Himalaya. *M. alba* typically showed high phenotypic variation along an altitudinal gradient. A small change in altitude in the trans-Himalaya leads to significant change in phenotype. The results suggested that a small increase in altitude lead to high adversity for plant to survive in the trans-Himalayan condition. This study suggests that observed phenotypic variation to some extent maintained by phenotypic plasticity of *M. alba*. Understanding the role of phenotypic plasticity is necessary for predicting how plants will respond to global warming or other environmental change in future. Phenotypic plasticity in *M. alba* in the trans-Himalaya may serve as a model system to study plant adaptation to future environmental changes. So further study in controlled environment is needed to completely unravel the phenotypic plasticity of *M. alba*. The Himalayan region is considered as centre of diversity for mulberry [Chen et al., 2010], so this study also has evolutionary significance, as it provides a basic profile of *M. alba* phenotypic plasticity in the trans-Himalayan region for future researchers.

CHAPTER 3

STRUCTURE AND GENETIC DIVERSITY OF NATURAL POPULATIONS OF *M. ALBA* L.

Abstract

Sequence-related amplified polymorphism (SRAP) markers were used to assess the genetic structure in three natural populations of *M. alba* L. from trans-Himalaya. Multi-location sampling was conducted across 3 populations. The overall genetic diversity estimates: percentage polymorphic loci (89.66%), Nei's gene diversity (0.2286) and Shannon's information index (0.2175) were high. At regional level, partitioning of variability assessed using analysis of molecular variance (AMOVA), revealed 80% variation within and 20% among collection sites. Pattern appeared in STRUCTURE, BARRIER and AMOVA results clearly demonstrated that there is gene flow between Indus and Suru population while there is geographical barrier between Indus-Suru and Nubra population which effectively hinders gene flow. The results showed significant genetic differentiation, population structure, high to restricted gene flow and high genetic diversity. Assumption that samples collected from the three valleys represent three different populations does not hold true. The fragmentation present in the trans-Himalaya was more natural and less anthropogenic.

3.1 Introduction

Understanding of population genetic structure is the basic prerequisite for conservation and management of biodiversity [Baverstock and Moritz, 1996; Allendorf and Luikart, 2007]**.** Habitat destruction is a major problem to the preservation of biodiversity in many terrestrial ecosystems [Young et al., 1996]. Population fragmentation leads to reduced genetic diversity and increased genetic differentiation because of high random genetic drift and inbreeding, and reduction in gene flow [Young et al., 1996; Sork et al., 1999; Lowe et al., 2005]. These effects can be visualized in light of traditional biogeography and meta-population theories [MacArthur and Wilson, 1967; Levins, 1969]. A number of studies have shown effects of fragmentation on the genetic diversity and population structure of plant species [Cardoso et al., 2005; Prentice et al., 2006; Yao et al., 2007]. Fragmentation of plant populations is caused not only by anthropogenic activities but also by climatic changes, which cause formation of natural barriers between populations like mountain ranges, deserts and other geographical barriers [Slatkin 1987]. Conservation of biodiversity within these fragmented landscapes is a major challenge for policy makers to device tools for effective conservation. It is therefore necessary to assess patterns of plant response to habitat fragmentation.

Molecular markers have been extensively used to characterize *Morus* species. RAPD and ISSR markers have previously been used to study the genetic relationships of Japanese and Indian *Morus* cultivars to assess molecular variability [Vijayan, 2004; Vijayan et al., 2004a). ISSRs are found to be the most commonly used marker system in mulberries [Vijayan and Chatterjee, 2003; Vijayan et al., 2004b, 2004c, 2006; Zhao et al., 2006, 2007a, 2007b; Kar et al., 2008]. DAMD, SPAR [Bhattacharya and Ranade, 2001; Bhattacharya et al, 2005] and SRAP markers have also been used to assess the genetic relationship among mulberry cultivars [Zhao et al., 2009]. However, majority of molecular marker based studies in *Morus* species are mainly restricted to characterization of accessions and cultivars. To our knowledge use of molecular marker for assessment of structure and genetic diversity of natural populations of *M. alba,* especially from the trans-Himalayan region has not been reported. The present study was therefore undertaken to answer some of the pertinent queries, such as, (i) How different populations of *M. alba* structured in the trans-Himalaya? (ii) What is the level of genetic differentiation? (iii) Is there any geographic tendency in genetic data? (iv) What is the level of

fragmentation revealed by population genetic structure? (v) How can the genetic information be used to establish effective conservation measures? We have used SRAP markers to answer the above mentioned questions.

3.2 Materials and methods

3.2.1 *Population sampling*

Representative samples from 56 sporadic wild *M. alba* trees, representing three natural populations, were collected from 14 different collection sites spread across Indus, Suru and Nubra valleys in the Indian trans- Himalaya during 2010 (Table 2.1 and Figure 2.1). Multi-location sampling was conducted across 3 populations. Two accessions of *M. alba* obtained from Central Sericulture Germplasm Resource Centre, Hosur, Tamil Nadu, India during 2004 and maintained at the horticulture field of DIHAR, Leh, were used for comparison.

3.2.2 *DNA extraction and PCR amplification*

Total genomic DNA was extracted from frozen leaves (5 g) by the CTAB method [Saghai-Maroof et al., 1984] with minor modifications, which includes the use of 200 mg polyvinyl pyrollidone per sample. SRAP markers developed by Zhao et al. [2009] were adapted in this study. Seventy two primer combinations using eight forward (Me 1-8) and nine reverse (Em1-9) (Table 3.1) were tested for amplification in duplicate PCR reactions to ensure reproducible banding pattern. Fifty five SRAP primer sets were selected based on proper

Table 3.1 Forward and reverse SRAP primers used in the present study

Forward primer		Reverse primer	
Me1:	TGAGTCCAAACCGGATA	Em1:	GACTGCGTACGAATTAAT
Me2:	TGAGTCCAAACCGGAGC	Em2:	GACTGCGTACGAATTTGC
Me3:	TGAGTCCAAACCGGAAT	Em3:	GACTGCGTACGAATTGAC
Me4:	TGAGTCCAAACCGGACC	Em4:	GACTGCGTACGAATTTGA
Me5:	TGAGTCCAAACCGGAAG	Em5:	GACTGCGTACGAATTAAC
Me6:	TGAGTCCAAACCGGTAA	Em6:	GACTGCGTACGAATTGCA
Me7:	TGAGTCCAAACCGGTCC	Em7:	GACTGCGTACGAATTGAG
Me8:	TGAGTCCAAACCGGTGC	Em8:	GACTGCGTACGAATTGCC
		Em9	GACTGCGTACGAATTTCA

amplification and reproducibility. Each 20 µl SRAP PCR reaction consisted of 2 mM dNTPs, 10mM of each forward and reverse primer, 0.75 U Taq polymerase, 25 ng template DNA, Taq buffer containing tris with 15 mM $MgCl_2$ and Milli-Q water. Amplification was carried out with the initial cycle at 94^{o}C for 5 min, 5 cycles of 94^{o}C for 1 min, 72^{o}C for 1 min and 72^{o}C for 1 min; followed by 35 cycles of 94^{o}C for 1 min, 50^{o}C for 1 min and 72^{o}C for 1 min, and the final extension at 72^{o}C for 5 min. Amplification products were electrophoresed on 1.5 % agarose gel and molecular size of amplicons was estimated using a 100 bp and 1 Kb DNA ladders.

3.2.3 *Data collection and analysis*

The banding patterns obtained from SRAP were scored as present (1) or absent (0), each of which was treated as an independent character. POPGENE version 1.32 [Yeh et al., 1997] was used to calculate the different genetic diversity parameters: Nei's genetic diversity (H), Shannon's information index (I), number of polymorphic loci (NPL), percentage polymorphic loci (PPL), gene diversity of total population (Ht) and average gene diversity of subpopulations (Hs). The partitioning of genetic variability at different levels was calculated by AMOVA (Analysis of Molecular Variance) using GenAlEx v. 6.3 [Peakall and Smouse, 2006] software. AMOVA was calculated at population and regional level.

Genetic differentiation coefficient G_{ST} and Φ_{PT} were calculated by GenAlEx v. 6.3v and POPGENE, respectively. Gene flow (Nm) was calculated on the basis of Φ_{PT} with formula [0.25(1- Φ_{PT})/ Φ_{PT}] [Wood and Gardner, 2007]. STRUCTURE version 2.3 [Pritchard et al., 2000; Falush et al., 2003 and 2007] was used to predict number of clusters (K) and probability of individual assignment to each cluster. The parameters sets assumed were admixture allele model with correlated allele frequencies and with no prior population location information. Number of clusters set from K=1 to 10 with four simulations for each K and for each simulation we have fixed burnin period of 100,000 steps followed by 250,000 Monte Carlo Markov chain replicates. Results obtained from STRUCTURE were interpreted by online available tool STRUCTURE HARVESTER [Earl et al., 2012] which implements Evanno's method [Evanno et al., 2005] for calculation of correct number of clusters (K). CLUMPP indfile obtained from STRUCTURE HARVESTER was used as input for CLUMPP [Jakobsson and Rosenberg, 2007] program which permutes replicated matrix into one representative matrix. CLUMPP output was visualized graphically by DISTRUCT [Rosenberg, 2004] program. To test the isolation by distance model (IBD) [Wright, 1943], Mantel test was performed by comparing matrices of genetic and

geographic distance using GenAlEx v. 6.3 software. Matrix of geographic distance was calculated by Universal Transverse Mercator (UTM) method using GPS coordinates. Null hypothesis for IBD model was no correlation between geographic and genetic distance matrices. A specific test was devised to suggest historical barriers to gene flow among collection sites using the program BARRIER version 2.2 [Manni et al., 2004], which uses the geographic coordinates of each collection site and the F_{ST} genetic distances calculated in AFLP-SURV [Vekemans, 2002] as input. Robustness of estimated barriers was tested by means of 100 bootstrapped distance matrices. Geographical map was constructed using GPS mapping software (http://www.eye4software.com).

3.3 Results

3.3.1 *Genetic Diversity*

A total of 72 SRAP primer pairs were initially screened. Fifty five primer-pairs that gave proper reproducible amplification were considered for further analysis. A total of 348 amplicons were produced, out of which 329 (94.3%) were polymorphic. The average number of bands produced by each primer was 6.33, however, polymorphic bands was 5.98 (Table 3.2). The different genetic diversity parameters at the level of sampling sites were highest in the case of PSY (NPL=178, PPL=51.15%, H=0.2027±0.2155, I=0.2961±0.3047) sampling site of Suru and lowest in CHK (NPL=74, PPL=21.26%, H=0.0828±0.1687, I=0.1215±0.2419) of Nubra valley (Table 3.3). Genetic diversity parameters at population level are presented in Table 3.4. The average gene diversity of subpopulations (Hs), which reveals the actual gene diversity present within population, was highest in Suru (0.1834±0.0241) and lowest in Nubra (0.1234±0.0121). However, total gene diversity of population (Ht) was highest in Nubra (0.2447±0.0385) and lowest in Indus (0.2064±0.0352).

3.3.2 *Putative genetic barrier prediction*

The barrier prediction analysis using Monmonier's maximum difference algorithm revealed three likely barriers to gene flow. We considered only those barriers which have 100% bootstrap value. First barrier (aa) was assigned isolating the peripheral HU collection site from rest of the collection site. Second barrier (bb) separated the Nubra valley population from Indus and Suru valley population. Third barrier (cc) was detected within Nubra population between TU-TY and CH-BK collection sites (Figure 3.1 and 3.2).

Table 3.2 Polymorphism revealed by fifty five SRAP primer combinations

Primer combination	Total band	Polymorphic band	Percentage of polymorphic band	Primer combination	Total band	Polymorphic band	Percentage of polymorphic band
Me1/Em1	5	5	100	Me4/Em4	4	4	100
Me1/Em2	7	7	100	Me4/Em5	4	4	100
Me1/Em3	4	3	75	Me4/Em6	5	5	100
Me1/Em4	8	8	100	Me4/Em7	7	7	100
Me1/Em5	6	6	100	Me4/Em8	5	5	100
Me1/Em6	5	3	60	Me4/Em9	6	6	100
Me1/Em7	4	4	100	Me5/Em1	9	9	100
Me1/Em8	4	3	75	Me5/Em2	7	7	100
Me1/Em9	6	6	100	Me5/Em3	7	7	100
Me2/Em1	7	7	100	Me5/Em4	6	5	83.33
Me2/Em2	10	10	100	Me5/Em5	9	7	77.78
Me2/Em3	9	8	88.89	Me5/Em6	5	5	100
Me2/Em4	7	7	100	Me5/Em8	2	2	100
Me2/Em6	9	8	88.89	Me5/Em9	4	4	100
Me2/Em7	7	6	85.71	Me6/Em1	6	6	100
Me2/Em9	10	9	90	Me6/Em2	4	3	75
Me3/Em1	8	8	100	Me6/Em3	4	4	100
Me3/Em2	4	4	100	Me6/Em5	9	9	100
Me3/Em3	6	6	100	Me6/Em6	8	7	87.50
Me3/Em4	10	8	80	Me6/Em7	7	7	100
Me3/Em5	7	7	100	Me8/Em3	6	6	100
Me3/Em6	8	7	87.50	Me8/Em4	7	7	100
Me3/Em7	7	7	100	Me8/Em5	7	7	100
Me3/Em8	5	5	100	Me8/Em6	8	8	100
Me3/Em9	4	3	75	Me8/Em7	6	5	83.33
Me4/Em1	5	5	100	Me8/Em8	9	9	100
Me4/Em2	4	3	75	Me8/Em9	8	8	100
Me4/Em3	3	3	100	Total	348	329	94.326
				Average	6.33	5.98	94.326

Table 3.3 Intra-population gene diversity of fourteen *M. alba* subpopulations belonging to three populations from Ladakh region

Population / Sub-population	Sample size	H (mean +SD)	I (mean +SD)	NPL	PPL
Indus					
SKB	4	0.1555±0.2112	0.2254±0.2991	132	37.93
DM	4	0.1460±0.2032	0.2131±0.2906	127	36.49
ACH	4	0.1447±0.1967	0.2142±0.2829	134	38.51
BM	4	0.1406±0.2069	0.2025±0.2943	115	33.05
SJ	4	0.1114±0.1880	0.1625±0.2699	96	27.59
Suru					
BT	4	0.1729±0.2176	0.2495±0.3075	144	41.38
AKM	4	0.1855±0.2032	0.2753±0.2915	173	49.71
PS	4	0.2027±0.2155	0.2961±0.3047	178	51.15
LO	4	0.1724±0.2068	0.2535±0.2956	155	44.54
Nubra					
CH	4	0.0828±0.1687	0.1215±0.2419	74	21.26
BK	4	0.1182±0.1947	0.1717±0.2771	101	29.02
TU	4	0.1534±0.2090	0.2226±0.2973	130	37.36
TY	4	0.1309±0.1975	0.1914±0.2823	115	33.05
HU	4	0.1316±0.1913	0.1951±0.2762	122	35.06

Table 3.4 Genetic diversity across 3 populations of *M. alba* in Ladakh

Populations	Sample size	NPL	PPL	Hs (mean±SD)	I (mean +SD)	Ht (mean +SD)	G_{ST}
Indus	20	233	66.95	0.1396±0.0183	0.2035±0.2874	0.2064±0.0352	0.3236
Suru	16	257	73.85	0.1834±0.0241	0.2685±0.2998	0.2360±0.0353	0.2231
Nubra	20	259	74.43	0.1234±0.0121	0.1805±0.2750	0.2447±0.0385	0.4958
Average		250	71.74	0.1488±0.2531	0.2175±0.2874	0.2290±0.0363	0.3475
Species level	56	312	89.66	0.2286±0.0262		0.2547±0.0320	0.1026

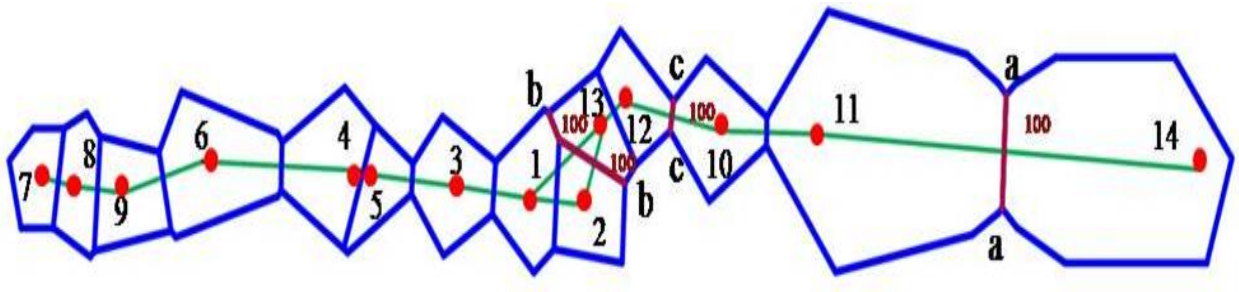

Figure 3.1 Genetic barriers predicted by BARRIER (ver. 2.2). The genetic barriers are shown in bold red lines with arrows, numbers indicate collection sites

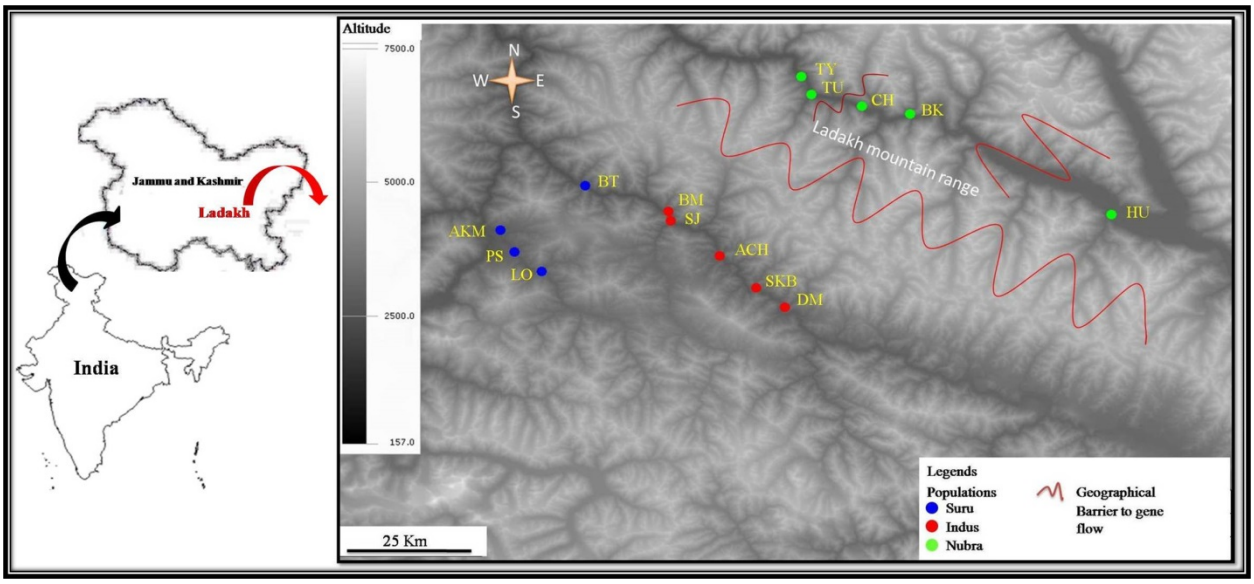

Figure 3.2 Genetic barriers to gene flow in Ladakh map

3.3.3 ***Genetic structure***

Mantel test (Rxy=0.409, *p*=0.02) (Figure 3.3) showed a weak positive correlation between genetic and geographic distance across the sampled region. Mantel test rejected null hypothesis as correlation between genetic and geographic distances was established.

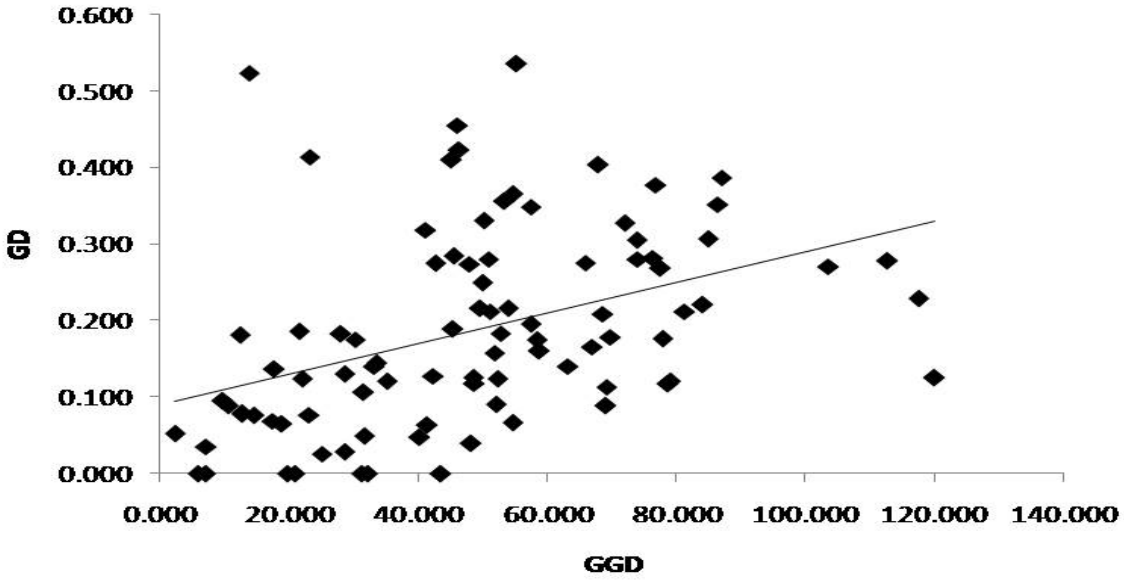

Figure 3.3 Mantel test showing correlation between geographic distance (X-axis) and genetic distance matrices (Y-axis) [Mantel Test (Rxy) =0.409 and *P* value =0.02]

AMOVA at population level based on sampling locations showed a peculiar pattern. Nubra population displayed 67% variability within sampling locations and 33% among sampling locations, whereas Indus and Suru population showed more than 90% variability within sampling sites (Table 3.5). AMOVA at regional level displayed 80% variability within sampling sites and 20% among sampling sites.

Table 3.5 Analysis of molecular variance (AMOVA) for fourteen subpopultions of *M. alba* distributed in three valleys of Ladakh

Group	Partitioning	d.f	SSD	MSD	Estimated variance	Variance (%)	Φ_{PT} (*p* value)	Nm
At population level								
Indus	Among collection sites	4	179.7	44.92	2.781	8%	0.076 (0.04)	3.04
	Within collection sites	15	507	33.8	33.8	92%		
	Total	19	686.7		36.58	100%		
Suru	Among collection sites	3	144.81	48.22	0.911	2%	0.02 (0.029)	12.25
	Within collection sites	12	535.5	44.62	44.62	98%		
	Total	15	680.31		45.54	100%		
Nubra	Among collection sites	4	389.35	97.33	16.03	33%	0.325 (0.01)	0.52
	Within collection sites	15	498.5	33.23	33.23	67%		
	Total	19	887.85		49.26	100%		
At regional level	Among collection sites	13	954.39	73.41	9.19	20%	0.2 (0.01)	
	Within collection sites	42	1539.5	36.65	36.55	80%		
	Total	55	2493.89		45.84	100%		

To investigate population structure at regional level, we applied Bayesian model based clustering algorithm implemented in the STRUCTURE program, which estimate the shared population

ancestry of individuals purely on the basis of genetic data without considering population location information. The log probability of data and Evanno's method of cluster determination,

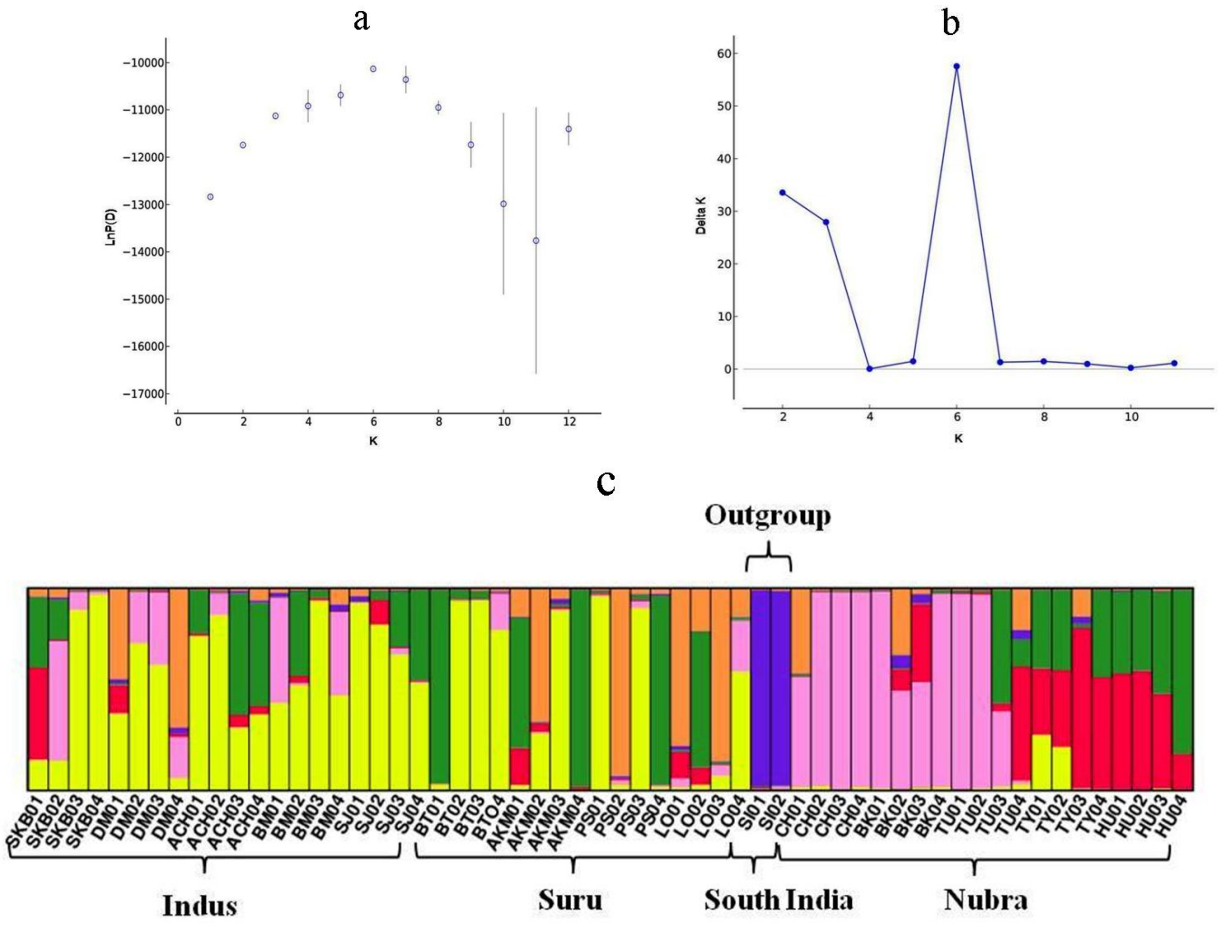

Figure 3.4 STRUCTURE analysis *M. alba* populations [(a) Relationship between K & LnP(D), (b) Relationship between K & delta K and (c) Membership probability of assigning individuals of the all populations to different clusters when K=6]

detected six (ΔK=6) clusters (Figure 3.4a and b), suggesting that the 58 individuals partitioned into six clusters (Figure 3.4c). STRUCTURE analysis separated samples from south India as outgroup and divided Ladakh populations into five clusters. Indus and Suru populations revealed admixture while Nubra displayed clear cut structuring.

3.3.4 ***Genetic differentiation and gene flow***

Overall genetic differentiation coefficients (Φ_{PT}=0.20 and G_{ST}=0.1026) showed high and significant differentiation. G_{ST} varies from 0.2231 (Suru) to 0.4958 (Nubra) and Φ_{PT} from 0.02 (Suru) to 0.325 (Nubra). Both genetic differentiation coefficients revealed higher fixation in

Nubra population and lower in Suru. Number of migrants per generation calculated on the basis of Φ_{PT} displayed higher migration rate in Suru population with 12 migrants per generation whereas no migrants was observed in Nubra population (Nm=0.52) (Table 3.4 and 3.5).

3.4 Discussion

3.4.1 *SRAP marker system*

SRAP marker system has several advantages over other molecular marker systems such as simplicity, reasonable throughput rate, disclosure of numerous co-dominant markers, ease of isolation of bands for sequencing and most importantly, targeting of open reading frame (ORFs) [Li and Quiros, 2001]. Budak et al. [2004] showed that SRAP analysis is more powerful than SSR, ISSR or RAPD markers for detection of genetic diversity among closely related cultivars. According to Ferriol et al. [2003], the information derived from SRAP marker is more concordant to the morphological variability and to the evolutionary history of the morphotypes than that of AFLP in genetic diversity analysis. This marker system has been recognized as a new and useful molecular tool in assessing population structure and genetic polymorphism in many plant species [Ding et al., 2008]. Therefore, SRAP markers were used in the present study to evaluate the genetic diversity and population genetic structure of wild *M. alba* populations. The 55 SRAP polymorphic markers used were highly informative. The level of polymorphism obtained (94.3%) was higher than previous reports using RAPD, ISSR and AFLP studies [Bhattacharya and Ranade, 2001; Vijayan et al., 2004c; Awasthi et al., 2004; Kafkas et al., 2008]. Fifty five combinations of primer pairs produced high number of polymorphic bands (329) among 56 individuals, which indicate utility of SRAP markers for genetic diversity evaluation of natural *M. alba* populations.

3.4.2 *Genetic diversity*

Accurate estimates of genetic diversity are useful for optimizing sampling strategies and for conservation and management of various trees species [Hamrick and Godt, 1996]. Analysis in the present study revealed overall high genetic diversity at species level meaning that there is large difference in intra-population gene diversity and total gene diversity. However, genetic diversity in Nubra population was low as revealed by Hs (average gene diversity of subpopulations). This signifies that Nubra population is structured. Indus population displayed low genetic diversity but higher than Nubra. Suru population showed the highest level of genetic diversity and least difference in Ht and Hs values.

High genetic diversity at species level was in accordance with previous studies [Cao et al., 2006; Zhou et al., 2012; Pometti et al., 2012]. Percentage of polymorphic loci (PPL) across studies revealed high genetic diversity within the species. Bhattacharya and Ranade [2001] reported 85% and 91% PPL using RAPD and DAMD marker, respectively, in various *Morus* species. Srivastava et al. [2004] reported 74.13% and 60.75% PPL in *M. alba* using ISSR and RAPD markers, respectively. Vijayan et al. [2004c] reported PPL=86%, Ht=0.27 and PPL=78%, Ht=0.23 using ISSR and RAPD markers, respectively, in *M. alba* and other *Morus* species. Studies in Turkey [Kafkas et al., 2008] and China [Zhao et al., 2004 and 2007a] also reported high genetic diversity. Genetic diversity within natural populations is directly linked to their mating system, life form, pollen dispersion syndrome (relative strength of pollen vs. seed dispersal), mechanism of seed dispersal, geographic range and also to their effective population size [Hamrick, 1983; Hamrick and Godt, 1996]. *M. alba* is outbreeding and wind pollinated species. The pollen release is fastest in the plant kingdom, which is about half the speed of sound [Taylor et al., 2006]. Outbreeding woody species have high within population genetic diversity than plants with inbreeding and herbaceous life forms [Hamrick and Godt, 1996]. The proportion of gene flow via pollen dispersal compared to seed dispersal is higher in wind pollinated trees [Squirrell et al., 2001]. Moreover, pollen often disperses farther than seeds and in greater quantities [Kremer et al., 2012]. Ability of plants to disperse pollen grains at very high velocities is an ancient evolutionary adaptation that enhances wind pollination in unfavorable environmental conditions [Taylor et al., 2006], such as the trans-Himalaya region which is characterized by prolonged sub zero temperatures, low precipitation, sandy soils with low water holding capacity, low relative humidity, intense solar radiation, low atmospheric pressure, high wind velocity and rugged terrain. Schuster et al. [1994] reported high genetic diversity in outbreeding tree species in cold and arid environments. Spatially the cold arid land is characterized by differences in substrate, soil structure, slope and aspect that create a mosaic of microhabitats and vegetation patterns [Parker, 1991]. Theoretical and empirical studies have indicated that such environmental heterogeneity can lead to maintenance of high levels of within population genetic variation [Hedrick et al., 1976; Ewing, 1979; Gillespie and Turelli, 1989; Yeaman and Jarvis, 2006]. So it is not unreasonable to believe that *M. alba* has maintained high level of genetic diversity due to environmental heterogeneity.

3.4.3 ***Population structure and geographical barrier***

Geographic isolation is one of the major constraints to gene flow via both pollen and seeds, which results in genetic differentiation of populations [Pfeifer and Jetschke, 2006]. Mantel test displayed weak positive correlation between genetic and geographic distance matrix. Therefore, isolation by distance model does not fit completely in the present study. Genetic clustering based on Bayesian model predicted six clusters; only five clusters were of interest as one cluster represents outgroup formed by south Indian genotypes. The five clusters displayed interesting pattern. Individuals from Indus and Suru populations show co-ancestry while Nubra population structured into subpopulations. Indus and Suru population clearly fits into admixture model which we considered during STRUCTURE analysis. Coancestry in Indus and Suru genotypes indicated presence of good migration pattern, which was confirmed by significant number of migrants per generation (Indus=3 and Suru=12 per generation). BARRIER programs predicted four gene pools; first consist of only HU collection site, second of Indus and Suru populations, third of BKD and CHK and fourth of TRK and TYK. When we clubbed STRUCTURE and BARRIER, then four genetic populations emerged. Indus and Suru populations merged into one populations and Nubra population structured into three genetic subpopulations namely: TRK-TYK, BKD-CHK and HUN. Structuring in Nubra was also confirmed by high genetic differentiation coefficient (Φ_{PT}=0.325 and G_{ST}=0.4958) and nil migrants (Nm=0.52) per generation. Nubra population showed isolation by distance, as nearby collection sites merged into same subpopulation but distant collection sites formed separate subpopulation. But Indus and Suru population showed co-ancestry due to which overall Mantel test correlation was weak. AMOVA results were also in concordance with STRUCTURE and BARRIER results. Indus and Suru populations exhibited over 90% variability within samples which displayed high gene flow and outbreeding, whereas Nubra population showed 33% variability among subpopulations and 67% within subpopulations, which indicate structuring in Nubra population. Pattern appeared in STRUCTURE, BARRIER and AMOVA results clearly demonstrated that there is gene flow between Indus and Suru population while there is geographical barrier between Indus-Suru and Nubra population that effectively hinders gene flow. Geographical barrier predicted between Indus-Suru and Nubra populations by BARRIER was supported by the existence of the Ladakh mountain range (Figure 3.2). The separation of Nubra and Indus-Suru population may be the result of past vicariance event. These results

showed that Ladakh mountain range is effective barrier to gene flow. Migration of Mulberry is also influenced by human migration pattern. Historically, Ladakh mountain range was great barrier to human movement. The mountain range therefore plays an important role in shaping genetic structure of *M. alba* in trans-Himalaya.

Divergence within Nubra valley population may be due to fact that HUN subpopulation is separated from other Nubra sampling sites by 40-60 Km distance. The long distance may act as geographical barrier, and hence isolation by distance was observed. BKD-CHK subpopulation is located on eastern side of Shyok River and TRK-TYK subpopulation site is on western side as shown in geographical map (Figure 3.2). Shyok River may be the natural barrier which corresponds to historical barrier predicted by BARRIER program and separates BKD-CHK and TRK-TYK subpopulations. This may be responsible for occurrence of structure and two geographical barriers among collection sites in Nubra valley. Our initial classification of three populations based on three valleys does not hold correct. Actually there are four gene pools or genetic populations of *M. alba*.

3.4.4 ***Genetic differentiation and gene flow***

Correct estimation of population differentiation is essential in conservation biology, as it gives an idea about how much populations are genetically isolated from each other and to what extent [Balloux and Lugon-Moulin, 2002]. In the present study we used two types of genetic differentiation coefficient to get correct approximation of genetic differentiation scenario. G_{ST} was derived by Nei [1973], which can be defined as the ratio of the inter-sub-populational gene diversity to the total gene diversity. Φ_{PT} [Peakall and Smouse, 2006] is a measure of population genetic differentiation for binary data and is analogous to Wright's F_{ST}.

Overall all genetic differentiation was high in this region. Especially, Nubra population showed greater genetic differentiation, which reveals high among population diversity in Nubra population. High differentiation in Nubra population confirmed the population sub structuring revealed by STRUCTURE, BARRIER and AMOVA. However, Suru population showed high gene flow within population with low genetic differentiation. According to Waples and Gaggiotti [2006], when Nm=1-5, genetic differentiation is strong to moderate. Therefore, gene flow also showed strong differentiation in Nubra population, moderate in Indus and negligible in Suru.

The main evolutionary forces leading to genetic differentiation between natural populations are generally considered to be natural selection, random genetic drift and limited

migration [Baines et al. 2004]. These results clearly showed role of restricted to moderate gene flow in contributing towards genetic differentiation. Natural selection can be assumed as these plants are growing in variety of microhabitats in this heterogeneous climate. Genetic drift is also one of the factors which cannot be ruled out in such unpredictable climate. Natural selection, genetic drift, and gene flow do not act in isolation in natural populations [Andrews, 2010]. So, the apparent genetic differentiation is the result of interplay between all three factors *viz*. natural selection, genetic drift and gene flow. Yeaman and Jarvis [2006] suggested that migration-selection balance is important factor in maintaining genetic diversity in heterogeneous environments. The present study revealed overall high genetic diversity and significant amount of differentiation. So it appears that genetic diversity is maintained because migration and genetic differentiation both are present and balancing each other. Andrews [2010] also stressed that balancing selection, in contrast to directional selection, maintains genetic polymorphism in populations. It means, despite significant structuring and differentiation, *M. alba* still maintains high level of genetic diversity, which is in concordance other similar studies on tree species [Suarez-Montes et al., 2011; Melendez-Ackerman et al., 2005].

3.5 Implications for conservation

The negative effect of fragmentation on genetic diversity is the main basis for conservation efforts made in recent past [Ouborg et al., 2006]. To formulate effective conservation strategies it is essential to study patterns of plant response to habitat fragmentation. The present study displayed high levels of genetic diversity with significant population genetic structure and genetic differentiation in *M. alba*. But genetic structure predominant in Nubra population and therefore population from Nubra valley should be given priority while devising management procedures. Considering significant genetic structure and limited gene flow in Nubra valley, conservation strategies should be aim at *in situ* conservation since habitat fragmentation and small population size make it vulnerable to the loss of genetic diversity caused by the effects of genetic drift and inbreeding. Besides, balance between genetic differentiation and migration proved to be important factor in maintaining genetic diversity. Conservation measure should therefore be such that it conserves both heterogeneous landscapes and historical levels of gene flow. All *M. alba* plants grow wild in trans-Himalayas with no conservation measures; so here we propose the establishment of new populations in areas like farmers field and under Governmental protection. Another important measure is to carry out surveys to

uncover more individuals and populations in other localities. Additionally, in some regions like Nubra valley where possibility of habitat destruction or exploitation of plants for local consumption are high, it is necessary to establish sustainable management plans through local government agencies and Non-Government Organizations (NGO's). Considering the critical situation of *M. alba*, probably the safest way to preserve the species is through all of the methods mentioned above.

3.6 Conclusion

In conclusion, mixed population structure appeared in this study. Nubra population clearly displayed structuring with three subpopulations. But Indus and Suru population did not display any significant structuring. Ladakh mountain range proved an important barrier to gene flow and shaped population genetic structure of *M. alba* in the trans-Himalaya. The results shed light on role of geographical barriers in speciation. The genetic diversity have important role in plants survival as it provides disease resistance, adaptability to a changing climate, or some other trait necessary to survive in the ever-changing world. The present study reported overall high genetic diversity at species level. There is balance between migration and genetic differentiation that helps in maintaining genetic diversity. These results also have evolutionary significance as Himalayan region is considered as centre of diversity for Mulberry. Furthermore, our results provide a basic genetic profile for conservation and responsible exploitation of the extant germplasm of this species to improve the genetic base for breeding. SRAP markers are highly reproducible and efficient markers for assessment of genetic structure and diversity among wild populations.

CHAPTER 4

DETECTING MOLECULAR SIGNATURES OF NATURAL SELECTION IN *M. ALBA* L. POPULATIONS

Abstract

Genome scan was done with 439 dominant marker loci to identify outlier loci in three populations of *M. alba* L. from the trans-Himalayan region. Employing two population genetics model *viz.* finite and hierarchical island model, we detected 30 (6.85%) loci under selection of which 3.64% was under directional selection and 3.19% under balancing selection. Hierarchical island model was efficient in detecting signatures of natural selection with low occurrence of false positives. Data showed significant spatial genetic structure (SGS), especially in population from Nubra valley with significantly high SGS. Bayesian approach revealed high genetic differentiation and inbreeding in Nubra population. Indus and Suru valleys populations showed comparatively low SGS, genetic differentiation and inbreeding. The study showed that *M. alba* falls in the range of mixed mating and outcrossing species. However, population from Nubra valley showed selfing with high SGS. Significant SGS strengthens the presence of natural selection in *M. alba* natural populations.

4.1 Introduction

Unleashing the genetic basis of adaptation to different environment is the central topic in evolutionary biology [Storz, 2005]. The detection of signatures of natural selection within the genome of organisms is key, since it may allow a greater understanding of what proportion of a genome or which genes are being shaped by ongoing natural selection [Joost et al., 2007]. Recent advances in the fields of molecular and computational biology techniques led to the emerging field of 'population genomics', whose main objective is to characterize the parts of the genome subject to natural selection [Foll and Gaggiotti, 2008]. Such studies are also of fundamental biological importance because they can reveal the very nature of adaptation and speciation [MacCallum and Hill, 2006]. Additionally, ignoring the effect of natural selection in evolutionary studies can lead to wrong estimates of the demographic history of species. Therefore, separating the effect of neutral drift and adaptive genetic differentiation is a necessary preliminary step in most analyses of genome wide data sets, and this distinction can also help us to understand speciation processes [Foll and Gaggiotti, 2008].

A wide variety of methods have been developed to reveal genomic regions that are likely to be under natural selection [Vasemagi and Primmer, 2005]. Some of them belong to 'candidate gene' approach and takes particular locus as a starting point and assess whether it has been affected by selection [Phillips, 2005; Wright and Gaut, 2005]. Another category of methods aims at identifying 'quantitative trait loci' (QTL) involved in expression of adaptive traits [Mackay, 2001; Erickson, 2004]. However, such approaches have proved helpful but their application is limited to relatively few well studied model species [Phillips, 2005]. Further, such approaches usually require prior information that may not be easily possible for non-model organisms [Joost et al., 2007]. But with the recent advancement in the field of population genomics, alternative strategies are now available which can identify regions of the genome that have been subjected to natural selection. The underlying principle of the methods based on population genomics data is that directional selection acting on a locus increases population divergence, while balancing selection keeps it low [Lewontin and Krauker, 1973]. Hence, methods based population genomics data identify loci that show atypical values of genetic differentiation (Wright's F_{ST}) coefficients that are significantly different from those expected under the neutral theory [Foll and Gaggiotti, 2008].

The most commonly used analytical approaches of genome scan for dominant markers is FDIST, which was originally developed by Beaumont and Nichols [1996]. It employs a classical Wright's island model to generate the expected neutral distribution of F_{ST} estimates [Wright, 1951; Helyar et al., 2011]. Besides, a Bayesian approach [Beaumont and Balding, 2004; Foll and Gaggiotti, 2008] was also proposed, which assumes that allele frequencies within population follow a multinomial Dirichlet distribution with F_{ST} parameters that are a function of population-specific components shared among all loci and of locus-specific components shared among all populations. However, the above two approaches does not considers hierarchical structure, if present in populations. Further, Bayesian methods explicitly allow sampled populations to receive unequal number of migrants from migrant pool, but still assume that migrant genes originate from the same pool [Excoffier et al., 2009]. In this study, we used hierarchical island model [Slatkin and Voelm, 1991] approach proposed by Excoffier et al. [2009] as an extension to FDIST, in which populations samples are assigned to different groups, and allowing for different migration rates between populations within groups and between groups. Excoffier et al. [2009] suggested that hierarchical structure, if present in populations should be considered since it reduces the level of false positives in outlier detection. They also pointed out that genetic structure can be determined by using program like STRUCTURE proposed by Pritchard et al. [2000]. Genetic structure is present in *M. alba* populations in the trans-Himalaya [Bajpai et al., 2014] therefore, we considered hierarchical island approach of Excoffier et al. [2009] more suitable for the present study.

Fine-scale spatial patterns of genetic variation in plant populations result from the complex interplay of local dispersal with genetic drift and other evolutionary processes such as natural selection [Slatkin, 1973; Epperson, 1990; Rousset, 2004]. Therefore, we explored the spatial genetic structure (SGS) present among *M. alba* individuals to study the effect of natural selection. The most common way of estimating SGS is *Sp* statistic. The development of the *Sp* statistic for quantification of SGS based on the decay of inter-individual kinship with spatial distance [Vekemans and Hardy, 2004] has eased the research into the factors governing SGS. The estimation of SGS from dominant marker data required prior information of inbreeding coefficient. So, in the present study we used Bayesian approach proposed by Holsinger et al. [2002] to calculate inbreeding and genetic differentiation.

In trans-Himalayan Ladakh region of India, *M. alba* is found at 2700-3200 m asl. The trans-Himalayan region is characterized by prolonged sub zero temperatures, low precipitation, sandy soils with low water holding capacity, low relative humidity, intense solar radiation, low atmospheric pressure, high wind velocity and rugged terrain. These harsh environmental conditions pose great amount of selective pressure for plant survival. Selection induces a very particular signature in terms of genetic diversity [Nielson, 2005; Storz, 2005]. The directional selection acting on a locus increases population divergence, while balancing selection keeps it low (Lewontin & Krauker, 1973). The Himalayan region is considered as the center of diversity for mulberry, so studying signatures of natural selection in mulberry population in this part of the world has evolutionary significance. Therefore in the present study we investigated signatures of selection in natural population of *M. alba* L. in trans-Himalayan region. To further explore the natural selection, we estimated SGS. Two dominant markers: ISSR [Zietkiewicz et al., 1994] and SRAP [Li and Quiros, 2001] were used for detection of outlier locus in *M. alba* genome. In this study we aimed to quantify loci which are under selection (directional and balancing selection) and secondly to estimate SGS present among *M. alba* individuals. To the best of our knowledge, this is the first report on detection of candidate loci under selection by genome scan in *M. alba*.

4.2 Materials & methods

4.2.1 *PCR amplification*

Forty ISSR primers were initially screened and twenty primers were selected based on amplification and reproducibility (Table 4.1). Each 20-µL PCR reaction consisted of 2 mmol/L dNTPs, 10 mmol/L of each forward and reverse primer, 0.75 U Taq polymerase, 25 ng template DNA, Taq buffer containing tris with 15 mmol/L $MgCl_2$ and Milli-Q water. Amplification condition for ISSR marker was: initial denaturation for 5 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at specific annealing temperature (±5°C of Tm), 2 min at 72 °C and a 10 min final extension step at 72°C. Amplification products were electrophoresed on 1.5% agarose gel and molecular size of amplicons was estimated using 100 bp and 1 Kb DNA ladders.

Table 4.1 List of primers used for ISSR amplification and level of polymorphism

Primers	Primer sequence (5'-3')	Total number of loci	Number of polymorphic loci	Percentage of polymorphic loci	Total number of fragments amplified
ISSR 2	$(AG)_8T$	3	0	0	180
ISSR 3	$(GA)_8T$	4	4	100	178
ISSR 4	$(CT)_8T$	5	4	80	240
ISSR 7	$(AC)_8T$	5	4	80	184
ISSR 8	$(TG)_8A$	4	4	100	118
ISSR10	$(AG)_8YT$	4	4	80	166
ISSR12	$(GA)_8YT$	7	7	100	335
ISSR15	$(ACC)_6$	5	5	100	182
ISSR16	$(CCG)_6$	5	5	100	272
ISSR21	$(GA)_8C$	5	5	100	182
ISSR22	$(TC)_8C$	9	9	100	321
ISSR23	$(AG)_8CT_2$	5	5	100	243
ISSR26	$(GACA)_4$	6	6	100	214
ISSR27	$(AGT)_2ACG(TC)_7$	1	1	100	50
ISSR 29	$(GA)_8A$	3	3	80	62
ISSR30	$(AC)_8C$	5	4	80	197
ISSR31	$(AG)_8YA$	4	4	100	210
ISSR32	$(GA)_8YC$	3	3	100	159
ISSR33	$(AC)_8YG$	5	5	100	169
ISSR34	$(TG)_8RC$	3	3	100	113
	Total	91	85	93.41	3775

4.2.2 ***Data collection and analysis***

4.2.2.1 ***Data collection***

The banding patterns obtained from ISSR and SRAP (chapter 3) were scored as present (1) or absent (0), each of which was treated as an independent character. The total numbers of loci obtained from ISSR and SRAP markers were pooled for analysis. We combined ISSR and SRAP markers to increase genome coverage, as two markers targets different region of genome. Several previous studies showed that ISSR and SRAP markers can be combined for population genetics studies (Budak et al., 2004; Tang et al., 2010; Liao et al., 2012; Wu et al., 2014) and some studies reported that integration of ISSR and SRAP is more effective in detecting genomic variation (Tang et al., 2010; Liao et al., 2014).

4.2.2.2 ***Genome scan analysis***

Genome scan analysis was performed to detect outlier loci under natural selection. The null hypothesis was that no loci under natural selection based on neutral allele model of Kimura [1968]. The outlier loci were detected under two genetic models *viz.* finite island model and hierarchical island model.

4.2.2.2.1 ***Finite island model***

FDIST approach of Beaumont and Nichols (1996) was commonly used to apply finite island model. MCHEZA [Antao and Beaumont, 2011] program was used to apply finite island model, it implements DFDIST approach, which is a modification of FDIST for dominant markers. MCHEZA is better than previous approaches as MCHEZA introduces support for multi-test correction based on false discovery rates (FDR) [Benjamini and Hochberg, 1995], as implemented in Chiurugwi et al. [2010]. Without such a correction there is a danger in over-estimating the proportion of loci that are under selection [Beaumont, 2008; Pérez-Figueroa et al., 2010]. In MCHEZA a total of 50 000 realizations was performed and maximum allowable allele frequency was 0.99. We chose 0.99 confidence intervals and set the significance level at 99%. The Benjamini and Hochberg false discovery rate (FDR) correction method was used to correct for the occurrence of false positives in loci identified as under selection [Benjamini and Hochberg, 1995]. Loci with significant *p*-values at FDR threshold of 0.01 were identified using the Benjamini and Hochberg [1995] method. The finite island model was also implemented in ARLEQUIN software package [Excoffier and Lischer, 2010]. Finite island model analysis was carried out by assuming the presence of 100 demes with 20,000 simulated loci.

4.2.2.2.2 ***Hierarchical island model***

The hierarchical island model was applied using approach of Excoffier et al. [2009], which is a modification of the model proposed by Beaumont and Nichols [1996]. In hierarchical island model we considered structure based on our previous study [Bajpai et al., 2014]. The four structures formed from populations of three valleys *viz.* Indus, Nubra and Suru are Indus-Suru, Chalunka-Bokdang, Turtuk-Tyakshi and Hunder. Indus-Suru is formed by merger of populations from Indus and Suru valleyes. Other three structures are formed by division of populations from Nubra valley. According to Excoffier et al. (2009), if the data are analyzed without taking the hierarchical structure into account, large false positives are obtained at all significance levels because of narrower simulated null distributions under the finite island model, especially loci

under balancing selection is considerably overestimated. Therefore, the hierarchical island model minimizes the number of false positives to a great extent. Hierarchical analysis was carried out by assuming the presence of 10 groups of 100 demes with 20,000 simulated loci. In ARLEQUIN outlier loci were detected at *p* values of 0.01 and 0.05. For finite island model we considered only those loci which were detected at $p \leq 0.01$ and for hierarchical island model at $p \leq 0.05$. To minimize the detection of likely false positives, we considered only those loci to be under selection which were found common in both outlier methods. Those loci identified by only one method were considered to be likely false positives. A separate analysis also carried out for Indus-Suru and Nubra populations to see extent of loci under directional and balancing selection.

4.2.2.3 ***Calculation of inbreeding and genetic differentiation***

Bayesian approach of Holsinger et al. [2002], which allows a direct estimate of F_{ST} and F_{IS} from dominant markers data was used. The f (analogous to F_{IS}) and θ^B (analogous to F_{ST}) statistics were calculated under different models implemented in the HICKORY version 1.1 program [Holsinger and Lewis, 2007]. We tested four models using Hickory version 1.1: (i) a full model with non-informative priors for f and θ^B, (ii) a model in which f=0 and (iii) a model in which θ^B=0 and (iv) f-free model. Several runs were conducted with default sampling parameters (burn-in=50 000, sample=250 000, thin=50) to ensure consistency of results [Tero et al., 2003]. The four models were compared using the deviance information criterion (DIC) [Holsinger and Wallace, 2004]. Model with smaller DIC is preferrable but a difference of >6 DIC units among different models is required to indicate that there is strong favoring of one model over another [Holsinger and Lewis, 2003]. Holsinger and Wallace [2004] suggested that there is need to pay attention not only to DIC, but also to Dbar and pD in deciding among models. Here we used pD (measure of model complexity) to decide among models when there is very little difference in DIC values of different models. A model with smaller pD was preferred.

4.2.2.4 ***Estimation of spatial genetic structure (SGS)***

The SGS was assessed using a multi-locus kinship coefficient. The analyses were performed using the SPAGeDi ver. 1.3 software [Hardy and Vekemans, 2002]. Kinship coefficient was estimated according to Hardy [2003]. SPAGeDi need a inbreeding coefficient to be provided. We calculated inbreeding coefficient using HICKORY ver. 1.1. In order to illustrate

the intensity of the SGS, we estimated *Sp* statistic [Vekemans and Hardy, 2004], $Sp=-b_1 / (1-f_{ij(1)})$, where b_1 is the slope of a log-linear regression between observed kinship and a distance between individuals, and $f_{(1)}$ is the average kinship for the first distance class. All standard errors were estimated by jackknife procedure over loci.

4.3 Results

4.3.1 *Genome scans for detection of outlier loci*

Genome scan was performed on combined 439 ISSR and SRAP loci (Table 3.2 and 4.1). MCHEZA detected total 90 loci under selection out of which 27 were under directional and the remaining 63 loci were under balancing selection (Figure 4.1 and Table 4.2). ARLEQUIN in finite island model detected total 61 loci under selection out of which 13 were under directional and 48 loci were under balancing selection (Figure 4.2 and Table 4.2). In hierarchical structure we detected 65 loci (14.8%) under selection of which 20 loci (4.56%) were under directional selection and 45 loci (10.25%) were under balancing selection (Figure 4.3, Table 4.2).

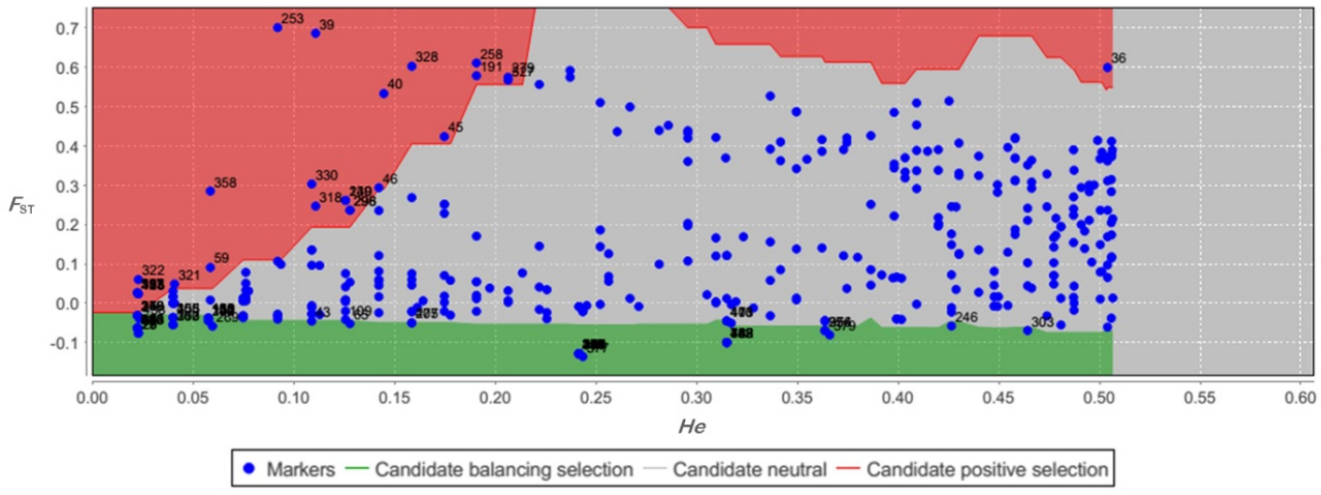

Figure 4.1 F_{ST}-outlier analysis performed using MCHEZA program under finite island model for the 439 SRAP+ISSR loci. Loci were identified as significant, corresponding to a 99% confidence interval and 0.01 false discovery rate (FDR). Each blue dot indicates marker loci; dots in gray areas represent neutral loci, whereas dots in red (under directional selection) and green (under balancing selection) areas represent outlier loci accompanied by their locus numbers.

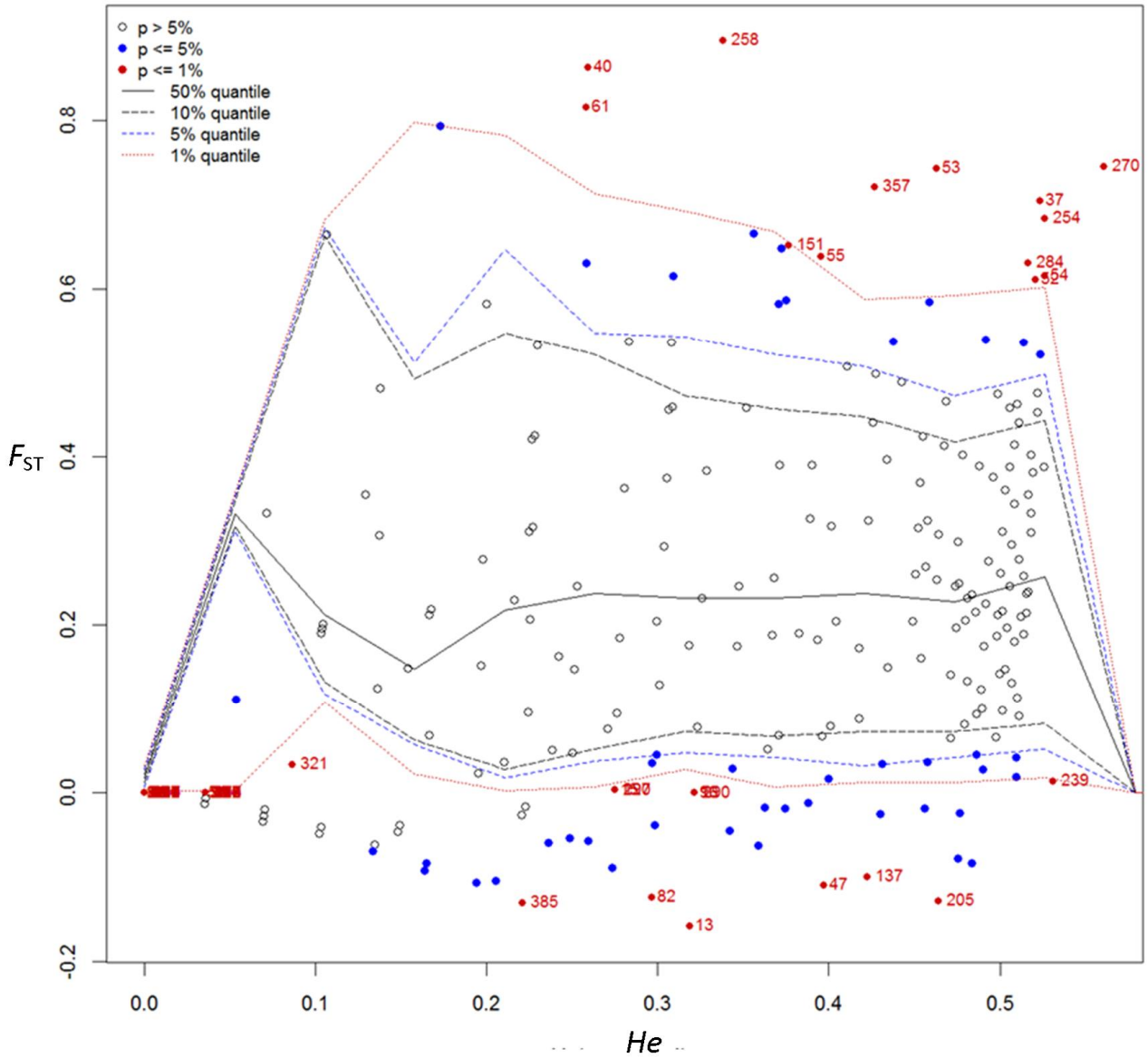

Figure 4.2 F_{ST}-outlier analysis performed using ARLEQUIN program under finite island model for the 439 SRAP+ISSR loci. The contour line indicates the $p \leq 0.05$ and $p \leq 0.01$ quantiles corresponding to the 95 and 99% confidence intervals. Observations outside these ranges were considered as having significantly higher or lower F_{ST} than expected under a neutral model indicating directional or balancing natural selection, respectively.

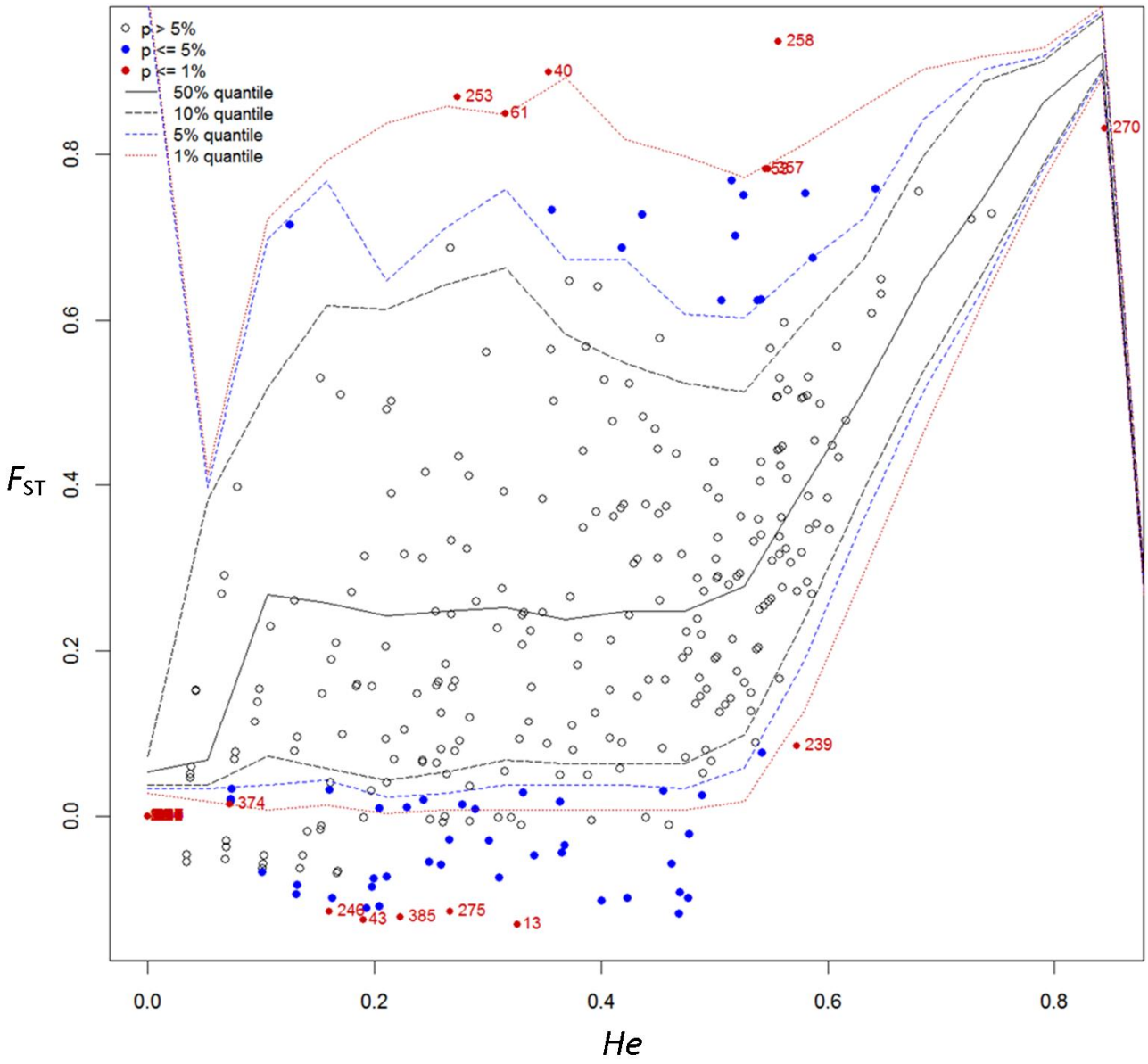

Figure 4.3 F_{ST}-outlier analysis performed using ARLEQUIN program under hierarchical island model for the 439 SRAP+ISSR loci. The contour line indicates the $p \leq 0.05$ and $p \leq 0.01$ quantiles corresponding to the 95 and 99% confidence intervals. Observations outside these ranges were considered as having significantly higher or lower F_{ST} than expected under a neutral model indicating directional or balancing natural selection, respectively.

Table 4.2 Detection of outlier loci under two different genetic models

SOFTWARE/MODEL/SELECTION	OUTLIER LOCUS IDENTIFIED
MCHEZA	
INFINITE ISLAND MODEL ($p \leq 0.01$)	
Directional	5, 36, 39, 40, 45, 46, 59, 125, 130, 157, 163, 191, 249, 253, 258, 279, 293, 296, 298, 317, 318, 321, 322, 327, 328, 330, 358=27
Balancing	20, 25 26, 27, 29, 34, 35, 43, 65, 74, 91, 94, 97, 99, 103, 107, 109, 132, 133, 146, 150, 155, 168, 171, 172, 178, 184, 188, 192, 202, 208, 210, 223, 224, 234, 246, 256, 269, 275, 278, 300, 303, 307, 344, 348, 349, 353, 354, 355, 363, 377, 379, 382, 386, 403, 407, 408, 412, 416, 425, 426, 430, 438=63
ARLEQUIN	
INFINITE ISLAND MODEL ($p \leq 0.01$)	
Directional	37, 40, 52, 53, 54, 55, 61, 151, 254, 258, 270, 284, 357=13
Balancing	5, 13, 15, 16, 20, 25, 34, 35, 47, 82, 90, 91, 94, 99, 103, 137, 146, 157, 172, 178, 184, 190, 198, 202, 205, 208, 223, 224, 227, 239, 278, 290, 293,300, 307, 317, 321, 334, 342, 344, 348, 349, 353, 356, 385, 386, 426, 430=48
HIERARCHICAL ISLAND MODEL ($p \leq 0.05$)	
Directional	36, 37, 39, 40, 45, 46, 53, 55, 59, 61, 62, 85, 127, 151, 247, 253, 258, 270, 284, 357=20
Balancing	4, 13, 16, 19, 23, 24, 43, 47, 65, 72, 82, 86, 89, 120, 137, 147, 160, 169, 173, 179, 201, 203, 205, 227, 233, 238, 239, 246, 275, 276, 282, 292, 294, 310, 333, 341, 357, 371, 374, 376, 379, 384, 385, 433, 437=45
COMMON LOCI	
Directional	36, 37, 39, 40, 45, 46, 53, 55, 59, 61, 151, 253, 258, 270, 284, 357=16
Balancing	13, 16, 43, 47, 65, 82, 137, 205, 227, 239, 246, 275, 379, 385=14

We found 30 common loci under selection that were present in hierarchical island model and also present in one of the finite island model analysis (Table 4.2). Out of 30 loci, 16 loci (3.64%) were under directional selection and remaining 14 loci (3.19%) were under balancing selection.

4.3.2 ***Inbreeding and population differentiation***

Using Bayesian approach, the *f* and θ^B values obtained from the four different models are presented in Table 4.3. At population level full model was preferred for Indus and Suru populations with DIC values of 3360.01 and 3150.66, respectively. But for Nubra population,

Table 4.3 Wright's *F* statistics calculated for populations of *M. alba* in the trans-Himalaya, India under different models based on a Bayesian approach without prior knowledge of inbreeding coefficient

Models	*f*				θ^B				DIC	pD
	Mean	SD	2.5 %	97.5 %	Mean	SD	2.5 %	97.5 %		
Indus										
Full model	0.319	0.169	0.052	0.732	0.077	0.015	0.05	0.109	3360.01	432.038
f =0 model	--	--	--	--	0.066	0.011	0.044	0.089	3388.98	469.206
θ=0 model	0.522	0.083	0.366	0.693	--	--	--	--	3587.64	241.479
f-free model	0.501	0.286	0.028	0.974	0.121	0.016	0.091	0.153	3392.04	525.911
Nubra										
Full model	0.185	0.124	0.011	0.479	0.295	0.022	0.254	0.341	3339.09	678.095
f =0 model	--	--	--	--	0.270	0.017	0.238	0.303	3364.81	714.367
θ=0 model	0.722	0.088	0.553	0.899	--	--	--	--	4731.48	264.124
f-free model	0.500	0.289	0.025	0.976	0.336	0.018	0.300	0.371	3339.52	660.202
Suru										
Full model	0.401	0.208	0.059	0.869	0.030	0.012	0.007	0.057	3150.66	316.962
f =0 model	--	--	--	--	0.025	0.01	0.007	0.045	3170.47	339.269
θ=0 model	0.872	0.075	0.710	0.989	--	--	--	--	3223.85	252.777
f-free model	0.504	0.29	0.026	0.978	0.071	0.014	0.044	0.099	3213.14	444.567

f =analogous to Wright's $F_{IS;}$ θ^B=analogous to Wright's F_{ST}, DIC =deviance information criterion; pD=measure of model complexity and SD=standard deviation.

two out of the four models (*f*-free model and full model) have same lower values of DIC and therefore model was selected based on pD value. The *f*-free model showed lower pD value (660.202) compare to full model, therefore *f*-free model was selected for Nubra population. The Nubra population had highest value of *f* and θ^B. The point estimate of *f* for Nubra population was 0.500 (SD=0.289) with 95% confidence interval 0.025-0.976. The posterior mean of θ^B over

Nubra population was 0.336 (SD=0.018) with 95% confidence interval of 0.300-0.371. The Indus population has lowest value of *f*=0.319 (SD=0.169) with 95% confidence interval of 0.052-0.0732. The mean estimate of θ^B for Indus population was 0.077 (SD=0015) with 95% confidence interval of 0.05-0.109. The Suru population has lowest mean estimate of θ^B=0.030 (SD=0.012), with 95% confidence interval of 0.007-0.057. The point estimate of *f* for Suru population was 0.401 (SD=0.208), with 95% confidence interval of 0.059-0.869.

4.3.3 ***Spatial genetic structure***

All descriptive measures of SGS were significantly different from zero (Table 4.4). Results showed that the clustering of related individuals was more intensive in Nubra than Indus and Suru populations.

Table 4.4 Summary statistics of the SGS estimated for the three study populations

Population	$F_{ij(1)}$	*b*	*Sp*
Indus	0.1987 (0.0383)	-0.0161 (0.0029)	0.0201 (0.0030)
Suru	-0.0401 (0.0312)	-0.0050 (0.0027)	0.0048 (0.0027)
Nubra	0.4715 (0.0574)	-0.0658 (0.0038)	0.1247 (0.0040)
Overall	0.1867 (0.0251)	-0.0381 (0.0023)	0.0468 (0.0024)

SGS: spatial genetic structure; $F_{ij(1)}$: the average kinship in the first distance interval; *b*:slope of the log linear regression between distance and kinship; *Sp*:descriptive measure of SGS intensity. The standard errors are in parentheses.

The average kinship coefficients between the neighboring individuals (as represented by $F_{ij(1)}$), which indicated the strength of the SGS was largely variable, with the highest value (0.4715) in the Nubra population (suggesting strong SGS) and the lowest (-0.0401) in the Suru population (weak SGS). Values of the regression slope *b* were all negative, with the highest values (-0.0050) in the Suru population and the lowest (-0.0658) in the Nubra population. The results suggested that individuals close to each other were genetically more related than individuals that were farther apart. The *Sp* statistic values varied from 0.0048 in the Suru population to 0.1247 in the Nubra population. These data indicated significant variation in spatial genetic patterns among

wild *M. alba* populations. Nubra population had a strong spatial structure and that of Suru population had a relatively weak spatial structure.

4.4 Discussion

We detected structuring in natural populations of *M. alba* in the trans-Himalaya [Bajpai et al., 2014] and we reasoned that visible genetic structure is due to presence of geographical barrier among different populations of *M. alba*. Existence of geographical barrier leads to genetic drift that separates population. The separated populations grow independently and mate among nearby individuals and with passage of time they accumulate variation.

Kimura [1968] proposed neutral allele model, according to which all new mutations are selectively neutral and does not affect fitness of organisms and observed genetic variation is only due to genetic drift. But in last decade, through comparative genomic studies there is growing body of evidence which suggested widespread selection in the genome of certain species [Barrett and Hoekstra, 2011]. So, in this study, we strive to find out signatures of natural selection in *M. alba* genome. To further ascertain the presence of natural selection we analyzed SGS, which is after effect of natural selection.

During the process of population divergence and speciation, genetic differentiation accumulates in some regions of the genome due to selection, genetic conflict, variable mutation rates, and chromosomal structure, but not in others because of the homogenizing effects of gene flow and/or insufficient time for random differentiation by genetic drift (Nosil et al., 2009). The resulting and variable pattern of genomic differentiation among populations has been referred to as 'heterogeneous genomic divergence' [Nosil et al., 2009]. We detected 30 loci under selection in this study by following stringent conditions ($p \leq 1\%$ for finite island model and $p \leq 5\%$ hierarchical island model analysis). We considered only those loci under selection which appeared selected in hierarchical island model and also present in one of the finite island model analysis. Our results rejected the null hypothesis based on neutral allele model [Kimura, 1968] which considers that the vast majority of segregating polymorphisms are selectively neutral and thus their evolutionary fate is determined by genetic drift. In the present study, hierarchical island model as implemented in ARLEQUIN revealed less number of loci under selection as compared to finite island model. Results revealed high number of loci under balancing selection

in finite island model. According to Excoffier et al. [2009], if data is analyzed without taking the hierarchical structure into account, large false positives is obtained at all significance levels because of narrower simulated null distributions under the finite island model, especially loci under balancing selection considerably overestimated. Therefore, hierarchical island model minimizes the number of false positives to a great extent. We detected 6.85% loci under selection of which 3.64% were under directional selection and another 3.19% loci were under balancing selection. Previous genome scan studies have identified putative directional selection at, on average, 5% (range: 1–15%) of the screened marker loci [Shimada et al., 2011]. The present study revealed lower number of loci under directional selection compared to previous studies. Detection of lower number of loci in the present study could be because we followed stringent condition. Directional selection is expected to decrease within population diversity and increase between-population differentiation in comparison to neutral expectations. In contrast, balancing selection tends to homogenize allele frequencies and increase the within-population diversity [Nielsen, 2005; Charlesworth, 2006]. Thus, genomic regions showing such patterns of genetic diversity could be considered as candidates for containing loci involved in evolutionary change [Schlötterer, 2003]. High value of genetic differentiation (θ^{B}) revealed in this study confirms this notion.

The SGS is the non-random distribution of genotypes within a population [Vekemans and Hardy, 2004]. This study showed significant SGS. The *Sp* statistics obtained for overall populations (*Sp*=0.0468) was higher than reported for outcrossing wind pollinated tree species (mean *Sp*=0.0304). It is between mixed mating (*Sp*=0.0529) and outcrossing species (*Sp*=0.0393). At population level, Nubra population (*Sp*=0.124) revealed high SGS similar to selfing species (*Sp*=0.123) [Vekemans and Hardy, 2004]. The Indus population revealed moderate SGS (*Sp*=0.0204) while Suru population revealed low SGS (*Sp*=0.0048) similar to wind pollinated tree species [Vekemans and Hardy, 2004]. These results showed that there is non-random distribution of individuals in Nubra population and gene flow is highly restricted. Whereas in Indus population distribution is near random and gene flow is moderate. However, distribution of individuals in Suru population is completely random and there is high gene flow. Bayesian approach of Holsinger et al. [2002] revealed high inbreeding and genetic differentiation in Nubra population, which is consistent with high SGS close to selfing species [Vekemans and

Hardy, 2004]. However, Suru population revealed low inbreeding and genetic differentiation which correspond well with SGS obtained for the population. According to Kalisz et al. [2001], the relative importance of local genetic drift versus local selection pressures as factors governing the formation and maintenance of within-population SGS has been at issue since even before the time of Fisher and Wright and is yet to be resolved. Under neutral allele model, genetic drift is considered as a sole force responsible for genetic divergence. If SGS is present, it is exclusively due to genetic drift. Grant and Linhart [1996] stressed the role of natural selection in formation of SGS. Natural selection has evolutionary advantage over genetic drift since it is the only evolutionary force which can produce adaptation [Wade, 2005]. The present study also emphasized the role of natural selection in formation of SGS.

Most of the theoretical studies on SGS were based on Wright's neighborhood concept, in which each individual is assumed to mate at random with its surrounding neighbors [Doligez and Joly, 1997]. The present study revealed non-random distribution of individuals in Nubra population. According to Doligez and Joly [1997], when SGS is present at the population level, it has important repercussions for selection programs and conservation strategies. It will lead to biased estimates of outcrossing rates, affect estimations of genetic variance as measured from open-pollinated progeny and influence sampling strategies in natural populations. So revelation of strong SGS in Nubra population will help in devising breeding programs and conservation strategies.

Detection of natural selection also sheds light on the local adaptation of an organism. Local adaptation can evolve only if the strength of directional selection overrides random genetic drift and homogenizing effect of gene flow among populations [Kawecki and Ebert, 2004]. Therefore, it is expected that genes and linked regions under the influence of directional selection will show elevated differentiation in comparison with selectively ‘neutral’ gene regions [Limborg et al., 2012]. This study revealed that 3.64% loci were under directional selection and 3.19% under balancing selection. Therefore, it indicates that some region of *M. alba* genome is under local selection.

4.5 Conclusion

This study display footprints of natural selection in *M. alba* genome. The natural selection was detected with high stringency in hierarchical island model. Overall, *M. alba* falls in the range of mixed mating and outcrossing species. But population from Nubra valley showed selfing with high SGS. The presence of natural selection is further evident by presence of SGS in *M. alba* natural populations. The knowledge of the spatial structure of populations is important when selecting natural populations for conservation and sampling for breeding programs. It should be taken into account in order to maximize diversity, and to avoid misrepresenting species and population diversity [Epperson and Allard, 1989; Shapcott, 1995]. Secondly, because spatial patterns change in a substantial, cumulative and sometimes characteristic manner due to the past effects of natural selection and other factors, spatial pattern analysis can help to detect the action of these factors [Epperson, 1993]. Further, this study sheds light on adaptability of *M. alba* in the trans-Himalaya and revealed that some region of *M. alba* genome is under local selection.

CHAPTER 5

CHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITIES OF *M. ALBA* L. FRUIT

Abstract

Mulberry is listed in modern Chinese Materia Medica for its medicinal uses. Our aim was to evaluate the antioxidant capacities and bioactive compounds of methanolic extract of *Morus alba* fruit from the trans-Himalaya. Thirty seven genotypes of *M. alba* with white, pink and black fruit were studied to find antioxidant capacities, total phenolic content (TPC), total flavonoid content (TFC), total proanthocyanidin content (TPAC) and total anthocyanin content (TAC) in fruit. We have identified and quantified the phyto-chemotypes present in the methanol extracts by gas chromatography/mass spectrometry (GC/MS) technique. We have also estimated mineral content of *M. alba* fruit by inductively coupled plasma/optical emission spectrometry (ICP/OES). The ferric reducing antioxidant potential (FRAP) ranged from 34.2 to 46.2 µM Fe(II)/g DW. A 1-25 fold variation in IC_{50} value was observed for DPPH radical scavenging capacity of extracts. However, the fold variation in IC_{50} by ABTS radical cation scavenging, lipid peroxide inhibition (LPI) and nitric oxide (NO) radical scavenging capacity of extracts was lower i.e 1–11.5, 1–5.6 and 1–3.4, respectively. The fruits were found to be rich in TPC ranging from 1.8 to 8.3 mg gallic acid equivalent (GAE)/g DW. The TFC and TPAC ranged from 0.4 to 0.9 mg quercetin equivalent/g DW and 0 to 0.02 mg catechin equivalent/g DW, respectively. Two way analysis of variance (ANOVA) revealed that fruit color has no significant relationship with TPC, TAC and TPAC. However, fruit color showed inconsistent effect on antioxidant capacities as determined by different methods. Sampling location has significant effect on antioxidant capacities, TPC, TFC and TAC. Interaction between fruit color and sampling location showed significant effect on antioxidant capacities and TAC. The study therefore showed that source of genotypes from different sampling location is more important as compare to fruit color in determining antioxidant capacities, TPC and TAC in *M. alba* fruit from the trans-Himalaya. GC/MS analysis revealed the presence of healthy ω-6 fatty-acids. It appeared from the results that mulberry is a good source of macro and micro nutrients.

5.1 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, 2007]. Along with others, phenolics, flavonoids, anthocyanin and proanthocyanidin present in fruit and vegetable have been reported to act as antioxidants. These results have stimulated researchers to characterize different types of plants with regards to their health promoting compounds.

Studies have been reported on the pharmacological activity and chemical composition of mulberry species from China, India, Japan, South Korea, Pakistan, Taiwan and Turkey [Gerasopoulos and Stav-Roulakis, 1997; Darias-Martin et al., 2003; Arabshahi-Delouee and Urooj, 2007; Ercisli and Orhan, 2007; Gungor and Sengul, 2008; Koca et al., 2008; Nikkhah et al., 2009; Imran et al., 2010; Kutlu et al., 2011; Du et al., 2008; Arfan et al., 2012; Calín-Sánchez et al., 2013]. Studies related to variability in health promoting compound contents in different colored fruits in *M. alba*, *M. rubra* and *M. nigra* have also been reported (Ercisli and Orhan, 2007; Ercisli et al., 2010; Calín-Sánchez et al., 2013). However, studies involving different color fruits within a species have not been reported. Besides, content of the health promoting compounds in mulberry growing in the harsh environmental condition such as the trans-Himalaya has not been investigated. Therefore, the objective of the present study was to investigate variability and relationship of fruit color and source of genotype from different sampling locations with antioxidant capacities, total phenolic content (TPC), total flavonoid content (TFC), total proanthocyanindin content (TPAC) and total anthocyanin content (TAC) in fruit from 37 genotypes from the trans-Himalayan region. Attempt was also made to identify phyto-chemotypes in methonolic fruit extract. Macronutrient and trace elements present in *M. alba* fruit was also determined.

5.2 Materials and Methods

5.2.1 *Sample collection*

Thirty seven genotypes of *M. alba* L. were sampled across the major distribution sites from the Indian trans-Himalaya in July 2010 (Table 5.1).

Table 5.1 Genotypes *M. alba* sampled for fruit from Indian trans-Himalaya

Sr. No	Genotype code	Skin color
1	SKB-01	White
2	SKB-04	Black
3	SKB-05	Black
4	SKB-06	Black
5	DMK-03	Black
6	DMK-05	Black
7	ACH-01	Pink
8	ACH-02	Black
9	BMA-01	White
10	BMA-02	Black
11	BMA-03	Black
12	BMA-04	Pink
13	SNJ-03	Pink
14	SNJ-04	Pink
15	BTK-01	White
16	BTK-02	White
17	BTK-04	Pink
18	BTK-05	Black
19	AKM-01	Black
20	AKM-02	White
21	AKM-03	Black
22	AKM-04	Black
23	PSY-01	Pink
24	PSY-02	White
25	PSY-04	Black
26	PSY-06	Black
27	LCM-01	White
28	LCM-02	White
29	LCM-03	Pink
30	CHK-02	Black
31	CHK-07	Black
32	BKD-03	White
33	BKD-04	White
34	TRK-11	Black
35	TRK-12	Black
36	TRK-13	Black
37	TRK-14	White

5.2.2 ***Chemicals***

1,1-diphenyl-2-picrylhydrazyl radical (DPPH*), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tripyridyl-*s*-triazine (TPTZ), butylated hydroxy toluene (BHT), butylated hydroxyl anisole (BHA), quercetin (Q) and catechin, α-tocopherol (TP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteus's phenol reagent and solvents were purchaed from Merck Chemical Supplies (Merck KGaA, Darmstadt, Germany). All the other chemicals used were of analytical grade.

5.2.3 ***Physicochemical characterization and extraction***

For each genotype, approximately 50 g fruits were collected. Skin color, fruit weight (FWT) and total soluble solids (TSS) were recorded for each genotype before pulping. Skin color was recorded based on visible coloration of fruit by naked eye. FWT was measured using a digital balance with a sensitivity of 0.001g. TSS was determined using a digital refractometer (Model RA-250. Kyoto Electronic Manufacturing Co. Ltd., Japan) at 22^{o}C and expressed as ^{o}Brix. Fruit was crushed and lyophilized using Laboratory freeze dryer (CHRIST Alpha 2-4 LD plus, Martin Christ, Germany) to obtain a dry mass. The fruit powder was taken for extraction in 100% methanol by Soxhlet apparatus (Borosil Glass Works Limited, Worli Mumbai, India). Extraction was carried out with 10 g of ground dried fruit powder at 40^{o}C and solid to solvent ratio was 1:8. Extraction was carried for 8 hours. The solvent was then removed by using rotary evaporator (Rotavapor® R-210, Büchi Labortechnik AG, Flawil, Switzerland) under reduced pressure. The stock solution of 1 mg/ml was prepared for further assays.

5.2.4 ***Bioactive content assays***

5.2.4.1 ***Total polyphenol content***

TPC was measured using Folin-Ciacalteu colorimetric method as described previously [Gao et al., 2000]. The 10 µL methanol fruit extract (0.1 mg/ml) was mixed with 20 µL of Folin-Ciocalteu reagent and 200 µL of water, and incubated at room temperature for 3 minutes. Following the addition of 100 µL of 20% sodium carbonate to the mixture, TPC was determined after 1 h of incubation at room temperature. The absorbance of the resulting blue color was measured at 765 nm using spectrophotometer (Spectramax M2^{e}, Molecular Devices, Germany).

Quantification was done with respect to the standard curve of gallic acid. TPC was expressed as gallic acid equivalent (GAE) using the equation based on the calibration curve: y=0.005x + 0.059, R^2=0.987, where x was the absorbance and y was the GAE at a final concentration of 100 µg/ml. The result was expressed as mg GAE /g dry weight (DW).

5.2.4.2 ***Total flavonoid content***

Estimation of the TFC in methanol extract was carried out using the method of Ordon et al. [2006]. Briefly, to 100 µL of sample (0.1 mg/ml), 100 µL of 2% $AlCl_3$ ethanol solution was added. The content was incubated for 1 h at room temperature and the absorbance was measured at 420 nm. TFC was expressed as quercetin equivalent (QE) of extract using the following equation based on the calibration curve: y = 0.011x + 0.038, R^2 = 0.984, where x was the absorbance and y was the QE (mg/g) at a final concentration of 0.1 mg/ml. Final content was expressed as mg QE/g DW.

5.2.4.3 ***Total proanthocyanidin content***

Total proanthocyanidin test was performed by vanillin-HCl assay according to the method of Price et al. [1978] with minor modification. Vanillin reagent (1%) was prepared in methanol and incubated at 30°C before use. The working reagent was prepared by mixing one part of 1% vanillin solution and one part of 8% HCl solution in methanol. The reaction mixture contained working vanillin reagent (100 µL) and 20 µL of extract (0.1 mg/ml). The absorbance at 500 nm was measured after 20 min at 30°C. TPAC was expressed as mg catechin equivalent (CE)/g DW using the equation based on the calibration curve: y = 0.288x + 0.041, R^2 = 0.987, where x was the absorbance and y was the CE (mg/g) at a final concentration of 0.1 mg/ml.

5.3.4 ***Total anthocyanin content***

TAC of the methanolic extract was measured by the pH-differential method as described previously [Rodriguez-Saona and Wrolstad, 2001; Teow et al., 2007]. Sample was diluted with two different solutions: potassium chloride (0.025 M), pH 1.0; and sodium acetate (0.4 M), pH 4.5. The pH was adjusted with concentrated hydrochloric acid. Sample (1 mg/ml) was diluted to give an absorbance at 530 nm of <1.2. Diluted sample was held for 15 min before measuring the absorbance. The absorbance was measured at 530 nm and 700 nm with distilled water as a blank.

The absorbance (A) difference between the pH 1.0 and pH 4.5 samples was calculated by following equation:

$$A = (A_{530nm} - A_{700nm})_{pH\ 1.0} - (A_{530nm} - A_{700nm})_{pH\ 4.5}$$

The monomeric anthocyanin content was calculated using the following equation:

$$\text{Monomeric anthocyanin content (mg/L)} = (A \times MW \times DF \times 1000) / (\varepsilon \times l)$$

MW=449.2 and ε=26,900 are, respectively, molecular weight and molar extinction coefficient of cyanidin-3-glucoside, which was used as a standard; DF is the dilution factor; l is the path length. The total monomeric anthocyanin content was expressed as mg cyanidin-3-glucoside equivalent (C3GE)/g DW.

5.2.5 *Antioxidant activity assays*

5.2.5.1 ***Ferric reducing antioxidant potential (FRAP)***

A modified method of Benzie and Strain [1996] was adopted for the FRAP assay. The stock solutions included 300 mM acetate buffer (3.1 g CH_3COONa and 16 ml CH_3OOH), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) solution in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3.6H_2O$. The 15 µL of extract (0.1 mg/ml) was allowed to react with 28 µL of the FRAP solution for 30 minute in dark. Observation of the colored product (ferrous tripyridyltriazine complex) was taken at 593 nm. The calibration curve was prepared from the equation y=0.001x - 0.058, R^2=0.993, where x was the absorbance and y was $FeSO_4$ concentration (molar). Linearity was achieved between 1×10^{-4} and 1×10^{-3} M $FeSO_4$. Results were expressed in µM Fe (II)/g DW.

5.2.5.2 ***ABTS radical cation scavenging assay***

The ABTS radical cation scavenging capacity of extracts was determined by method of Re et al. [1999]. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate (PPS) solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml $ABTS^{*+}$ solution with 60 ml of methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm. The concentrations of plant extracts was 100–1000 µg/ml. Plant extracts (100 µL) were allowed to react with 100 µL of the $ABTS^{*+}$ solution and the absorbance was taken at 734 nm after 7 minute incubation at 25°C in a 96 well plate. The percentage inhibition was calculated as follows:

ABTS radical cation scavenging capacity (%) = $[(Abs_{control} - Abs_{sample})] / (Abs_{control})] \times 100$

where, $Abs_{control}$ is the absorbance of ABTS radical cation + methanol; Abs_{sample} is the absorbance of ABTS radical cation with sample extract.

The inhibitory concentration (IC_{50}) was estimated and calculated as described by Kroyer [2004].

5.2.5.3 *DPPH radical scavenging assay*

The effect of extract on DPPH radical was determined using the method of Liyana-Pathiranan and Shahidi [2005]. A solution of 0.135 mM DPPH* in methanol was prepared and 100µL of the solution was mixed with 100 µL of methanolic extract. The concentration of plant extracts was 100–1000 µg/ml. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 minute. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The ability to scavenge DPPH* was calculated by the using the equation:

DPPH radical scavenging capacity (%) = $[(Abs_{control} - Abs_{sample})] / (Abs_{control})] \times 100$

where, $Abs_{control}$ is the absorbance of DPPH radical + methanol; Abs_{sample} is the absorbance of DPPH radical with sample extract or the standards.

The inhibitory concentration (IC_{50}) was estimated and calculated as described above.

5.2.5.4 *Nitric oxide radical scavenging assay*

The method of Green et al. [1982] was used to assay the scavenging activity of mulberry fruit extracts on nitric oxide radical. The reaction solution (300 µL) containing 250 µL of 10 mM sodium nitroprusside in PBS (pH 7.0) was mixed with 50 µL mulberry extracts at different concentrations (100 to 1000 µg/ml) followed by incubation at 37^{o}C for 1 h. A 125 µL aliquot was then mixed with 125 µL Griess reagent. The absorbance was measured at 540 nm and BHA was used as standard.

Percent inhibition of nitric oxide radical generated was measured by comparing with the absorbance value of control (10 mM sodium nitroprusside and PBS).

Nitric oxide (NO) radical scavenging capacity (%) = $[(Abs_{control} - Abs_{sample})] / (Abs_{control})] \times 100$

where $Abs_{control}$ is the absorbance of NO radical + methanol; Abs_{sample} is the absorbance of nitric oxide radical radical with sample extract or the standards.

The inhibitory concentration (IC_{50}) was estimated and calculated as described above.

5.3.5 *Lipid peroxidation inhibition assay*

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxidation inhibition ability of extract. The egg yolk homogenates was used as lipid-rich media as described by Miguel et al. [2004]. In this assay readymade egg yolk emulsion (Himedia Lab. Pvt. Ltd., Mumbai, India) was used, which was diluted to 10% v/v with 1.15% w/v KCl and mixed thoroughly. The reaction solution (400 µL) consist of 50 µl egg yolk emulsion, 50 µl of sample solution in different concentrations (100 to 1000 µg/ml), 150 µl of 20% (aq) trichloroacetic acid and 150 µl of 0.67% w/v thiobarbituric acid. The whole reaction solution was vortexed thoroughly and incubated at 95^{o}C for 1 hour. After cooling, reaction mixture was centrifuged at 3000 rpm for 10 minute. The absorbance of the upper layer was measured at 532 nm. The percentage inhibition was calculated using the formula:

% inhibition of lipid peroxidation = (1-t/c) x 100

where, c being the absorbance of fully peroxidized control and t the absorbance of test sample. The inhibitory concentration (IC_{50}) was estimated and calculated as described above.

5.2.6 *Gas chromatography/mass spectrometry (GC/MS) analysis*

5.2.6.1 *Preparation of sample for GC/MS analysis*

Twenty five mg of concentrated extract was redissolved in the methanol, vortexed properly and filtered through 0.22 mm syringe filter (Millipore Corp., Bedford, MA, USA). One microlitre aliquot of the sample solution was injected into the GC/MS MS system for the requisite analysis.

5.2.6.2 *Instrumentation and chromatographic conditions*

GC/MS analysis was carried out on a Thermo Finnigan Polaris Q Ion Trap GC/MS MS system comprising of an AS2000 liquid autosampler (Thermo Finnigan, Thermo Electron Corporation, Austin, TX, USA). The gas chromatograph was interfaced to a mass spectrometer instrument employing the following conditions *viz*. Durabond DB-5 ms column (30 m 60.25 mm 60.25 mm) operating in electron impact [electron ionisation positive (EI+)] mode at 70 eV, helium (99.999%) was used as carrier gas at a constant flow of 1 ml/min, an injection volume of 0.5 EI was employed (split ratio of 10:1), injector temperature 280^{o} C, and transfer line

temperature 300^{o} C. The oven temperature was programmed from 50^{o} C (isothermal for 2 min), with gradual increase in steps of 10^{o} C /min, to 300^{o} C. Mass spectra were taken at 70 eV, a scan interval of 0.5 s, and full mass scan range from 25 m/z to 1000 m/z. The data acquisition was performed on Finnigan Xcalibur data acquisition and processing software version 2.0 (ThermoQuest, LC and LC/MS Division, San Jose, California,USA).

5.2.6.3 ***Identification of components***

Interpretation of mass spectrum of GC/MS was done using the NIST/EPA/NIH Mass Spectral Database (NIST11), with NIST MS search program v.2.0g [National Institute Standard and Technology (NIST), Scientific Instrument Services, Inc., NJ, USA]. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

5.2.7 ***Inductively coupled plasma-optical emission spectrometry (ICP-OES) analysis***

5.2.7.1 ***Sample digestion for ICP-OES analysis***

Dried fruit powder (500 mg) was mixed with 8 ml of 6:2 HNO_3/H_2O_2 mixtures on the hot block. The sample was heated on a hot block at 100-150^{o} C for about 2 hours with a gentle boil. During this time the sample was refluxed so the mixture was not allowed to dry. After digestion, sample was cooled and 10 ml of distilled water was added on the sample and mixed. The residue was filtered through filter paper and diluted to 50 ml with distilled water. Metal contents of final solution were determined by ICP-OES.

5.2.7.2 ***ICP-OES Instrumentation***

A multi-elemental standard solution of 1000 mg/ml containing all analysed element supplied by Merck (Darmstadt, Germany) was used for calibration. Detrminations was carried out using a Perkin Elmer model-Optima 5300 DV spectrometer (Perkin Elmer, USA). ICP-OES equipped with a Ultrasonic nebulizer CETAC U-6000AT+ (CETAC, USA) and auto sampler AS 93-plus. Argon (purity higher than 99.995%) was used to sustain plasma and as carrier gas. The operating condition employed for ICP–OES determination were 1300–1350W RF power, 15 L

min^{-1} plasma flow, 2 L min^{-1} auxiliary flow, 0.8 L min^{-1} nebulizer flow, 1.5 ml min^{-1} sample uptake rate.

5.2.8 ***Statistical analysis***

All the experiments were performed in triplicate. The experimental results were expressed as mean±standard deviation (SD) using statistical analysis with SPSS (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL) version. One way analysis of variance (ANOVA) and post hoc analysis with 2-sided Tukey HSD at $p \leq 0.05$ level were performed. A two-way ANOVA was used to test the effect of fruit color, source of genotypes from different sampling sites and their interaction on fruit quality characteristics, total bioactive content and antioxidant capacities. Pearson's correlation analysis and principal component analysis (PCA) were performed to compare the data. Box plots were produced using MS excel.

5.3 Results and discussion

5.3.1 *TSS and fruit weight*

TSS varied from 12.6 to 38.1 ºBrix (Figure 5.1a) among the 37 genotypes and thus three fold differences were observed. A similar range of TSS (17.8-30.7%) has been reported in mulberry genotypes from Turkey [Ercisli and Orhan, 2007; Yilmaz et al., 2012]. FWT ranged from 0.6 to 3.6 g, therefore six fold differences was observed (Figure 5.1b). FWT of the trans-Himalayan origin is similar to that of Turkey (0.66-3.07 g) as reported by Yilmaz et al. [2012]. However, Ercisli and Orhan [2007] reported higher fruit weight (3.49 g) from Turkey. No significant difference ($p \leq 0.05$) was observed in FWT and TSS among the white, pink and black fruit of *M. alba* (Table 5.2, Figure 5.2a and b). Significant difference in FWT and TSS has been reported in mulberry fruit with different color belonging to different *Morus* species. Ercisli and Orhan [2007] found that FWT of white (*M. alba*), black (*M. nigra*) and red (*M. rubra*) was 3.49, 4.37 and 2.14 g, respectively while the TSS content was 20.4, 16.7 and 15.9%, respectively. Similarly, Calín-Sánchez et al. [2013] reported higher TSS in *M. nigra* than *M. alba*. Therefore significant difference in FWT and TSS in fruit with different color in mulberry is a function of the species.

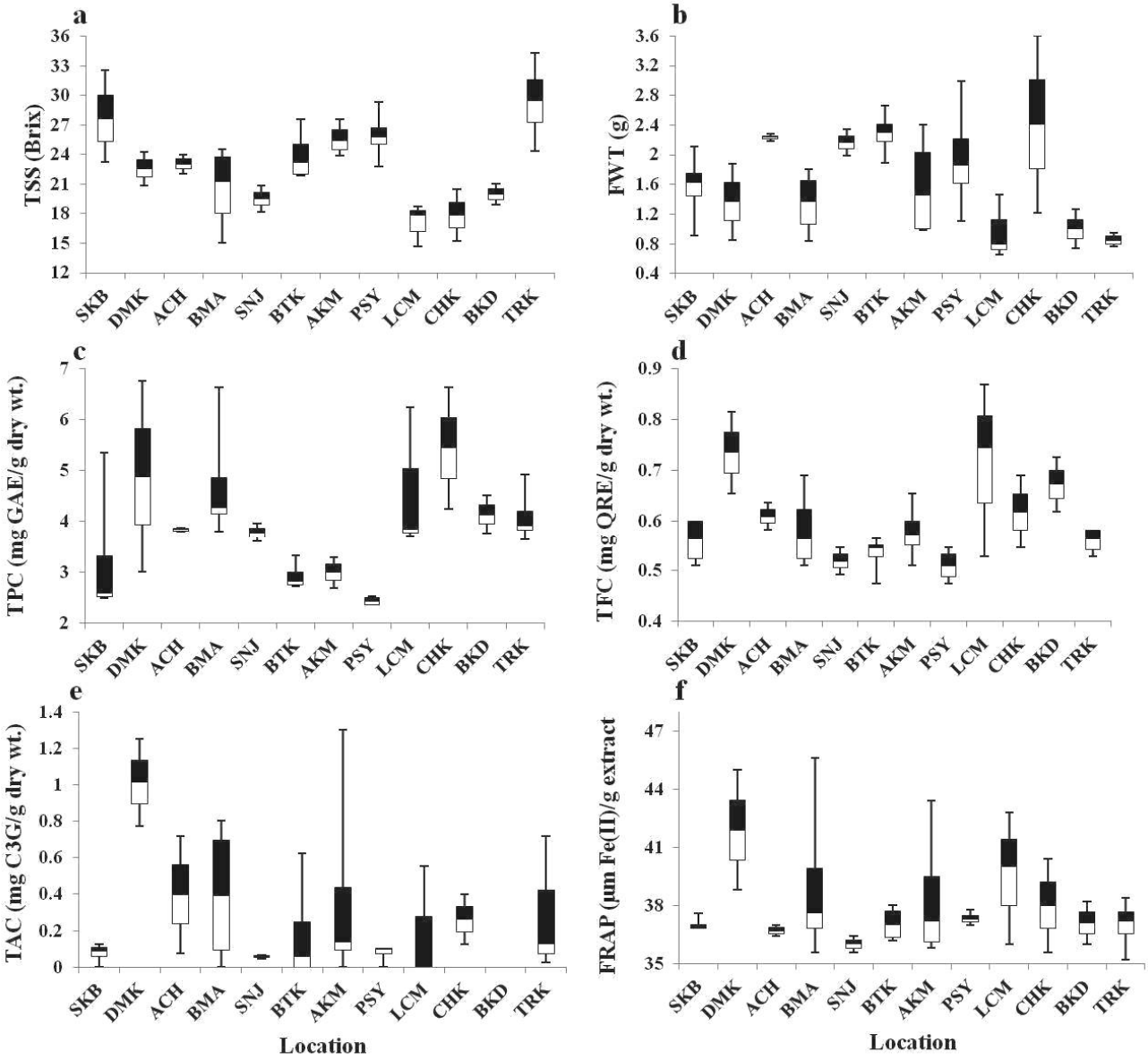

Figure 5.1 Box plot showing minimum, 1st quartile (blank box), median, 3rd quartile (black box) and maximum values of physiochemical properties and bioactive contents of methanolic fruit extract of *M.alba* from 12 different locations in trans-Himalaya.

5.3.2 ***Total polyphenol content and antioxidant capacity***

TPC in fruit of 37 genotypes is presented in Figure 5.1c. High variability within and among genotypes from different sampling locations was observed. The TPC data mostly distributed at lower quartile and ranged from 1.8 to 8.3mg GAE/g DW between genotypes. Therefore, a

Table 5.2 Fruit quality characteristics, antioxidant capacity and bioactive compounds in white, pink and black fruit of *M. alba* from trans-Himalaya.

Parameters	Unit	Fruit color			Overall
		White	Pink	Black	
[1]FWT	G	1.34±0.68[a]	1.8±0.68[a]	1.7±1.16[a]	1.6±0.9
[2]TSS	°Brix	22.59±5.77[a]	21.89±3.05[a]	24.65±5.56[a]	23.51±5.34
[3]FRAP	µM Fe(II)/g DW	37.09±1.73[a]	37.63±2.42[a]	38.47±3.31[a]	37.9±2.8
[4]DPPH	*IC_{50}	4.35±2.85[a]	4.05±2.5[a]	4.12±2.5[a]	4.17±2.6
[5]ABTS	*IC_{50}	1.17±0.46[a]	1.24±0.36[a]	1.06±0.5[a]	1.13±0.47
[6]LPI	*IC_{50}	1.61±0.41[ab]	1.71±0.38[b]	1.46±0.41[a]	1.55±0.42
[7]NO	*IC_{50}	1.43±0.24[b]	1.25±0.32[a]	1.53±0.32[b]	1.45±0.31
[8]TPC	mg GAE/g DW	3.47±0.92[a]	3.87±1.34[a]	3.7±1.44[a]	3.67±1.28
[9]TFC	mg QE/g DW	0.58±0.1[a]	0.6±01.2[a]	0.59±0.09[a]	0.59±0.1
[10]TPAC	mg CE/g DW	0.0007±0.003[a]	0.0006±0.0018[a]	0.0035±0.0053[b]	0.002±0.0044
[11]TAC	mg C3GE/g DW	0.244±0.28[a]	0.19±0.24[a]	0.23±0.39[a]	0.22±0.34

Values represented as mean ± SD; for each row, different lowercase letters indicate significantly different at $p<0.05$, as measured by 2-sided Tukey's HSD between different colored fruit.

[1]FWT: Fruit weight, [2]TSS: Total soluble solids, [3]FRAP: Ferric reducing antioxidant potential expressed as, [4]DPPH: Inhibitory conc. to scavenge DPPH free radical, [5]ABTS: Inhibitory conc. required to scavenge ABTS free radical, [6]LPI: Inhibitory conc. required to inhibit lipid peroxidation, [7]NO: Inhibitory conc. required to scavenge nitric oxide (NO) free radical, [8]TPC: Total phenolic content expressed as gallic acid equivalent (GAE), [9]TFC: Total flavonoid content expressed as quercetin equivalent (QE), [10]TPAC: Total proanthocyanidin content expressed as catechin equivalent (CE), [11]TAC: Total anthocyanin content expressed as cyanidin-3-glucoside equivalent (C3GE), *IC_{50}: Inhibitory concentration needed to scavenge 50% radical

variation of 1–4.6 fold in TPC was observed. At location level, the highest mean TPC (5.4 mg GAE/g DW) was observed in CHK while the lowest was observed in PSY location (2.4 mg GAE/g DW). The overall mean TPC was 3.8±0.9 mg GAE/g DW. In comparison Bae and Suh [2007] reported 0.96–2.57 mg GAE/g DW in five Korean cultivars of *M. alba*. Lin and Tang [2007] reported higher TPC (15.16 mg GAE/g fresh matter) in a deep colored *M. alba* from Taiwan. Ercisli and Orhan [2007] reported 1.81 mg GAE/g of fresh material in *M. alba* in Turkey. Radojković et al. [2012] found 4.133 mg chlorogenic acid equivalents/g dry extract of *M. alba* fruit from Serbia. Imran et al. [2010] reported 16.5 mg tannic acid equivalent/g fresh weight. The mean TPC in white, pink and black mulberry fruit was 3.5, 3.9 and 3.7 mg GAE/g DW, respectively. Black fruit showed greater variability in TPC as compare to that of pink and white fruit genotypes (Figure 5.2c). However, the difference in TPC in mulberry based on fruit color was not significant ($p \leq 0.05$) (Table 5.2). Ercisli and Orhan [2007] reported significantly

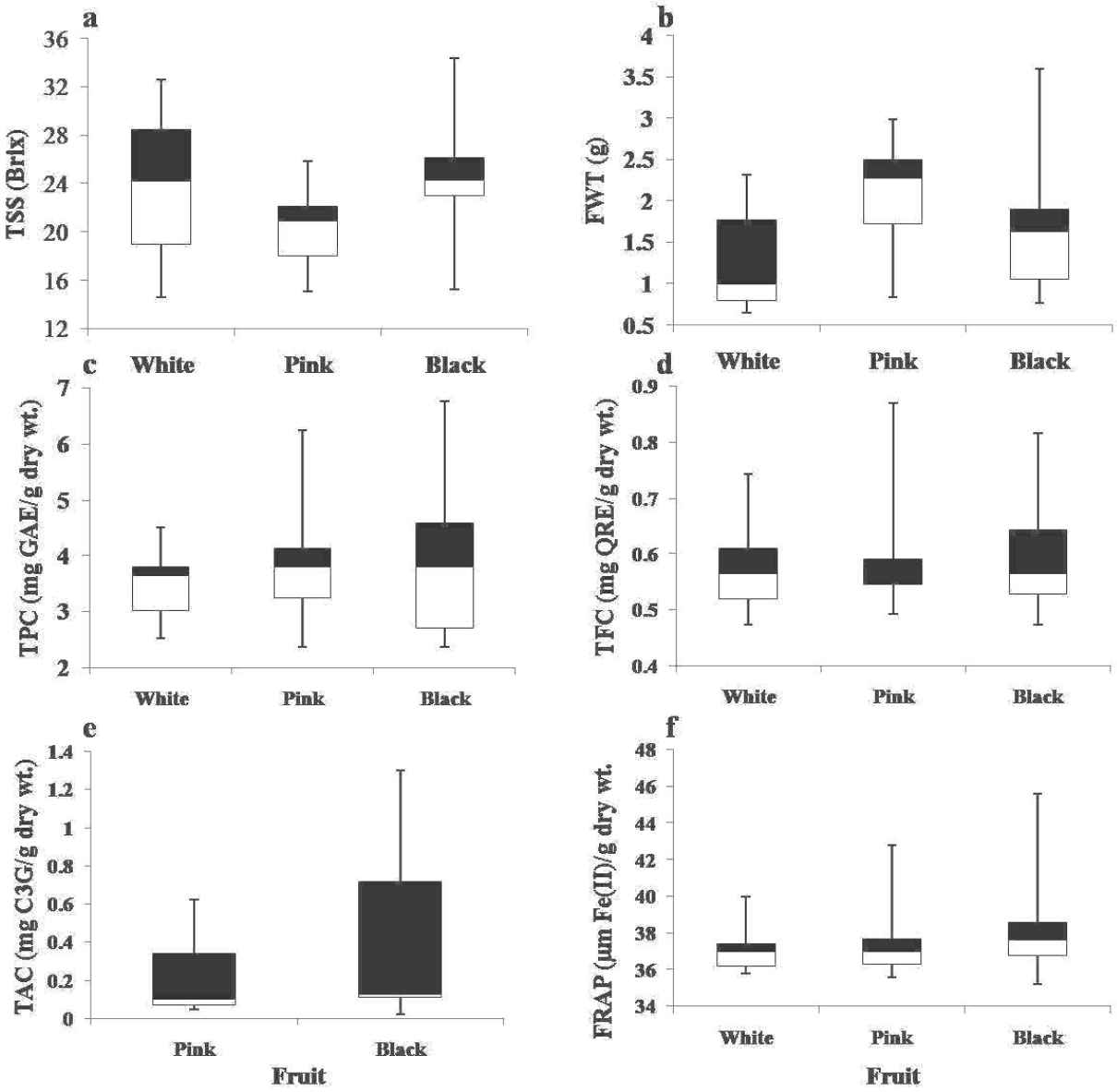

Figure 5.2 Box plot showing minimum, 1st quartile (blank box), median, 3rd quartile (black box) and maximum values of physiochemical properties and bioactive contents of methanolic fruit extract of three differently colored *M.alba* from trans-Himalaya.

higher TPC in *Morus* sp. with black (*M. nigra*) followed by red (*M. rubra*) and white fruit (*M. alba*). Similar results have also been reported by Calín-Sánchez et al. [2013] in *M. nigra* and *M. alba*. However, Imran et al. [2010] reported higher total phenol in *M. alba* as compare to *M. nigra* and also reported higher total phenol in white fruit as compare to black fruit of *M. laevigata*.

The ferric reducing antioxidant potential of extracts ranged from 34.2 to 46.2 µM Fe (II)/g DW (Figure 5.1f). The difference in FRAP value between the genotypes showing the highest and lowest value was 1-1.4 folds. At sampling location level, the highest mean value was 41.9 µM Fe (II)/g DW (DMK) while the lowest was 36.0 µM Fe (II)/g DW (SNJ). The overall mean FRAP value was 38.0±1.6 µM Fe (II)/g DW. The result suggested that there is not much variability in FRAP value among different locations. The mean FRAP value in white, pink and black mulberry fruit was 37.1, 37.6 and 38.5 µM Fe (II)/g DW, respectively (Table 5.2 and Figure 5.2f). Therefore, difference in FRAP value based on fruit color was not significant ($p \leq 0.05$).

Free radical scavenging capacity of extracts expressed as IC_{50} using four different assays showed variable results.

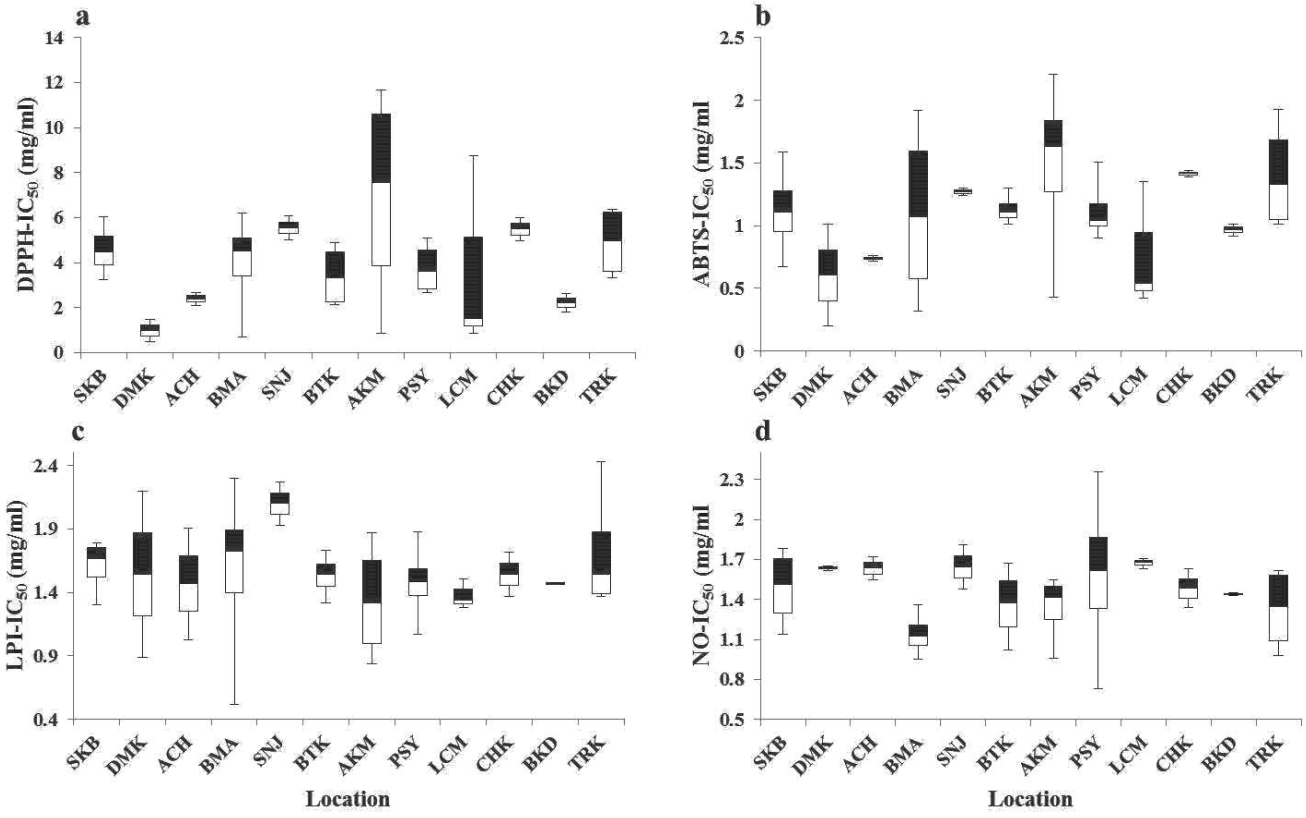

Figure 5.3 Box plot showing minimum, 1st quartile (blank box), median, 3rd quartile (black box) and maximum values of antioxidant activities of methanolic fruit extract of *M. alba* from 12 different locations in trans-Himalaya: (a) DPPH free radical scavenging capacity, (b) ABTS free radical scavenging capacity, (c) Lipid peroxidation inhibition capacity and (d) Nitric oxide radical scavenging capacity.

A 1-25 fold variation in IC_{50} value was observed for DPPH* scavenging capacity of extracts. However, the fold variation in IC_{50} by ABTS*$^{+}$ scavenging, LPI and NO* scavenging capacity of extracts was lower i.e 1–11.5, 1–5.6 and 1–3.4, respectively. Significant variation was observed between the individual within a sampling location suggesting genotypic effect on free radical scavenging capacity of extracts in mulberry (Table 5.2 and Figure 5.3). High fold variation in IC_{50} values highlighted unexploited variability among the mulberry genotypes from the trans-Himalayan region. The order of sampling location in terms of free radical scavenging capacity of extracts by the four different antioxidant assays was different which could be due to presence of compounds having different affinity to react with DPPH*, ABTS*$^{+}$, lipid* and NO*. Similar results have been reported earlier [Korekar et al., 2011; Korekar et al., 2014].

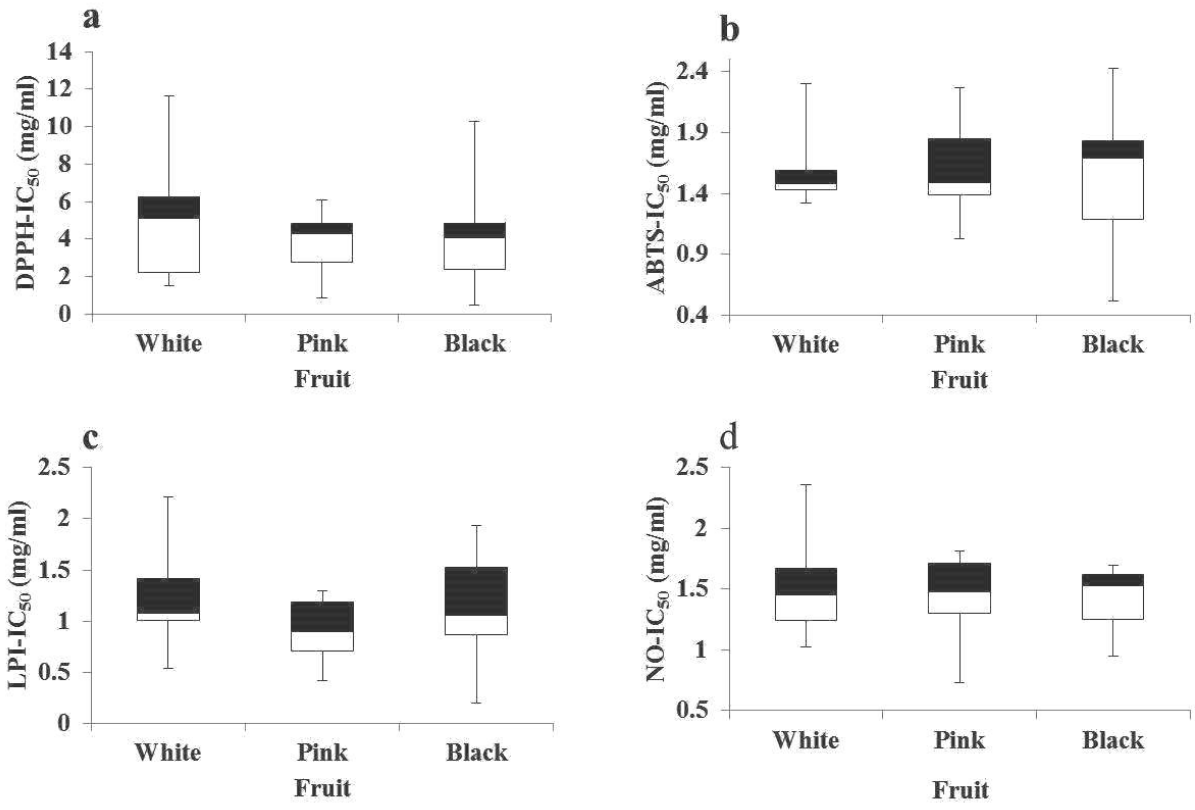

Figure 5.4 Box plot showing minimum, 1st quartile (blank box), median, 3rd quartile (black box) and maximum values ofantioxidant activities of methanolic fruit extract of of three differently colored *M. alba* from trans-Himalaya: (a) DPPH free radical scavenging capacity, (b) ABTS free radical scavenging capacity, (c) Lipid peroxidation inhibition capacity and (d) Nitric oxide radical scavenging capacity.

The difference in mean IC_{50} value in white, pink and black mulberry fruit determined by DPPH* and ABTS*$^{+}$ was not significant ($p \leq 0.05$) (Table 5.2 and Figure 5.4). However, Calín-Sánchez et al. [2013] reported that between different mulberry species, the black fruit species (*M. nigra*) contain significantly higher total antioxidant activity than that of white fruit species (*M. alba*).

5.3.3 ***Total flavonoid, proanthocyanidin and anthocyanin content***

Variability in TFC and TAC are shown in Table 5.2, Figure 5.1 and Figure 5.2 respectively. The TFC ranged from 0.4 to 0.9 mg QE/g DW and thus 1–2.2 fold variation was found. The mean TFC value was 0.6±0.1 mg QE/g DW. Significant variability in TFC was found between the different sampling locations. Within a sampling location significant variability between genotype was observed in six out of the 12 locations. In comparison *M. alba* from Turkey and Taiwan contains 0.29 and 2.5 mg QE/g fresh weight, respectively [Ercisli and Orhan, 2007; Lin, & Tang, 2007]. Radojkovic et al. [2012] reported 0.89 mg rutin equivalent/g dry extract in mulberry from Serbia. A mean TFC value for white, pink and black mulberry fruit were similar i.e 0.6 mg GAE/g DW and hence fruit color has no significant ($p \leq 0.05$) effect on TFC within *M. alba*. However, Ercisli and Orhan [2007] found that between different mulberry species, the black fruit species (*M. nigra*) contain significantly higher TFC than that of red (*M. rubra*) and white fruit species (*M. alba*).

The TPAC ranged from 0 to 0.02 mg CE/g DW. The mean TPAC of black mulberry fruit (0.0035 mg CE/g DW) was significantly higher as compare to that of white (0.0007 mg CE/g DW) and pink (0.0006 mg CE/g DW) fruit (Table 5.2). However, fruit color has no significant effect on TAC ($p \leq 0.05$). In comparison TPAC of temperate fruit such as pear, plum and apricot are 0.319, 2.159 and 0.156 mg/g FW, respectively [Gu et al., 2004]. Therefore, mulberry is not a rich source of proanthocyanidin. TAC in *M. alba* fruit from the trans-Himalaya ranged from 0 to 1.2 mg C3GE/g DW. In comparison Ercisli et al. [2010] reported 0.674–0.787 and 0.081–1.32 mg C3GE/g FW in *M. nigra* and *M. rubra*, respectively. Bae and Suh [2007] reported 0.1373–2.057 mg malvidin-3-glucoside equivalents/g extract in *M. alba* from Korea.

5.3.4 ***Two way analysis of variance***

Two-way ANOVA was carried to visualize the relationship of fruit color, source of genotypes from different sampling locations and their interaction with fruit quality parameters, antioxidant capacities and bioactive content of fruit extract (Table 5.3). The results showed that

fruit color has no significant relationship with FWT and TSS while sampling location showed significant effect on FWT and TSS. Sampling location accounted 22.8% and 48.5% variation in FWT and TSS, respectively. It was observed that fruit color has no significant relationship with TPC and TAC. However, fruit color showed inconsistent effect on antioxidant capacity of extracts as determined by different methods. Sampling location has significant effect on antioxidant capacity of extracts, TPC, TFC and TAC.

Table 5.3 Two-way ANOVA for fruit quality characteristics, bioactive contents and antioxidant capacities of *M alba* fruit with sampling location and fruit color as main effects.

Source	d.f	*F*	η^2	*F*	η^2	*F*	η^2
		DPPH		FRAP		TAC	
Location	11	8.36***	0.389	2.5**	0.209	6.837***	0.383
Color	2	11.2***	0.095	3.05	0.046	1.448	0.015
Loc x Col	9	3.8***	0.144	1.123	0.077	3.35***	0.154
		ABTS		TPC		TSS	
Location	11	5.38***	0.313	7.39***	0.432	10.01***	0.485
Color	2	6.28**	0.066	1.40	0.015	2.9	0.026
Loc x Col	9	3.26**	0.155	1.77	0.085	2.56*	0.1
		LPI		TFC		FWT	
Location	11	2.21*	0.158	10.52***	0.497	2.76**	0.228
Color	2	1.49	0.019	4.47*	0.038	1.29	0.019
Loc x Col	9	4.28***	0.25	2.25*	0.086	1.325	0.09
		NO		TPAC			
Location	11	8.6***	0.295	1.80	0.172		
Color	2	9.29***	0.058	1.7	0.029		
Loc x Col	9	13.28***	0.37	0.43	0.034		

The *F* ratio (*F*) is presented for each factor; d.f: Degrees of freedom, η^2: Eta squared as a measure of effect size

*Significant at $p \leq 0.05$, **Significant at $p \leq 0.01$, ***Significant at $p \leq 0.001$

DPPH=DPPH free radical scavenging capacity, ABTS=ABTS free radical scavenging capacity, LPI= lipid peroxidation inhibitory capacity, NO=nitric oxide radical scavenging capacity TPC=total polyphenol content, TFC=total flavonoid content, TPAC=total proanthocyanidin content, TAC=total anthocyanin content, FRAP=ferric reducing antioxidant potential, FWT=fruit weight; TSS=total soluble solids

Sampling location contributed 38.9%, 31.3%, 15.8%, 29.5%, 20.9%, 43.2, 49.7%, 38.3% variation in DPPH* scavenging capacity of extracts, $ABTS^{*+}$ scavenging capacity of extracts, LPI capacity of extracts, NO* scavenging capacity of extracts, FRAP, TPC, TFC and TAC, respectively. Interaction between fruit color and sampling location showed significant effect on antioxidant capacity of extracts and TAC. The interaction accounted 14.4%, 15.5%, 25%, 37%, 15.4% variation in DPPH* scavenging activity of extracts, $ABTS^{*+}$ scavenging capacity of extracts, LPI capacity of extracts, NO* scavenging capacity of extracts and TAC, respectively. The study therefore showed that source of genotypes from sampling location is more important as compare to fruit color in determining FRAP, TPC and TAC in *M. alba* fruit from the trans-Himalaya. The significant sampling location effect could be because of the varying micro-climatic condition of the sampling sites, which varies significantly in the trans-Himalayan region within a short distance.

5.3.5 ***Correlation analysis***

Table 5.4 displayed the correlation between fruit quality characteristics, antioxidant capacities (IC_{50}) and bioactive content of extracts. TPC was significantly correlated with TFC (0.595, $p \leq 0.01$) and TSS (0.5, $p \leq 0.01$). DPPH* scavenging capacity of extracts (IC_{50}) was significantly correlated with $ABTS^{*+}$ scavenging capacity of extracts (0.66, $p \leq 0.01$) and FRAP (-0.446, $p \leq 0.01$). Anthocyanins and flavonoids are considered very good antioxidant agent and showed significant negative correlation with $ABTS^{*+}$ and DPPH* scavenging capacity of extracts (IC_{50}) and a positive correlation with FRAP ($p \leq 0.01$). The result suggested that both TAC and TFC contribute significantly towards antioxidant capacity in *M. alba* fruit extracts. 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 mulberry fruit. Similar result was observed in seabuckthorn fruit from the trans-Himalaya (Korekar et al., 2014).

Table 5.4 Pearson's correlation coefficients of fruit quality characteristics, antioxidant capacities and total bioactive compounds.

Variable	DPPH	ABTS	LPI	NO	FRAP	TPC	TFC	TPAC	TAC	FWT	TSS
DPPH											
ABTS	0.66**										
LPI	-0.187	-0.197									
NO	0.17	0.159	0.157								
FRAP	-0.446**	-0.684**	-0.14	-0.198							
TPC	-0.03	-0.18	-0.156	0.028	0.334*						
TFC	-0.4*	-0.635**	0.012	-0.108	0.755**	0.595**					
TPAC	-0.36	-0.356*	0.187	0.143	0.417*	-0.087	0.174				
TAC	-0.358*	-0.484**	-0.122	-0.159	0.719**	0.149	0.577**	0.386*			
FWT	0.287	0.04	-0.208	0.11	0.102	0.141	0.147	-0.099	0.066		
TSS	0.089	0.195	0.25	0.053	-0.28	-0.5**	-0.49**	0.09	-0.194	-0.045	

*****Significant at $p \leq 0.05$

******Significant at $p \leq 0.01$

DPPH=DPPH free radical scavenging capacity, ABTS=ABTS free radical scavenging capacity, LPI=lipid peroxidation inhibitory capacity, NO=nitric oxide radical scavenging capacity TPC=total polyphenol content, TFC=total flavonoid content, TPAC=total proanthocyanidin content, TAC=total anthocyanin content, FRAP=ferric reducing antioxidant potential, FWT=fruit weight; TSS=total soluble solids.

5.3.6 ***Principal component analysis***

Principal component analysis (PCA) provides an objective way of finding indices that can be accounted for variation in the data set as concisely as possible (Sarbu and Pop, 2005). PCA was used to understand how antioxidant capacity of extracts, TPC, TAC, FWT and TSS contribute to variability among the 37 genotypes. PCA showed that first four PCs explained 27.8, 19.2, 11.2 and 10.7% of the variation respectively, making a total of 69% (Table 5.5). PC1 was strongly correlated with TPC (0.604), TFC (0.824), TAC (0.735), FRAP (0.787) and TSS (-0.519). The highly correlated variables with PC2 were DPPH* scavenging capacity of extracts (0.768), ABTS*$^{+}$ scavenging capacity of extracts (0.904) and LPI (0.634) of extracts. PC3 was highly correlated with NO* scavenging activity of extracts (0.758) and FWT (-0.518) while PC4 was dominated by TPC (-0.559) (Table 5.6).

Table 5.5 Principal component analysis for all eleven parameters.

Components	Eigen value	Variance (%)	Cumulative variance (%)
1	3.488	31.706	31.706
2	1.550	14.094	45.799
3	1.158	10.527	56.326
4	0.932	8.469	64.795
5	0.877	7.975	72.770
6	0.852	7.750	80.520
7	0.714	6.494	87.014
8	0.567	5.153	92.167
9	0.351	3.195	95.362
10	0.303	2.757	98.120
11	0.207	1.880	100

Table 5.6 Source of variation for first three principal components (PC).

Variable	PC1	PC2	PC3
DPPH	-0.608	0.114	0.451
ABTS	-0.784	0.033	0.297
LPI	-0.511	0.463	-0.082
NO	0.133	0.232	0.621
TPC	0.422	0.698	0.147
TFC	0.729	0.347	0.122
TPAC	0.416	-0.400	0.034
TAC	0.727	-0.179	0.146
FRAP	0.834	-0.103	0.055
FWT	-0.246	0.113	-0.566
TSS	-0.244	-0.304	0.304

DPPH=DPPH free radical scavenging capacity, ABTS=ABTS free radical scavenging capacity, LPI=lipid peroxidation inhibitory capacity, NO=nitric oxide radical scavenging capacity TPC=total polyphenol content, TFC=total flavonoid content, TPAC=total proanthocyanidin content, TAC=total anthocyanin content, FRAP=ferric reducing antioxidant potential, FWT=fruit weight; TSS=total soluble solids

5.3.7 ***GC-MS Analysis***

This is the first study which revealed detailed GC/MS profile of methanol fruit extract of *M. alba*. GC/MS analysis was carried for only two extracts, which showed high antioxidant capacity and bioactive content *viz*. BMA-03 and DMK-03. This study revealed presence of 20 and 22 different chemotypes in BMA-03 and DMK-03 extract, respectively (Table 5.7 and Figure 5.5). In BMA-03 extract there were 3 unique chemotypes (4- Hydroxypyridine-1-oxide, Ethyl, 4-methyl-3-Ketovalearate and Acetic acid 3- Acetoxy-1-ethyl-2-nitro butyl ester) which were not present in DMK-03. In DMK-03 there were 5 unique chemotypes (citraconic anhydride, 2,3-dimethyl fumaric acid, acetic acid 5-hydroxy-4-nitrotetrahydopyran-3-yl ester, sitosterol and 2,4-dihydroxy-2,5 -dimethyl-3(2H)-furan-3-one) which were not detected in BMA-03. The remaining 17 chemotypes were common in both extracts. In BMA-03 there were 6 major chemotypes which occupied more than 5% peak area each *viz*. methyl linoleate (21.5%), 5-(hydroxymethyl)-2-furancarboxaldehyde (HMF) (16.6%), ethyl linoleate (11.8%), methyl 3-hydroxyhexanoate, palmitic acid (5.4%) and dodecanoic acid 3-hydroxy (5.3%). In DMK-03 there were 9 major chemotypes which occupied more than 5% peak area each *viz*. 5-(hydroxymethyl)-2-furancarboxaldehyde (24.1%), methyl linoleate (10.6%), palmitic acid (7.7%), linolein1-mono (7.5%), ethyl linoleate (7.1%), methyl 3-hydroxyhexanoate (6.8%), acetic acid 5-hydroxy-4-nitrotetrahydropyran-3yl ester (6.39%), 2,3-dihydro-3,5-dihydroxy-6methyl-4H-pyran-4-one (6%) and dodecanoic acid 3-hydroxy (5.1%). HMF was found in both the extracts in high quantity, which is reported to have good biological activities such as anti-inflammatory activity and bacteriostatic action. Recent studies found that HMF has great protection effect to the CCl_4 liver injury and the vascular endothelial cell (Ouyang et al. 2011). Essential fatty acids (EFA) are a group of unsaturated fatty acids which are not produced in humans, can be obtained in diet and are necessary for the proper functioning of the human body. The two main families of EFAs are the ω-6 and the ω-3 groups. Linolenic and linoleic acids are essential fatty acids which belong to the omega 3 and omega 6 fatty acids respectively. The *M. alba* fruit extracts revealed presence of linoleic acid esters. Fruit extract was also rich in palmitic acid which is reported to have antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant, antiandrogenic, flavor, hemolytic 5-alphareductase inhibitor properties. Overall methanol fruit extract have good bioactive compound profile. Several flavor and aromatic compounds (e.g Methyl 3-

hydroxyhexanoate, 3-hydroxy-Dodecanoic acid 3-Furaldehyde and 4-methoxy-2-butanone) were also detected in fruit extract.

Table 5.7 Phyto-chemotypes identified in the methanol fruit extract of two *M. alba* samples (BMA-03, DMK-03) by GC/MS.

	BMA-03				DMK-03			
SN	Peak RT (min)	Peak Area	Peak Area %	Compound detected	Peak RT (min)	Peak Area	Peak Area %	Compound detected
1	5.9	406782	0.2	3-Furaldehyde	5.9	1002342	0.6	3-Furaldehyde
2	0.2	906794	0.4	2-Butanone,4-Methoxy	7.1	1759741	1.1	4-Methoxy-2-Butanone,
3	8.7	2617014	1.3	4-Hydroxypyridine-1-oxide	8.5	432394	0.3	Citraconic anhydride
4	10.8	591258	0.9	Methyl Acetoxyacetate	10.8	1634153	1.0	Methyl Acetoxyacetate
5	12.7	4538478	2.2	Ethyl, 4-methyl-3-Ketovalerate	11.5	3940554	2.5	2,3-dimethylfumaric acid
6	12.9	6586178	3.2	2,3-dihydro-3,5-dihydroxy-6methyl-4H-pyran-4-one	12.9	9509566	6.0	2,3-dihydro-3,5-dihydroxy-6methyl-4H-pyran-4-one
7	14.6	31677293	16.6	HMF / 5-(hydroxymethyl)-2-Furancarboxaldehyde	14.5	38333434	24.1	HMF / 5-(hydroxymethyl)-2-Furancarboxaldehyde,
8	15.0	9895739	4.9	Acetic acid 3-Acetoxy-1-ethyl-2-nitro butyl ester	14.9	10166215	6.4	Acetic acid 5-hydroxy-4-nitrotetrahydropyran-3yl ester
9	16.0	11470350	5.6	Methyl 3-hydroxyhexanoate	15.9	10825474	6.8	Methyl 3-hydroxyhexanoate
10	16.9	4894980	2.4	1,1-diethoxy-2-propanone	16.9	2378417	1.5	1,1-diethoxy-2-propanone
11	17.4	5755675	2.8	3-hydroxy Dodecanoic acid	17.4	8155679	5.1	3-hydroxy-Dodecanoic acid
12	19.4	4018032	2.0	D-Glucopyranoside	19.1	798310	0.5	D-Glucopyranoside
13	20.6	6918084	3.4	Decanoic acid 9-oxo-methyl ester	20.6	632055	0.4	Decanoic acid 9-oxo-methyl ester
14	22.1	10750758	5.3	Dodecanoic acid 3-hydroxy	21.9	4814290	3.0	Dodecanoic acid 3-hydroxy
15	22.3	5557986	2.7	Nonanoic acid 2-acetyl, ethyl ester	22.3	3134447	2.0	Nonanoic acid 2-acetyl, ethyl ester
16	25.0	8967518	4.4	Methyl Palmitate (Hexadecanoic acid methyl ester)	25.0	6090353	3.8	Methyl Palmitate (Hexadecanoic acid methyl ester)
17	25.4	9233218	5.4	Palmitic acid (Hexadecanoic acid)	25.4	12305890	7.7	Palmitic acid (Hexadecanoic acid)
18	27.1	41533471	21.4	Methyl Linoleate	27.0	16793633	10.6	Methyl Linoleate
19	27.5	21955290	11.8	Ethyl Linoleate	27.5	9882095	7.1	Ethyl Linoleate
20	27.8	5411053	2.7	Linolein, 1-mono	27.8	11864687	7.5	Linolein, 1-mono
21	--	--	--	--	42.3	1480648	0.9	Sitosterol
22	--	--	--	--	9.3	1590400	1.0	2,4-dihydroxy-2,5-dimethyl-3(2H) -furan-3-one

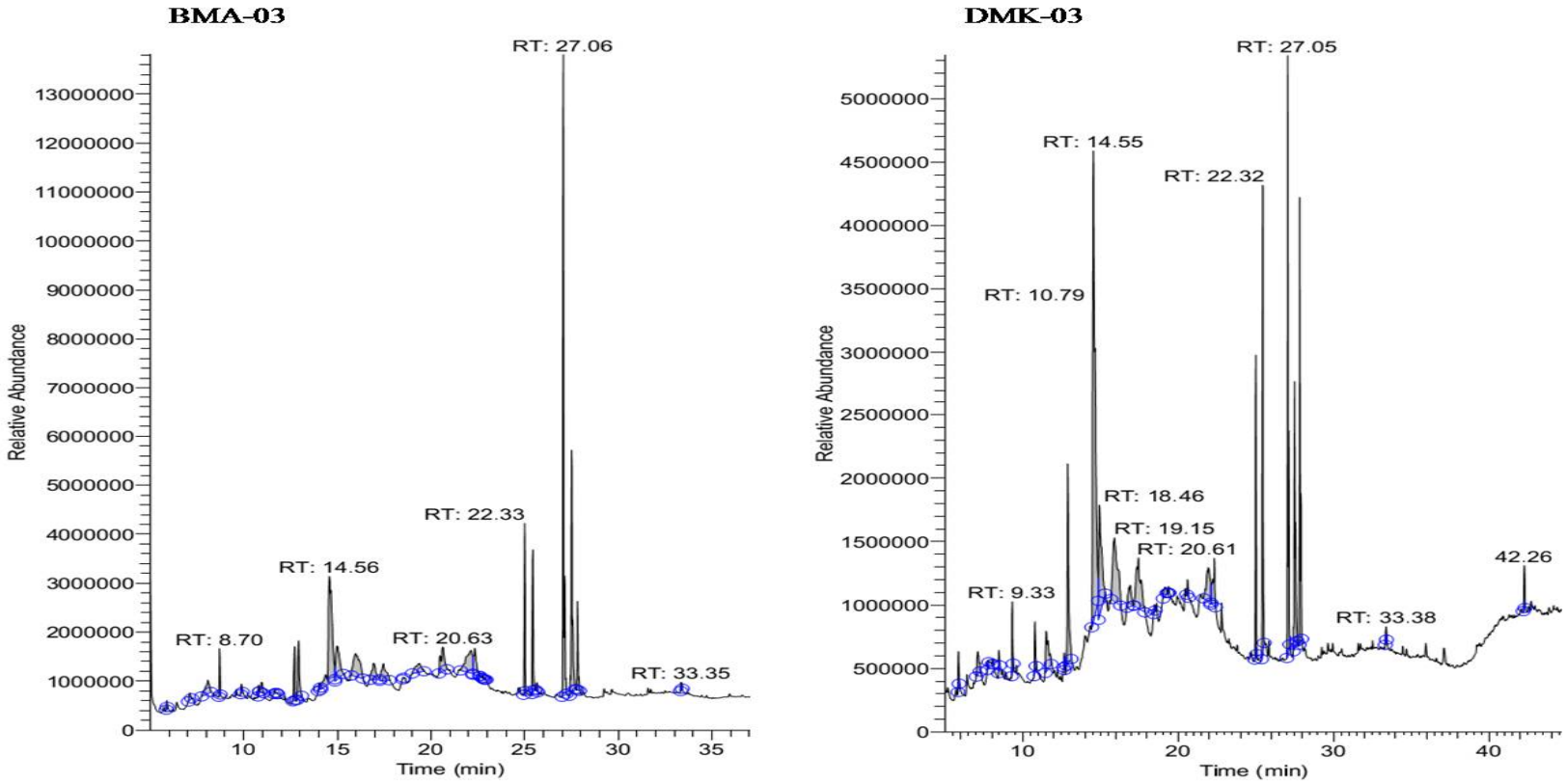

Figure 5.5 GC/MS chromatogram of methanol fruit extract of two *M. alba* L. samples.

5.3.8 ***Mineral analysis***

Accurate and adequate food composition data are invaluable for estimating the adequacy of intakes of essential nutrients and assessing exposure risks from intake of toxic non-essential elements (Onianwa et al. 2001). This is the first study in *M. alba*, which reported presence of 13 essential and trace elements. Previous studies reported only 10-11 elements (Ercisli and Orhan, 2007; Imran et al. 2010). We analyzed 13 essential macro and micro (trace) elements in *M. alba* fruit collected from 37 genotypes belonging to twelve locations. Out of thirteen elements 10 elements (Al, B, Ca, P, Mg, Na, K, Fe, Cr and Cu) were detected in all 37 genotypes (Table 5.8 and 5.9). Among other minerals Mn was detected in 24 genotypes, Ni in 22 and Se in 12.

Table 5.8 Essential macronutrients composition (mg/100g of dry weight) in *M. alba* L. fruit.

Location	Na	K	Ca	P	Mg
SKB	62.9±64.7^{a} (3.2-121.6)	1177.3±348.1^{a} (867.3-1635.5)	634.6±198.6^{a} (421.8-842.9)	254.9±110.2^{ab} (175-414.3	193.9±44.6^{a} (138-232.5)
DMK	581.7±352.4^{c} (332.5-830.9)	1124.2±105.4^{a} (1049.6-1198.7)	840.3±168.2^{ab} (721.4-959.3)	336.8±113.8^{ab} (256.3-417.3)	229.7±24.4^{a} (212.5-247)
ACH	119.3±122.6^{a} (32.6-206.0)	1253.5±280.1^{a} (1055.4-1451.6)	608.8±283.7^{a} (408.2-809.4)	211.3±46.7^{ab} (178.3-244.3)	206.9±4.4^{a} (203.8-210)
BMA	58.6±13.2^{a} (46.8-73.1)	1890.7±793.4^{a} (993.6-2905.6)	774.2±210.8^{ab} (548.4-1055)	414.7±156.47^{abc} (211.6-582.7)	227.4±55.2^{a} (192.1-309.5)
SNJ	183.8±65.1^{ab} (137.7-229.8)	1094.1±219.6^{a} (938.9-1249.4)	867.9±188.2^{ab} (734.9-1001)	601.3±19.8^{c} (587.3-615.3)	308.2±31.7^{ab} (285.8-330.6)
BTK	41.4±37.5^{a} (11.6-90.0)	798.8±118.1^{a} (686.1-956)	494.5±103.1^{a} (409.8-644.2)	174.8±24.0^{a} (152.8-207.4)	192.0±17.2^{a} (177.9-215.9)
AKM	26.4±29.1^{a} (0.7-60.4)	740.6±80.3^{a} (641.4-815)	564.3±148.2^{a} (415.3-704.4)	147.7±8.1^{a} (138.1-157.1)	170.9±37.4^{a} (131.4-206)
PSY	46.5±38.3^{a} (0.4-80.4)	838.4±152.5^{a} (673.9-1024.6)	378.6±62.3^{a} (293.9-441.1)	155.8±24.9^{a} (135.2-186)	179.1±18.6^{a} (163.8-206.1)
LCM	528.2±319.3^{bc} (320.5-895.8)	1354.3±250.2^{a} (1174.2-1640)	1407.6±731.2^{b} (575.8-1949)	339.9±94.6^{abc} (243.3-432.3)	388.7±163.4^{b} (201.9-505.2)
CHK	371.4±274.6^{abc} (177.2-565.6)	1686.7±1027.1^{a} (960.4-2413)	832.1±208.6)^{ab} (684.6-979.6)	484±83.0^{bc} (425.3-542.7)	323.4±60.3^{ab} (280.8-366.1)
BKD	61.5±7.6^{a} (56.1-66.9)	1716.9±878.4^{a} (1095.8-2338)	412.3±45.7^{a} (380-444.6)	493.8±7.8^{bc} (488.3-499.3)	224.4±5.2^{a} (220.8-228.1)
TRK	74.1±50.0^{a} (24.2-124.8)	1255.2±91.3^{a} (1162.6-1363.6)	494.8±78.6^{a} (421.5±595)	456±164.3^{bc} (212.1-563.3)	189.6±15.2^{a} (177.8-210.8)

Data are mean ± standard deviation, range given in brackets

a-c=Means in a column with the same superscript letter are not significantly different (p=0.05), as measured by 2 sided Tukey's-b post-hoc range test between genotypes

Table 5.9 Concentration (mg/100g of dry weight) of trace elements in *M. alba* L fruit.

Location	Fe	Cr	Cu	Ni	Mn	Se	Al	B
SKB	23.6±18.7[a] (11.9-51.5)	3.4±1.2[ab] (1.6-4.1)	1.7±0.7[a] (0.6-2.3)	0.9	1.5±0.8[ab] (0.35-2.1)	125.6	10.8±2.0[a] (9.2-13.8)	4.0±0.4[a] (3.7-4.6)
DMK	26.9±2.0[a] (25.5-28.4)	0.74±0.01[a] (0.73-0.74)	1.3±0.6[a] (0.9-1.7)	0.6±0.0[a] (0.6-0.6)	0.87	79.9±24.1[a] (62.8-96.9)	7.7±1.8[a] (6.4-8.9)	3.7±0.1[a] (3.6-3.7)
ACH	13.8±2.9[a] (11.8-15.8)	0.8±0.2[a] (0.6-0.9)	1.9±1.8[a] (0.6-3.1)	1.1±0.0[a] (1.1-1.1)	ND	ND	3.7±0.7[a] (3.2-4.3)	5.6±0.3[a] (5.41-5.83)
BMA	21.9±7.1[a] (14.9-31.5)	1.7±1.7[ab] (0.8-4.3)	1.9±0.7[a] (1.2-2.9)	1.2±0.1[a] (1.1-1.2)	1.4±1.6[ab] (0.3-2.6)	ND	7.6±3.3[a] (4.3-12.0)	7.2±1.9[a] (4.7-8.9)
SNJ	20.8±4.2[a] (17.8-23.7)	0.9±0.1[a] (0.8-0.9)	3.6±0.8[a] (3.0-4.2)	0.9±0.2[a] (0.7-1.0)	ND	45.4±20.2[a] (31.2-59.7)	4.7±0.9[a] (4.0-5.3)	4.9±1.1[a] (4.0-5.7)
BTK	20.5±6.4[a] (16.6-30.1)	4.1±0.2[b] (3.9-4.3)	3.0±1[a] (1.9-4.3)	ND	1.9±1.0.3[b] (1.7-2.3)	ND	13.3±2.4[a] (11.4-16.8)	6.03±4.48[a] (3.4-12.7)
AKM	16.1±3.2[a] (12.0-19.8)	3.1±1.6[ab] (0.7-3.9)	2.3±0.2[a] (2-2.5)	1.4	2.0±0.3[b] (1.8-2.4)	ND	12.4±4.5[a] (6.3-17)	3.5±0.4[a] (3.0-4.0)
PSY	26.5±17.3[a] (16-52.2)	4.1±0.2[b] (3.9-4.3)	2.4±1.4[a] (1.5-4.4)	ND	2.0±0.4[b] (1.6-2.6)	ND	17.4±8.8[a] (11.9-30.4)	5.5±2[a] (2.75-7.4)
LCM	39.7±14.5[a] (22.87-48.09)	1.2±0.2[a] (1.1-1.5)	2.2±2.7[a] (0.5-5.3)	1.0±0.6[a] (0.6-1.7)	2.1±0.3[b] (1.9-2.3)	93.4±41.6[a] (47.6-129.9)	25.6±21.8[a] (7.8-5)	5.4±1.6[a] (3.6-6.7)
CHK	40.7±8.5[a] (34.7-46.7)	1.2±0.2[a] (1-1.3)	0.8±0.2[a] (0.7-1.0)	2.1±0.5[b] (1.8-2.5)	0.6	62.7±85.1[a] (2.5-122.9)	16.3±8.3[a] (10.5-22.1)	5.7±0.2[a] (5.6-5.9)
BKD	26.5±0.1[a] (26.4-26.6)	0.9±0.0[a] (0.9-1.0)	0.75±0.1[a] (0.7-0.8)	1.3±0.2[ab] (1.1-1.5)	0.1±0.0[a] (0.04-0.1)	166.9	7.8±0.3[a] (7.6-8.1)	5.4±1.7[a] (4.2-6.7)
TRK	27.5±4.6[a] (24.1-34.0)	1.2±0.2[a] (1.0-1.5)	3.1±1.6[a] (1.8-5.4)	0.8±0.2[a] (0.5-1.0	0.2	101.4	7.7±3.2[a] (5.6-12.5)	8.6±4.9[a] (4.3-13.9)

Data are mean ± standard deviation; range given in brackets; data without standard deviation and range indicates single individual with respective element in that location; ND=element not detected in that location

Minerals content was expressed in mg/100g of dry weight. Among essential macronutrients Na ranged from 0.4 to 895.8 mg/100g dry weight, K ranged from 641.1 to 2905.6 mg/100g dry weight, Ca ranged from 293.9 to 1949 mg/100g dry weight, P from 135.2 to 615.3 mg/100g dry weight and Mg from 131 to 505.1 mg/100g dry weight. Out of five essential elements, Na, Ca and P had significantly different mean ($p \leq 0.05$).

Among micronutrients Fe ranged from 11.8 to 52.2 mg/100g dry weight, Cr from 0.7 to 4.3 mg/100g dry weight, Cu from 0.5 to 5.4 mg/100g dry weight, Ni from 0.5 to 2.5 mg/100g dry weight, Mn from 0.0 to 2.6 mg/100g dry weight, Se from 2.5 to 166.9 mg/100g dry weight, Al from 3.2 to 50 mg/100g dry weight and B from 2.7 to 13.9 mg/100g dry weight. Among macronutrients, higher content of K, Na and P was obtained in the present study as compared to that of previous studies [Ercisli and Orhan, 2007; Imran et al. 2010; Karlidag et al. 2011]. As far as essential micro-nutrients are concern, this study revealed higher concentration of Cu and Ni in comparison to previous studies; other micro-nutrients fell within range as reported earlier.

5.4 Conclusion

The influence of plant genetic background and fruit color on antioxidant capacities, TPC, TFC, TPAC and TAC in *M. alba* fruit extracts was demonstrated. Source of genotypes from different sampling sites has significant effect on the studied health promoting compound content. Contrary to the findings that different colored fruit belonging to different species of *Morus* has significant effect on the content of health promoting compounds, we did not find significant effect of fruit color within *M. alba*. Results obtained in this study can be considered for selection of genotype for breeding purpose to improve health promoting compounds in *M. alba* from the trans-Himalaya. GC/MS analysis revealed the presence of healthy ω-6 fatty-acids. It appeared from the results that mulberry is a good source of macro and micro nutrients.

CONCLUSIONS

Phenotypic variation in *Morus alba* L. along altitudinal gradient

The present study revealed significant variation in morphological characters among *M. alba* populations in the trans-Himalaya. *M. alba* typically showed high phenotypic variation along an altitudinal gradient. A small change in altitude in the trans-Himalaya leads to significant change in phenotype. The results suggested that a small increase in altitude lead to high adversity for plant to survive in the trans-Himalayan condition. This study suggests that observed phenotypic variation to some extent maintained by phenotypic plasticity of *M. alba*. Understanding the role of phenotypic plasticity is necessary for predicting how plants will respond to global warming or other environmental change in future. Phenotypic plasticity in *M. alba* in the trans-Himalaya may serve as a model system to study plant adaptation to future environmental changes. So further study in controlled environment is needed to completely unravel the phenotypic plasticity of *M. alba*. The Himalayan region is considered as centre of diversity for Mulberry [Chen et al., 2010], so this study also has evolutionary significance, as it provides a basic profile of *M. alba* phenotypic plasticity in the trans-Himalayan region for future researchers.

Structure and genetic diversity of natural populations of *M. alba* L

In conclusion, mixed population structure appeared in this study. Nubra population clearly displayed structuring with three subpopulations. But Indus and Suru population did not display any significant structuring. Ladakh mountain range proved an important barrier to gene flow and shaped population genetic structure of *M. alba* in trans-Himalaya. The results shed light on role of geographical barriers in speciation. The genetic diversity have important role in plants survival as it provides disease resistance, adaptability to a changing climate, or some other trait necessary to survive in the ever-changing world. The present study reported overall high genetic diversity at species level. There is balance between migration and genetic differentiation that helps in maintaining genetic diversity. These results also have evolutionary significance as Himalayan region is considered as centre of diversity for Mulberry. Furthermore, our results provide a basic genetic profile for conservation and responsible exploitation of the extant germplasm of this species to improve the genetic base for breeding. SRAP markers are highly reproducible and efficient markers for assessment of genetic structure and diversity among wild populations.

Detecting molecular signatures of natural selection in *M. alba* L. populations

This study display footprints of natural selection in *M. alba* genome. The natural selection was detected with high stringency in hierarchical island model. Overall, *M. alba* falls in the range of mixed mating and outcrossing species. But population from Nubra valley showed selfing with high SGS. The presence of natural selection is further evident by presence of SGS in *M. alba* natural populations. The knowledge of the spatial structure of populations is important when selecting natural populations for conservation and sampling for breeding programs. It should be taken into account in order to maximize diversity, and to avoid misrepresenting species and population diversity [Epperson and Allard, 1989; Shapcott, 1995]. Secondly, because spatial patterns change in a substantial, cumulative and sometimes characteristic manner due to the past effects of natural selection and other factors, spatial pattern analysis can help to detect the action of these factors [Epperson, 1993]. Further, this study sheds light on adaptability of *M. alba* in trans-Himalaya and revealed that some region of *M. alba* genome is under local selection.

Chemical composition and antioxidant activities *M. alba* L. fruit

The influence of plant genetic background and fruit colour on antioxidant capacity, TPC, TAC, TFC, TPAC and TAC in *M. alba* fruit was demonstrated. Source of genotypes from different sampling sites has significant effect on the studied health promoting compound content. Contrary to the findings that different colored fruit belonging to different species of *Morus* has significant effect on the content of health promoting compounds, we did not find significant effect of fruit color within *M. alba*. Results obtained in this study can be considered for selection of genotype for breeding purpose to improve health promoting compounds in *M. alba* from the trans-Himalaya. GC/MS analysis revealed the presence of healthy ω-6 fatty-acids. It appeared from the results that mulberry is a good source of macro and micro nutrients.

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1. **Prabodh K. Bajpai**, Ashish R. Warghat, Ram Kumar Sharma, Ashish Yadav, Anil K. Thakur, Ravi B. Srivastava, Tsering Stobdan (2014). Structure and genetic diversity of natural populations of *Morus alba* L. in trans-Himalayan Ladakh region. *Biochemical Genetics*, vol. 52, pp. 137-152. **Impact factor- 0.938**
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