High Phenotypic Variation in *Morus alba* L. along an Altitudinal Gradient in the Indian Trans-Himalaya

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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 above the sea level (asl). Coefficient of variation (CV) showed high phenotypic variation in *M. alba*. Linear regression analysis revealed that leaf and fruit size decreases with an increase in altitude. High CV was observed for leaf length, leaf width, petiole length, leaf area, inter-nodal distance, number of nodes, bud length, fruit length, fruit width and fruit weight. Similarly, a high phenotypic plasticity index was observed for bud length, leaf length, leaf width, petiole length, leaf area, inter-nodal distance, number of nodes, fruit length, fruit width and fruit weight. 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 the altitude caused significant change in the plant morphological characteristics. The present investigation represents to our knowledge the first study addressing phenotypic variation in mulberry along an altitudinal gradient.

Keywords: Adaptation; Ladakh; Leaf; Morphometry; Mulberry; *Morus alba*; Stress

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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 characteristics 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; Stenström et al. 2002). Plant adjustment to environment 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 plant adjustment to environment 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; Valldares et al. 2006). Study of plant adjustment to environment 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 gradients are particularly relevant in order to study plants phenological responses to temperature since they provide a wide temperature range over 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).

Morus alba L. (Moraceae) is a small to medium-sized dioecious, occasionally monoecious, perennial, heterogeneous tree, distributed in tropical, sub-tropical and temperate zones in Asia, Europe, North east America, Africa and South west America (Kafkas et al. 2008). It is a wind pollinated and out breeding species (Awasthi et al. 2004). Fruiting occurs in the trans-Himalayan region in July and fruit colour varies from white, purple, or pink to red. The fruit is botanically called a sorosis and it is juicy and has a sweet taste with some sourness that is more prominent in the less mature fruits. In India, there are mainly four species of *Morus* viz. *M. alba, M. indica*, *M. serrata* and *M. laevigata*. Among them, *M. alba* is the most important species from the sericulture point of view. In the trans-Himalayan Ladakh region of India, *M. alba* is found at 2700-3300 m asl. A long spell of subzero temperatures force *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 (Bajpai et al. 2014).

Mulberry is a promising plant for studying phenotypic plasticity. The genus is cosmopolitan in nature, easily adapted to different ecological conditions, and easily hybridized both naturally and artificially, which creates 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, no study sheds light on the phenotypic response of mulberry along altitudinal gradients 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 (Debat and David, 2001). The present investigation was undertaken to study the effect of altitudinal gradient on the phenotypic variation of *M. alba* in the trans-Himalayan Ladakh region. Our working hypothesis is: Is there a statistically-significant effect of altitude on phenotypic variation of *M. alba*? The present investigation represents what is to our knowledge the first study addressing phenotypic variation in mulberry along an altitudinal gradient.

1 Materials and Methods

1.1 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 2009-2010 (Table 1). The valley divisions were considered as separate populations and collection sites as sub-populations. The altitude of collection sites ranged from 2815 - 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 34°08'15" N, 77°34'18" E, in the region was 18.9±9.5°C and -5.8±9.8°C, 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.

Table 1 **Geographical location and sampling site of *Morus alba* L. in the trans-Himalaya**

Valley	Sub Pop (ID)	No.	Alt	Lat	Lon
Indus	Skurbuchan (SKB)	4	2895	34°25'N	76°43'E
	Domkhar (DMK)	4	3177	34°19'N	76°45'E
	Achinathang (ACH)	4	2892	34°30'N	76°37'E
	Beama (BMA)	4	2840	34°36'N	76°30'E
	Sanjok (SNJ)	4	2928	34°34'N	76°31'E
Suru	Batalik (BTK)	4	2815	34°39'N	76°20'E
	Achkamal (AKM)	4	2901	34°33'N	76°09'E
	Paschym (PSY)	4	2873	34°31'N	76°10'E
	Lochum (LCM)	4	3034	34°28'N	76°15'E
Nubra	Chalunka (CHK)	4	2991	34°49'N	76°58'E
	Bokdang (BKD)	4	3010	34°48'N	77°02'E
	Turtuk (TRK)	4	2816	34°50'N	76°49'E
	Tyakshi (TYK)	4	2971	34°53'N	76°48'E
	Hunder (HUN)	4	3177	34°19'N	77°27'E

Notes: Sub Pop - Sub Populations; ID - Population ID; No. - No. of samples; Alt - Altitude (m asl); Lat - Latitude; Lon – Longitude.

1.2 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. Four trees per sub-population were sampled 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 0.001 g.

1.3 Statistical analysis

Minimum, first quartile, median, third quartile and maximum values were calculated for each data set. Tukey's honestly significant difference test was used to assume equal variances with $p \le 0.05$. 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 fitted with 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. released in 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 among population by following formula: PPI = maximum mean - minimum mean/maximum mean (Balaguer et al. 2001; Gratani et al. 2003; Valladares et al. 2006).

2 Results

2.1 Variation in morphological characters

Morphological characters showed high variation (Figures 1 and 2). 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,

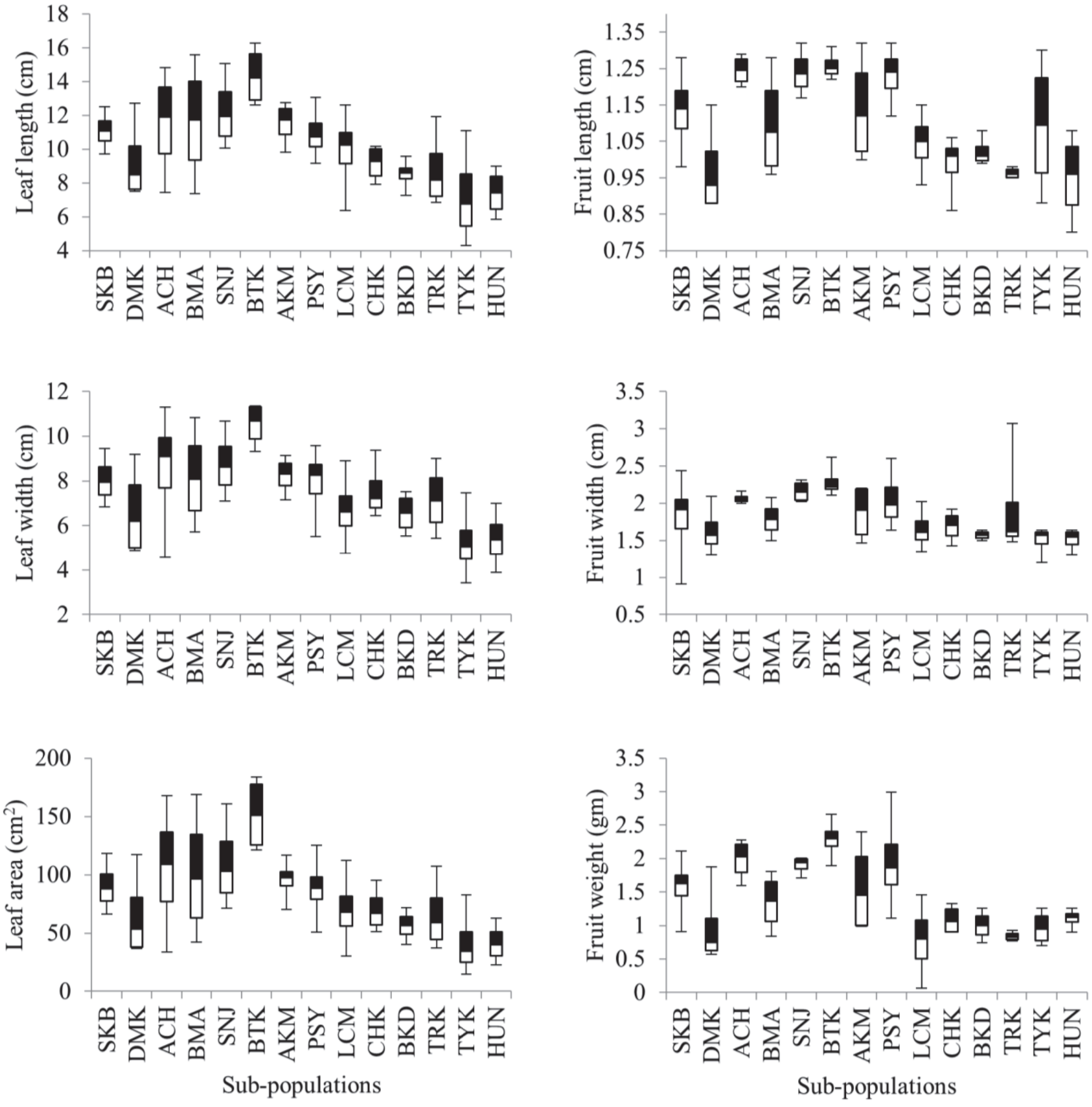

Figure 1 Box plot showing minimum, 1st quartile (blank box), median, 3rd quartile (black box) and maximum values of leaf and fruit characters in 14 sub-populations of *M. alba* (SKB: Skurbuchan, DMK: Domkhar, ACH: Achinathang, BMA: Beama, SNJ: Sanjok, BTK: Batalik, AKM: Achkamal, PSY: Paschym, LCM: Lochum, CHK: Chalunka, BKD: Bokdang, TRK: Turtuk, TYK: Tyakshi, HUN: Hunder).

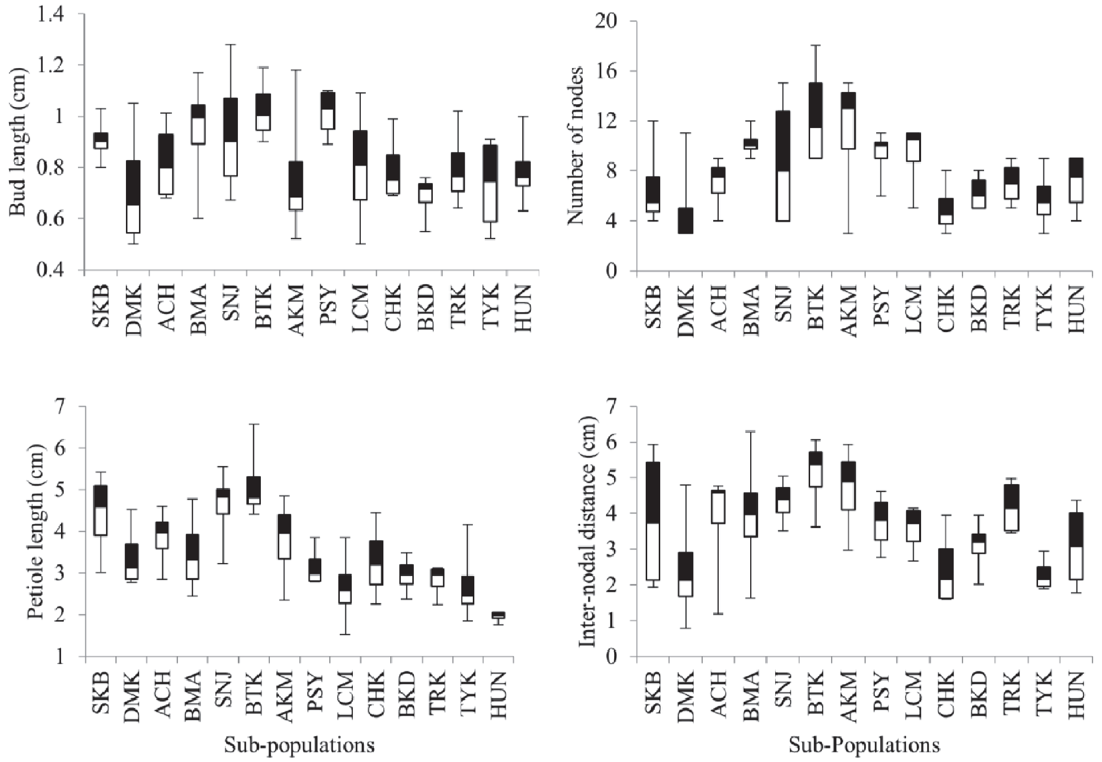

Figure 2 Box plot showing minimum, 1st quartile (blank box), median, 3rd quartile (black box) and maximum values of bud length, petiole length, number of nodes and inter-nodal distance in 14 sub-populations of *M. alba*.

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 Figures 1 and 2, representing variation in different morphological characters.

The PCA based on a 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). Overall, variability in PC1 was explained mainly by leaf traits while in PC2 the variability was explained mainly by fruit traits (Figure 3).

PPI for 10 morphological characters is presented in Figure 4. 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).

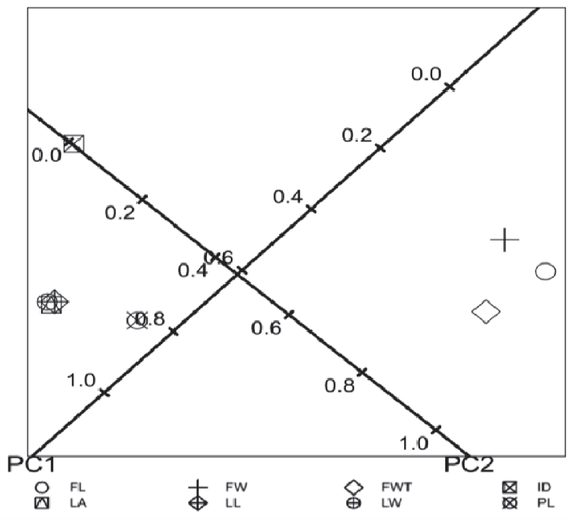

Figure 3 Biplot showing first two components of principal component analysis (LL: leaf length; LW: leaf width; PL: petiole length; LA: leaf area; ID: inter-nodal distance; FL: fruit length; FW: fruit width; FWT: fruit weight).

2.2 Effect of altitudinal gradient on morphology

The ANCOVA was carried out to see the effect of population and altitude on morphological characters. ANCOVA clearly revealed predominant effects of altitude on morphology. Population and interaction between population and altitude showed effect on petiole length (Table 2). Altitudinal variations present in eight morphological characters viz. leaf length, leaf width, leaf area, petiole length, number of nodes, inter-nodal distance, fruit width and fruit weight are presented in Figure 5. Leaf length, leaf width and leaf area showed 1 cm, 0.8 cm and 16.6 cm² decrease per 100 m increase in altitude, respectively. Petiole length, inter-nodal distance and number of nodes displayed an inverse relationship with altitude. Fruit size decreased significantly with increase in altitude, especially fruit width.

Table 2 One way ANCOVA analysis for morphological characters with population fixed factor and altitude as covariate

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

Notes: *F* - F ratio; d.f - Degrees of freedom; *Significant at $p \le 0.05$; **Significant at $p \le 0.01$; ***Significant at $p \le 0.001$.

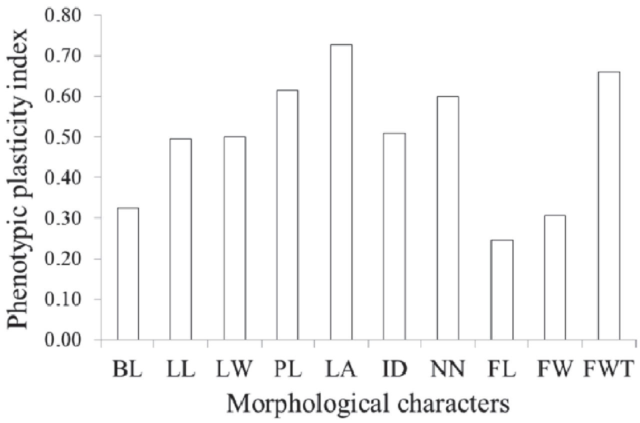

Figure 4 Phenotypic plasticity index of ten morphological characters (BL: bud length; LL: leaf length; LW: leaf width; PL: petiole length; LA: leaf area; NN: number of nodes; ID: inter-nodal distance; FL: fruit length; FW: fruit width; FWT: fruit weight).

2.3 Correlation among morphological characters

Pearson correlation among different morphological characters is presented in Table 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).

3 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 any empirical evidence that 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 the 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 was observed.

Coefficient of variation is a simple and common measure of phenotypic variation used in

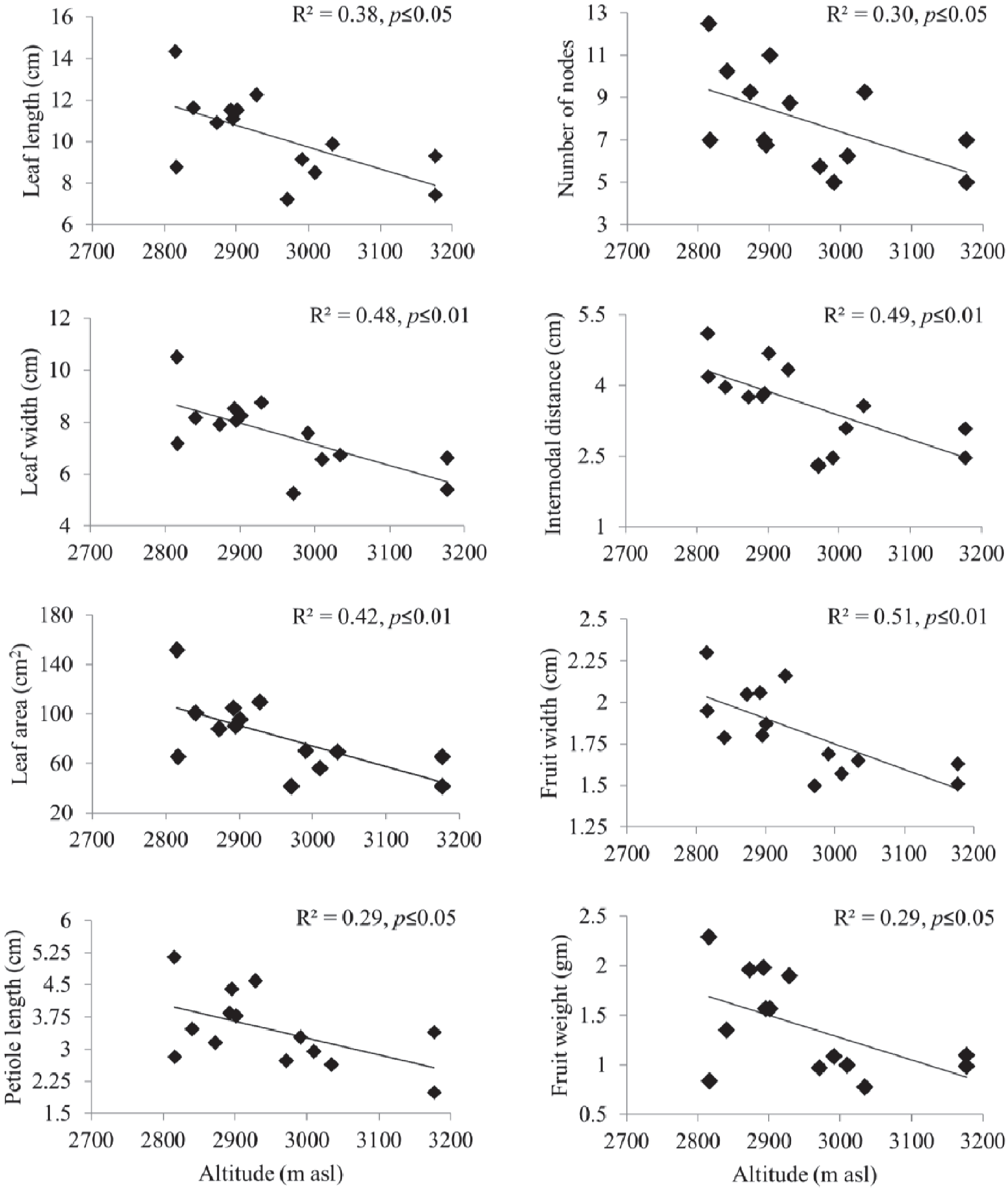

Figure 5 Altitudinal variation in leaf length, leaf width, leaf area, petiole length, number of nodes, inter-nodal distance, fruit width and fruit weight. Each point in graph represents four individuals.

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 (26.6%), 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%) 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

Table 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										

Notes: *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

al. (2006) found 29.8%, 27.9% and 44.6% 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 among population in terms of 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 manifest in phenotype.

These 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 their phenotypic features. *M. alba* adjusts itself to varying habitat conditions by modifying leaf size, petiole length, number of nodes and 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 plants 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 shadowed trans-Himalayan region. Plants decrease the size of their parts to reduce water loss through transpiration. Another possibility is that colder soils reduce the water uptake of the root system and induce water stress (Magnani and Borghetti 1995). Fruit morphological characteristics such as length, width and weight also decreased significantly with increased in altitude. Fruit morphological characteristics 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).

4 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 plants to survive in the trans-Himalayan conditions. This study suggests that observed phenotypic variation to some extent is maintained by phenotypic plasticity. 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. Further study in a controlled environment is needed to unravel the phenotypic plasticity of *M. alba*. The Himalayan region is considered to be a 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 variation in the trans-Himalayan region for future research.

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