Morphological trait variations in the west Himalayan (India) populations of *Arabidopsis thaliana* along altitudinal gradients

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Morphological trait variations in four populations of *Arabidopsis thaliana* that grow along altitudinal gradients (~700 to ~3500 m amsl) are described. A total of 38 traits were analysed from each of the four populations. Most of the quantitative traits were significantly correlated with each other among the four populations, but biomass-related traits were negatively correlated with altitude. There was significant correlation between geographical distance and mean pairwise distance of phenotypic traits among the populations. Overall our data suggest there is significant variation in phenotypic traits of the four populations along altitudinal gradients.

Keywords: Altitudinal gradients, *Arabidopsis thaliana*, genomic resources, morphological traits.

ARABIDOPSIS THALIANA (L.) Heynh. ($2n = 10$), commonly known as mouse ear cress or wild thale, belongs to the mustard family Brassicaceae. It is an important model plant species for studies in plant molecular biology, physiology, genetics and evolutionary biology and ecology[1–4]. Its use as a model species is facilitated by its short life cycle in the laboratory, production of large number of seeds and self-fertilization. Being a weed, *A. thaliana* is frequently found in human-disturbed areas such as roadsides, agricultural fields, river banks, mountainous slopes, etc. *A. thaliana* shows a wide range of traits and genetic variations among wild-type lines collected from the field. Moreover, there is unparalleled availability of genomic resources, which makes it highly suitable for studies of natural variations.

The genus *Arabidopsis* comprises nine species and eight subspecies. Among them, *A. thaliana* can be distinguished by morphological characteristics such as fruit and seed shape. The nine species of the genus *Arabidopsis* are primarily found in Europe. Two species are found in Asia and one in North America, but only *A. thaliana* has a worldwide distribution[5]. *A. thaliana* reproduces almost exclusively through selfing[6,7], though there are reports of cross-pollination to an extent of 1–3% (refs 8, 9).

There are several studies on phenotypic trait variations in *A. thaliana*[10–26]. Most of these reports were based on plants grown under controlled growth conditions[11,12,14,15,17–22,26–28] or common garden experiments[29–33]. There are also reports on latitudinal as well as altitudinal trait variations in *A. thaliana* from both native and non-native regions of the species[14,18,20–22,25,26,34,35]. Most of these studies employed artificial controlled growth conditions for phenological and morphological characterization of populations. However, this simulation of natural environment cannot account for all the variability present in their natural habitats. A phenotypic trait expression is best represented when it is measured in actual habitats of the plants. Thus, it has been shown that when grown under field and controlled conditions, plants differ in their light response and leaf morphology[36]. To the best of our knowledge, there is no report on any detailed study on *A. thaliana* from India, which is currently represented by only two accessions, viz. Kas-1 and Kas-2 in the two *Arabidopsis* seed stock centres (ABRC, Ohio State University, USA and NASC, University of Nottingham, UK). However, concerns have been raised about the origin of these accessions, they stand aloof from Asian accessions in phylogeny[37]. Further, its genomic signatures show higher affinity towards European accessions and is believed to have been transported from Europe to India sometime in the 19th century[38].

Presence of *Arabidopsis* species in the Western Himalaya has been mentioned in the *Floras of India*[39–44] with specimens preserved in some herbaria, viz. National Central Herbarium, Botanical Survey of India, Kolkata, Forest Research Institute, Dehradun, and Botanical Survey of India, Dehradun. However, no detailed studies on this model species have been conducted from the region. Here, we describe the morphological trait variations of the natural populations of *A. thaliana* that grow at different altitudinal gradients. Further, we assess the relationship

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between altitudinal gradients and observed variations in phenotypic traits in these populations.

Material and methods

Sample collection and phenotypic data

Field visits were conducted coinciding with the early flowering stage of plants at each of the four sites, viz. Dehradun (second week of February), Munsyari (first week of April), Sangla and Chitkul (third week of May). Details of geographical locations of the population are given in Table 1. These four populations are hereafter referred to as Deh, Mun, Sang and Chit respectively. The habitat of the Deh population was in highly disturbed open lawn, whereas the Mun population was found in undisturbed fertile hill slope. The habitat of Sang population was along the country roadside and Chit habitat was represented by undisturbed mountainous slopes along riverside. The representative habitats are shown in Figure 1. To minimize the bias due to impact of microclimate on plant growth, area within each population was selected that was maximally represented by plants of almost uniform growth and exposed to uniform sunlight. Each site was then divided into four patches for the ease of sampling. From each such patch around 7–10 individuals were randomly selected and morphological data were recorded for each plant. Each patch was considered as a replicate for statistical analysis. For recording morphological traits, plants with first flower open (principal growth stage 6.00)[45] to plants having not more than 10 green fruits were selected. A total of 121 plants were phenotyped. Both quantitative and qualitative phenotypic data were recorded for 38 traits per plant. All leaf traits were measured from three recently fully expanded leaves per individual and their averages were considered. Details of other trait measurements are given in Table S1 (see Supplementary material online). All the data were recorded in the field, except the microscopic data, viz. density of trichome, stomata and type of trichome, which were recorded from the same individuals after bringing them back to the laboratory. To record trichome density and stomatal density, one fully expanded mature leaf from each plant and 8–10 plants from each site were selected. To measure the stomatal density epidermal impression of the lower surface of the leaf was taken by applying a thin layer of transparent fingernail polish. After allowing it to dry for 10 min, the imprint was removed from the leaf with the help of a clear adhesive tape and glued onto the microscopic slide. The imprints were visualized on light microscope at a magnification of 20×. This magnification was kept constant and the microscopic field area was taken as a unit area. About five portions of each leaf were observed and stomata were counted manually. The stomatal density was calculated as the number of stomata per unit area (microscopic field).

Climatic data

The surveyed areas are located at remote places and have no weather stations, except for Dehradun. Therefore, to extract climatic data, we used the climate data interpolation method as described by Hijmans *et al.*[46]. This dataset besides having a high spatial resolution (1.0 sq. km), considers more weather stations and more accurate global elevation dataset and can be used for studies in mountain environments[46]. Several other studies have also used these data to interpret morphological trait variations at different climatic conditions[13,25]. Further, we compared the exact climatic data available for Dehradun (1901–2000; www.imd.gov.in/section/nhac/mean/Dehradun.htm) with the interpolated data and found no significant differences between the two (results not shown). The climatic variables were obtained from the WorldClim database (www.worldclim.org) for the period of 50 years from 1950 to 2000 and data were extracted using the DIVA-GIS program (www.diva-gis.org). Data for climatic variables were obtained with a spatial resolution of 30 arc sec or 1 sq. km and were generated on the basis of monthly temperature and rainfall values averaged for the period from 1950 to 2000. Nineteen bioclimatic variables were obtained from these data, including temperature and precipitation. The data for the solar radiation were taken from the global climatological database, meteonorm (www.meteonorm.com), which is a combination of climate database, a spatial interpolation tool and a stochastic weather generator. Meteonorm version 7 was used to extract the radiation data from the period 1986 to 2005. Details of the climatic data for the four study site are given in Table S2 (see Supplementary material online). We considered annual mean climatic parameter data instead of growing period data for our analyses. This is because germination timing of *A. thaliana* is highly influenced by environmental cue as well as vernalization requirement in some cases, which in turn may affect the phenotypes of plants[47]. Therefore, mean annual climatic conditions may provide appropriate effect on *A. thaliana* growth rather than considering only growing period climatic data. The climatic data were correlated with each altitude using univariate regression analysis.

Statistical analysis

To find out the correlation between climatic variables and altitude five variables, viz. annual mean maximum temperature, annual mean minimum temperature, annual precipitation, diffused light intensity and light duration were considered and regressed with altitudes using GraphPad Prism version 5.01. To explain the multivariate climatic variation, principal component analysis (PCA) was performed using StatSoft Statistica 8, involving 21 climatic variables and altitude. This multivariate relationship

Table 1. Details of geographic locations of four sites representing four populations of *Arabidopsis thaliana*

Site	Abbreviation	Coordinates		Altitude (m amsl)
		Latitude	Longitude	
Dehradun	Deh	30.33 N	77.98 E	700.0
Munsiyari	Mun	30.12 N	80.35 E	1829.0–2016.5
Sangla	Sang	31.41 N	78.25 E	2600.0
Chitkul	Chit	31.37 N	78.43 E	3430.0–3453.0

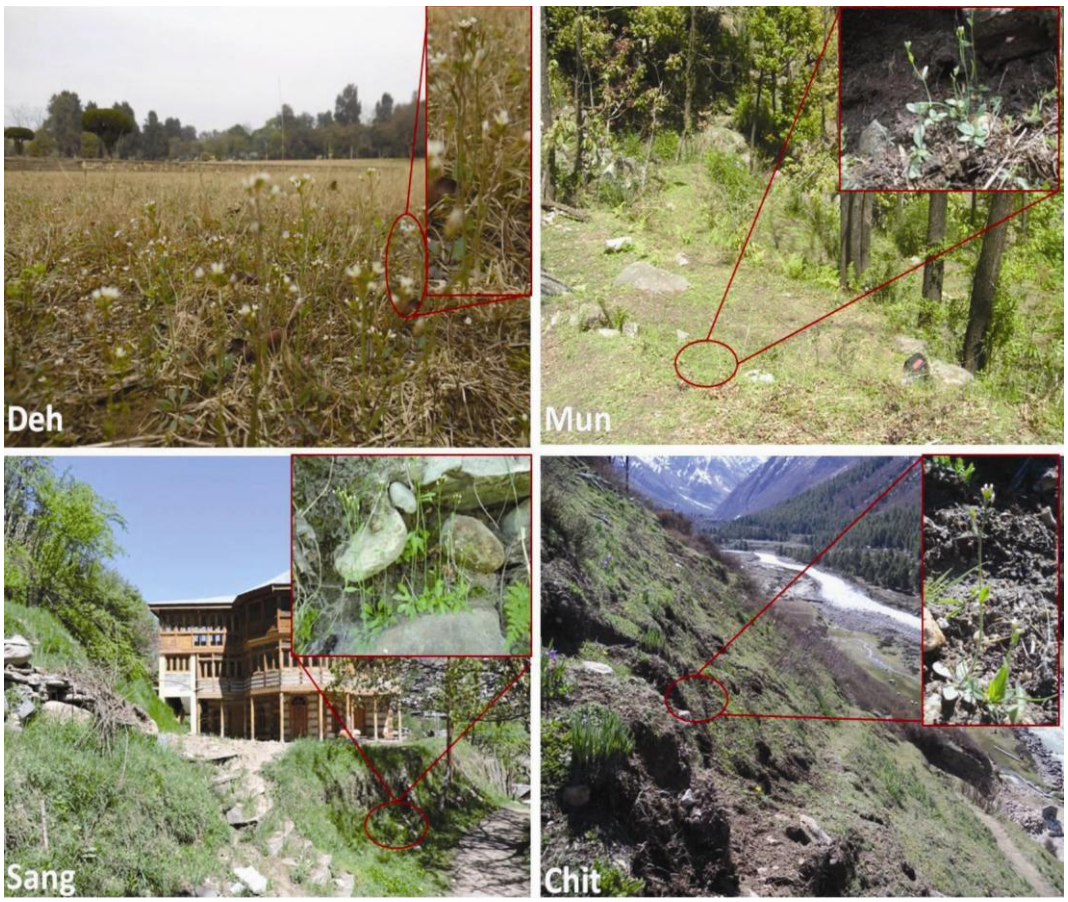

Figure 1. Representative habitats of the four sites. (Inset) A closer view of the population in each case.

between the climatic variables and altitude was used to test the extent to which one can treat the climatic gradient observed as a function of altitude. We used both Kaiser criterion[48] and scree test[49] to determine the number of meaningful climate axes. Based on this, two principal components (PC) were selected and PC score obtained here was regressed with the traits as dependent variable. The PC score of the climatic principle components was calculated using weighted sum method.

Analysis of variance (ANOVA) was performed to assess the significance of the variations observed in the quantitative phenotypic traits between populations using GLM (general linear model). Post hoc Bonferroni's multiple comparison test was used to find the differences between two populations. Multivariate regression analysis was performed using only the traits which were significantly different as indicated by ANOVA, considering traits as dependent variables and altitude as independent variable. This analysis was performed using 'multivariate' statement of GLM in SPSS 22.0 (trail version-IBM Corp., released 2013, IBM SPSS Statistics for Windows, Version 22.0. IBM Corp, Armonk, NY). To further assess the independent contribution of these traits to the multivariate correlation with altitude, canonical correlation analysis was performed[45]. As altitude was considered as a single variable in this analysis, only one canonical variate

was obtained. To explain the proportion of total variation explained by altitude, squared canonical correlation coefficient was used. This analysis was performed using 'STATS CANCORR' statement under 'MANOVA' in SPSS 22.0 (trail version – IBM Corp., released 2013, IBM SPSS Statistics for Windows, Version 22.0, IBM Corp., Armonk, NY).

Further, to test the correlation between geographic distance and mean pairwise distance based on morphological characters, Mantel test was performed. To generate geographic distance matrix, the GPS data of the sample sites were converted into geographic distance matrix using WGS84 (World Geodetic System) model of Geographic Distance Matrix Generator[50]. The mean pairwise distance matrix of phenotypic traits was generated using all the measured morphological data of the four populations after character-coding the traits (in the form of numbers). Distance matrix was generated using PAUP*4.0 (ref. 51).

Results

Climatic conditions along the altitudinal gradient

There were significant variations in overall climatic conditions of the selected sites (Table S2, see Supplementary material online). The minimum temperature for the coldest month was –10.9°C for the highest altitude, Chitkul and 6.2°C for the lowest altitude, Dehradun. The maximum temperature for the warmest month was 16.2°C and 36.6°C for Chitkul and Dehradun respectively. The mean temperature range during the growth period of the plants at different sites was: 9°C to 22.4°C for Dehradun (January to March); 9.3°C to 21.7°C for Munsyari (March to May); 7.1°C to 17.3°C for Sangla (April to June), and 2.1°C to 12.5°C for Chitkul (April to June), and mean precipitation during the growing period was 51.6, 64.0, 61.0 and 52.3 mm respectively. It was not possible to determine the exact timing of germination of seeds in natural condition to determine the growth period from germination to fruit senescence. Therefore, the growth period was determined from four leaf stages to fruit senescence as the optimum growth period for all the sites. In some places it was possible only after making two consecutive visits at the beginning as well as at the end of the growing period, to determine the exact timing of the growth period. Both minimum and maximum yearly mean temperature decreased with increase in the altitude (for $t_{min} - P = 0.0053$; $R^2 = 0.989$, for $t_{max} - P = 0.0061$; $R^2 = 0.987$) along with marginally significant decrease in precipitation ($P = 0.0684$; $R^2 = 0.864$). The diffused radiation in different sites ranged from 719 to 578 kWh/m^2, and was found to increase significantly with the altitude ($P = 0.0376$; $R^2 = 0.926$). The duration of sunshine was not significantly associated with altitude. Figure 2 displays scatter plots showing regression equations of the climatic variables.

PCA using 22 parameters (19 bioclimatic variables, and light intensity, light duration and elevation above sea level) yielded three PCs. All the three PCs were significant to interpret according to both the Kaiser criterion and the scree test method. The three PCs were able to account for 83.31%, 11.38% and 5.29% of the total variability. The first two PCs alone were able to account for 94.7% of the variability, and we interpreted the first two components only (Figure 3). The eigenvectors of correlation matrix of PC1 for the precipitation-related terms varied from 0.19 to 0.23 and were mostly positive, except for the variables that describe precipitation variability during a particular period (BIO14, BIO17 and BIO19). For PC1, the eigenvectors for the temperature-related variables were all positive, except for the temperature seasonality (–0.08) and ranged from 0.08 to 0.22. The variables describing temperature variability (BIO2, BIO3, BIO4 and BIO7) were more strongly and positively (except BIO3) loaded on climate PC2. The variable for solar radiation (light intensity and duration) showed negative eigenvectors of 0.21 to 0.22 for the climate PC1. The altitude was loaded negatively on climate PC1 (–0.22) with almost zero eigenvector loading on PC2.

Phenotypic variations among populations

The correlation matrix constructed using all the quantitative characters showed that most of the traits were significantly correlated with each other. At population level, the Deh population was characterized by higher leaf count, more profuse branching, more number of inflorescence and larger siliques compared to the Mun, Sang and Chit populations (Table S1, see Supplementary material online). The leaves of Chit population were more fleshy and thick compared to Deh population (data not shown). Deh and Mun populations had moderately elongated leaves, while for Chit population leaves were more rounded in shape. Further, the Deh population had lower trichome and stomatal density compared to the other three populations. The sepals of Chit population had trichomes, but it was absent or rarely present in the populations of Deh and Mun. Bifid and trifid types of trichomes were predominant in leaves, while largely unbranched trichomes were present on the stems and the sepals in all populations (Figure 4). The mean of each of the quantitative traits along with standard error of each of the four populations is shown in Figure 5. ANOVA analysis showed 11 out of 21 quantitative traits differed significantly among the populations (Table 2). Results of its post-hoc Bonferroni's comparison test are given in Table S3 (see Supplementary material online).

There were marked differences in colouration of sepals in the populations. The sepals in Deh and Mun populations were green, whereas those of Chit and Sang populations were pale yellow in colour. However, there were no

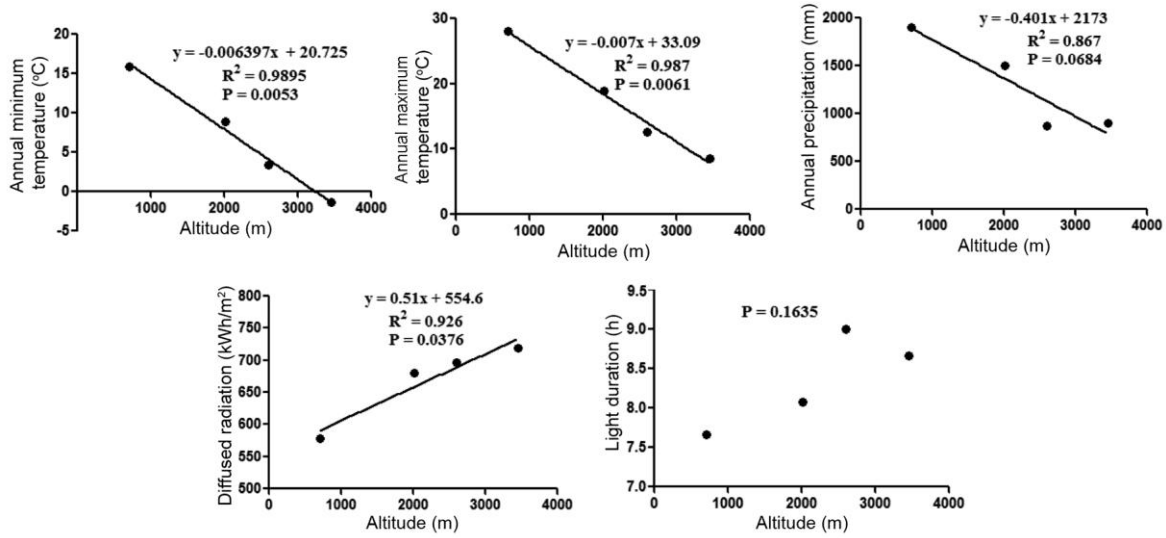

Figure 2. Scatter plots with fitted regression lines for five climatic parameters as dependent variable and altitude as independent variable. *P*-value, R^2 value and regression equation are also shown. Regression lines were drawn only for significant parameters ($P < 0.10$).

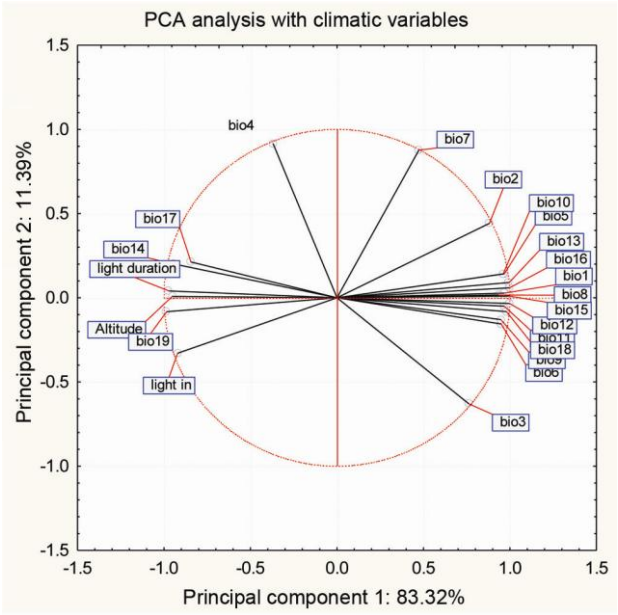

Figure 3. Plot showing loadings of the 21 climatic variables and altitude for the four collection sites on first two principal components, explaining a total of 94.71% of variability.

differences in petals and anther colour, which was white and pale to bright yellow respectively, in all the four populations. The seeds of Chit and Sang population were darker compared to Deh and Mun populations. Further, purple tinge in the leaves and sepals of Chit and Sang populations was observed, which was not prominent in the leaves and sepals of Deh and Mun populations.

Phenotypic variation versus altitude versus climate

The phenotypic variation in the populations was significantly associated with altitude, as indicated by the multivariate regression analysis of significantly different traits combined with altitude (Wilk's lambda = 0.051; $F = 7.387$; $P = 0.034$). Altitude explained 94% of the total multivariate trait (varying significantly among populations) variation as described by the canonical correlation analysis ($R_c^2 = 0.94863$, Wilk's lambda = 0.05137).

To observe the relationship between climatic variables along altitude and phenotypic traits of the respective population, PC scores were derived from PCA analysis as described above. The PC scores were treated as independent variables against phenotypic trait as dependent variable for regression analysis. The PC1 score was able to predict the values for the number of branches, number of leaves after bolting, rosette area, number of basal inflorescence, number of lateral inflorescence, length of pedicel of inflorescence and length of siliques, while the PC2 score was able to predict the number of stems from base, number of nodes, number of leaves after bolting, leaf shape, length of pedicel of flower, length of pedicel of inflorescence and length of pedicel of fruit (Table S4, see Supplementary material online). Mantel test between the geographic and pairwise distance matrix of phenotypic traits suggested a significant spatial relationship of the populations with their respective sites ($P < 0.0001$; $r = 0.274$).

Discussion

A. thaliana is known for its wide range of adaptability. This is mainly due to its large phenotypic plasticity and response to differences in environmental conditions by acclimation[52]. Field studies are rare because the photoperiod, temperature and light intensity, etc. are not controlled. To minimize the variations in traits, experiments are mostly conducted under controlled set-up. However, this cannot truly mimic the natural conditions to which the

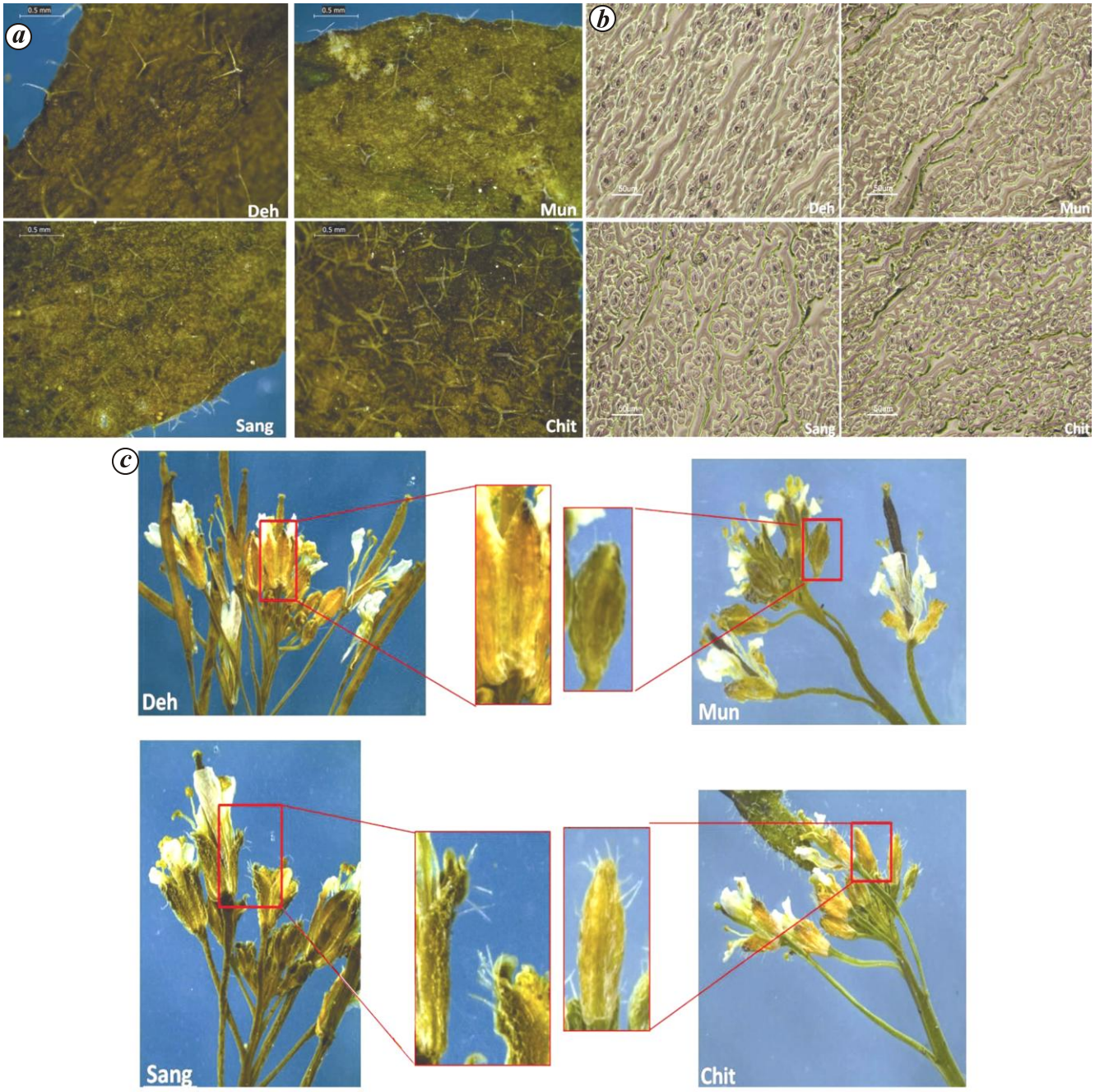

Figure 4 *a–c*. Morphological variations observed in populations of the four different sites. ***a***, Trichome density on leaves; ***b***, Stomatal density on lower side of the leaves; ***c***, Presence of trichomes on sepals of flower.

plants are adapted. Therefore, it is important to study the in-field traits that shape the plants architecture to a particular environmental condition.

Western Himalaya, the site of the *A. thaliana* populations in the present study, is associated with a wide range of minimum and maximum temperatures, precipitation, radiation, etc. depending on altitude. Accordingly, the life cycle of *A. thaliana* is completed at different seasons of the year. While Deh populations germinate in January and seed shedding occurs during early March, Mun populations germinate during March and seed shedding starts during May. In Sang and Chit populations, seed germination occurs during April and seeds shed during June. This difference in flowering time is in response to different environmental factors. Phenotype–environment association analyses earlier identified strong altitudinal clines (0–2600 m) in flowering-related traits. Among the other factors, minimum winter temperature and precipitation were the main climatic factors that might act as selective pressures on flowering traits[53]. This is also in accordance with our observations that there are wide range of variations in minimum temperature and precipitation among the

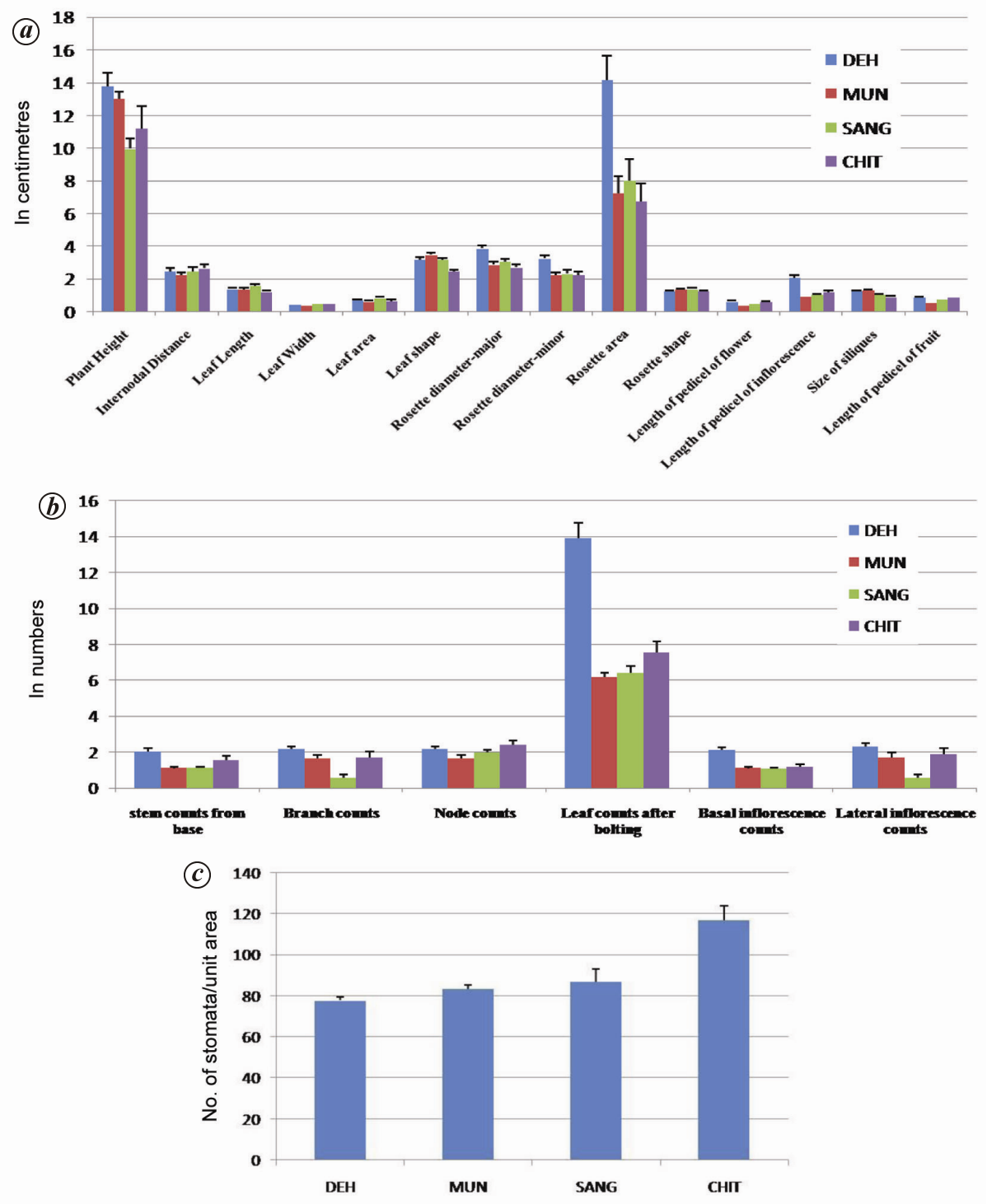

Figure 5 ***a–c.*** Graphical representation of quantitative morphological traits showing mean of each population. Line above the bar represents standard error. ***a***, Traits that are measured in centimeters; ***b***, Countable traits (in numbers); ***c***, Stomatal density.

different sites. We observed that the West Himalayan populations are of summer annual types, which complete all steps of life cycle in one season in contrast to winter annuals mostly prevalent in European countries, germinating in late summer or autumn and over winter as rosette and reproductive phase starts in spring. The observation on decrease in biomass-related phenotypic traits, particularly leaf shape and size as altitude increases has wider implications in plant adaptability. It has been shown that size and shape are governed by, among other factors, light intensity, and photoperiod and photosynthetic signals may influence leaf morphology[36]. The lower number

Table 2. Result of ANOVA testing for differences in morphological traits between populations from different altitudes

Traits	DF	*F*-value	*P*-value	Significance
Plant height	(3, 12)	1.184	0.3568	Ns
Stem counts from base	(3, 12)	4.673	**0.0219**	*
Branch counts	(3, 12)	11.33	**0.0008**	***
Node counts	(3, 12)	2.762	0.0880	Ns
Internodal distance	(3, 12)	0.4633	0.7131	Ns
Leaf counts after bolting	(3, 12)	16.68	**0.0001**	***
Leaf length	(3, 12)	1.305	0.3181	Ns
Leaf width	(3, 12)	1.246	0.3364	Ns
Leaf area (length × width)	(3, 12)	0.5296	0.6704	Ns
Leaf shape (length/width)	(3, 12)	8.779	**0.0022**	**
Rosette diameter–major	(3, 12)	2.581	0.1021	Ns
Rosette diameter–minor	(3, 12)	2.338	0.1251	Ns
Rosette area	(3, 12)	2.597	0.1007	Ns
Rosette shape	(3, 12)	0.3668	0.7783	Ns
Basal inflorescence counts	(3, 12)	10.30	**0.0012**	**
Lateral inflorescence counts	(3, 12)	7.195	**0.0051**	**
Length of pedicel of flower	(3, 12)	3.679	**0.0435**	*
Length of pedicel of inflorescence	(3, 12)	20.26	<**0.0001**	***
Size of siliques	(3, 12)	10.21	**0.0013**	**
Length of pedicel of fruit	(3, 12)	50.46	<**0.0001**	***
Stomata counts/unit area	(3, 35)	15.12	<**0.0001**	***

The *P*-values of significant trait are in boldface (at $P < 0.05$); Ns, Nonsignificant.

of rosette leaves and lesser number of branches for plants at high altitude may result from the balance between tolerance and ability to grow at low temperatures[54]. The increase in biomass-related traits in low-altitude population relative to high-altitude population is consistent with a previous study[55]. Further, it has been shown earlier that smaller leaf size is associated with cold[56], hot[57], dry[58] and high light intensity[59] and/or a combination of these factors[58], as observed in this study. The high altitude (Chitkul) area experiences all these climatic factors and might contribute in shaping the observed leaf morphology and hence biomass. The increase in stomatal density in populations of high altitude compared to low altitude may be attributed to decrease in atmospheric pressure at high altitude to counteract photosynthetic potential, which is further limited by increased UV radiation[60]. Similarly, increase in trichome density both in leaves and sepals in the populations of higher altitude suggests its role in response to extreme environmental conditions at high altitude. Trichomes play a role in protection against UV-B radiation, and UV-B induces the formation of trichomes through the expression of key trichome initiation regulator GL3 (GLABRA3)[61]. Trichome formation in *Arabidopsis* is suppressed after bolting, but phytohormones cytokinin and gibberellins can stimulate the induction of trichome on stem and inflorescence[62,63]; however, its regulation by the exogenous environmental clues is still not well established. The observed increase in trichome density may be attributed to the strategy of plants to cope with the high solar radiation which is prevalent at high altitudes. Although these differential morphological traits in *A. thaliana* from different environmental conditions have been well explained, there have been no reports on morphological trait variations in Indian *A. thaliana* populations from the Western Himalaya.

Our study further indicates that there is positive correlation between geographical distance and mean pairwise distance (based on morphological traits) of the populations. Though there are mixed reports on the relationship between geographical distance and genetic distance in *A. thaliana* populations[64–68], to the best of our knowledge, there are no reports on the relationship between morphological traits of *A. thaliana* populations along altitude and geographic distance. However, our observations may be limited due to small sample size, consideration of traits, etc. Further studies involving a large number of populations are needed to confirm the results.

Conclusion

Studies on natural populations for trait variations along altitudinal gradient (which in turn provide climatic gradient) give clues on how plants response to a particular environment or the changes they adopt over time. The significantly variable morphological traits observed in the four natural populations might be a result of sub-optimal growth in more stressful environment at higher altitude. The decrease in biomass-related traits as altitude increases may be due to conditions of low temperature, high radiation, low precipitation and low partial pressure of CO_2, etc. existing over maximum period of the year. The understanding of the strategies adopted in response to

different environmental conditions may provide us clues about how plants may cope with the climate change phenomenon.

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