

PHENOTYPIC VARIATION AND LEAF FLUCTUATING
ASYMMETRY IN NATURAL POPULATIONS OF *PARROTIA PERSICA*
(HAMAMELIDACEAE), AN ENDEMIC SPECIES FROM THE
HYRCANIAN FOREST (IRAN)

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ABSTRACT

Phenotypic variation in four natural populations of *Parrotia persica* (Hamamelidaceae), a species considered a living fossil endemic to the Hyrcanian forest, was evaluated through leaf morphometric and multivariate analysis. Furthermore, plasticity and leaf fluctuating asymmetry (LFA) were studied. Our findings clearly displayed significant divergence among sites. The smallest lamina size, the highest plasticity, and the lowest LFA values were recorded in the eastern and more xeric sites (Ghorogh and Daland), thus it seems these sites endure a larger environmental stress. Although our findings showed that multivariate and leaf morphometric analyses are suitable instruments to detect levels of phenotypic variability among *P. persica* natural populations, further study of allozyme and DNA diversity is necessary.

Key words: leaf morphometrics, multivariate analysis, natural populations, plasticity.

RESUMEN

Se evaluó la variación fenotípica en cuatro poblaciones naturales de *Parrotia persica* (Hamamelidaceae), una especie endémica considerada como un fósil viviente del bosque

hircaniano, a través de un análisis morfométrico y multivariado de las hojas. Asimismo, se estudiaron la plasticidad y la asimetría foliar fluctuante (AFF). Nuestros hallazgos mostraron claramente una divergencia significativa entre sitios. El tamaño más pequeño de la lámina foliar, la plasticidad más grande y los valores más pequeños de AFF fueron registrados en los sitios más xéricos de la parte oriental (Ghorogh y Daland), por lo que parece que estos sitios están sujetos a un mayor estrés ambiental. Obtuvimos resultados interesantes, pero hace falta realizar nuevos estudios de la diversidad de aloenzimas y ADN.

Palabras clave: análisis multivariado, morfometría foliar, plasticidad, poblaciones naturales.

INTRODUCTION

Parrotia persica C. A. Mey. (Persian ironwood tree) is a biological treasure of the forest ecosystems of Northern Iran, where it is considered as one of the endemic trees to the Hyrcanian forest region. This species is the last representative of a widespread and species-rich genus that originated in the Tertiary (Mosaddegh, 2006). Unfortunately, forest managers usually overlook *P. persica* because this species does not produce valuable timber. Conversely, it is widely used by indigenous people for charcoal and firewood production. The wood is also used by wood tuners and for weaving telephone poles. Browicz (1982) noted that wood of *P. persica* was “remarkably hard, heavy, dense and durable”, which is why it is called the iron tree. Although *P. persica* is not considered to be at risk of extinction, presently it faces conservation problems. Certainly, if the destructive process continues, genetic erosion of the species is likely. For this reason, it is important to collect more information about the adaptation of this species and its ability to respond to different types and patterns of disturbance.

The capability of plants to modify their morphology and physiology in response to environmental conditions is called phenotypic plasticity (Bradshaw, 1965). 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 adjustment to resource availability. A further source of variation and developmental stability, is the inability of an organism to grow and mature in a steady manner, according to its phenotypic potential, under a wide variety of environmental conditions (Palmer, 1994), and has been mostly studied as fluctuating asymmetry (FA). The idea is that low-quality individuals, with respect to reproductive fitness, are developmentally less stable and unable to develop traits with precisely one value equal on the left and right sides. According to Møller & Swaddle (1997), increase in the variability of cellular growth rates under sub-optimal conditions is a source

of developmental instability and it might induce asymmetric growth in plant organs with bilateral symmetry. Hence, any deviation from the genetically programmed phenotype may indicate the existence of stress during development, which in turn may be related to environmental quality (Leary & Allendorf, 1989). For example, increased FA has been observed both in animals and plants in response to pollution, parasitism, grazing, competition, and drought (Freeman et al., 1995; Kozlov et al., 1996; Alados et al., 2003; Hódar, 2002, Graham et al., 2010).

The central objective of this study was to analyze patterns of morphological diversity and trait asymmetry in four wild populations of *P. persica*, chosen to cover a wide enough rainfall range to admit predictions of shifts in relative growth optima. Topography and climate features have an important influence on plant diversity and richness of Iran. In particular, drought coinciding with the thermal maximum is a general factor that plays a decisive role in the selection of species that make up the natural vegetation in the Hyrcanian forest (Akhani et al., 2010). A first test was performed to verify whether there were significant morphological differences among the populations and whether these were related to environmental characteristics. Secondly, we examined if there were significant differences in levels of FA among populations. Our expectation was for increasing FA away from an optimum especially at higher drought levels.

MATERIALS AND METHODS

Sampling

We sampled four natural populations of *Parrotia persica* in the Hyrcanian Forest (Northern Iran), including the Sisangan and Noor populations in the western Hyrcanian forest, and Ghorogh and Daland populations in its eastern portion. The geographical location of the sampling sites and their characteristics are shown in Figure 1 and Table 1, respectively. Rainfall in the region decreases from west to east (Mohajer, 2007). All four study sites have been impacted by humans, especially by tourists for fuel. On May 27 2010, we selected twenty individual trees from each site. To minimize the possibility of intraspecific crossing, at each site the trees were chosen to be at least 50 m apart from each other (Zarafshar et al., 2010). Similarly, in order to minimize positional variability, 20 leaves were collected within the crown from the external part of the canopy on the aspect facing south and always in the middle part of the first flush shoot. Ten leaves were chosen at random from each subsample after elimination of broken, incomplete, or damaged leaves.



Fig. 1. Location of sampling sites in northern Iran (Hyrcanian forest). Distance between Sisangan and Noor: 40 km. Distance between Noor and Ghorogh: 250 km. Distance between Ghorogh and Daland: 80 km.

Table 1. Location and characteristics of *Parrotia persica* study sites.

Site	Longitude	Latitude	Altitude (m)	Temperature (°C)	Precipitation (mm)	Soil type
Sisangan	51°30' E	36°39' N	-20.9	16.4	1293.5	Forest brown compacted
Noor	52°19'12" E	36°33'46" N	-15	16.5	850.9	Forest brown compacted
Ghorogh	54°42'14" E	36°53'16" N	139	17.8	601.0	Forest brown compacted
Daland	55°03' E	37°02'27" N	73	18.2	357.1	Forest brown compacted

Annual mean is reported for temperature and precipitation.

Morphological measurements

The parameters measured included four morphological characters: lamina length (LL), lamina width (LW), petiole length (PL), and number of veins (V) (count variable). The leaf length/leaf width (LL/LW) and leaf length/petiole length (LL/PL) ratios were calculated, because ratios relate to shape rather than size variation, and hence may provide additional information (Dickinson et al., 1987; Frampton & Ward, 1990). Fig. 2.

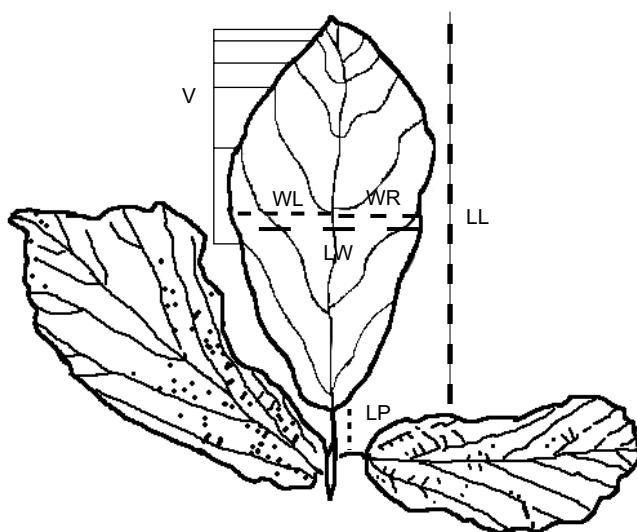


Fig. 2. Presentation of the assessed leaf morphological variables: lamina length (LL), lamina width (LW), petiole length (PL), width in left (WL), width in right (WR), number of veins (V).

Analysis

We calculated a measure of leaf overall size as the root square of the total leaf length \times blade width product to correct for allometric effects (Blue & Jensen, 1988). This new variable was regressed against leaf parameters and the residuals were used as input in the successive analyses. Thus, for each character i and OTU j (Operational Taxonomic Units) size-adjusted variables Y_{ij} were determined according to the formula:

$$Y_{ij \text{ adj.}} = Y_{ij} - Y_{ij\text{-cap}}$$

where $Y_{ij\text{-cap}}$ was the expected value of Y_{ij} given the size of OTU j .

Descriptive statistics were computed for the four study populations. Assumptions of normality were checked for all variables with Shapiro-Wilk's test. Principal Components Analysis (PCA) with standardized varimax rotation was used to separate interrelationships into statistically independent basic components (Bruschi, 2010). In PCA, eigenvectors were calculated to determine the contribution of each variable to the separation of the populations. An Analysis of Variance (One-way ANOVA) was performed in order to test the main effects of populations on the scores of the first three factors extracted in the PCA. A Pearson correlation analysis was performed to identify the significant climatic variables (predictors) affecting PC scores extracted.

In addition, we performed a Discriminant Analysis on the total data set to investigate the multivariate relationships among the morphometric traits, and how they change among the populations. The scatter plot of the discriminant scores corresponding to each case of each population in the multivariate space, as defined by the first two discriminant functions, was obtained to help visualize multivariate phenotypic variations. The statistical program SPSS (version 11.5 and 16) was used for all the analyses.

Plasticity

Total within-population plasticity (PI) was calculated for each parameter using the smallest (x) and greatest (X) mean values for any given measure, as $PI=1-(x/X)$ (Ashton et al., 1998; Bruschi et al., 2003).

Leaf fluctuating asymmetry

Signed fluctuating asymmetry (R-L) was estimated on the maximum width of lamina (MW). As suggested by Palmer (1994), distributions of signed asymmetries in all samples were tested for normality (Kolmogorov-Smirnov test) and a mean of zero (one-sample t-test) to detect the presence of antisymmetry and directional asymmetry. Skewness and Kurtosis were also calculated for all signed differences between sides in each of the leaf traits. Given that there was no evidence of antisymmetry (Kolmogorov-Smirnov test: $P > 0.10$) or directional asymmetry (t-test: $P = 0.12$), the observed variation in leaf size of *P. persica*, expressed as the difference between right and left side, was considered to be a measure of FA (Palmer & Strobeck, 1986). FA levels were measured as the absolute difference between sides (FA1) (Palmer, 1994). The mean for a trait in each population was calculated by summing the individual FA values and dividing the total by the number of individuals in that population (Palmer & Strobeck, 1986). Linear regressions of $|R-L|$, against the mean of

the two sides $(R+L)/2$, were calculated in all samples to test the linear dependency of the asymmetry on trait character size. Because some relationship between individual trait size and asymmetry was observed, we used $(|R-L|/0.5(R+L))$ as the FA index, controlled for trait size (Palmer & Strobeck, 1986). A One-way ANOVA was performed to test the main effects of populations on FA values.

RESULTS

Morphological measurements

Means and standard errors (SE) for studied leaf parameters in *P. persica* trees of four natural populations are shown in Table 2. The highest mean leaf size was observed at western, wetter and lower sites (Sisangan and Noor), whereas the lowest mean values were measured at the eastern, higher and drier sites (Ghorogh and Daland). Leaf length/leaf width and leaf length/petiole length ratios showed the highest values for Ghorogh and Daland. Coefficients of variation and plasticity were generally lower at the western sites (Sisangan and Noor), while higher values were measured at the eastern sites (Table 2). The Sisangan population, with higher mean annual precipitation, seems to have on average a more homogeneous morphology ($CV = 17.35 \% \pm 5.16$, and $P = 0.56 \pm 0.14$).

Table 2. Mean (\pm SE), coefficient of variation (CV) and plasticity (\pm SE) of each variable in each population; (lamina length (LL), lamina width (LW), petiole length (PL), width in left (WL), width in right (WR), number of veins (V)).

Variable	Sisangan			Noor			Ghorogh			Daland		
	Mean	SE	CV	Mean	SE	CV	Mean	SE	CV	Mean	SE	CV
LL (cm)	10.05	0.1	14.14	9.70	0.1	14.02	9.07	0.1	16.49	8.42	0.1	15.74
LW (cm)	6.75	0.1	23.70	6.65	0.1	26.91	5.40	0.8	30.21	5.32	0.9	30.03
PL (cm)	0.49	0.1	15.71	0.44	0.1	16.69	0.41	0.1	16.14	0.37	0.1	17.15
Wr (cm)	3.39	0.9	17.11	3.31	0.7	17.57	2.70	0.4	18.82	2.64	0.4	17.87
Wl (cm)	3.36	0.07	17.94	3.36	0.08	18.81	2.70	0.05	20.87	2.68	0.05	18.64
V	7.52	0.1	11.99	6.95	0.1	13.12	6.90	0.09	11.57	6.34	0.07	10.78
LL/LW	1.50	0.02	12.10	1.46	0.02	10.53	1.68	0.03	11.72	3.21	0.03	10.90
LL/PL	21.45	0.86	26.10	23.03	1.03	28.13	23.21	0.93	30.01	23.84	0.97	29.22
Plasticity	0.56	0.14		0.61	0.13		0.64	0.10		0.62	0.13	

Among all leaf characters measured, the greatest plasticity was found for petiole length and LL/LP (0.76 ± 0.02 and 0.78 ± 0.02 , respectively) while the lowest plasticity was observed for LL/LW (0.46 ± 0.05) (Fig 3).

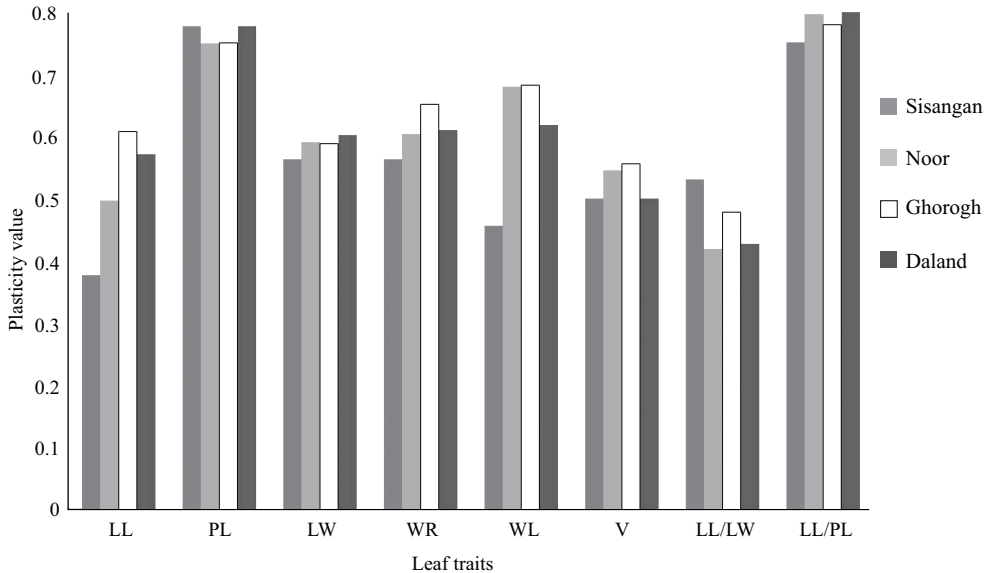


Fig. 3. Plasticity of *Parrotia persica* in the four study sites. (Lamina length (LL), lamina width (LW), petiole length (PL), width in left (WL), width in right (WR), number of veins (V)).

To find the most important traits responsible for differences between the different populations studied, a PCA was performed on all nine leaf traits. The eigenvalues, proportion of variance and cumulative proportion of the principal components are presented in Table 3. Regarding PCA, the first three principal components explained $> 80\%$ of the total variance of all traits, whereas the other components comprised a small percentage of total variation (18%). The first component explained more than 50% of the total variance and showed high eigenvector values for all leaf size traits (lamina length, lamina width, petiole length, width in right, width in left, and number of veins). PC2 was positively and highly related with leaf length/petiole length only. The number of the veins (V) trait contributed positively to PC3. Also, the LFA parameter contributed highly and positively to PC3.

An ANOVA showed that variation due to populations was significant in the first and third principal components extracted (Table 4). A post-hoc analysis (LSD

test) showed that mean factor scores of the first principal component were significantly different between Daland and all other populations ($P < 0.05$), and between

Table 3. Principal Components Analysis of leaf traits of *Parrotia persica* at four sites. Shown are eigenvalues, eigenvectors and percent of variation accounted by the first three PCs. (bold numbers present the highest eigenvectors).

Leaf trait	Factor 1	Factor 2	Factor 3
LL (cm)	0.875	0.082	0.070
LW(cm)	0.936	0.222	-0.070
PL(cm)	0.573	-0.797	0.020
Wr	0.936	0.203	-0.151
Wl	0.917	0.216	0.016
V	0.655	-0.025	0.373
LL/LW	-0.712	0.003	-0.105
LL/PL	-0.195	0.960	0.038
LFA	-0.209	0.029	0.922
Eigenvalue	4.7	1.7	1.03
Explained variance	0.52	0.19	0.11
Cumulative variance (%)	52	71	82

Table 4. One-way Analysis of Variance carried out on first and third factor extracted (these factors explained $> 80\%$ of the total variance). SS = sum of squares, MS = mean of squares, G = degrees of freedom, F = F, P = P_level.

	SS	G	MS	F	P
Factor 1					
Population	2.188	3	0.729	10.748	0.000
Error	5.156	76	0.068		
Factor 2					
Population	33.688	3	11.229	0.630	0.598
Error	1355.735	76	17.839		
Factor 3					
Population	544.765	3	181.588	55.609	0.000
Error	248.173	76	3.265		

Sisangan and Noor ($P < 0.05$). Moreover, leaves from Ghorogh trees were morphologically more similar to Sisangan and Noor than to Daland (Table 5). Mean factor scores of the third principal component were significantly different among all populations ($P < 0.05$) (Table 5). A Pearson correlation analysis indicated that all environmental variables considered (i.e. altitude, rainfall and temperature) may explain the morphological variation among studied populations. The population average score values of PC1 were positively related to altitude ($R = 0.37$, $P < 0.05$) and temperature ($R = 0.59$, $P < 0.05$), but negatively related to rainfall ($R = -0.58$, $P < 0.05$). This suggests a significant pattern of environmental variation for the morphological traits in PC1, according to which populations from higher altitudes, growing in wetter stands, have a larger leaf size.

Table 5. Post-hoc comparison based on LSD (Least Significant Differences) test. (P-value was reported).

	Sisangan	Noor	Ghorogh	Daland
Factor 1				
Sisangan		0.03	0.43	0.04
Noor	0.03		0.16	0.00
Ghorogh	0.43	0.16		0.00
Daland	0.04	0.00	0.00	
Factor 2				
Sisangan		0.04	0.00	0.00
Noor	0.04		0.00	0.00
Ghorogh	0.00	0.00		0.00
Daland	0.00	0.00	0.00	

The results of the discriminant analysis showed that first two roots (discriminant functions) had significantly associated eigenvalues (Table 6). Function 1, accounting for more than 50% of total variance, clearly separates the Daland site from the other three (Fig. 3). Function 2, representing 20% of the total variance, separates the Ghorogh site from the remaining three (Fig. 3). The matrix of cases correctly classified by the discriminant analysis shows that only the Daland site had the highest discriminating power (100% of cases correctly classified). Contrastingly, the Noor site showed the lowest discriminating power (50% of cases

Table 6. Standardized coefficients and characteristics of the discriminant analysis for the first two discriminant functions (DF1, DF2).

Trait	DF1	DF2
LL (cm)	-2.542	-0.499
LW(cm)	1.013	-1.091
PL(cm)	0.100	0.067
Wr	1.283	1.379
Wl	0.381	1.071
V	0.101	0.331
LL/LW	2.352	0.172
LL/PL	0.137	-0.201
LFA	-0.003	0.088
Eigenvalue	156.761	1.068
Wilks' lambda	0.002	0.309
χ^2	452.128	85.199
P-level	0.000	0.000

correctly classified). Generally, results of the discriminant analysis classification showed a high percentage of correctly classified cases to all actual sites (in total 75%: Table 7).

Fluctuating asymmetry

Table 7 shows the means and SE of |R-L|, as well as the relative (R-L) and absolute (|R-L|) asymmetries for the different populations. Based on ANOVA, there were no between-population differences in *Parrotia persica* FA index (g.l. =3; F = 0.93; P = 0.42).

DISCUSSION

To date, information on the morphological variation among and within *P. persica* natural populations is scarce. Yosefzadeh et al. (2010) examined morphology of this species along an altitudinal gradient and found that leaf size variation in this species was significantly affected by altitude. However, the authors did not determine which environmental factors contributed to influence this trend. In this study,

multivariate analyses showed significant differences among sampled populations and that these differences probably reflect morphological adaptations to different climatic conditions. Indeed, both rainfall and temperature appeared to be significantly correlated with PC1, suggesting that climate variables have a significant influence on the leaf size of the populations analyzed. On the other hand, discriminant analysis revealed a structure in which the Daland population is well differentiated from more mesic sites (Table 7, Fig. 4) suggesting a morphological adaptation to different moisture conditions. Mean data values showed that leaves from the western sites (Sisangan and Noor) were bigger than those from the drier eastern sites (Ghorogh and Daland). The smallest leaf size was measured in the Daland population where the lowest mean annual precipitation and highest mean annual temperature were observed.

Our results agree with other studies showing that environmental heterogeneity is a major source of variation in leaf morphology (Geeske et al., 1994; Velázquez-Rosas et al., 2002; Mcpherson et al., 2004; Xu et al., 2009; Bruschi, 2010). However, common garden experiments are needed to determine whether morphological differences among these populations remain when individuals are reared under identical environmental conditions, and to separate genotypic from environmental effects. Differences in plasticity were found among sites. Particularly, the two more xeric sites (Ghorogh and Daland) displayed higher plasticity values than the two mesic ones (Sisangan and Noor). Among all studied characteristics, petiole length and the LL/PL ratio displayed the largest plasticity values while LL/LW showed the smallest one. Zarafshar et al. (2010) and Bruschi et al. (2003), in studying leaf morphology of natural populations of *Castanea sativa* and *Quercus petraea*, respectively, reported high plasticity for petiole length. It can be hypothesized that petiole length is strongly associated with environmental conditions and petiole length variation could play an important adaptive role by influencing the spatial distribution of leaves and therefore light interception. Differently, Yousefzadeh et al. (2010) reported the lowest plasticity value for the number of vein pairs and base angle in *Parrotia persica* along an elevational gradient.

We also evaluated Leaf Fluctuating Asymmetry because this trait has been shown to be positively associated with environmental stresses such as pollution load (Kozlov et al. 1996), high elevation (Wilsey et al. 1998), cold climate (Valkama & Kozlov 2001), and drought (Alados et al., 2001; Hódar, 2002; Fair & Breshears, 2005). The idea that environmental stress can increase FA makes it a potential tool to be used for bio-monitoring. As suggested by Palmer (1994), knowledge of baseline levels of FA in natural populations is crucial to application of FA to conservation

efforts. FA in *Quercus petraea* leaves (mean absolute FA= 0.127 cm) was within the reported range for a number of other woody species (Wilsey et al., 1998; Valkama & Kozlov, 2001; Hódar, 2002).

Table 7. The classificatory discriminant analysis of *Parrotia persica* trees from four sites of its native habitat.

	Sisangan	Noor	Ghorogh	Daland	% of correct cases
Sisangan	14	5	1	0	70
Noor	5	10	5	0	50
Ghorogh	2	2	16	0	80
Daland	0	0	0	20	100
Total	21	17	22	20	75

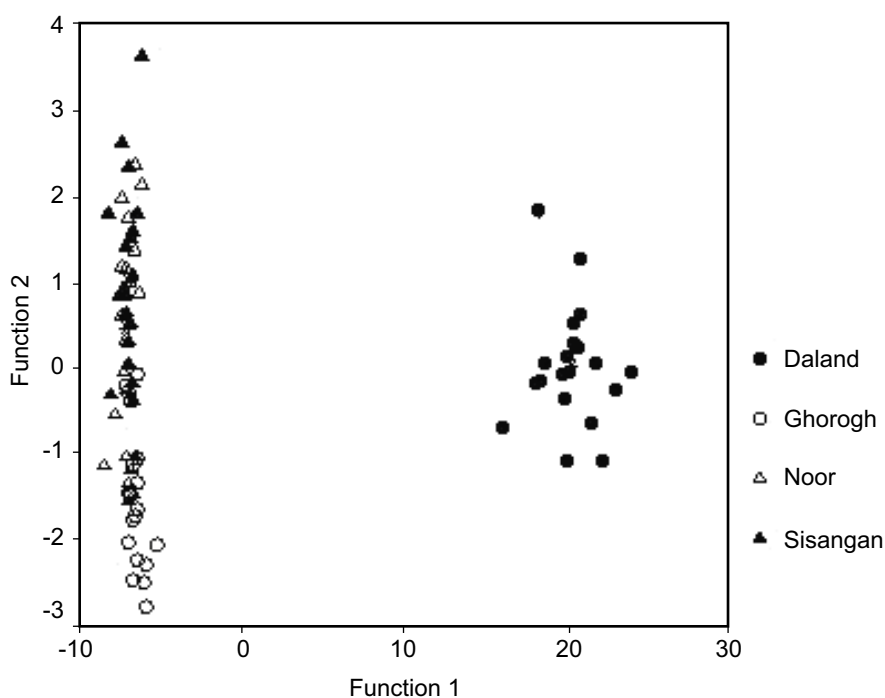


Fig. 4. Discriminant function analysis scores for leaf morphology of *Parrotia persica* from four populations in the Hyrcanian Forest.

In this study we did not find any significant difference among populations. *P. persica* seems capable of resisting environmental changes, since no effects of climatic influences were detected. Probably, physiological plasticity buffered the environmental stress conditions, allowing the maintenance of the developmental program that is exhibited under optimum or normal conditions. As shown in Table 8, leaves from wetter sites were more asymmetrical than those of drier sites. Some evidence suggests that FA does not always increase in drier environments, although this may depend on the traits and species being investigated and on the selection history experienced by different populations. For example, in a study conducted during a period of over 8 yr, Valkama & Kozlov (2001) did not find any effect of the amount of rainfall on FA of birch leaves. Llorens et al. (2002) found that drought treatment increased leaf area FA on *Vaccinium myrtillus* while it did not affect leaf width FA. An analysis of FA in *Quercus ilex* leaves from areas under different precipitation regimes (Hódar, 2002) showed that plants living in stressful sites were more symmetrical, and that leaves from non-stressful sites were prone to FA in drought years. Moreover, field conditions expose plants to several stresses, the relative roles of which on these populations are not known in this study. Clarke (1993) emphasized that, for developmental stability studies, the ‘use of natural populations is fraught with danger unless there is a good understanding of the genetic structure and evolutionary history of the populations under examination’.

Table 8. One-way Analysis of LFA parameters.

Population	FA				FA ass		FA correct
	Mean	Std. error	Skewness	Kurtosis	Mean	Std. error	Mean
Sisangan	0.033	0.038	-0.207	-0.658	0.447	0.020	0.131
Noor	-0.049	0.035	0.000	-0.935	0.422	0.018	0.127
Ghorogh	0.004	0.031	0.124	-0.778	0.371	0.016	0.140
Daland	-0.036	0.027	1.458	-0.682	0.286	0.018	0.110

In conclusion, our results indicated considerable leaf variation in the sites studied. Moreover, we showed that multivariate and leaf morphometric analyses are suitable instruments to detect levels of phenotypic variability among *P. persica* natural populations.

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