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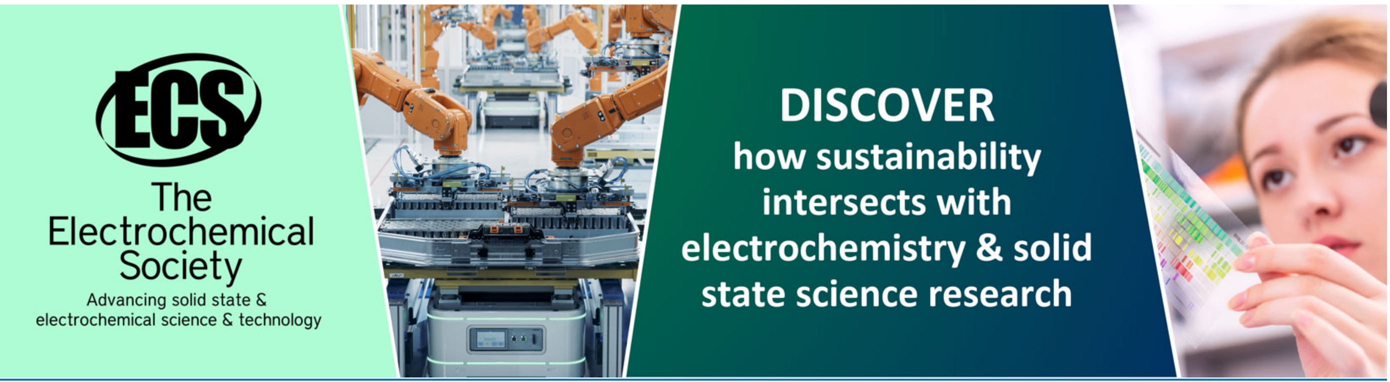

Bilateral Asymmetry and Shape of Lamina Plantago major L

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Abstract. The aim of the work was to answer the question whether pollution by vehicles and the geographical location of populations affect the asymmetry and shape of the leaf plate *Plantago major*. The Generalized Procrustes Analysis was provided and the difference in the variance of paired landmarks was indicated. Based on vector coordinates, the symmetry and asymmetry covariance matrices of two types were created, and canonical covariance analysis was performed. The difference was found using the distances between the centers of the coordinate sets, the so-called Mahalanobis distances. The overall pool of leaves showed the presence of fluctuating asymmetry at the level of leaf blades in roadside populations. In the control, a mixture of two types of asymmetry: fluctuating and directional was traced. Canonical variation analysis showed the difference between the center of sets ($p < 0.0001$). A correlation was obtained between the symmetric and asymmetric components of the shape. An allometric ratio of size/asymmetry and size/shape of the sheet plate were not obtained. The decrease in the number of landmarks from 52 to 26 did not affect the results. Thus motor transport, as shown by most of the samples (4 out of 6), had a significant impact on both the shape and asymmetry of the leaf blades. The geographical difference also affected both type of variability, as the difference in the asymmetry and shape of the plantain leaf blades increased over the geographical extent from Vladimir to Moscow on a distance about 200km.

1. Introduction

Fluctuating asymmetry (FA), as a weak non-directional deviation from strict asymmetry with a normal distribution of the difference between the left and right values, is an intriguing phenomenon used in environmental and epigenetic studies [1, 2, 3].

Studies in which the FA value is associated with environmental stress are very numerous, performed with various plant species, but often show mixed results. Even hanging birch is a recognized bioindication species, due to its genotypic diversity, morphological heterogeneity and multifactorial environmental impact; it is often called into question as a reliable biological indicator. For example, environmental pollution by heavy metals did not produce a major change in asymmetry, but changed the morphological characteristics of the sheet plate [4, 5]. At the same time, the study of the influence of relief height and geographical location showed a noticeable effect on the shape of the plant leaf blade [6, 7, 8].

The different genetic status of the studied populations yields non-coincident trajectories of the ontogenetic canalization trajectory. The hormesis effect and the paradoxical effect of the organism's reaction to toxins creates an additional difficultly factor load requiring special study [9]. However, the very definition of "environment" also raises questions. Modeling the development of genetically cloned samples *in vitro* helps to answer some questions [10, 5].

Most often, the FA value is evaluated in an easily accessible leaf blade. The method of geometric morphometrics advantages in the integral assessment of the leaf contour. The basis of geometric morphometrics is the alignment of the configuration of all samples of the leaf blade along the axis of symmetry, which is specified by two or three labels. This approach is match for plants with linear or arched venation of leaves, including representatives of family Cereals and Plantain. The testing of the FA value is carried out using Two-factor Analysis of Variance in Procrustes shape space. Procrustes distances serve as a measure of measurement, and statistical significance is evaluated using the Goodall criterion F, an analogue of the Fisher criterion.

Large plantain (*Plantago major,* Broadleaf plantain) is a perennial herb with hard leaves, and characteristic parallel venation of leaves. Plantain is a commonly encountered plant, which is one of the requirements for a bioindicator species. In references, the contradiction results have been found, indicating both the absence of the effect of stress on FA and the increase in FA only in some dimensional traits [11, 12]. The size of the *Plantago major* leaf plate depended on the technogenic impact in the plantain harvesting area. The intense load reduced both the size and weight of the leaves [13]. High plasticity and adaptability are pronounced in low affect to salinization or other harsh physical and chemical factors [14]. The trouble is the choice of bioindication trait, as this demonstrated by numerous studies in FA testing woody plants leaf. For example leaf width should be measured strictly normal to the tangent to the edge of the leaf blade or perpendicular to the midrib, otherwise a high measurement error seriously skews the measurement results. Some researchers are prone to the possibility of using covariance patterns, that is, a set of characters that have morphophysiological bioindication sense, for example, certain veins or areas at the base or apex of the leaf [12, 15]. This approach provides an evolutionary understanding of morphological processes in ontogenesis and phylogenesis and is actively developing in morphometry [16, 15, 17, 18]. Important issue is the nature of the frequency distribution of left and right values. In such cases, it is important to test the coefficient of variation of one or another bilaterally symmetrical trait. Therefore the testing stability of the development comes to a standstill due to the variety of characters and their unequal or unknown reaction to stress. The method of geometric morphometrics solves some issues, as regular arrangement of labels along the contour makes it possible to work with them as bilaterally symmetrical features. At the same time, there are limitations, since such labels are not true landmarks in contrast to clearly expressed structures; therefore, special methods for processing image coordinates are developed for semilandmarks [19, 20].

The aim of the work was to determine whether pollution by vehicles affects the asymmetry and shape of the sheet plate. The second task was to determine these differences depending on the geographical location of the populations.

2. Methods and site of collection

Three geographic locations were chosen that are located c. on the same latitude. One is in Vladimirskaya (56 ° 15 ′ 81.74 ″ N, 40 ° 46 ′ 58.51 ″ E) and two are in the Moscow region (Orekhovo-Zuevo, 55 ° 47'48.4 "N 39 ° 00'46.8" E and Izmailovsky district of Moscow, 55 ° 46'46.9 "N 37 ° 44'01.7" E). There were two sites at each collection point: experimental – 50-100m from the road and control – at least 300m away from the road. The areas where the herbarium was collected (with an area of about 1 km^2) in the city developed simultaneously, therefore, we consider the control and experimental populations as subpopulations with fairly uniform genotype and with similar trajectory of ontogenetic canalization. The experimental and control sites had the same physical and chemical conditions of the soil and lighting. The sites were open areas occupied by ordinary ruderal urban vegetation (quinoa, chicory, bluegrass, and wheat grass).

Plantain was collected along the edge of paved paths or paths, where its projective cover was 30-50% per 1 m^2. From each point, 60 leaf blades were collected, three laminas from each of twenty plant.

3. Measurement and statistics

Sheet plates with a half-sheet width of 6.0 ± 0.1cm were collected in August-September and were photographed twice. Using a screen digitizer, first two true landmarks (LMs) were applied at the base and the top (apex) of the plate, 50 semilandmarks were labeled using a curve along the contour of the sheet plate. All LMs were labeled out twice, the first time the curve was built clockwise, and the second time is in the opposite direction. All LMs were assumed as true ones. The first two served as unpaired landmarks and lay on the axis of symmetry, and the other 25 pairs represented homologous paired characters (traits) (figure 1).

Figure1. Two unpaired (1, 2) and 50 landmarks.

TPS files were created with the coordinates XY for each of the 6 (sub)populations in two repeats. The resulting data were used in the MorphoJ soft to assess shape and asymmetry variability. TPS and MorphoJ programs are freely available on the morphogeometric community website https://life.bio.sunysb.edu/morph/index.html. The essence of Procrustes generalized analysis of variance (GPA) is to build a consensus shape by removing the difference in the size of the samples and is in the determining the difference in the variance of pair landmarks along axis of symmetry [21, 16]. The GPA resembles a two-factor analysis of variance, which is widely used to test the fluctuating asymmetry and directional asymmetry (asymmetric dominance in one of the sides, DA).

Based on the fact that developmental stability is a population characteristic, attention was focused on the FA value of the population (individual × side). FA was also determined at the level of the leaf blade (leaf × side). The covariance matrices of two types were created: symmetry matrices and asymmetry matrices, and canonical covariance analysis was performed. If the symmetry matrix showed a difference in coordinates in the sample of the same labels, the asymmetry matrix showed a difference in homologous labels on both sides of the axis of symmetry. Thus, the first matrix helped to identify differences in the shape of the plate. The second one is the difference in bilateral asymmetry. The difference was found using the distances between the centers of the coordinate sets, the so-called Mahalanobis distances. A correlation analysis between the two types of matrices was also carried out. The statistically significant difference between the asymmetric and symmetric components in the samples was determined using multivariate factor analysis (MANOVA) using the Pillai trace value. For statistical evaluation, a significance level of α = 95% was used. The permutation procedure maintained the association between the X and Y coordinates of each landmark was carried out 10,000 times.

4. Results

4.1. Bilateral asymmetry

The GPA results showed differences in the shape of the plates (source of variation "individual") in three samples (Vladimir, Orekhovo and Moscow; p <0.01; p <0.0001). Directional asymmetry ("side" is not significant) was absent only in populations along the road in Vladimir and in Orekhovo. Therefore, a "pure" FA was obtained here (p is statistically significant in the interaction of two factors; p <0.0001). In the control "pure FA" was not found, because of a mixture of two types of asymmetry – directional and fluctuating.

Table 1. Results of General Procrustes analysis.

	Vladimir							
	Roadside				*Control*			
Source	*SS*	*MS*	*df*	*F*	*SS*	*MS*	*df*	*F*
Individual	0.471	0.0002	2650	1.09*	0.541	0.0002	2450	1.77**
Side	0.006	0.0001	50	0.75ns	0.016	0.0003	50	2.51**
Individual × Side	0.433	0.0002	2650	16.29**	0.306	0.0001	2450	7.45**
Residuals	0.379	0.0000	37800		0.910	0.0000	54200	
Leaf	0.648	0.0001	5400	1.17**	0.924	0.0001	7350	1.17**
Side	0.006	0.0001	50	1.2ns	0.016	0.0003	50	2.93**
Leaf × Side	0.552	0.0001	5400	40.14**	0.788	0.0001	7350	105.46**
Residuals	0.082	0.0000	32300		0.045	0.0000	44400	
	Orechovo							
	Roadside				*Control*			
Individual	0.533	0.0002	2650	1.82**	0.615	0.0002	2900	1.46**
Side	0.003	0.0001	50	0.54ns	0.021	0.0004	50	2.87**
Individual × Side	0.293	0.0001	2650	4.7**	0.422	0.0001	2900	6.08**
Residuals	1.389	0.0000	59000		1.554	0.0000	64900	
Leaf	1.232	0.0002	8000	1.42**	1.360	0.0002	8800	1.2**
Side	0.003	0.0001	50	0.55ns	0.021	0.0004	50	3.24**
Leaf × Side	0.869	0.0001	8000	46.19**	1.134	0.0001	8800	70.81**
Residuals	0.114	0.0000	48300		0.097	0.0000	53100	
	Moscow							
	Roadside				*Control*			
Individual	0.414	0.0003	1500	24.93**	0.426	0.0003	1650	2.01**
Side	0.004	0.0001	50	4.54**	0.011	0.0002	50	1.76**
Individual × Side	0.029	0.0000	1500	1.21**	0.211	0.0001	1650	4.87**
Residuals	0.369	0.0000	34100		0.975	0.0000	37000	
Leaf	0.492	0.0001	4600	18.83**	0.941	0.0002	5000	1.94**
Side	0.003	0.0001	50	10.5**	0.011	0.0002	50	2.33**
Leaf × Side	0.026	0.0000	4600	0.44ns	0.486	0.0001	5000	15.9**
Residuals	0.361	0.0000	27900		0.185	0.0000	30300	

* p <0.0001;
**– p <0.0001; ns – not statistically significant.

No FA was obtained in the Moscow experimental population, the significant directional asymmetry was in control, F = 0.44; p >0.05. In a whole in populations under the influence of stress, developmental instability was stronger than in control populations in which a mixture of both types of asymmetry was uniquely present. The total leaf pool showed the presence of FA at the level of leaf blades in roadside populations. In the control the mixture of both types of asymmetry was typical.

4.2. Leaf shape

As was said, the overall outline of the sheet plate contour consisted of a symmetry component and an asymmetry component. Therefore a comparison of symmetrical components, i.e. label variances around the axis of symmetry, and then a comparison of asymmetric components, i.e. variances of paired labels was provided. Canonical variate analysis showed a difference between the centers of the sets (p <0.0001). Table 2 displays comparative data in the shape and asymmetry in control and populations along roadside.

Table 2. Comparison of symmetric/asymmetric components in the distances of Mahalanobis.

	Control				Roadside		
	Vladimir	*Orechovo*	*Moscow*		*Vladimir*	*Orechovo*	*Moscow*
Vladimir	x			*Vladimir*	x		
Orechovo	1.57**/1.23**	x		*Orechovo*	2.07**/0.97**	x	
Moscow	1.85*/1.19**	1.56**/1.03**	x	*Moscow*	2.65**/1.19**	2.06**/1.06**	x

The distances of Mahalanobis increased in samples from Vladimir to Moscow, in geographic extent in latitude from west to east. There was no so contrast change in the asymmetric component comparing to variation in shape. The values of distances in shape matrix in the control were proportionally removing from Vladimir: 1.57 (Orekhovo), and 1.85 (Moscow). Accordingly, in the experimental populations: 2.07 (Orekhovo), and 2.65 (Moscow). Multivariate factor analysis showed differences in the values of Pillay (p <0.0001) as a product of the interaction of two factors at the individual level (figure 2).

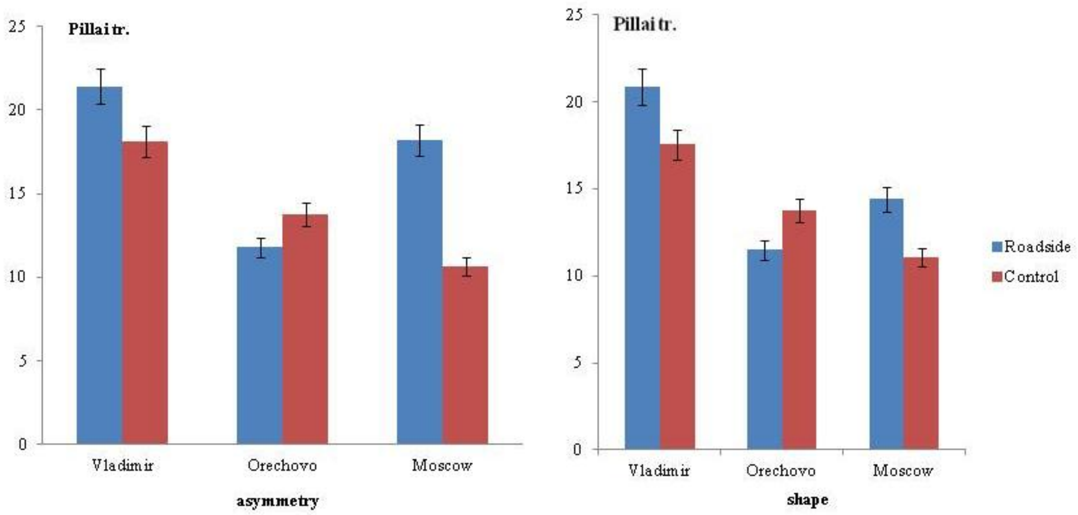

Figure 2. The difference in asymmetry and shape. Error bars with relative errors.

Multivariate analysis takes into account the properties of dependent quantities, i.e. Procrustes distances. In our case, both the asymmetric and asymmetric component prevailed in the populations by the road, i.e. variation in shape and asymmetry was more pronounced under the influence of stress.

Thus, motor transport, as shown by most of the samples (4 out of 6), had a significant impact on both the shape and asymmetry of the leaf blades. The geographical difference also had an effect on both kinds of variability: asymmetry variability and asymmetry variability.

5. Conclusion

Only two of the three populations (Vladimir, Orekhovo) showed an increased value of fluctuating asymmetry under the influence of the stress factor, which was an automobile emission. This made it possible to consider plantain a satisfactory indicator of development stability. High morphological plasticity of this species is the reason why it cannot be considered a reliable indicator (90-100% of positive bioindication results). The difference in the asymmetry and shape of the plantain leaf blades increased over the geographical extent from Vladimir to Moscow, i.e. at a distance of 200km. A correlation relationship was obtained between the shape and asymmetric variability. No allometric relationship was obtained between the lamina size and its asymmetry or its shape. In our opinion, the adaptive morphological ability of plantain dominated the variability of asymmetry. The directional asymmetry as an indicator of stress, as well as the hidden directional asymmetry, at the lower analytical categories the leaf and its image [22-24] deserves attention. Testing at the leaf plate level cross of all the samples collected along the road showed a significant FA ("leaf × side": $F = 5.05$; $p < 0.0001$) with statistically insignificant directional asymmetry. The leaf control pool consistently showed directional asymmetry at all test levels. In our case, at the leaf level (but not at the population level), we can confidently talk about plantain as a reliable indicator; although at present the development stability is justified as a population characteristics. Further work is associated with multidimensional testing, to study asymmetry and shape for several years.

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