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## On the issue of developmental stability of urban tree populations

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**Abstract.** Fluctuating asymmetry as an indirect deviation in the difference between the right and the left bilaterally symmetrical traits is considered an indicative symptom of stress. The results of urban populations studying *Betula pendula*, *Tilia cordata*, *Quercus robur* and *Acer platanoides* using the conventional normalizing method and the geometric morphometrics are presented. The relationship between the environmental stress of an urbanized area and asymmetry values was analyzed. 8-10% of the samples  $|R - L| / (R + L)$  had a normal distribution (K-S test,  $p < 0.01$ ). Only one trait in birch and one in linden significantly ( $p < 10^{-4}$ ) reflected the stress effect in a direct relationship. *Q. robur* and *A. platanoides* had a wider range of FA, and the traits reflecting stress were different. No correlation was found between the FA and GMFA integral indices. High heterogeneity of values  $(R - L)$  was accompanied by the significance of directional asymmetry. The GM method extinguished the kurtosis in trait and showed a significant value of GMFA at kurtosis values  $\gamma < 2.5$ . The high heterogeneity ( $\gamma > 2.5$ ) accompanied by insignificant fluctuating asymmetry in Procrustes ANOVA. The authors emphasize that the toxic effect of pollutants can be associated with the relief and protection from winds in urbanized areas. The heterogeneity of the genotype of plantings from different nurseries increases the factor diversity, which makes the results of the analysis of developmental stability much more critical.

### 1. Introduction

The search for bioindicator species that adequately and clearly reflects environmental stress with a high probability was carried out for a long time. Preference was given to easy-to-determine properties: size, morphological characteristics and functional properties. The bilateral asymmetry of the leaf blades turned out to be such a convenient property. Fluctuating asymmetry (FA), as a non-directional deviation in difference between the right (R) and the left (L) sides, is considered an indication of stress. A large number of articles were published indicated an increase in the FA index, which was calculated using the formula of the normalizing difference  $FA2 = |R - L| / (R + L)$  depending on the increase in anthropogenic load and other types of stress [1-6]. Doubt in the results obtained has been discussed in



detail in some articles, for example [7-9]. The skeptical attitude comes from the following serious arguments:

- nonparametric frequency distribution of the samples used;
- absence of samples along the transect of the gradient of distribution of pollutant;
- publication only positive results;
- lack of genotypically close control samples;
- ignoring the heterogeneity of metric characteristics, leading to serious errors.

The article, on a large value of data, shows the results obtained using the conventional normalizing method and the method of geometric morphometrics. The relationship between the environmental stress of an urbanized area, the FA values and the characteristics of the plastic (metric) traits used is analyzed. Main attention is paid to the heterogeneity of the magnitude of plastic linear traits, i.e. how they affect the result, if they are not taken into account.

## 2. Materials and methods

About 30,000 of the leaf blades were used, collected in 2003-2010 in the Vladimir and Moscow urban areas (Russia), from 300 populations (150 – birch (*Betula pendula* Roth.), 100 populations of linden (*Tilia cordata* Mill.) and 25 oak (*Quercus robur*) and maple (*Acer platanoides*). Each sample contained 100 leaf blades, collected by 10 leaf specimens from 10 trees according to method of V.M. Zakharov [10]. The stress was understood as the impact of anthropogenic factors in the urbanized area was ranged from 1 to 5 points. The following stress factors were taken into account: the urbanization of the territory (thousand inhabitants), the degree of compaction and disturbance of the soil cover (also in points), as well as emissions from vehicles and industrial enterprises ( $\text{g} / \text{m}^3$ ).

The normalizing formula was used to determine the correspondence of the fluctuating asymmetry value to the level of the general stress load. We used from 4 to 6 homologous traits of leaf blades [11]. The normal distribution of the FA2 index was checked by the Kolmogorov-Smirnov test. Allometric properties were checked by the correlation the value of the trait and FA2. The sample characteristics were determined, such as kurtosis and a variance. Directional asymmetry (DA) was tested on the deviation from zero variance ( $R - L$ ) in  $t$ -test. In the method of geometric morphometrics the marks, as true landmarks of the first type, were applied in the SAGE (Marquez, 2014) twice along the contour of a leaf blade from 20 (maple, oak) to 50 (birch, linden), and FA was determined by the deviation of coordinates from the consensus. The Procrustes alignment operation included mirroring the left and right landmarks and averaging the difference in magnitude of linear sizes. The value of FA (GMFA) was calculated in Procrustes ANOVA as the mean square of the interaction of the factors "side  $\times$  leaf" both for the aggregate of landmarks and for their homologous pairs. Directional asymmetry was tested according to the significance of the "side" factor. Permuting multiplication of samples up to 1000 iterations and statistical evaluate on 95% probability level were used.

## 3. Results and discussion

### 3.1. Normalizing method

Only the 8-10% of samples  $|R - L| / (R + L)$  had a normal distribution (K-S test,  $p > 0.01$ ). Only one trait in birch (leaf width) and one in linden (distance between the base of the first and second veins) in the direct relationship significantly ( $p < 10^{-4}$ ) reflected the stress effect. 4-5% of samples of populations of birch and linden and 3% of oak and maple responded to high stress with an increased FA value. *Quercus robur* and *Acer platanoides* (facultative heliophytes) had a wider range of fluctuating asymmetry values, and varied from traits to trait.

The reasons for the lack of a possible response to stress could be:

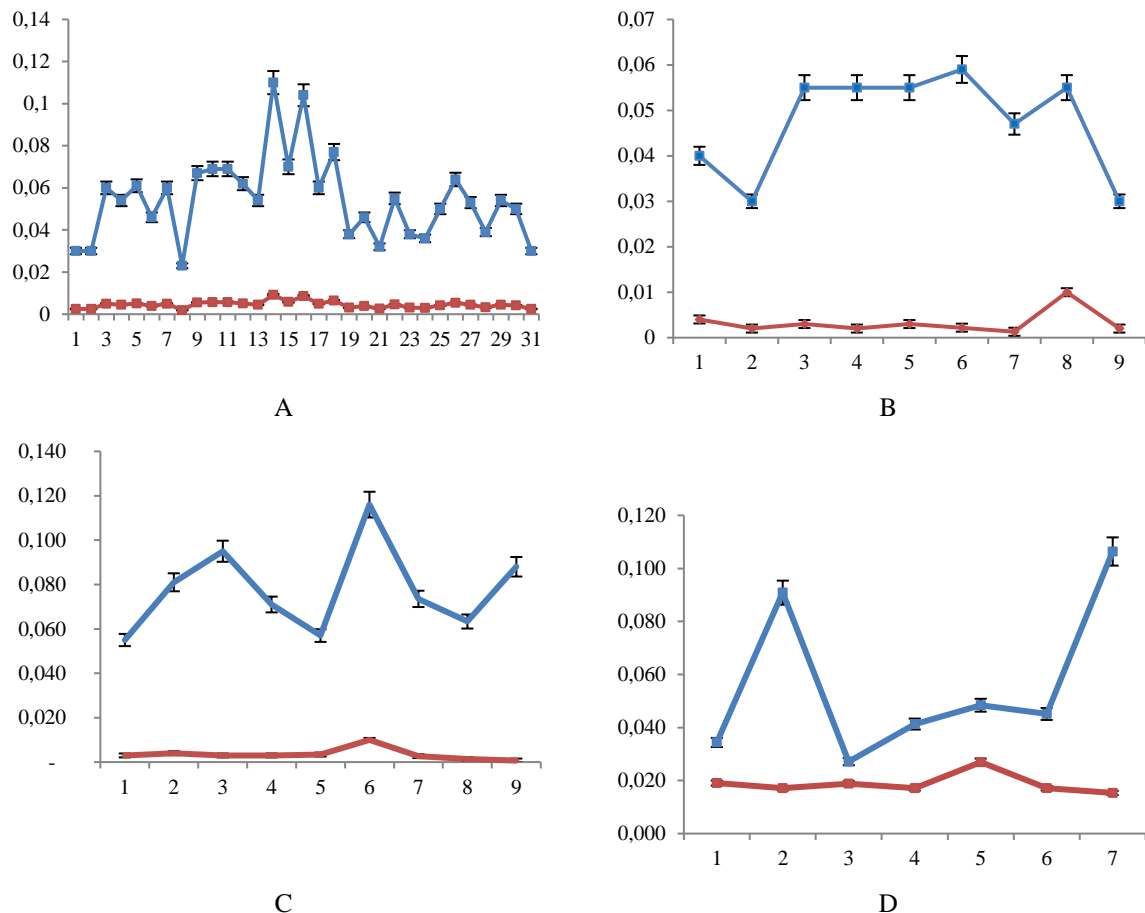
- genotypically determined path of developmental canalization;
- epigenetic effect of homeostasis;
- the mutually neutralizing effect of factors, including unknown ones;
- the phenomena of hormesis and paradoxical effect, which increase or, conversely,

decrease the plant's response in indirect relationship to the degree of pollutant load [12];

- genetic heterogeneity of samples and hidden (unavailable) micro biotic and molecular biological causes.

### 3.2. Geometric morphometrics

The comparison of the GMFA value with the integral FA2 index showed a weak relationship (Spearman's correlation coefficient  $r = 0.07$ ;  $p < 0.05$ , figure 1).



**Figure 1.** Correlation of two indices (FA2 – above and statistically significant GMFA – below): A – *Betula pendula* Roth, B – *Tilia cordata* Mill, C – *Quercus robur*, D – *Acer platanoides*

Thus, the index GMFA referred to shape asymmetry was not comparable to the integral fluctuating asymmetry index in normalized method. An increase in the individual variability ( $MS_i$ ) of the leaf led to an increase in the  $MS_{IS}$  value i.e. the GMFA index. In other words the high variability of the R and L values, indicating the variability of the size of the trait, contributed to an increase in the value of the GMFA index. The variance of the GMFA values was significantly lower than the variance of the FA2 index, and the standard error of the GMFA index was 10 times less. A significant correlation between the FA of the trait of leaf width and integral GMFA of average strength was obtained only for silver birch and small-leaved linden ( $r = 0.58-0.6$ ;  $p < 0.05$ ).

### 3.3. Directional asymmetry

The directional asymmetry is one of the types of bilateral asymmetry that makes it difficult to determine the value of FA. In the case of the presence of DA, the trait, according to the recommendations, is excluded, the sample size decreases, and an undesirable "skew" in the value of the integral FA occurs [13]. It is the "pure" It is well known that only pure FA reports the level of developmental stability of

populations; therefore, we compared DA in separate linear traits with the  $MS_s$  value obtained in the GM method for the corresponding pairs of landmarks (table 1).

**Table 1.** The comparison of DA in  $t$ -test (some traits) and in Procrustes ANOVA.

№	Spices	DA ( $t$ - test, $p$ )	Procrustes ANOVA	
			$MS_s$	$p$
1	<i>Betula pendula</i>	$10^{-2} - 10^{-7}$	0.003	$10^{-2}$
2	--	$10^{-2}$	0.004	ns
3	--	$10^{-4}$	0.002	$10^{-3}$
4	--	$10^{-2} - 10^{-3}$	0.004	$10^{-6}$
5	--	-	0.001	$10^{-3}$
6	--	-	0.005	$10^{-5}$
7	--	$10^{-5} - 10^{-10}$	0.006	$10^{-5}$
8	--	$10^{-6}$	ns	ns
9	--	$10^{-6} - 10^{-8}$	0.005	$10^{-6}$
10	<i>Acer platanoides</i>	$10^{-2}$	0.001	ns
11	--	$10^{-8}; 10^{-2}$	0.007	$10^{-7}$
12	--	$10^{-3}$	0.008	$10^{-6}$
13	<i>Quercus robur</i>	$10^{-4}; 10^{-7}$	5.17	$10^{-6}$
14	--	$10^{-3}$	0.006	ns
15	--	$10^{-2}; 10^{-3}$	0.01	$10^{-4}$
16	<i>Tilia cordata</i>	$10^{-12}; 10^{-3}$	--	$10^{-6}$
17	--	$10^{-2}$	1.03	ns
18	--	$10^{-2}$	1.04	ns

$MS_s$  – mean square «side»; ns – not significant

The highest DA values with a high  $p$  level were typical of both the normalizing method and GM analysis (for example lines №№9 and 11). In cases with low directional asymmetry of traits, the value of the  $MS_s$  in the GM method also had low means or they were statistically insignificant (for example, lines №№ 1, 17 and 18).

So the DA in traits was not vanished in the shape if its probability level was high enough, for example,  $p < 10^{-2}$ . When there were two traits with DA in the leaf blade, the geometric DA could both increase (row 4) and decrease (row 1). This is understandable. Two traits can have differently directed deviations (one has a right-sided, and the other a left-sided directional asymmetry).

### 3.4. Antisymmetry and frequency distribution heterogeneity

It is known that a positive kurtosis in the distribution ( $R - L$ ) indicates the presence of genetically determined heterogeneity and is expressed in the leptokurtic frequency distribution of ( $R - L$ ) [13]. The negative kurtosis values were not found ( $t$ -test) i.e. no statistically significant presence of antisymmetry was found. Permutation multiplication of samples, provided in the SAGE package, artificially normalized the studied samples in the GM analysis. This led to: a) a decrease in the statistical significance of DA and: b) an increase in the homogeneity of the variance. We analyzed the high values of kurtosis  $\gamma$  in the samples ( $R - L$ ) of individual characters and the results of the integral GMFA of the shape (table 2).

**Table 2.** Examples of kurtosis values and Procrustes ANOVA result.

№	Kurtosis (descriptive statistics), $\gamma$ (high FA2)	Procrustes ANOVA			
		no iteration		1000 iterations	
		$MS_s$ , $p$	$MS_{IS}$ , $p$	$MS_s$ , $p$	$MS_{IS}$ , $p$
1	4>	$10^{-6}$	$10^{-6}$	$10^{-2}$	$10^{-3}$
2	5.33; 6.14	ns	ns	ns	ns
3	3.2	$10^{-5}$	ns	$10^{-2}$	ns
4	2.5	$10^{-2}$	$10^{-6}$	$10^{-2}$	$10^{-3}$
5	2.9	ns	$10^{-6}$	ns	$10^{-3}$
6	3>	$10^{-5}$	$10^{-2}$	ns	$10^{-3}$
7	2.9-3.2	$10^{-6}$	$10^{-2}$	$10^{-2}$	$10^{-3}$
8	2.9-3.2	$10^{-2}$	$10^{-6}$	$10^{-2}$	$10^{-3}$
9	2.0-3.0	$10^{-2}$	$10^{-6}$	$10^{-2}$	$10^{-4}$

$MS_s$ , – mean square «side»;  $MS_{IS}$  – mean square «leaf × side»; 1-6: *B. pendula*; 7-9: *T. cordata*; ns – not significant

At high kurtosis values, GMFA value was not statistically significant (lines 2 and 3). After 1000 iterations, the  $MS_{IS}$   $p$  probability level changed to a less significant side (from  $10^{-6}$  to  $10^{-3}$ - $10^{-4}$ , lines 4, 8 and 9). The high heterogeneity of samples (high kurtosis) was accompanied by the significance of directional asymmetry, which either decreased (lines 1, 2, 6 and 7), or remained at the level of significance as in linear traits (lines 4, 8 and 9). Notably the high kurtosis did not show GMFA in the absence of directional asymmetry, even after permuting multiplication (line 2). Based on the foregoing, the multiplication of samples is convenient for testing the presence of statistically significant fluctuating asymmetry. At the same time, the GM method leveled (smoothed) the kurtosis ( $R - L$ ) (in terms of geometric morphometrics – Procrustean distances) only at kurtosis values of no more than 2.5.

At higher kurtosis values, the statistical significance of GMFA was declined even after normalizing reproduction of samples. This fact must be taken into account, since in the normalizing method a high kurtosis value contributes to an overestimated FA, which distorts the result of testing developmental stability.

#### 4. Conclusion

In urbanized areas, the accumulation of toxins, for example, in the soil, is associated with the features of the relief, for example, with the slope of the rainwater runoff. Wind protection or, on the contrary, wind turbulence plays a significant role in the morphology and physiology of trees and leaf blades. The diversity of plantings from different nurseries increases the factorial diversity. The foregoing forces us to be much more critical to the results of the developmental stability testing.

The linear leaf traits showed the presence of fluctuating asymmetry in 8-10% of cases. While a third of this number showed an increase in FA in response to the degree of urbanization, cover compaction, and environmental pollution, the other two-thirds of the samples showed no increase or decreased FA value. The method of geometric morphometrics “smoothed” the heterogeneity of the variance and facilitated a robust assessment of the FA value. The metric traits (lengths and angles between veins) possessing directional asymmetry was expressed as significant DA in Procrustes ANOVA. A serious remark is that when using the normalizing formula, researchers often do not take into account the heterogeneity of the variance of the difference  $R$  minus  $L$ , which leads to an unclear increase in FA.

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