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Use of Morphogeometric Method for Study Fluctuating Asymmetry in Leaves *Tilia cordata* under Industrial Pollution

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ABSTRACT

Morphogeometric study showed the low mean value of fluctuating asymmetry (FA) of leafy plates Tilia cordata in Procrustes ANOVA in compare to trivial normalized method. The low variance of FA index in Procrustes ANOVA and correlation with conventional two-way ANOVA was also observed. The stability of development was influenced by traffic density. The lowest developmental stability and high fluctuating asymmetry were detected along the main towns' roads.

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INTRODUCTION

Tilia cordata Mill is one of the species most commonly used to determine the stability of development by using fluctuating asymmetry, a pattern of variation of the difference between the right and left bilaterally symmetrical sides (R-L) where the variation is normally distributed about a mean of zero [15,23,24].

In compared to known species-bioindicators of birch, linden is characterized more stable genotype, as not inclined to intraspecific hybridization. Linden is part of the green landscape of cities, dramatically reacts to air contaminants, so Tilia chordata is a convenient target for biomonitoring.

A large body of works on evaluation as industrial well as natural factors on the variability of fluctuating asymmetry linden were performed. The research in this area is ongoing [3,4,21,8,7].

In the cities of central Russia with a relatively homogeneous landscape, as well as with a predominance of the hills, the difference in the nature of the soil, the definition of FA seems tedious, but interesting task.

Mass transfer of toxic substances is affected with location of buildings, movement of air masses from the roads, green areas health and the whole climate of the city. Microclimate of the city depends on the density of buildings, on the number of inhabitants and on the character of industrial enterprises.

The most accessible and widespread method of determining of FA is the testing on difference between mean value of left-right metric traits of the leaf blade [24].

The idea of morphological geometric definition FA is the use of differences in the values of the point coordinates of left and right homologous organs.

Endpoints (landmark) commonly used are located on the oak or maple leaf veins, at the base and at the tip of the leaflet lamina, on sinus, on the lobes or other places. Benefit of the method is the use of two-dimensional space. The value of fluctuating asymmetry is determined by taking into account the shape of the leaf plates.

Earlier software applications used for these purposes, mainly used to analyze some laboratory models of Drosophila, form of fossil bones and other biological objects, such as representatives of the class Pisces. In botanical studies shape analysis is widely used in taxonomy and cladistics.

The seminal works in the field of morphometry was performed by Rohlf and, later by Klingenberg, including papers in the area of statistical analysis of morphometric characteristics of some plants.

The growing popularity of morphological geometric analysis can be traced on the example of work performed in the study of morphological features leaf plates of linden alder [11,2,22] and tormentil *(Potentilla)* [18,9].

Variability in the form of the leaf blade of many habitats and biotopes has been studied in population of oak [12].

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Variability of morphological characters according to industrial impact was studied on a birch [19,25,7] on linden [21] and on maple [7].

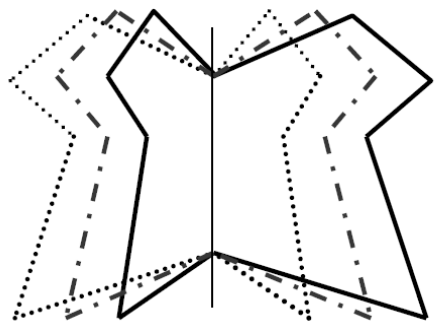


Fig. 1: Displaying of the original figure (solid line) on the basis of the Procrustean alignment [10].

Dotted line is a mirror-image copy for each half. Dash-dotted line is the symmetrical average figure forming on the base of least squares method (superimposition). Procrustes FA is the difference between the landmarks coordinates of the original figure and symmetric average standard, or consensus.

One of the approaches is the method of Procrutes averaging (Fig.1). The Procrustes analysis includes the original and mirrored configurations of a sample combined, and superimposes all of them simultaneously. For averaging consensus method of least squares is used [17,16].

Methods of geometric morphometry have limitations because they are algebraic, not random and not related to descriptive statistic laws.

To perform the FA two-factor analysis of variance the values of the XY coordinates of homologous bilaterally symmetrical points displayed in the tangent space are taking into account.

The fundamental advance of morphogeometric approach is in the way landmarks carry data on the shape and form difference of the homologous symmetrical structures.

Thus the value of angles and characters of shape is taking into account as well.

In comparing with trivial two-way ANOVA assay (individual x side) the Procrustes analyse includes more freedom of degree in the (2k + l - 2) times, where: k - number pairs of landmarks; l - number of single landmarks on the midline. Therefore, the Fisher level of probability is increased, as this criterion testing the null hypothesis is sensitive to the degree of freedom [9].

Initially two-way Procrustes ANOVA was adopted for FA testing of Drosophila metric traits.

For the plants this kind of analyse of variance was used on digitalised data for leaf plates of oak and flowers from some families' representatives [1,6].

In these study MorphoJ TPS, PAST и SAGE soft packages were used.

The latter was developed to FA analyse the by Marquez in 2006 and is designed to work with the file format TPS.

The program is easy for the user, allows not only identify the integral value of FA in the Procrustean analysis, but allows also to test a statistical difference in fluctuating asymmetry of individual landmarks.

Based on this, the decision was taken to compare the values FA derived in two ways normalized difference and using Procrustes analysis.

MATERIALS AND METHODS

Collecting Samples And Primary Treating:

The leaf linden plates were collected in Vladimir and other inhabited locations of Vladimirskaya oblast (Russia) during 2009 in accordance with standard procedure [23].

An essential complement to this was that the plates were gathered to the width of the leaf bade 3-4 cm. The purpose is to eliminate variation in size, hence, reduce the number of discarded samples, due to the possible relationship between the magnitudes of the leaf size of FA value. The aim pursued the goal to reduce allometric variability, associated with size scaling leafy plates.

Directional asymmetry (NA) in (R-L) samples has been detected by 2 tailed paired t-test. With probability p less than 0.05 the sample has been rejected as a sample containing NA.

Antisymmetry (AS) was tested by finding the values of kurtosis in samples (R-L). Significant deviation from the range (-2 ÷ 2) indicated the presence of AS. The significance of kurtosis was tested also by *t* criterion. Both functions (*t* - test and kurtosis) were carried out in a medium Excel (tab "function").

A total number leafy plates analysed was 100-150 from each of the forty five populations. The samples in which all traits contained AC or DA were precluded.

It was assumed that the samples represent a portion of the population with normally distributed values, and hence retain the functions of the normal distribution.

There was not identified strong correlation between trait size and FA trait value (Pierson $r < 0.7$; $p > 0.05$).

FA index was found with a trivial way normalizing difference (R - L), that is:

$FA = |R - L| / (R + L)$

where, *R* and *L* are means of right and left bilaterally symmetrical homologies traits [[14]].

Numerical score the environmental quality was assessed using a modified scale developed by Mokrov and Gelashvili in 1999.

Morphogeometric Analysis:

The leaves were scanned by Colour Page-Vivid 1200XE, 300-400 dpi resolution or leaves were photographed (Panasonic DMC-FZ100) and stored in JPEG.

Labelling was performed using the program TPSdig [17].

Marks were plotted in accordance with conventional dimensional characteristics used to determine the trivial FA (Fig. 2).

Labelling was performed twice (for finding errors of measurement) for each the leaf blade.

Selected landmarks were classified as homologous marks type I, as fixed points in the venation points [16]. Files with the XY coordinates stored in the format TPS.

After that the files were combined in pairs in the program TPSutil [17].

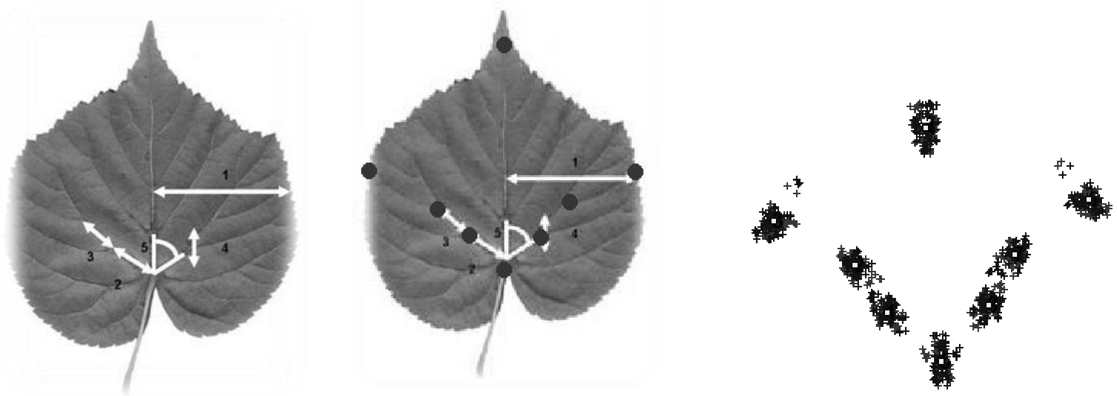
All data as a TPS file contained the coordinates for the eight points (three paired and two lying on the midline).

Procrustes two-way ANOVA was performed using SAGE (Symmetry and Asymmetry in Geometric Data) soft package. SAGE provides the following isometric transformations: shift and rotation. Thus landmarks labelling is performed accordingly with saving proportions among all landmarks.

Index Procrustes ANOVA was calculated as the difference between mean squares (MS) side x individuals and measurement error.

Significance of factor “side” showed the presence of directional asymmetry.

Antisymmetry was detected with F-Goodal test (null hypothesis of fit landmarks configuration to centroid (consensus) [10].



a b c

Fig. 2: Arrangment of landmarks on leaf *Tilia cordata.*

a - traits involved in trivial testing FA; b - landmarks used to morphogeometric testing; c - Procrustes fit of original and reflected data (screenshot SAGE package). Constellations of labels after double mirroring reflection, n = 60-80. Light marks in center of every constellation mean perfect bilaterally symmetrical centroid (consensus)

RESULTS AND DISCUSSION

Results of two methods trivial and Procrustes FA are performed in table 1.

The large values of FA were observed in small regional cities and habitations up to 50 000 dwellers.

The populations of linden reveal a high index FA nearby big auto traces (Fig.3).

The largest deviation (FA more 0.064) was identified in largest cities as Vladimir and Kovrov (Fig.4).

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Google maps showed high level of FA along the roads whiles FA values are less in the parks and green squares, protected from air contaminants.

Table 1: Range estimation of environment health, Vladimirskaya oblast (2009).

Mark	Trivial FA	Procrustes FA	Characteristics	Population, site
I	< 0.038	< 0.001	conventional norm	Vladimir: Dzerzhinskya st.,
				Ubilejnayst, Park VlSU
				Vyaznikovsky region, Edon
				Gus-Chrustalny region, Krasnoe echo, Urshel
				Kirzhach
				Muromsky region, Molotici
				Sudogodsky region, Aksenovo
II	0.038-0.046	0.001 - 0.002	weak stress factor	Kirzhach
				Kolchugino
				Vladimir, Spaskay st.
				Vladimir, Lenina st.
				Melenki
III	0.047-0.055	0.002 - 0.003	polluted area	Vladimir
				Peganovo, near Vladimir
				Vladimir, Pichugina st.
				Sobinka
IV	0.056-0.064	0.003 - 0.004	strong polluted area	Vladimir, Streleckay st., park
				Vladimir,Gorkogo st.
				Sudogda, Lenina st.
				Melenkovsky region, Papulino
V	> 0.064	> 0.004	critical value, unfavourable conditions	Kovrov, industrial zone
				Vladimir, st. Niznay Dubrova

Almost every population contained DA (mostly on first trait) or antisymmetry presented samples.

AS presence has been tested by *t*-test with Bonferonni correction. Detection was released across all five metric traits per sample.

As a rule a kurtosis is over 2.3 signalled as a significant ($p < 0.05$). About 50% of samples (R - L) contained significantly high means of kurtosis on one or other trait. So those samples were sort off next finding normalized index FA. There were not met high negative kurtosis values.

Rejection of AS- and DA- contained samples either increased or decreased the value of an integrated value FA, however, no significant difference was found for the totality of samples before and after rejection ($n = 25$; $p > 0.05$).

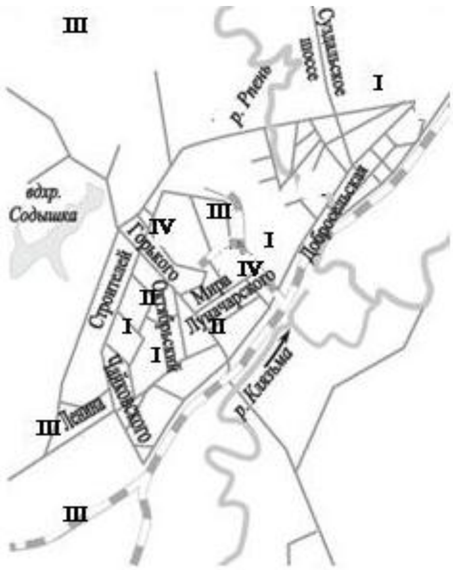

Fig. 3: The main Vehicle Trails in the centre of city of Vladimir (st.Gorkogo and Mira) with a strong and critical contamination levels (IV-V-th mark). At a distance of 1-2 km were recorded I-st and II-nd mark of contamination

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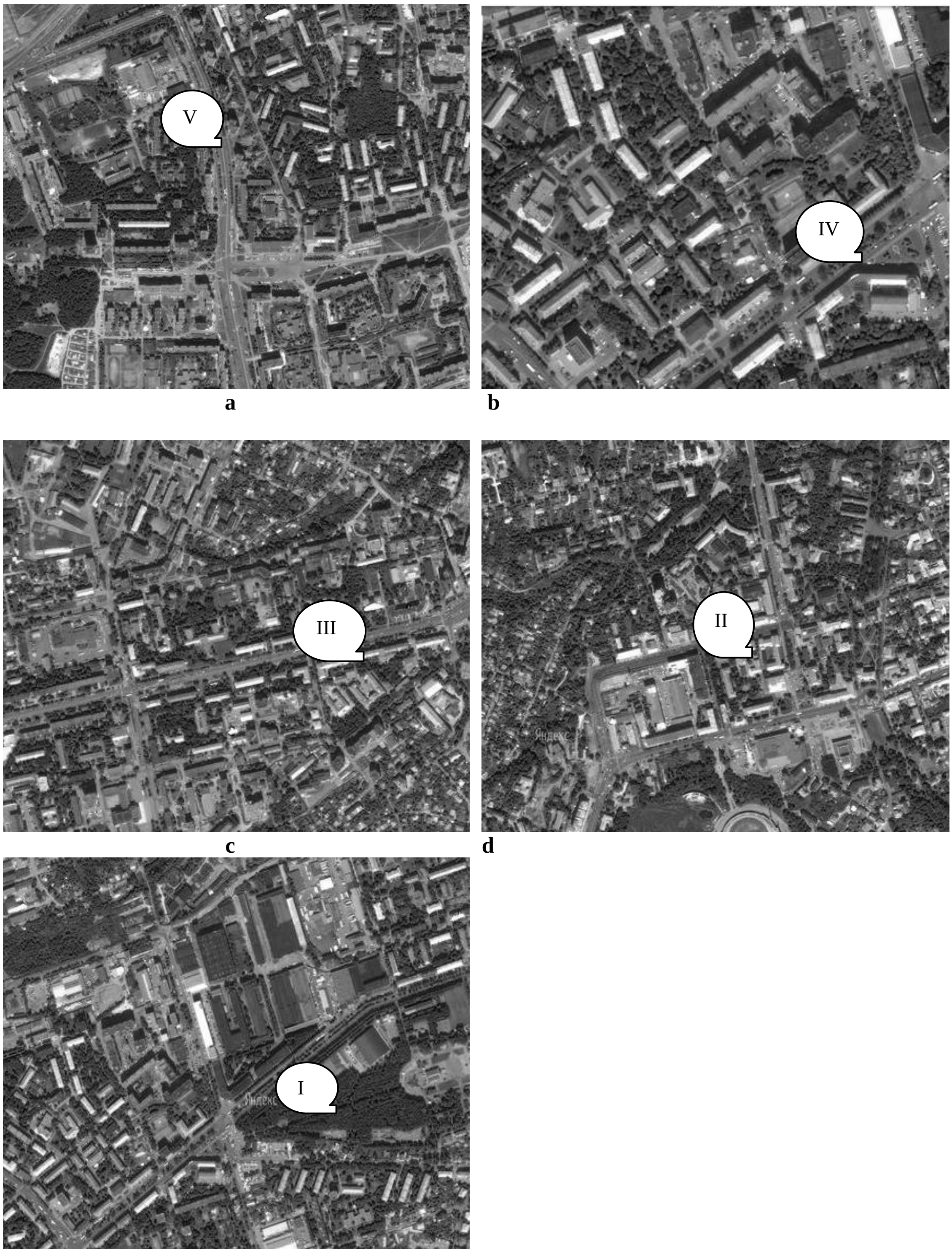

Fig. 4: Marking distribution FA index in Vladimir. The stability of development was influenced by traffic density: a - st. Niznay Dubrova: (V); b - st. Mira: (IV); c - pr. Lenina: (III); d - st. Spaskay: (II); e - Park: (I)

The results of morphogeometric method have shown a rather low variance of means square MS values “side” and “individual” in compare with a trivial method normalized difference.

In whole Procrustes ANOVA results corresponds to values, detected by trivial two-way ANOVA. The MS values in Procrustes ANOVA were 10 times less than in trivial two-way ANOVA(Tab.2).

Table 2: Example of results Procrustes and trivial (conventional) ANOVA, Melenkovsky region, n = 51; FA = MS (individual x side) - MS (error). Trivial FA = 0.007, df = (n – 1); Procrustes FA = 0.001, df = (n – 1)(2k + l – 2).

trivial 2 way ANOVA				Procrustes ANOVA		
	MS	*df*	*p*	*MS*	*df*	*p*
individual	0.219	50	0.000	0.005	300	0.000
side	0.008	1	0.322	0.003	6	0.045
individual x side	0.008	50	0.000	0.001	300	0.000
error	0.001	204		0.000	612	

Upon identifying mean FA one of the serious questions is raising the question of rejection samples with premixes DA and AS.

When finding integrated FA we are faced to averaging some FA traits, sometimes final result depends on only one trait.

However the traits are not equivalent on FA value, therefore the relevancy of integrating FA usage is doubtful [3,5,7].

In this regard clearly visibly the advantage of using Procrustes FA index determined with powerful 2-factor analysis using a landmark coordinates informing about the geometry of two-dimensional character of the leaf blade.

With regard to antisymmetry which caused by high or low kurtosis mean, there are many approaches which come down to two polar views of either removal of samples, or ignoring the presence of the high / low kurtosis.

Besides the high positive kurtosis is a very characteristic feature of (R-L) value distribution.

This property has led some researchers to classify the FA with AS premix to a certain kind of fluctuating asymmetry [15].

Pure fluctuating asymmetry as a phenomenon based on the definition of FA, as a small random deviation from zero within the normal distribution (R - L) could not be referring to the frequent occurrence.

There are a number of approaches that allow AS testing in geometric morphometry [10,18]. In this study AS presence was tested with F-Goodall test (TPSreg).

It tests the null hypothesis on the difference between shapes of the samples and is based on analyse of Procrustes distances from each specimen to the reference and from each specimen to the configurations predicted by linear regression.

For example in Melenkovsky region F-Goodle criterion was equal 253.4; df = 192.1; p = 0.00, that told about absence difference between the coordinates of landmarks and consensus.

For testing normal distribution of coordinates of landmarks the permutation test (99 iterations) is also envisaged.

If the permutation test shows insignificant mean MS (individual x side) it says on deviation from parametric distribution (R-L) sample value.

The shortcoming Procrustes analysis appears low variability of the Procrustes FA index. This can be explained by compensation FA value with some traits. If the FA value is higher for one pair landmarks, another pair compensates this with lower FA value.

So averaging mean Procrustes FA lead for the low variability and variance among samples. However, even this index ought to be perceived as an indicator of developmental stability, despite the fact that the low variability causes difficulty in scaling of the study area and environmental assessment.

Procrustes analysis method is fast and accurate and gives an objective assessment of developmental stability, as revealed from a number of works in field morphometry and developmental stability, rapidly increasing in recent years.

At the same time method describes the shape of the object, which gives learning opportunities to study the form factor.

Method of geometric morphometrics has serious methodological bases, avoiding the difficulties associated with the directional asymmetry and antisymmetry as conventional satellites fluctuating asymmetry.

Directional asymmetry is easily detectable in Procrustes ANOVA. Significance of factor “side” signalizes on presence DA.

In this work correlation between DA, tested by t-test and DA tested in Procrustes ANOVA was not found.

This can be explained by methodical procedure for the determination of FA based on the spatial arrangement and on the analysis of set of marks that reduce the chances for DA of some metric traits. This picture can be expanded on AS contained traits as well.

Conclusion:

Morphogeometric method described above involves the use of standardized size leaf plates.

The question of allometric variation (size - developmental stability) however, is an essential and to be solved with frontier methodological approaches.

The question of choice landmarks that adequately reflect the fluctuating asymmetry of the leaf plates is essential as well.

A contemporary approach includes both tasks: determining of stability of development and testing of variability of morphological characters.

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