Three methods testing the asymmetry of the leaf blades of the small-leaved lime

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Abstract. Three methods were used to determine the fluctuating asymmetry (FA) of leaf blades and the developmental stability of small-leaved linden (*Tilia cordata* Mill): the normalizing difference method, the sample normalization method, and the two-factor analysis method (FA10). The collection of leaf blades took place along the latitudinal zonality gradient from north to south in five cities from Murmansk (68°58′ N) to Mozhaisk (55°30′ N), including the cities of Apatity, Petrozavodsk and Saint Petersburg. In all analyses, the highest FA value was obtained in the population of Petrozavodsk, the lowest in the population of Mozhaisk. In other cities, the FA indices differed, except for the populations in Apatity and Petrozavodsk with FA10 indices, respectively, 0.02 (p=0.02) and 0.03 (p=0.01). In the northernmost population (Murmansk), no decrease in developmental stability was noted. The stability of the development of *Tilia cordata* was more strongly influenced by the climatic and geographical conditions of the area and the technogenic state of the environment than by the geographic latitude. The authors note that a non-parametric sample distribution is advisable to use the two-factor analysis of variance, since normality difference and normalization lead to a non-equivalent deviation of the results. Bonferroni's adjustments for measurement error were convenient for accurately distributing FA values at significance level.

1 Inroduction

When comparing different populations in terms of developmental stability, the size of the compared leaf blades is important. With a high latitudinal zonality gradient, the leaf blades differ both in size and in the dispersion of the values of the traits of the leaf blade, for example, the lengths of the veins, the distances between them. In previous works, the factors of altitudinal zonality, the nature of pollutants, the intensity of sources of

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technogenic pollution, and the climatic features of the collection site were considered [1–3]. From the obtained results, it follows that the adaptation of introduced species, such as small-leaved linden, proceeds from year to year with a high degree of morphological variability. If there is no direct positive correlation between the values of asymmetry and the value of a trait, then the trait does not affect the asymmetry, including fluctuating asymmetry (FA), and comparison of populations is possible even with different sizes of the trait. One should also take into account the property of plastic variability, which often (but not always) correlates with the FA value [2]. An important problem facing the researcher is the normality of sample values. If the samples do not have a normal distribution, then it makes no sense to compare the average FA values. In this case, two-way analysis of variance can and should be used, since it compares variances, not arithmetic means. Therefore, when comparing samples with different leaf sizes and variability of variance, it is important to test different approaches to determining fluctuating asymmetry and developmental stability. We used small-leaved lime, small-leaved linden (*Tilia cordata* Mill) as a model.

Previous work has shown that the population variability of the FA index was higher in the northern latitudes (Kola Peninsula) compared to central Russia. A higher fluctuating asymmetry was indicated by the method of geometric morphometry, based on a set of landmarks along the contour of the leaf blade [3]. The method of geometric morphometrics operates with high degrees of freedom, often (but not always) shows a directional asymmetry (DA) as a dominant deviation to the side, right or left. Therefore, fluctuating asymmetry is a phenomenon that is much less common, as represented using the normalizing difference L–R, where L and R are the values of the corresponding signs of the right and left. The manifestation of latent directional asymmetry is especially characteristic at low functional levels of the experiment, for example, at the level of leaf blades [4]. Among the northern populations (60-67 degrees north latitude), the fourth trait was the most variable - the distance between the bases of the first and second leaf veins. We decided to study this trait in order to determine the FA in a measured way using the normalizing difference method, taking into account the specifics of the frequency distribution of the FA value at the level of population variability. For comparison, we used the method of normalizing samples by their random generation with normal distribution and two-factor analysis of variance [5]. For a more accurate calculation of FA, the Bonferroni correction method was used [6]. Thus, the aim of this study was to compare the level of developmental stability in geographically distant populations, as well as methods for determining FA when working with samples with different frequency heterogeneity. The working hypothesis assumed the influence of geographic latitude on the developmental stability of the small-leaved linden.

2 Sites and their characteristics

The analysis of FA was carried out on the field material of five populations located in different geographical zones with different physical - geographical relief and environmental ambient. Thus, the city of Murmansk (Murm; 68°58′00″ N) had a complex air pollution index of 2.99; The city of Apatity (Apatit; 67°34′03″ N) is more prosperous in terms of air quality (complex air pollution index of the city is 1.25, which is below the national average); the city of Petrozavodsk (Pzv; 61°47′46″ N) is characterized by lead and zinc pollution in excess of the maximum allowable concentration, especially in the upper soil layers along highways and railways; St. Petersburg (SPb; 59°57′ N), as the most densely populated metropolis with a humid climate, had a specific urban environment with an uneven distribution of pollutants andthe sources of technogenic stress (Figure 1).

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Fig.1. Localities of study.

For comparison, we used the population of the city of Mozhaisk, Moscow Region (Moz; 55°30′ N), which is characterized by the most favorable ecological and climatic conditions for the growth of small-leaved linden. We collected leaf blades in August-September 2022 from the lower part of the crown of trees of the generative stage of development of typical sizes for the studied populations. The collection of leaf blades was carried out 2 times with a break of 1-2 weeks, 5 blades from each of ten trees from all sides of the crown, a total of 500 leaf sheets (5 × 2 × 5 × 10). The sites for collection were selected with the influence of anthropogenic stress (urban development) and without it (parks, botanical gardens).The obviously asymmetric blades were excluded.

3 Methods

The leaves were scanned and the fourth dimensional trait was measured (TPSdig2, Rholf, 2017), which differed the most in FA. The measurement results (in cm) were first entered into Excel spreadsheets, then into STATISTICA 10 (Statsoft Ink.), where analyzes of descriptive statistics and basic tests were performed. The correlation coefficient between the values of the trait and FA2=|L–R|/(L+R) was found using the Pearson formula for normally distributed samples per tree variable. We tested normality using the Kolmogorov-Smirnov test (K-S test). The samples with a nonparametric distribution were tested for the homogeneity of the variance by the Levin test [7]. Normalization in Excel was carried out based on the samples, taking into account the mean and standard deviation. Three variables are generated and one sample with average values is obtained from them. The Bonferroni correction was carried out taking into account the measurement error in percent, and the FA10 index was determined by the ratio of the mean square MS of the interaction of factors leaf × side, subtracting the MS error of two-way analysis. In most cases, we took the significance level of the null hypothesis as 95%.

4 Results and discussion

4.1 Trait size

There was no difference in the trait's value between repeated samplings (t-test, p>0.05), but the value of the trait varied significantly among the populations. Levin's test for the homogeneity of variance showed a significant difference between the populations of Mozhaisk and Petrozavodsk (*p* <<0.000; standard deviation SD=0.24 in Mozhaisk and SD=0.30 in the population of Petrozavodsk). It these populations, the samples had a normal distribution (K-S test; *p* >0.2). A statistically significant mean was obtained (p<0.000). In other samples, the means also differed, despite the homogeneity of the variances (Levin's test; *p* >0.05). Characteristically, the populations of Mozhaisk and St. Petersburg did not differ in the trait's value, although the heterogeneity of the samples was high (Table 1).

Table1. Size trait statistics.

Site	Mean, cm	Mean, *p*	Variance, *p*	Levene, *p*	K-S, *p*	Lilliefors, *p*	Correlation (L+R)/2 and FA2, *r*	SE, *r*
Murm	1.467	0.000	0.267	0.631	*p*<0.10	*p*<0.01	-0.21	0.18
Apatit	1.020	0.000	0.054	0.168	*p*<0.01	*p*<0.01	-0.15	0.17
Pzv	1.375	0.000	0.000	0.000	*p*>0.20	*p*<0.05	-0.14	0.12
SPb	1.280	0.146	0.027	0.000	*p*<0.01	*p*<0.01	-0.02	0.17
Moz (contr.)	1.301	-	-	-	-		0.13	0.15

Note: *p*-value show the result in comparison to Moz, as a control site; SE – standard error

The leaf blades on the Kola Peninsula did not dominate and were not inferior in size to the trait, for example, in Mozhaisk, the average value of the trait is 1.3 cm, in Petrozavodsk, 1.38 cm, while in Murmansk, the northernmost region, this trait exceeded the rest (1.47 cm).

The correlation of the trait – FA2 was weakly negative or insignificant, for example, in the population of Mozhaisk, the Pearson r correlation was –0.07, as well as the Spearman correlation (–0.07; *p* =0.5).

4.2 Fluctuating asymmetry

The leaf blades varied in size, especially in the northern regions. The null hypothesis of the equality of the left and right half was rejected at the error probability more than 5% (*p* = 0.6-0.9). The FA2 values calculated by the normalizing difference formula, and other descriptive statistics were characterizing the samples (n =74) distributed according to a nonparametric law, as the Kolmogorov test showed a high value of normality deviation (Table 2).

Table 2. Characteristics of |L–R|/(L+R) variables.

Site	Mean	Standard Error	Variance	Kurtosis	Skew	K-S test, *d, p*
Pzv	0.077	0.007	0.004	2.115	1.347	0.14(<0.01)
Apatit	0.062	0.007	0.003	1.407	1.304	0.15(<0.01)
Spb	0.055	0.005	0.002	2.155	1.389	0.18(<0.05)
Murm	0.028	0.003	0.001	6.186	2.216	0.17(<0.01)
Moz (contr.)	0.069	0.006	0.003	-0.260	0.898	0.15(<0.01)

To normalize the samples, we used the random number generation based on the sample mean and standard deviation. The values of outliers with high FA2 values were removed, since linden leaves are characterized by left-sided directional asymmetry; therefore, the sample size was reduced to n =67.

After normalization, the values of kurtosis and skew were within acceptable limits [-2; ÷ 2]. The normality of the distribution was confirmed by the Kolmogorov test, since everywhere the deviation from the model sample showed no difference ($p>0.2$; Table 3).

Table 3. Characteristics of |L–R|/(L+R) variables after normalization.

Site	Variance	Mean	SD	SE	Skewness	Kurtosis
Ptz	0.001	0.080	0.04	0.004	0.06	-0.17
Apat	0.001	0.068	0.03	0.004	-0.38	-0.01
SPb	0.001	0.056	0.02	0.003	-0.21	-0.72
Murm	0.000	0.038	0.02	0.002	-0.08	-0.53
Moz (contr.)	0.001	0.061	0.03	0.003	0.04	-0.55

It follows from the table that the highest value of the FA2 mean was in the population Petrozavodsk, and the lowest in the population of Mozhaisk, as with normalized samples. The lowest FA value was in the population of Murmansk. Compared to the control (Mozhaisk), we found differences in all samples. A pair of samples from Apatity and Petrozavodsk showed no difference in the t-test (df =132; p =0.033).

In two-way ANOVA, the coded variables were the factors "leaf" (df =95) and "side" (df= 1). The FA10 index calculated as MS sample × side minus MS errors. The index of fluctuating asymmetry F calculated as the ratio of MS leaf × side to MS error [6].The measurement error in % was determined by the ratio of MS error to MS leaf × side. For the significance level p =0.05, the population with the lowest value of F fluctuating asymmetry selected, i.e., the least significance level p of the leaf × side interaction, in our case it was the population of Mozhaisk (Table 4).

Table 4. Evaluates of (R–L) values and data FA10.

Site	2 way ANOVA *p* values						Measurement error % from variance difference L and R	Bonferroni α	FA10	*df*
	side		leaf		side a× leaf					
	F	*p*	*F*	*p*	*F*	*p*				
Ptz	0.23	0.63	5.54	0.00	11.60	0.00	8.62	0.010	0.025	79.19
Murm	0.21	0.65	13.59	0.00	11.14	0.00	8.97	0.013	0.007	78.56
Apat	0.29	0.59	4.42	0.00	10.45	0.00	9.57	0.017	0.016	77.52
SPb	0.22	0.64	7.53	0.00	8.19	0.00	12.21	0.025	0.014	72.95
Moz (contr.)	0.00	0.971	5.04	0.00	1.85	0.00	54.07	0.050	0.009	18.75

The Bonferroni adjustment showed that the population of Petrozavodsk had the most significant level of statistical significance of FA (α =0.01). Further in descending order of importance: Murmansk, Apatity, St. Petersburg and Mozhaisk. The highest FA10=0.025 index (Petrozavodsk) corresponded to the smallest FA error (8.6%). Accordingly, the smallest value FA10=0.009 (Mozhaisk) corresponded to the largest measurement error in % of the difference between L and R (54.1%).

Each population had a Bonferroni significance level, reflecting the F value (side × sample) in the two-way analysis. The populations of Petrozavodsk and Apatity had the highest FA10 values (0.025 and 0.016), which were confirmed by the FA2 values obtained after normalization of the samples. We explain the highest FA values on the Kola Peninsula by the high plastic variability of the linear traits of leaf blades.

5 Conclusions

1. Two methods – normalizing and two-factor analysis allow to find a gradation in the value of FA and development stability, which can be used in mapping territories by the level of development stability.
2. The measurement error and correction make it possible to line up the FA values in terms of magnitude and significance level. The normalized samples can only serve as a guide and comparison for a more detailed two-way analysis.
3. The population of Murmansk, despite its extreme northern location, could not be characterized by low developmental stability. We associated low level with special climatic conditions. Thus, the hypothesis direct influence of geographic latitude on the developmental stability was not confirmed. The latitude can only be considered as one of the climatic components of the overall environmental impact.
4. The small-leaved linden population in Petrozavodsk had the highest FA2 and FA10 indices and was characterized by reduced developmental stability, which is presumably due to increased technogenic stress at this collection site.
5. The population of Mozhaisk with the lowest FA10 value (but not FA2) can be characterized as a population (cenopopulation) with high developmental stability due to favorable climatic and environmental conditions.

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