Evaluation of shape and asymmetry in rye leaf

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Abstract. The variability of bilateral structures is associated with the stability of development; the populations attribute which characterizes homeostasis of plant development at the epigenetic level. A violation in the functioning of regulatory proteins creates a deviation from the norm in fluctuating asymmetry, as a measure of ontogenetic noise. To test the variability of the shape and asymmetry of winter rye leaf plates depending on the dose of mineral fertilizer, we used the Generalized Procrustes analysis and the thin splines method. A small dose (N⁹₀P⁹₀K⁹₀) caused the apical growth and the fluctuating asymmetry of the leaf plate, indicating a reduced development stability of the population (p <0.0001), the “side” factor was statistically insignificant. The interaction side × individual was statistically significant at both doses of fertilizer. The bending energy of the thin spline was higher (p = 0.001) in population with an increased dose of fertilizer (N¹₂₀P¹₂₀K¹₂₀) and high directional asymmetry (“side”; p <0.0001). The authors consider that a high dose of fertilizer contributed to the synthesis of cells in the medial part of the leaf blade and caused asymmetry in a mix of fluctuating and directional asymmetry. Thus, an increased dose of mineral fertilizer enhanced the growth of the lateral part of the leaf blade and led to directional asymmetry, which reduced the asymmetric variability and increased the variability of the shape in the leaf blade.

1. Introduction

The phenotypic integration based on the modular structure of organism traits is related to the evolutionary theory of plants and animals [1, 2]. The variability of morphological characters, including bilateral structures, is of interest to morphologists and geneticists [3-5]. A disturbance in the functioning of regulatory proteins creates a deviation from the norm in fluctuating asymmetry (FA), as a measure of ontogenetic noise. Determination of differences in fluctuating asymmetry allows to indirectly creating an epigenetic landscape of population differences that reflect epigenetic disturbances during ontogenesis of a population and evolutionary phylogenesis of cultivars [6]. At the same time, the stability of development also depends on the morphofunctional unity of the parts of the body, i.e. modules, some of which are connected with the integrated structure; others are less connected with it and more independent [7]. It is assumed that morphological integration is the result of both genetic and
environmental influences. The convenience of bilateral asymmetry as a research tool is that FA is considered as the result of environmental exposure to the epigenetic mechanisms of homeostasis, while directional asymmetry (DA, the dominance one of the side), as an inherited characteristics is the result of the functioning of some genes at the genotypic level [8-10].

The FA/DA ratio remains the subject of discussion on ecological and genetic nature of character variability, including their plastic variability [5, 11]. Phenotypic integration was studied using the modular ontogenesis of a leaf plate [12-14] using various methods [15-16]. The work was mainly carried out on the structures of the wings of insects and rodents [10, 17-18]. Methods of geometric morphometrics and applied soft on the base of multidimensional processing marks coordinates and visualization of shape elements have been developed [19-21].

We applied the method of geometric morphometrics for the quantitative analysis of the plant sheet plate shape (symmetric component) and the asymmetry component – the variability of paired homologous characters, which served as paired labels placed along the margin of the plate.

Thus, the subject of the proposed study was the shape and asymmetry of winter rye leaf blade, and the goal of the work was to evaluate the components of the shape and asymmetry depending on the dose of fertilizer. An assumption was made about the effect of fertilizer on the asymmetry and shape of the leaf blade and on the relationship of these categories and developmental stability.

2. Materials and methods

2.1. Collecting of herbarium and primary treat

The studies were conducted on gray forest medium loamy soil on a flat, slightly wavy relief of the Suzdal district of the Vladimir region (56.2157° N; 40.5166° E). Two doses of mineral fertilizer nitroammophos were used: low – N90P90K90, and high – N120P120K120. The plants winter rye (Secale cereale L.) Cultivar Nemchinovka was used. This is a bushy cereal 45-50 cm tall with a specific leaf blade longitudinal venation. The measured length was 10.0 cm ± 0.42. Both populations were located on adjacent sites with standard tillage and accepted crop rotations. In the first decade of July 2019 (stage of wax ripeness) 20-25 plant specimens, 4-6 stems of each plant, the upper (flag) leaf blades were randomly selected from each of site area of 35 m². The smallest and largest plates, as well as plates with mechanical damage, were rejected. After 1-2 days, the plates were placed in a solution of household detergent for 30-40 minutes, dried in filter paper and scanned twice.

2.2. Labeling and processing of statistical data

Images of the plates were saved in JPEG format. To digitize the images, we used the Dig2 digitizer in TPS software package (Rohlf, 2017). First, the true landmarks were set, one at the base of the sheet and the second in the apical part (№ 1 and № 2). Then 48 paired semi landmarks (24 on each side) were set clockwise. All landmarks were placed in 2-fold repeats, so each sheet had 4 replicas. For regular placement of landmarks, the curve was automatically divided into equal segments: first, a curve was formed, and then 48 semi landmarks were labeled. Each file was saved in TPS format, a common file with the coordinates of 50 true landmarks was formed, which was later used in the MorphoJ.

2.3. Statistical and visual assessment

The shape and two types of asymmetry were evaluated in a Generalized Procrustes Analysis of variance (GPA). To do this, the common TPS file was used. The outliers were rejected, which allowed us to avoid a possible presence of antisymmetry in the sample, as a kind of asymmetry which biases the FA testing. Mirroring and averaging were also carried out in the MorphoJ program and a consensus shape was built. The labeling counterclockwise did not give a significant difference in GPA results. The shape was studied using the centroid value, i.e. the square root of the sum squares coordinates of the landmarks along the X- and Y axes. Classifiers “individual” and “leaf” served as an experimental unit. The databases represented Procrustes coordinates and distances within the space same name after moving, rotating, and scaling leaf blade’s shape elements. Procrustes distance is the distance between the shapes,
numerically equal to the square root of the sum of the differences between the coordinates of the landmarks after optimal alignment using the least squares method. The statistical significance of the interaction “leaf × side” and “individual × side” indicated the presence of fluctuating asymmetries in the plate, and, accordingly, a deviation in development stability [19, 22, 23]. On the databases for each of the four populations, covariance matrices were generated for both the symmetric and asymmetric components of the shape. The analysis of the main components of variation (CVA) was carried out and diagrams were built. For analysis of the shape the TPSsplin soft was used. Procrustes superimposed shape was compared with a consensus sample. The difference in the shape of leaf blades between the samples was evaluated using the values of the bending energy (Bₚ) of splines and Procrustes distances in a non-parametric analysis of variance (Friedman ANOVA). Traditional methods of variation statistics were used (α=95%) with permutation round n = 10 000.

3. Results and discussion

The GPA was carried out separately for 2 populations (doses of fertilizer). A significant difference (p<0.0001) was obtained in the shape at the population level (the classifier is an individual plant), at the level of the leaf and at the level of image (table 1).

| Source | Low dose (№1) | High dose (№2) |
|--------|--------------|----------------|
|        | % | SS | MS | df | F | % | SS | MS | df | F |
| Individual | 12.61 | 0.020 | 0.00 | 480 | 2.08¹ | 27.50 | 0.051 | 0.000 | 816 | 2.11¹ |
| side | 0.57 | 0.001 | 0.00 | 48 | 0.94α | 15.58 | 0.029 | 0.001 | 48 | 20.35¹ |
| individual × side | 6.06 | 0.010 | 0.00 | 480 | 1.63¹ | 13.01 | 0.024 | 0.000 | 816 | 5.93¹ |
| residuals | 80.77 | 0.131 | 0.00 | 10464 | - | 43.90 | 0.081 | 0.000 | 16320 | - |
| total, % | 100 | 0.163 | - | - | 100.00 | 0.185 | - | - | - |
| Leaf | 47.92 | 0.045 | 0.00 | 1392 | 2.5¹ | 27.50 | 0.051 | 0.000 | 816 | 2.11¹ |
| side | 0.54 | 0.001 | 0.00 | 48 | 0.81α | 15.58 | 0.029 | 0.001 | 48 | 20.35¹ |
| leaf × side | 19.15 | 0.018 | 0.00 | 1392 | 3.67¹ | 13.01 | 0.024 | 0.000 | 816 | 5.93¹ |
| residuals | 32.39 | 0.030 | 0.00 | 8640 | - | 43.90 | 0.081 | 0.000 | 16320 | - |
| total, % | 100 | 0.094 | - | - | 100.00 | 0.185 | - | - | - |
| Image | 48.31 | 0.045 | 0.00 | 2832 | 2.29¹ | 48.15 | 0.089 | 0.000 | 4464 | 1.34¹ |
| side | 0.54 | 0.001 | 0.00 | 48 | 1.5α | 15.58 | 0.029 | 0.001 | 48 | 40.33¹ |
| image × side | 21.10 | 0.020 | 0.00 | 2832 | 1.43¹ | 35.93 | 0.067 | 0.000 | 4464 | 208.46¹ |
| residuals | 30.06 | 0.028 | 0.00 | 5760 | - | 0.35 | 0.001 | 0.000 | 9024 | - |
| total, % | 100 | 0.094 | - | - | 100.00 | 0.186 | - | - | - |

¹p<0.0001;  
α= not significant;  
* p = 0.015

Regression the centroid size – shape did not show significance (p> 0.05), i.e. no serious allometric dependence was obtained. The high variability in bilateral asymmetry was obtained (interaction of two factors – p <0.0001). The first population (low dose of fertilizer) was not expressing DA (“side” – p> 0.05), therefore a “pure” fluctuating asymmetry was obtained at the biosystem levels “individual” and “leaf” (p <0.0001). The source of variation “image” showed a tendency directional asymmetry emergence, as the probability level of the “side” factor expressed a low statistically significant value of p = 0.015. In the second population directional asymmetry was pronounced (the “side” factor at both biosystem levels was highly statistically significant; p <0.0001). Procrustes residuals as the sum of the squared lengths of vectors between the centroid and each of the shape reached the high values, we consider as the norm for plants with a high tendency to phototaxis. With an increase df at the “image”
level the amount of residuals from total $SS$ decreased up to 30.1% in the first population and up to 0.35% in the second.

Thus, the first population was characterized by increased fluctuating asymmetry, and the second one by a mixture of both types of asymmetry. The first principal components of the asymmetry matrix showed visually also a high deviation from consensus in the first sample with high fluctuating asymmetry (figure 1):

![Figure 1](image1.png)

**Figure 1.** First principal components (LM – blue colored, consensus – not filled). A – low dose (FA); B – high dose (FA+DA).

At high FA, a high variation in the base and apical parts of the leaf blade (figure 1, A) was peculiar. At the second, increased dose (B), the dispersion of the Procrustes distances was less, with the center of the sets of landmarks in the medial part shifting to one side (in the figure 1,B – to the down). The first principal components (PC1) filled most of the variance (low dose – 60%, high dose – 45%; fi.2, A, B).

![Figure 2](image2.png)

**Figure 2.** Diagram of distribution % of variance in principal components in matrix of asymmetry: A – low dose (FA); B – high dose (FA+DA).

The second diagram (figure 2, B) shows the uniform variance distribution the first two main components. Thus, asymmetry was more pronounced in the first sample than in the second. The symmetry matrices showed opposite results: first low dose – a uniform decrease in dispersion, with a
low percentage of coverage in the first principal component (44%). At the second dose, an uneven dispersion with a high concentration in the first principal component was about 65% (figure 3).

Figure 3. Diagram of % distribution of variance in principal components in matrix of symmetry: A – low dose (FA); B – high dose (FA+DA).

Thus, the first dose contributed to a set of asymmetrical shapes, and the second dose – symmetrical ones. To test the statistical differences in the bending energy of thin splines (Be) on all landmarks, a pairwise comparison was carried out. The energy of the deformation in a thin spline prevailed at the second dose (statistically significant difference in Friedman ANOVA; \( p < 10^{-5} \); \( p = 0.001 \)). The difference in Procrustes distances and angles was also significant (\( p << 10^{-5} \)). Procrustes distances and angles were higher in the first dose at high asymmetry. The characteristics with increased (+) or reduced values (−) are displayed in the table 2:

| Characteristics                  | Low dose | High dose |
|----------------------------------|----------|-----------|
| FA                               | +        | −         |
| DA                               | −        | +         |
| PC1 asym. component              | +        | −         |
| PC1 symm. component              | −        | +         |
| B_e                              | −        | +         |
| Procrustes distance              | +        | −         |
| Angle                            | +        | −         |

Thus fluctuating asymmetry was associated with asymmetric shape, and increased Procrustes distances. Directional symmetry was associated with a low shape variation and compensated FA. We explain the high directional dispersion of labels in the two-dimensional Procruste space by enhanced mineral nutrition of plant by fluctuation growth leaf blade attributed to the high dose of fertilizer.

4. Conclusion

The results of the Generalized Procrustes Analysis were confirmed by visualization methods. The input of a high dose of N\(_{120}\)P\(_{120}\)K\(_{120}\) promoted cell synthesis in the medial part of the leaf blade, while at dose № 1, deformation of the base and apex of the plate and the presence of fluctuating asymmetry were markedly indicating a decrease in developmental stability. At the first, low dose, the asymmetric component had a high dispersion of the vector directions. Procrustes analysis at lower level of classifier showed the presence of hidden directional asymmetry, a phenomenon depending on the heterogeneity of the sample [8]. The given example does not mean a forecast of increased development stability at
higher doses of fertilizer, but only shows the variability of the leaf blade given cultivar. Individual differences in the shape were observed at all biosystem levels (p <0.0001). In general, the presence of DA in rye leaves we consider a regular phenomenon for cultivated cereals. The further studies will be carried out using various cultivars and doses of fertilizer to obtain a picture of the factors decreasing developmental stability.

References
[1] Pozdnykov A A 2019 Epigenetic theory of evolution: prior ideas, problems, and perspectives Russian ornithological journal 28 4051-77
[2] Klingenberg C P 2005 Developmental constraints, modules, and evolvability In: Variation Academic Press pp 219-47
[3] Migicovsky Z, Li M, Chitwood D H and Myles S 2018 Morphometrics reveals complex and heritable apple leaf shapes Frontiers in plant science 8(2185) 219-47
[4] Ustyuzhanina O A, Sokolova L A, Golofteeva A S and Burlutskiy V A 2017 The effect of different mineral backgrounds on the crop yield and the coefficient of fluctuating asymmetry for the winter and spring wheat Regional Environmental Issues 3 99-102
[5] Baranov S G, Zykov I E, Kuznetsova D D, Vinokurov I Y and Fedorova L V 2020 Environmental factors influencing expression of bilateral symmetrical traits IOP Conf. Ser.: Earth Environ. Sci. 421(5) 052029
[6] Vasil’ev A G, Vasil’eva I A and Shkurikhin A O 2018 Geometric morphometrics: from theory to practice (Moscow: KMK Scientific Press LTD) p 471
[7] Vasil’ev A G and Vasil’eva I A 2009 Homological variability of morphological structures and epigenetic divergence among taxa: principles of population meronomy (Moscow: KMK Scientific Press LTD) p 511
[8] Klingenberg C 2015 Analyzing fluctuating asymmetry with geometric morphometrics: concepts, methods, and applications Symmetry 7(2) 834-43
[9] Klingenberg C P and Marugán-Lobón J 2013 Evolutionary covariation in geometric morphometric data: analyzing integration, modularity, and allometry in a phylogenetic context Systematic biology 1 62(4) 591-610
[10] Klingenberg C P 2008 Morphological integration and developmental modularity Annual review of ecology, evolution, and systematics 39 115-32
[11] Baranov S, Vinokurov I and Fedorova L 2019 Environmental Factors Affecting the Expression of Bilateral-Symmetrical Traits in Plants In: Gene Expression and Phenotypic Traits IntechOpen 1-13
[12] Damián X, Fornoni J, Domínguez C A and Boege K 2018 Ontogenetic changes in the phenotypic integration and modularity of leaf functional traits Funct. Ecol. 32 234-46
[13] Melo D and Marroig G 2015 Directional selection can drive the evolution of modularity in complex traits Proc. Natl. Acad. Sci. 112(2) 470-5
[14] Koenig D and Sinha N 2010 Evolution of leaf shape: a pattern emerges In: Curr. Top. Dev. Biol. 91 169-83 Academic Press
[15] Adams D C 2016 Evaluating modularity in morphometric data: challenges with the RV coefficient and a new test measure Methods Ecol. Evol. 7(5) 565-72
[16] Klingenberg C P 2014 Studying morphological integration and modularity at multiple levels: concepts and analysis Philosophical Transactions of the Royal Society B: Biological Sciences 19 369(1649) 20130249
[17] Benítez H A, Lemic D, Bažok R, Bravi R, Buketa M and Püschel T 2014 Morphological integration and modularity in Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae) hind wings Zoologischer Anzeiger-A Journal of Comparative Zoology 253(6) 461-8
[18] Klingenberg C P 2009 Morphometric integration and modularity in configurations of landmarks: tools for evaluating a priori hypotheses Evol. Dev. 11(4) 405-21
[19] Klingenberg C P 2011 MorphoJ: an integrated software package for geometric morphometrics. *Molec. Ecol. Resources.* 11 353-7

[20] Klingenberg C P 2013 Visualizations in geometric morphometrics: how to read and how to make graphs showing shape changes. *Hystrix, the Italian J. Mammalogy* 24(1) 15-24

[21] Adams D C, Rohlf F J and Slice D E 2013 A field comes of age: geometric morphometrics in the 21st century. *Hystrix, the Italian J. of Mammalogy* 24(1) 7-14

[22] Auffray J C, Debat V and Alibert P 1999 Shape asymmetry and developmental stability. In: *On growth and form: spatio-temporal pattern formation in biology* eds M A J Chaplain, G D Singh et al. (Wiley Chichester UK) pp 309-24

[23] Klingenberg C P 2015 Analyzing fluctuating asymmetry with geometric morphometrics: concepts, methods, and applications. *Symmetry* 7(2) 834-43