Asymmetry and shape in leaf blade red clover

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Abstract. The plastic variability, shape and asymmetry of the leaf plates of meadow clover (Trifolium pratense L) depending on various doses of fertilizer and soil cultivation methods were studied. A correlation between the index of plastic variability and the value fluctuating asymmetry was obtained ($r = 0.23-0.63$; $p < 0.05$). Using the geometric morphometrics, the presence of directional asymmetry was confirmed, and a highly statistically significant variation in the shape of the sheet plate was obtained. In only one sample, a pure fluctuating asymmetry was obtained; in remain cases – a mixture of two types of asymmetry. The authors explain the effect of the declining developmental stability by a low dose of fertilizer, as well as a crop rotation scheme with a relatively low content of trace elements in the soil. A high dole of directional asymmetry verified the influence of genotypic variation on the asymmetry and shape of leaf blade and, therefore, a high dependence of phenotypic variation on the genotype.

1. Introduction
It is known that homozygosity contributes to an increase in the fluctuating asymmetry of bilateral structures and to a decrease in the developmental stability. Agricultural plants have a relatively high purity of the genotype, high homozygosity, therefore, are the convenient model for the differential study of phenotypic and genotypic properties. One of the goals of phenogenetics as a branch of epigenetics is to identify varieties with high or low developmental stability and to predict the effect of the genotype – environment interaction. Clover meadow Trifolium pratense (variety "Mars", var. praccx) is a reliable model for determining phenotypic, including plastic variability. Firstly, this herbaceous perennial plant is convenient for collecting and conducting morphometric studies. Secondly, it is a relatively genetically homogeneous species that responds to many environmental factors, including various doses of mineral fertilizers [1], geographical location [2, 3], and environmental pollution, for example, oil contamination of soil [4].

High plastic variability was demonstrated in the study of the influence of geographic extent, minerals [5, 6] and biotic factors, both on clone plants [7] and on single and double-crop varieties [8, 9]. Thus, the morphological variability of the dimensional characteristics of meadow clover varies greatly depending on the variety and cultivation methods [10, 11].
The aim of the study was to answer the next question. Does the asymmetry and shape of the clover leaf plate depend on such factors as: fertilizer dose, crop rotation stage and type of soil tillage?

2. Materials and methods
The study was conducted at 12 experimental sites. Clover sowing was carried out in autumn 2016-2017 on gray forest soil, pH = 5.3 (Suzdal district, Vladimir region, Russia). Four types of soil tillage were used. The first is conventional soil treatment tilting the soil with polishing of the ground and rising with plough to the depth of 20-22 cm. The second type is combined energy-saving, with processing at a depth of 10-12 cm. The third type is a combined longline, and the fourth is anti-erosion with deep loosening at a depth of 25-27 cm. In each type of tillage, three levels of fertilizing ammonium (NPK) were envisaged: supportive, medium, and intensive according to the adopted crop rotation scheme. Directly under clover the first year the fertilizer was not entered. The intensity levels depended on total fertilizer application for crop rotation: from (N_{230}P_{100}K_{240} – supporting, to N_{405-495}P_{245-345}K_{345-385} – high-intensity).

In May-June of the following year, the mowing was carried out, 20 clover plants were randomly taken from each plot, per three leaf plates from each plant. After photographing each leaf twice the image processing were performed using the TPS software package (Rohlf, 2019). Five metric traits were used (a-e, figure 1).

![Figure 1](image-url) Figure 1. The metric traits used to value the FA evaluation (a–e) and landmarks for geometric morphometrics (1-10), on the middle leaflet – 50 landmarks (TPSDig2).

For geometric morphometrics, the landmarks (LMs) corresponding to dimensional traits were used (1-10, figure 1) plus 50 semilandmarks on the middle leaflet. The obtained TPS files were used to find the consensus shape of the sheet plate to determine the variance of the landmarks around the superimposed coordinates. FA value was calculated by the normalizing formula: \( FA = \frac{R - L}{R + L} \), where \( R \) and \( L \) are the values of the right and left homologous traits, integrative FA mean was calculated as well. The geomorphometric alternative for FA was the same formula, where \( R \) and \( L \) were variances of coordinates \( XY \) of the right and left LMs. The measurement error after three measurements randomly selected traits did not exceed 10% of the value of those traits. Plastic variability is a phenotypic property that reflects the ability to adapt and reveals the variability of bilaterally symmetrical traits value. The magnitude of this type of variability was determined by the formula: \( PL = 1 - \left( \frac{x}{X} \right) \), where \( x \) is the minimum size of the right and left homologous bilaterally symmetric trait \( R + L \) / 2, and \( X \) is its maximum value. The shape variability was determined using the procrustes distances of thin splines in TPSsplin. For an indirect testing of normality, the \( t \)-test was used. The \( F \)-test was preliminarily carried out, and then the \( t \)-test was used for samples with the same or different variances, the level of statistical significance \( \alpha = 95\% \) was used.
3. Results and discussion

Of interest was the nature of the relationship between plastic variability and the size of the metric trait. Only the first (a) and fourth (d) metric traits indicate a weak correlation between PL and the size of the trait ($r = 0.23–0.33; p <0.05$). Analysis of the entire pool data showed a correlation with the mean strength (Pearson’s $r = 0.63$). Thus, a relationship was obtained between the two types of phenotypic variation.

Between the first and fourth doses of fertilizer, a decrease in the value of FA was observed ($p <0.05$) only in 2017. The high value of FA this year was associated with a high value of leaf plate integrative dimensional features (figure 2).

![Figure 2](image)

**Figure 2.** The value of FA depending on the dose of fertilizer (1-4).

Each crop rotation involved the use of two doses of fertilizer. A decrease in FA ($0.053 \pm 0.01$) and increased development stability was characteristic of the 4th crop rotation scheme with 3-4 doses of fertilizer in 2017 (figure 3):

![Figure 3](image)

**Figure 3.** The dependence of FA on crop rotation, in brackets is the dose of NPK.
The intensive and highly intensive method of applying fertilizers in the dump method of tillage for the fourth crop rotation IV (3,4) showed an increase in development stability. The described effect was obtained under the climatic conditions of 2017 for plants of the first year sowing. The cool summer 2017 contributed to an increase in FA (0.08 ± 0.01) of clover leaf plates against the background of anti-erosion tillage (№ 4; figure 4).

![Figure 4. Dependence of FA on 4 types of tillage (1–4, OX axis).](image)

In 2018, the maximum FA value (0.052 ± 0.01) was found on combined energy-saving processing. In 2018, second year plants there was a tendency the aligning values of FA. A clearly defined minimum was not obtained, which we explain by the removal of nutrients by herbs 1st year sowing.

4. Conclusion

2017 was colder and wetter than 2018. The average temperature in May-August 2017 was 16-18% lower, and the humidity was 2-3% higher compare to 2016 and 2018. Cool summer 2017 contributed to an increase in the FA of clover leaf plates by various kind of tillage. The highest FA value was observed in anti-erosion tillage. In 2018, the maximum FA value was found in combined energy-saving processing. When using 4 doses of fertilizer, in 3 cases the presence of DA was observed, i.e. normal distribution of signed values (R – L) from zero to the left or to the right side (p <0.05). This result was confirmed by geometric morphometrics and obtained a statistically significant deviation of coordinates in two-dimensional space from the averaged landmarks of the consensual shape with aligned coordinates (p <0.001). The bending energy of thin splines did not show a difference in the shape of sheet plates, procrustes distances and vector angle of deviation from consensus showed a statistical difference (p <0.05).

The climatic factor played a crucial role in plastic variability, which positively correlated with the FA value ($r > 0.5$). We explain the effect of reducing the development stability by a low dose of fertilizer when using the first crop rotation scheme with a relatively low trace mineral content in the soil. A high proportion of directional asymmetry confirmed to a high proportion of genotypic variability in asymmetry in the shape of the leaf clover of meadow and a high dependence of phenotypic variability on the genotype.

References

[1] Bibik T S, Schukin I M and Baranov S G 2017 Effect of mineral fertilizers on red clover developmental stability Advances in current natural sciences 3 51-5
[2] Welham C V, Turkington R and Sayre C 2002 Morphological plasticity of white clover (Trifolium repens L.) in response to spatial and temporal resource heterogeneity Oecologia 130(2) 231-8

[3] Fazlioglu F, Wan J and Bonser Sp 2018 Phenotypic plasticity and specialization along an altitudinal gradient in Trifolium repens Turkish Journal of Botany 42(4) 440-7

[4] Svetlakova T N, Mandritsa S A, Boronnikova S V and Suslonov A V 2010 Estimation of Trifolium pratense L. morphological character mobility in conditions of oil contamination of soil Problems of Contemporary Science and Practice. Vernadsky University 1(3) 16-22

[5] Caradus J R, Hay M J, Mackay A D, Thomas V J, Dunlop J, Lambert M G, Hart A L, Bosch J and Wewala S 1993 Variation within white clover (Trifolium repens L.) for phenotypic plasticity of morphological and yield related characters, induced by phosphorus supply New Phytologist 123(1) 175-84

[6] Varin S, Leveel B, Lemauviel-Lavenant S and Cliquet J B 2009 Does the white clover response to sulphur availability correspond to phenotypic or ontogenetic plasticity? Acta Oecologica 1 35(3) 452-7

[7] Gonzalez A P, Chrtek J, Dobrev P I, Dumalasova V, Fehrer J, Mraz P and Latzel V 2016 Stress, induced memory alters growth of clonal offspring of white clover (Trifolium repens). American journal of botany 103(9) 1567-74

[8] Dyckova T A, Rekashus E S, Prudnikov A D, Konova A M and Kurdakova O V 2015 Ecological plasticity and stability of the red clover samples in the Smolensk region. Int Res J 11(42) 56-60

[9] Akmanaev E D and Bogatyreva A S 2016 Influence of abiotic conditions on the yield of single and double-crop clover meadow in middle preduralie Perm Agrarian Journal 17 12-8

[10] Ergon A and Bakken A K 2016 Red clover traits under selection in mixtures with grasses versus pure stands Proc of the 26th General Meeting of the European Grassland Federation (Trondheim: NIBIO) pp 811–3

[11] Abakumova M, Zobel K, Lepik A and Semchenko M 2016 Plasticity in plant functional traits is shaped by variability in neighborhood species composition. New Phytologist 211(2) 455-63