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Phenogenetic Monitoring of the Weeping Birch (*Betula pendula* Roth.) in the Middle Urals: Testing a New Method for Assessing Developmental Instability in Higher Plants

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Abstract—Variation of bilateral leaf structures in Middle Ural cenopopulations of the weeping birch (*Betula pendula* Roth.) growing in gradients of industrial pollution was studied to test the efficiency of a new method of population and individual phenogenetic monitoring allowing an indirect assessment of developmental destabilization by segregating the variance of total asymmetry (TA_R^2) into its additive components, the variances of fluctuating asymmetry (FA_R^2) and directional asymmetry (DA_R^2). The method was tested in the impact zones of two copper-smelting plants in the Middle Urals. The degree of impact was characterized by the index of technogenic pollution (*ITP*) reflecting the average total contents of 15 water-soluble pollutants in snow samples. The level of asymmetry was estimated from the numbers of denticles with incoming veins (dentovenal elements) on the left and right leaf margins. Spearman's coefficient of rank correlation between the group values of variance in fluctuating asymmetry FA_R^2 and corresponding *ITP* values reached $r_S = 0.914$ ($p < 0.001$), providing evidence that the method is highly appropriate for ecological indication of the phenogenetic response of trees to environmental pollution.

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Key words: fluctuating asymmetry, developmental stability, monitoring, birch.

In recent years, increasing attention has been devoted to the problem of rapid assessment of environmental quality by methods based on responses of the main biotic components of ecosystems to the impact of industrial pollutants, since these methods make it possible to determine tolerance limits for different species and to develop valid ecological norms for industrial pollution of the environment (Vorobeichik et al., 1992; Vorobeichik, 2004). Specialists in this field widely employ the method of indirect assessment of environmental quality based on indices of developmental stability such as manifestations of fluctuating asymmetry (FA) in bilateral characters (Zakharov, 1982, 1987; Palmer and Strobeck, 1986; Parsons, 1992; Zakharov et al., 1993; Kozlov et. al., 1996; Graham et al., 1998; Valkama and Kozlov, 2001; etc.). In parallel, statistical methods of FA assessment are being developed and improved (Zakharov, 1992; Palmer, 1994; Möller and Swaddle, 1997; Leung et. al., 2000; Gelashvili et al., 2004; Freeman et al., 2005; etc.). However, there are still numerous problems and inconsistencies providing fuel for continuous debates on the phenomenon of *FA* and its interpretation (Palmer, 1996; Möller and Swaddle, 1997; Debat and David, 2001; etc.).

Today, 15 indices for FA assessment are known, with 13 of them being listed in the paper by Palmer (1994). It should be noted that, as a rule, specialists use group indices characterizing both individual characters and their combination within the sample as a whole. Gelashvili et al. (2004) proposed an original nonlinear method of FA assessment in which normalization is performed simultaneously with the convolution procedure. The indices of FA that allow estimation of developmental stability at the individual level have not been used until recently, although one such index is well known. This is the index of overall nonmetric fluctuating asymmetry (FAnm) (Zakharov, 1987; Markowski, 1995) calculated as the frequency of nonmetric or meristic bilateral characters asymmetrically manifested in an individual; however, its value is then averaged for the whole sample (Zakharov and Clarke, 1993). A major task of segregating the components of directional and fluctuating asymmetries from total asymmetry is accomplished by means of mixed two-way ANOVA (Palmer and Strobeck, 1986; Palmer, 1994). The assessment of FA is usually performed with bilateral metric characters pertaining mainly to changes in the shape of biological objects, while structural changes are often ignored; in other words, attention is devoted to size and shape rather than to structure. Hence, it is important to estimate whether the indices of FA in not only metric but also in structural characters are suitable for the purposes of biomonitoring.

Birch species of the section Albae Regel. are often used as model objects in studies on FA (Kryazheva et al., 1996; Kozlov et al., 1996; Valkama and Kozlov, 2001; etc.). Hence, our study in the Middle Urals was also performed with the weeping birch (*Betula pendula* Roth.) growing in areas exposed to emissions from two large copper-smelting works, the Kirovgrad Copper-Smelting Plant (KCSP) and the Middle Ural Copper-Smelting Plant (MUCSP), and also in a relatively clean area of the Visim State Biosphere Reserve. We analyzed manifestations of bilateral structural disturbances in leaf venation at the levels of individual trees and of samples from areas with different degrees of industrial pollution.

The purpose of this study was to perform phenogenetic monitoring of the weeping birch in both protected and technogenically disturbed areas in order to develop and test the method for assessing the variances of directional and fluctuating asymmetries (and their ratio) as individual components of the variance of total asymmetry in the pattern of leaf veins and denticles.

MATERIAL AND METHODS

The leaves of *B. pendula* were collected from brachyblasts located at a height of up to 2 m, ten leaves per tree. In July and August 2002, leaf samples were taken in three sites located at different distances from the MUCSP and designated MUCSP-impact (0.5 km), MUCSP-buffer (3 km), and MUCSP-control (30 km). In July 2003, the leaves were collected near Makarovskoe Lake, a source of potable water for Yekaterinburg (they were used as an additional control sample). In the zone exposed to emissions from the KCSP and other industries operating in Kirovgrad, leaf samples were taken in July 2004 (during one week) in 13 sites located along the highway running from Kirovgrad westward, to the Visim Reserve. These sites were designated KCSP, with numerical indices showing the distance from the plant: (1) KCSP-40 (i.e., 40 km from the KCSP); (2) KCSP-35A, on the northern side of the highway; (3) KCSP-35B, 150 m from the previous site, on the southern side of the highway; (4) KCSP-30; (5) KCSP-25; (6) KCSP-20; (7) KCSP-15, near a high-voltage power line; (8) KCSP-8; (9) KCSP-5; (10) KCSP-4; (11) KCSP-3; (12) KCSP-2; and (13) KCSP-1. In the same period (July 2004), birch leaves were also collected in five sites within the area adjoining the KCSP from the northeast. These samples were designated as follows: (14) KCSP-0, in the immediate vicinity of the plant; (15) KCSP-01, 1 km; (16) KCSP-02, 2 km; (17) KCSP-03, 3 km; and (18) KCSP-04, 4 km from the plant. On the wholeÇ we studied 1469 leaves from 151 1
B. pendula trees (damaged leaves were excluded from analysis).

Data on pollution in the vicinity of the MUCSP were taken from relevant publications (Vorobeichik et al., 1992; Vorobeichik, 2004). To estimate the relative pollution level in the zone exposed to emissions from the KCSP, snow samples from sites 1–18 were taken in March 2003 and analyzed for the gross contents of 15 major technogenic pollutants, both soluble and insoluble (Al, Cd, Sr, Zn, SO_4^{2-}, F^-, As, Na, Ni, Pb, K, Ca, Mg, Mn, and Cu). The analysis was kindly performed by specialists of the chemical analytical laboratory of the Ural Electrochemical Integrated Plant (Novouralsk). Using data on the contents of individual movable ions, the index of technogenic pollution (*ITP*) was calculated as the average total content (mg/l) of all soluble pollutants deposited over winter months and accumulated in the snow.

An analysis of variation in leaf venation was performed with regard to the results of previous studies (Korona and Vasil'ev, 2000). According to the terminology used by these authors, marginal leaf denticles with incoming veins are named **d**ento-venal **e**lements (DVELs). In our case, the pattern of DVELs in a birch leaf is formed by the midrib and the first-, second-, and third-order leaf veins ending in corresponding denticles on the leaf margin. Leaf segments delimited by first-order veins extending from the midrib (below, referred to as intervenal segments) were numbered beginning from the lower leaf margin. In each of the first four (basal) intervenal segments, we calculated the number of DVELs on the left and right sides of the leaf blade. Thus, asymmetry was assessed with respect to five structural meristic characters: the total number of first-order leaf veins (intervenal segments) and the numbers of DVELs in each of the four basal intervenal segments (Fig. 1).

Second- and third-order DVELs were first calculated on the lower leaf margin, from the midrib to the tip of the first first-order vein, and then in the intervals between the first and second, the second and third, and the third and fourth first-order veins (denticles at the tips of first-order veins were not taken into account). The leaf shown in Fig. 1 has six first-order veins (intervenal segments) on the left and seven veins (segments) on the right; in the first segment, there are 8 DVELs on the left and ten DVELs on the right; in the second segment, five and three DVELs, respectively. Note that DVELs in the same segment may belong to different veins of the same order: in the second segment on the left, for example, one of the five DVELs belongs to the first vein (of the first order), while the remaining four belong to the second first-order vein. Differences between the values of the five test characters on the left and right leaf sides were used to calculate "individual" variances of asymmetry for each leaf as a whole (the R-method) or variances of individual characters (the C-method).

To solve the problem of assessing asymmetry in the pattern of DVELs, we considered it expedient to use Penrose's size and shape measures recommended by Sneath and Sokal (1973) for taxonomic purposes. In this way, it is possible to calculate the variance of total asymmetry (TA^2) and the variances of its two components, directional asymmetry (DA^2) and fluctuating asymmetry (FA^2). Having designated the numbers of leaf segments and DVELs on the left and right sides as *s* (sinister) and *d* (dexter), we calculated the variance of total asymmetry for each leaf by the formula

$$TA^2 = \frac{1}{r}\sum_{i=1}^{r}(s_i - d_i)^2,$$

where r is the number of test characters (in our case, five) in calculations by the R-method or the number of leaves in the sample if the C-method is used. This formula corresponds to that for Palmer and Strobeck's FA5 index (Palmer and Strobeck, 1986; Palmer, 1994). Considering the advantages and drawbacks of this index, these authors note that, theoretically, its value should shift if there is directional asymmetry or antisymmetry. However, it allows us to differentiate contributions of directional and fluctuating asymmetries.

The variance of directional asymmetry, the first component of the total variance of asymmetry in the pattern of DVELs, was calculated by the formula

$$DA^2 = \frac{1}{r^2}\left[\sum_{i=1}^{r}(s_i - d_i)\right]^2.$$

Since $TA^2 = DA^2 + FA^2$ (Sneath and Sokal, 1973), the variance of fluctuating asymmetry (the second component) was determined as $FA^2 = TA^2 - DA^2$. The same formulas can be used to obtain group average estimates of TA^2, DA^2 and FA^2 in individual characters by the C-method. We used both methods, since they complement each other and provide more complete information. The values of TA^2 obtained by both methods should coincide, and differences in DA^2 or FA^2 values are indicative of unequal correlation of values in cells of the matrix containing the data on "leaves" arranged in lines and the data on "characters" arranged in columns. Below, designations of variances have indices indicating the method of calculations: e.g., FA^2_R and FA^2_C for the variances of fluctuating asymmetry calculated by R- and C-methods.

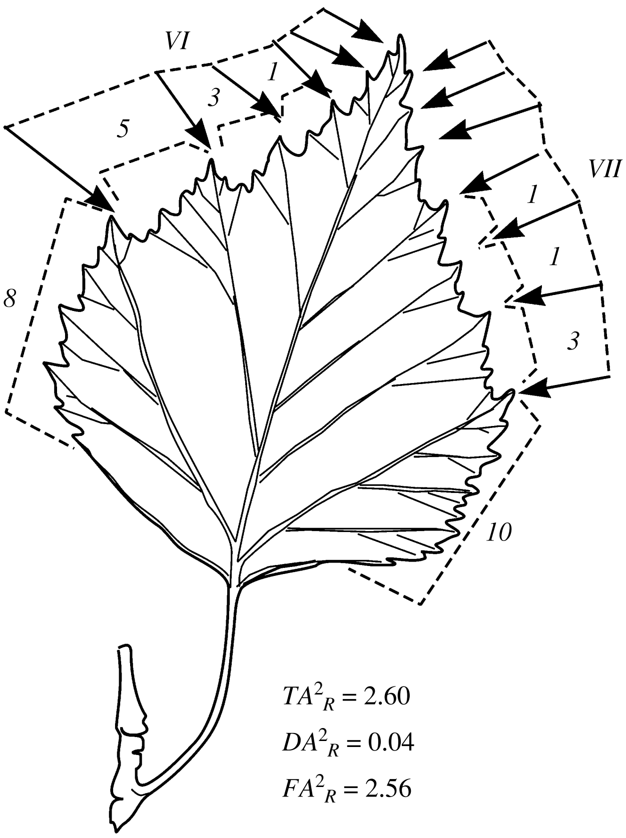

Fig. 1. The pattern of DVELs in a *B. pendula* leaf sampled 3 km from the KCSP and an example of calculating the individual variance of asymmetry in their manifestation in the first four antimeric leaf segments and in all leaf segments. *Arrows* indicate antimeric leaf segments (beginning from the lower leaf margin), *broken lines* delimit intervals in which second- and third-order DVELs were counted in each segment, *Arabic numerals* show the numbers of these DVELs in the first four leaf segments, and *Roman numerals* show the numbers of segments delimited by first-order veins on each side of the leaf. TA^2_R, DA^2_R, and FA^2_R are "individual" variances of total, directional, and fluctuating asymmetry in the pattern of DVELs, respectively.

The presence of antisymmetry in certain bilateral characters is traditionally revealed by means of ANOVA (see Palmer, 1994), but we propose a simpler method based on the use of Pearson's correlation coefficient (r) in the formula $An = (1 - r)/2$. A negative coefficient of correlation between character values on different sides is evidence for possible antisymmetry. At $r = 0, An = 0.5$; therefore, the necessity of estimating the effect of antisymmetry and its significance arises at $An > 0.5$.

Table 1. Comparison of the variances of total (TA^2), directional (DA^2), and fluctuating (FA^2) asymmetry in the numbers of intervenal leaf segments and of DVELs in four basal segments of *B. pendula* leaves collected in the gradient of technogenic pollution from the MUCSP as calculated for the leaf as a whole (R-method) and by individual characters (C-method)

Variance of asymmetry	MUCSP-control		MUCSP-buffer		MUCSP-impact	
	leaf	character	leaf	character	leaf	character
TA^2	0.407	0.407	0.759	0.759	1.219	1.219
DA^2	0.081	0.004	0.179	0.008	0.274	0.005
FA^2	0.326	0.403	0.580	0.751	0.945	1.214
Leaf length, mm	51.52 ± 0.82 (n = 140)		43.28 ± 0.74 (n = 82)		37.88 ± 0.52 (n = 140)	

RESULTS AND DISCUSSION

Variation in the DVEL pattern of *B. pendula* leaves was initially studied in the zone exposed to emissions from the MUCSP. We first compared estimates made by the R-method for individual leaves, which were then averaged for each tree and sampling site), and by the C-method for individual characters, which were also averaged in the same way. As follows from Table 1, the variances of total asymmetry (TA^2) calculated by the two methods did tot differ, but the variance of directional asymmetry (DA^2) in individual characters (columns) could be one or two orders of magnitude lower than that calculated for leaves (lines). The group variance of fluctuating asymmetry (FA^2) calculated by individual leaves was also slightly lower. In our calculations, *An* never approached 0.5, varying from 0.08 to 0.31; therefore, antisymmetry could have no effect on the results.

The comparison of FA parameters in the gradient of pollution in the zone MUCSP (Table 1) showed that asymmetry of the DVEL pattern was lower in the control sample and markedly increased in the impact sample. In the case of FA_R^2, the corresponding values differed by a factor of three, with the value for the buffer sample being intermediate. FA_R^2 in the sample MUCSP-control was almost the same as in the additional control sample from the vicinity of Makarovskoe Lake (0.326 and 0.387, respectively). In general, estimated FA values were in agreement with published data on the levels of technogenic pollution in areas around the MUCSP (Vorobeichik et al., 1992; Vorobeichik, 2004). The variance of directional asymmetry calculated for the whole leaves (DA_R^2) also depended on the level of environmental pollution, being lower in the control group and increasing in the impact group. Its value provides additional information on coordination of DVEL morphogenesis on different sides of the leaf, and this result shows that this coordination in the impact site is disturbed to a greater extent than in the control. In calculations by the traditional method (by individual characters), the variance of fluctuating asymmetry FA_C^2 was almost equal to TA_C^2 and the variances of directional asymmetry DA_C^2 in different MUCSP samples were similar. Hence, we subsequently relied on more informative estimates made by the R-method.

After this preliminary adjustment of the method, we compared FA_R^2 in samples from the sites located between the Visim Reserve and the KCSP (Table 2). Near the reserve (sites 1–5), its values ranged from 0.309 to 0.387, being similar to those in two reference control sites (MUCSP-control and Makarovskoe Lake). In the impact zone of the KCSP (sites 13–18), FA_R^2 values were significantly higher: they ranged from 0.614 to 0.898, with the highest value approaching that in the MUCSP-impact sample. Sites 6–12 formed a kind of buffer zone between the reserve and KCSP with intermediate FA_R^2 values (0.400–0.614) comparable to that in the MUCSP-buffer sample.

We did not reveal any significant correlation of TA_R^2 and FA_R^2 with leaf size. In different samples, Spearman's rank correlation coefficient varied from –0.19 to 0.14. The rank correlation between DA_R^2 and leaf blade length proved to be significant in only one case, in the Visim-5 sample: $r_S = -0.27$ ($p = 0.027$). However, there was no such correlation in the pooled sample: $r_S = 0.03$ ($p = 0.441$).

In comparisons between sites 1–18, the Kruskal–Wallis test revealed significant differences between them in the average FA_R^2 values for individual trees ($Hc = 224.4$; $df = 17$, $p < 0.001$; $N = 151$ trees) and for leaf samples considered regardless of source trees ($Hc = 2156$; $df = 17$, $p < 0.001$; $N = 1469$ leaves). The Leven test revealed significant heterogeneity of

Table 2. Variances of total (TA^2), directional (DA^2), and fluctuating (FA^2) asymmetry in leaf characters and the lengths of *B. pendula* leaves from sites with different indices of technogenic pollution (*ITP*) in the KCSP zone

Site	*ITP*, mg/l	Number of leaves (trees)	Leaf length, mm	Average individual variance		
				TA_R^2	DA_R^2	FA_R^2
1. KCSP-40	5.89	83 (9)	42	0.436	0.095	0.341
2. KCSP-35A	2.05	70 (8)	43	0.431	0.091	0.340
3. KCSP-35B	2.05	48 (10)	48	0.446	0.094	0.352
4. KCSP-30	2.68	120 (9)	42	0.412	0.103	0.309
5. KCSP-25	2.40	66 (9)	45	0.509	0.122	0.387
6. KCSP-20	2.82	87 (9)	42	0.499	0.126	0.373
7. KCSP-15	2.69	46 (6)	46	0.617	0.217	0.400
8. KCSP-8	8.20	86 (9)	50	0.600	0.104	0.496
9. KCSP-5	5.01	58 (7)	43	0.610	0.134	0.476
10. KCSP-4	6.62	98 (9)	50	0.600	0.136	0.464
11. KCSP-3	5.39	82 (8)	50	0.590	0.123	0.467
12. KCSP-2	6.76	101 (10)	49	0.651	0.141	0.510
13. KCSP-1	11.39	50 (7)	44	0.820	0.206	0.614
14. KCSP-0	14.78	147 (10)	47	0.838	0.182	0.656
15. KCSP-01	13.15	72 (7)	39	0.844	0.199	0.646
16. KCSP-02	19.90	94 (6)	47	0.977	0.260	0.717
17. KCSP-03	21.77	96 (10)	52	1.119	0.221	0.898
18. KCSP-04	14.76	65 (7)	45	0.834	0.166	0.668

sample variances FA_R^2 in the former variant ($F = 5.87$; $df_1 = 17$, $df_2 = 133$; $p < 0.001$) as well as in the latter variant ($F = 10.65$; $df_1 = 17$, $df_2 = 1451$; $p < 0.001$). The degree of this heterogeneity showed a positive correlation with the group average FA_R^2 values: $r_S = 0.56$, $p = 0.015$ in the former variant and $r = 0.73$, $p < 0.001$ in the latter variant. In other words, not only instability in DVEL pattern formation but also the diversity of morphogenetic reactions of birch leaves and trees increase along the gradient of technogenic pollution.

The recorded FA_R^2 values agreed well with indices of technogenic pollution (*ITP*) for the same sites (Table 2). Changes in FA_R^2 and *ITP* had almost the same trend. Spearman's coefficient of rank correlation between these parameters for 18 sites was $r_S = 0.914$ ($p \ll 0.001$). As the total asymmetry increased, both its components also increased almost proportionally to it, but this proportionality was disturbed in some cases. A noteworthy example is a sharp increase in DA_R^2 value in site 6, compared to those in neighboring sites, which could be attributed to the influence of high-voltage power line on leaf morphogenesis.

The samples arranged in order of increasing FA_R^2 values form an ascending series closely reflecting the levels of technogenic environmental pollution (Fig. 2).

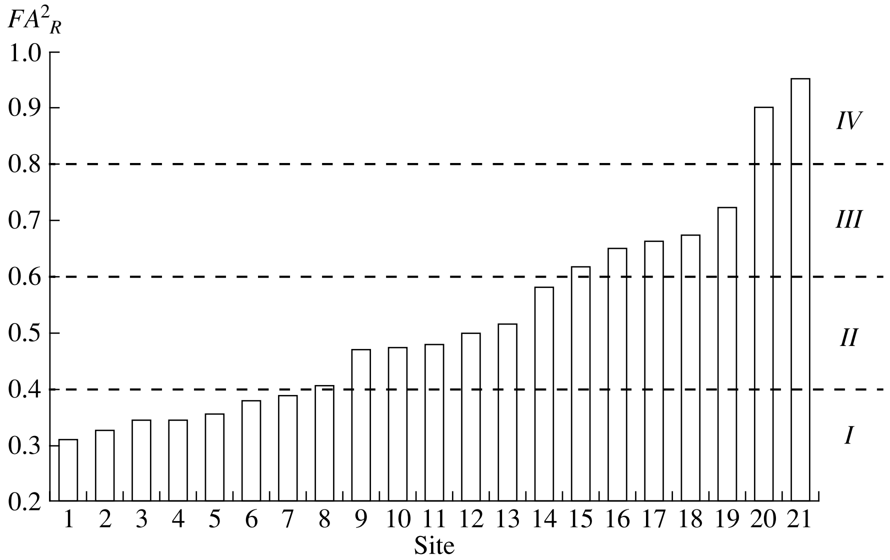

Fig. 2. Samples of *Betula pendula* leaves from the Visim Reserve and the zones of KCSP and MUCSP ranked with respect to the level of fluctuating asymmetry (FA^2_R) in the pattern of DVELs. Sampling sites: (*1*) KCSP-4, (*2*) MUCSP-control, (*3*) KCSP-35A, (*4*) KCSP-40, (*5*) KCSP-35B, (*6*) KCSP-20, (*7*) KCSP-25, (*8*) KCSP-15, (*9*) KCSP-4, (*10*) KCSP-3, (*11*) KCSP-5, (*12*) KCSP-8, (*13*) KCSP-2, (*14*) MUCSP-buffer, (*15*) KCSP-1, (*16*) KCSP-01, (*17*) KCSP-0, (*18*) KCSP-04, (*19*) KCSP-02, (*20*) KCSP-03, and (*21*) MUCSP-impact. Broken lines show FA^2_R levels empirically estimated for birch in (I) control, (II) buffer, and (III–IV) impact areas in the Middle Urals.

The series begins with control samples and samples from the Visim Reserve and its immediate surroundings. The rightmost position is occupied by the MUCSP-impact sample; next follows the KCSP-03 sample from the most polluted site near this plant, with other KCSP samples being located farther left. Closer to the middle is the MUCSP-buffer sample, which neighbors the samples taken midway between the reserve and the KCSP.

CONCLUSIONS

The advantage of the new approach is not only in segregation of the variances of fluctuating and directional asymmetries but also in the possibility of obtaining individual estimates of these components (in this case, for individual leaves as plant metameres). Obviously, the scope of its applications is not limited to plant leaves: this method can be used for analyzing any meristic or metric characters of plants and animals.

This method of phenogenetic monitoring is no more labor-intensive than the method based on linear and angular measurements of birch leaves (Kryazheva et al., 1996; Kozlov et al., 1996; et al.). Moreover, it has proved reliable in reflecting the overall level of technogenic pollution, which results in significant disturbances of leaf structure formation in trees exposed to emissions from the two largest copper-smelting plants in the Urals. In the impact zone of the MUCSP and the most polluted sites near the KCSP, the variance of directional asymmetry in the DVEL pattern of leaves (DA^2_R) increases (while DA^2_C remains almost unchanged); i.e., coordination of DVEL development within each half of the leaf increases, but their development on different leaf sides becomes less coordinated, as follows from increasing variances of fluctuating asymmetry (FA^2_R and FA^2_C). Differences are also observed in growth responses. Unlike in the MUCSP zone, the growth of leaves near the KCSP is not inhibited. A probable explanation to this fact is that the Visim Reserve is located at higher elevation than Kirovgrad, which accounts for differences in birch phenology, including the period of growth and life span of leaves: in sites 1–6, birch leaves develop later and fall earlier than near the KCSP and do not reach a large size. It would be interesting to estimate the contributions of phenological and ontogenetic components to the dimensional and structural variation of leaves, with reference to the technogenic component. The variance of fluctuating asymmetry in the DVEL pattern of *B. pendula* leaves may be used as a basis for developing a scale indicating the level of developmental instability in an individual tree or a group of trees (see Fig. 2): an excess over the critical value $FA^2_R = 0.40$ indicates unfavorable conditions for tree morphogenesis, and

FA_R^2 above 0.70–0.80 is evidence that the tree grows in the zone of heavy technogenic impact.

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