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**Can the use of landmarks improve the suitability of fluctuating asymmetry in plant leaves as an indicator of stress?**

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## Abstract

Fluctuating asymmetry (FA), defined as the random deviations from symmetry in morphological traits, has repeatedly been suggested as a handy indicator of genetic and environmental stress experienced by organisms during their development. However, the value of FA for applied ecological and environmental research is now questioned by a growing number of studies that have found no increases in FA under stress. We used geometric morphometrics to test the hypothesis that FA measures based on naturally defined landmarks are better suited for detection of deviations from leaf symmetry than are traditional measures of FA. We compared two sets of mountain birch (*Betula pubescens* ssp. *czerepanovii*) leaves, obtained from trees growing in industrially polluted natural populations and from experimentally stressed seedlings, by examining three measures of FA: 1) a traditional distance-based measure calculated from the width of the left and right halves of a leaf at the middle of the lamina (FA<sub>width</sub>), 2) a distance-based measure reflecting differences in vein length calculated from three landmarks (FA<sub>veins</sub>), and 3) a multivariate FA measure describing asymmetry in leaf shape based on five landmarks (FA<sub>shape</sub>). These three FA measures were poorly (albeit positively) correlated with each other. The two distance-based measures reflected different parts of the multivariate measure FA<sub>shape</sub>, which had the highest sensitivity for detection of deviations from leaf symmetry. FA<sub>shape</sub> was also the only FA measure that weakly confirmed the expected increase in FA under experimental stress, and particularly under stress caused by application of heavy metals. However, very high levels of within-individual variation in asymmetry remain to be explained, and even FA<sub>shape</sub> did not show the expected effects of industrial pollution on leaf symmetry. We conclude that FA in birch leaves cannot be regarded as a reliable indicator of environmental stress, even when quantified using modern methods.

**Keywords:** Geometric morphometrics, *Betula pubescens* ssp. *czerepanovii*, stress, pollution, research methodology, drought, heavy metals, within-individual variation

## 1. Introduction

Fluctuating asymmetry (FA) is defined as the random, generally subtle, deviations from symmetry in symmetric traits (Palmer & Strobeck 1986). When symmetry is a normal result of ontogeny, deviations from symmetry can reflect genetic or environmental disturbances of development (Palmer & Strobeck 1986, Freeman et al. 1993). For this reason, FA is commonly interpreted as a result of developmental instability (Møller & Shykoff 1999), and

this has led to the suggestion that FA could represent a universal indicator of stress (Leary & Allendorf 1989, Freeman et al. 1993, Zakharov et al. 2000). However, at least in plants, this application of FA is strongly questioned by a large number of field studies (reviewed by Graham et al. 2010 and Kozlov 2017) and controlled experiments (Roy & Stanton 1999, Andalo et al. 2000, Sandner & Matthies 2017, Zverev et al. 2018) that have failed to detect the expected effects of stress on FA. Nevertheless, scientific questions remain regarding how environmental and genetic factors influence FA (Graham et al. 2010, Klingenberg 2015).

Plants seem to be ideal subjects for studying FA, as they generally consist of multiple repeating modules (e.g., leaves) that allow replicated measurements within the same individual (Freeman et al. 1993). However, the shape and symmetry of plant modules (and of leaves, in particular) also show large phenotypic plasticity, and this can reduce their suitability for use in measurements of developmental instability (Palmer & Strobeck 2003, Tucić et al. 2017). In addition, environmental effects on FA can be masked by measurement error (ME), which has properties similar to FA, as it can also lead to random deviations from symmetry in either direction (Palmer & Strobeck 1986). In leaf measurements, ME accounts for around 25% of variation in the measured values of the widths, areas and weights of leaf halves (Kozlov et al. 2017a). Therefore, any increases in FA due to environmental stress could be obscured by either phenotypic plasticity or ME.

The suitability of FA for the detection of the effects of stress can also be influenced by the method used to quantify FA, and the conclusions of the study may vary depending on the choice of the measured trait (Llorens et al. 2002, Ivanov et al. 2015). We hypothesize that developmental instability may be most clearly reflected by measurements that are based on naturally defined landmarks (i.e. developmentally homologous points of the studied symmetric organ: Rohlf & Marcus 1993, Graham et al. 2010). Examples include the intersections of the veins of fly wings (Klingenberg & McIntyre 1998), the morphological structures on the mite exoskeleton (Radwan 2003), the tips of opposing lobes of some leaves (Black-Samuelsson & Andersson 2003, Sinclair & Hoffmann 2003, Cuevas-Reyes et al. 2011) or the tips of opposing shoots or leaves in plants with regular branching (Kozlov et al. 2001, Sandner & Matthies 2017). In contrast, the most frequently used measure of FA in plant leaves is based on the distances from the midrib of a leaf to the leaf margin at some arbitrary position, like the middle of the lamina (Black-Samuelsson & Andersson 2003, Kozlov et al. 2017a) or the widest point of the lamina (Hochwender & Fritz 1999, Sandner & Matthies 2017). When the symmetric growth of a leaf is disturbed, two opposing veins may differ in

length; however, only part of this asymmetry will be reflected as a difference in the distances to the margins from the middle of the leaf, whereas another part will result in shifts of the two homologous points (= tips of the veins) along the leaf margins. FA based on naturally defined landmarks thus can be better interpreted from biological points of view (Rohlf & Marcus 1993) and may be better suited to detect environmentally induced deviations from perfect symmetry than FA based on differences at an arbitrarily defined point of a leaf.

Birches (*Betula* spp.) are widely distributed trees in the temperate zone that can persist in polluted sites, and their leaves are frequently used in studies of environmental impacts on FA. However, birch leaves have serrate margins, which contribute a high ME in traditional measures of FA based on the widths of left and right leaf halves (Kozlov et al. 2017a). FA in birches has also been quantified based on the number of teeth (Vasil'ev et al. 2008) and on the lengths of veins and angles between veins (Kryazheva et al. 1996), but no single study has yet compared distance- and landmark-based methods. In the present study, we obtained landmark-based measures of FA from scanned images of birch leaves collected during a field study on the effects of long-lasting, severe industrial pollution and from an experiment addressing the impacts of heavy metals and drought on birch performance. In both data sets, distance-based measures failed to detect the expected increase in FA (Kozlov and Zverev 2018, Zverev et al. 2018). The current study addressed the following questions: (1) Are landmark- and distance-based measures of FA correlated? (2) Are landmark-based FA measures, in contrast to distance-based FA measures, increased by environmental pollution or experimental stress?

## **2. Methods**

### *2.1. Study species*

Mountain birch (*Betula pubescens* ssp. *czerepanovii* (Orlova) Hämet-Ahti) is a thin-leaved deciduous tree-line species and one of the main forest-forming trees in subarctic Europe. This birch is the only woody plant that is still relatively abundant in the extremely contaminated habitats surrounding the smelters at Monchegorsk and Nickel, where it accounts for up to 99% of the total foliar biomass in the most disturbed sites (Kozlov et al. 2009). The leaves of mountain birch are simple, generally 3 to 5 cm long and have a characteristically oval leaf base; the edges of the leaves are serrated or saw-toothed (Fig. 1).

Leaf FA of different birch species was reported to increase in industrially polluted areas (Kozlov et al. 1996, Ivanov et al. 2015) and in the years with low early summer temperatures (Valkama & Kozlov 2001, Kozlov & Zverev 2018), as well as with previous-year herbivory (Hagen et al. 2004, Kozlov & Zverev 2018), altitude of the study site and with birch hybridisation (Wilsey et al. 1998). By contrast, other studies have indicated no change in birch FA along strong pollution gradients (Kozlov et al. 2009, Koroteeva et al. 2015), after heavy grazing (Berteaux et al. 2007) or in response to heavy metals, drought (Zverev et al. 2018) and nutrient stress (Black-Samuelsson & Andersson 2003), and no association of birch FA with either leaf growth rate (Kozlov 2003) or the date of leaf fall (Kozlov 2004).

## 2.2. Field sampling

The first set of measured leaves was collected from eight sites located at different distances from the nickel–copper smelter at the town of Monchegorsk (67°56' N, 32°49' E) in the north-west of Russia, next to Finland and Norway, to the north of the Polar Circle. This smelter was one of the largest polluters in the Northern hemisphere for decades. The smelter began production in 1937–1938 and had no air-cleaning facilities until 1968. The annual emissions of sulphur dioxide reached a maximum of 278,000 metric tons (t) in 1983, steadily declined to about 100,000 t by the mid-1990s, dropped to 45,000 t in 1999 and have remained at about this level since then. Metal emissions during the 1980s–1990s were 3000–8000 t of nickel and 1000–6000 t of copper annually and then declined in concert with declines in sulphur dioxide. For the history of pollution impacts on the study region and the levels of environmental degradation consult Kozlov and Barcan (2000) and Kozlov et al. (2009).

For the present study, we used leaves that were collected in the last days of June 2016 from four unpolluted sites located 35 km S, 40 km S, 49 km SE and 64 km SE from the Monchegorsk smelter in pristine Norway spruce (*Picea abies* (L.) Karst.) forests (Fig. S1) and from four severely deteriorated sites (industrial barrens; Fig. S2) located 3 km N, 1 km NE, 6 km S and 8 km S from the same smelter (for locality data consult Kozlov et al. 2017b). We collected the largest leaf from each of 10 haphazardly selected short shoots across the entire canopy of five low-stature mature birch trees, which were tagged in each site in 1993 (Kozlov & Zverev 2018), resulting in a total of 395 leaves (8 sites × 5 trees × 10 leaves, for one tree only 5 leaves). The largest leaf was sampled as a way of standardization to reduce variation and to reduce a potential bias of unconsciously selecting the most (or least) symmetric leaves of a tree. The leaves used in the present study represent a subset of leaves collected during a

long-term (1993–2017) monitoring program that involves a total of 21 study sites (Kozlov & Zverev 2018).

Concentrations of the main metal pollutants (nickel and copper) are 15–25 times higher in birch foliage from industrial barrens than from unpolluted sites (Kozlov 2005). Birches persisting in barren sites represent 5–15% of the original population, so these individuals have survived extreme pollution impact for decades. Nevertheless, they continue to decline, despite a substantial decrease in aerial emissions from the smelter (Zverev 2009). No natural revegetation has yet been observed in our barren sites.

### *2.3. Greenhouse experiment*

The second set of measured leaves was collected from six-month-old seedlings of mountain birch grown in a phytotron at the University of Tromsø, Norway. The seeds for this experiment were collected in October 2002 from five mother trees in each of 18 study sites around the Kola Peninsula (for locality data consult Eränen et al. 2009). Eight of these sites were located in heavily polluted areas near the Monchegorsk and Nikel smelters, and ten sites were located in pristine (unpolluted) habitats. Seeds were germinated in March 2003, and the experiment was initiated on 13 May 2003. Five days a week, the seedlings in the control and heavy metal stress treatments were watered with 30 mL of tap water, whereas seedlings in the drought stress treatment received 15 mL. The water used to irrigate the seedlings in the heavy metal treatment included added nickel and copper sulphates to give concentrations of 5 mg L<sup>-1</sup> copper and 10 mg L<sup>-1</sup> nickel. On the last 2 days of every week, all plants were irrigated with clean tap water in the same quantities as mentioned above. The stress treatments strongly affected plant performance, as seedlings exposed to heavy metals and drought were smaller in height and leaf size and their leaf photosynthetic efficiency was reduced compared to control seedlings (for more details, consult Eränen et al. 2009). Due to low germination in one progeny, the total number of mother trees used in the experiment was 79, with 711 seedlings in total, i.e. three seedlings from each mother tree per treatment. The leaves used for this study represent a random subset of 150 leaves chosen from 1331 leaves collected on 8–9 August 2003 from the top of each seedling. Whenever possible, we sampled two leaves per plant; however, some seedlings had died prior to sampling, or had only a single green leaf remaining by the time of sampling..

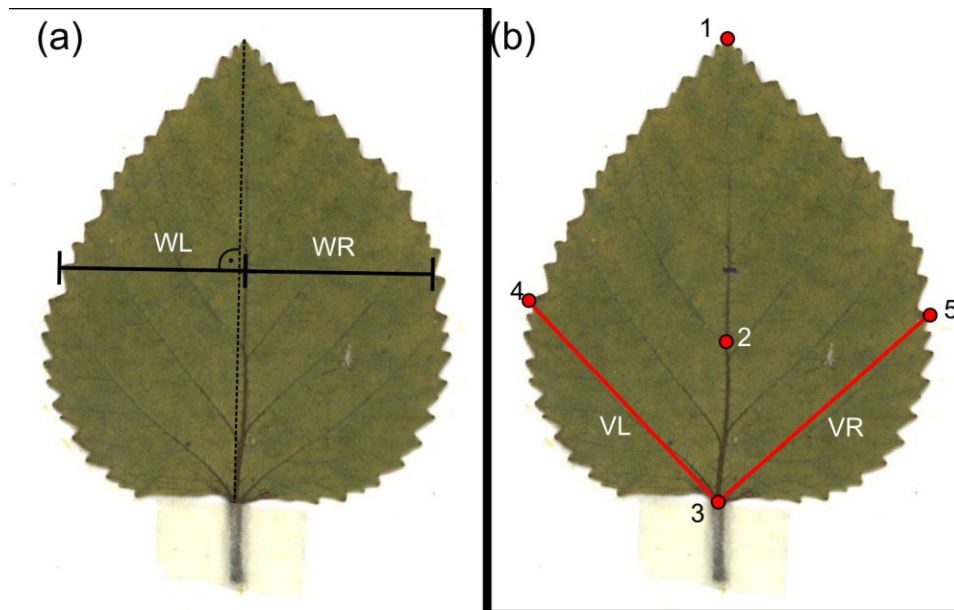
#### *2.4. Traditional distance measurements*

The collected leaves were press-dried between sheets of filter paper and then mounted as ordinary herbarium specimens. For each leaf, we measured the width of the left and right halves (WL and WR, respectively) at the midpoint between the base and the apex of the leaf lamina (Fig. 1a). The measurements were conducted with a ruler to the nearest 0.5 mm; the perpendicularity of the measurement line to the midrib was controlled visually. This protocol was identical to one used in many earlier studies (Kozlov et al. 1996, Valkama & Kozlov 2001; low accuracy measurements in Zverev et al. 2018). All leaves were measured twice, independently and blindly.

#### *2.5. Landmark measurements*

The mounted leaves were scanned at 400 dpi, and the obtained images were used by the first author (TS) to obtain coordinates of landmarks using ImageJ software (Rasband 2017). The five landmarks were: (1) the apex of the leaf, (2) the point where the sixth lateral vein diverges from the midrib as a landmark close to the centre of the lamina, (3) the base of the leaf (i.e. the point where the petiole joins the lamina), and (4 and 5) the two points where the first two lateral veins arrive at the leaf margin, (Fig. 1b). The relatively low number of landmarks used in the present study is consistent with our aim to find an efficient method that with lowest effort (= few landmarks) can reveal meaningful differences in leaf shape of birch. All leaves were coded in a blinded fashion, so the leaf origin was not known during the measurements. For a subset of 200 leaves representing four of the eight natural populations, landmarks were placed twice independently to estimate the ME. Three of the five landmarks were used to calculate the distances VR and VL, which correspond approximately to the lengths of the right and left first lateral veins (Fig. 1b).





**Fig. 1.** The two approaches for quantifying leaf FA in the present study: (a) traditional measurement of distances from the midrib to the left and right margins in the middle of the lamina (WL and WR, respectively) and (b) use of landmarks (circles) to calculate lengths of the first left and right lateral veins (VL and VR, respectively) and for multivariate analysis of leaf shape (see text for details).

### 2.6. Measurement error and FA in distance-based measures

The traditional measurements of leaf width from the pollution gradient resulted in a ME (index ME1, calculated according to Palmer & Strobeck 2003) nearly as large (76%) as the mean absolute difference in width between left and right leaf halves (mean  $\pm$  S.E.:  $0.74 \pm 0.03$  mm vs.  $0.98 \pm 0.04$  mm). The width measurements of the leaves from the stress experiment also resulted in a large (62%) ME when compared to the mean difference in leaf width ( $0.97 \pm 0.11$  mm vs.  $1.57 \pm 0.11$  mm). In both cases, the two measurements were averaged to minimize the effect of ME on our results. The measurements of vein length of the 200 leaves from the field-grown birches measured twice resulted in a very small ME (4.7%) compared to mean difference in vein length ( $1.04 \pm 0.05$  pixels vs.  $22.04 \pm 0.91$  pixels, corresponding to  $0.066$  mm vs.  $1.40$  mm). An individual  $\times$  side ANOVAs (Palmer & Strobeck 2003) of the two measurements of these 200 leaves did not indicate any directional asymmetry in the width of leaf halves ( $F_{1,199} = 2.0$ ,  $p = 0.16$ ; Table S1) and length of basal veins ( $F_{1,199} = 0.17$ ,  $p = 0.68$ ; Table S2), but provided strong evidence for FA ( $p < 0.001$ ; Tables S1, S2).

The WR-WL differences in leaf width and the VR-VL differences in vein length in both data sets were normally distributed (no indication of antisymmetry, kurtosis  $> 1$ ; Table S3), and in

most cases, the mean was not statistically different from zero (no directional asymmetry; Table S3). Only the mean of the WR-WL differences in leaves from the pollution gradient was slightly larger than zero. Similarly, indications of directional asymmetry were evident in the low accuracy measurements, but not in the high accuracy measurements of FA in birch leaves from the greenhouse experiment (Zverev et al. 2018). This effect can easily occur when perpendicularity of the measurement line to the midrib is controlled visually and is thus subjective (Sandner & Matthies 2017 Supplement). The unsigned right minus left ( $|R-L|$  hereafter) differences increased with an increase in average size of the measured trait (Table S3), and a relative (size-corrected) FA index was therefore calculated as follows:  $FA = 2 \times |R-L| / (R+L)$ . This correction for the absolute size of a leaf removed the positive correlation between FA and trait size (Table S3). These two FA indices ( $FA_{width}$  and  $FA_{veins}$ ) were square-root transformed to achieve normally distributed residuals and homoscedasticity in analyses.

### *2.7. Measurement error and FA in multivariate measures of leaf shape*

Variation in leaf shape was analysed using the MorphoJ software (Klingenberg 2011). This software carries out Procrustes superimposition, a transformation that changes the size, orientation and rotation of objects to minimize the squared deviations between all individual landmarks and their means (Palmer & Strobeck 2003). Objects are then mirrored along their symmetry axis and the variation in shape can be divided into symmetric components (variation among individual leaves) and asymmetric components (variation in the position of the landmarks on both sides of a leaf; Klingenberg et al. 2002). The covariance matrix that represented the correlated changes in the arrangement of the landmarks was analysed using principal component analysis (PCA). The three principal components (PCs hereafter) were z-transformed, squared and summed to form a Mahalanobis FA measure ( $FA_{shape}$ ) of the asymmetric components of leaf shape, corrected for non-isotropic variation (Klingenberg & Monteiro 2005). The particularly high values of  $FA_{shape}$  observed in 13 leaves from the greenhouse experiment were associated with a three-dimensional deformation of the lamina that led to folding during pressing of the leaves. Removing these leaves from the analysis did not qualitatively change the results, so we decided to retain them in this analysis, and to regard them as normal extremes of asymmetry.

For the 200 leaves that had landmarks placed twice, a Procrustes ANOVA in MorphoJ revealed no directional asymmetry (side:  $F_{3, 597} = 0.55$ ,  $p = 0.65$ ; Table S4), but significant FA (individual  $\times$  side:  $F_{597, 1200} = 33.12$ ,  $p < 0.001$ ). The ME based on Procrustes ANOVA (ME5,

calculated according to Palmer & Strobeck 2003) was 1.3% of the total variation in shape. The  $FA_{\text{shape}}$  values based on two measurements were highly correlated with the  $FA_{\text{shape}}$  values based on only the first measurement ( $r = 0.98$ ,  $p < 0.001$ ). The  $FA_{\text{shape}}$  values were square-root transformed to achieve normally distributed residuals and homoscedasticity in analyses.

### *2.8. Comparisons between FA measures*

The relationship between the three FA measures was explored by computing Pearson linear correlations for all leaves from the pollution gradient and the greenhouse experiment. In addition,  $FA_{\text{width}}$  and  $FA_{\text{veins}}$  were correlated with the absolute values of the three principal components ( $|PC1| - |PC3|$ ), which together form the multivariate measure  $FA_{\text{shape}}$ .

We compared the variance components of FA using the 200 leaves of mature mountain birches from the pollution gradient for which landmarks were placed twice, and we calculated the three FA measures based on width, vein length and leaf shape for each individual measurement. Variance components were then computed for the effects of the random factors of site ( $n=4$ ), tree ( $n=20$ ; 5 trees per site), leaf ( $n=200$ ; 10 leaves per tree) and ME using restricted maximum likelihood methods in IBM SPSS 23.0. From this analysis, we calculated the proportion of the variance in FA explained by each of the four hierarchical levels.

### *2.9. Effects of industrial pollution and experimental stress on FA measures*

We tested for the effects of pollution at a site on three FA measures in the leaves collected from natural populations by performing hierarchical ANOVAs (type I sums of squares). The effect of pollution was tested against the effect of site, which was tested against the effect of tree.

In the set of leaves of plants grown under experimental stress, the progenies of some mother plants were represented with more than one leaf per stress treatment. In those cases, the FA values were averaged per mother  $\times$  treatment. An ANOVA using the remaining set of 109 values included the site of seed origin (random) to account for variation in FA among sites before testing the effect of treatment (fixed). The treatment effect (2 df) was split into the orthogonal contrasts ‘control vs. stress’ (1 df) and ‘drought vs. metals’ (1 df). The site  $\times$  treatment interaction was not tested because we were not interested in this interactive effect; moreover, not every site  $\times$  treatment combination was present in the subset of 150 leaves randomly chosen from the 1332 leaves available for our study. Including this interaction or

the mother plant in the analysis did not qualitatively change the results in any case (data not shown).

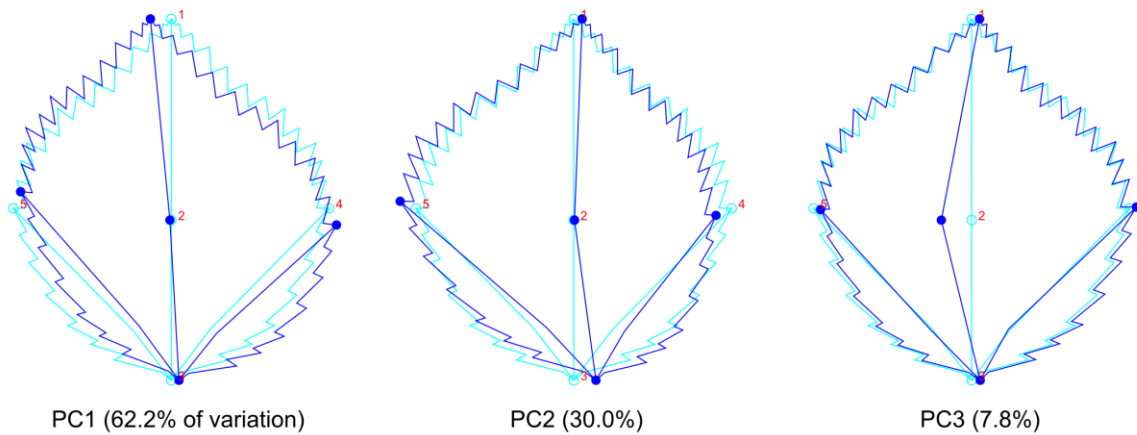
We tested the hypothesis that FA is lowest under control conditions but highest under metal stress (control < drought < metals; Zverev et al. 2018) by performing an ordered-heterogeneity test (Rice & Gaines 1994). This test combines an ANOVA with a test of the order of the factor means. Therefore, the complement of the p-value of the treatment effect in the ANOVA (1-p) was multiplied by the Spearman's rank correlation coefficient between the expected and the observed order of treatment means, and the significance of the resulting  $r_s P_c$  statistic was looked up in a statistical table (Rice & Gaines 1994).

### **3. Results**

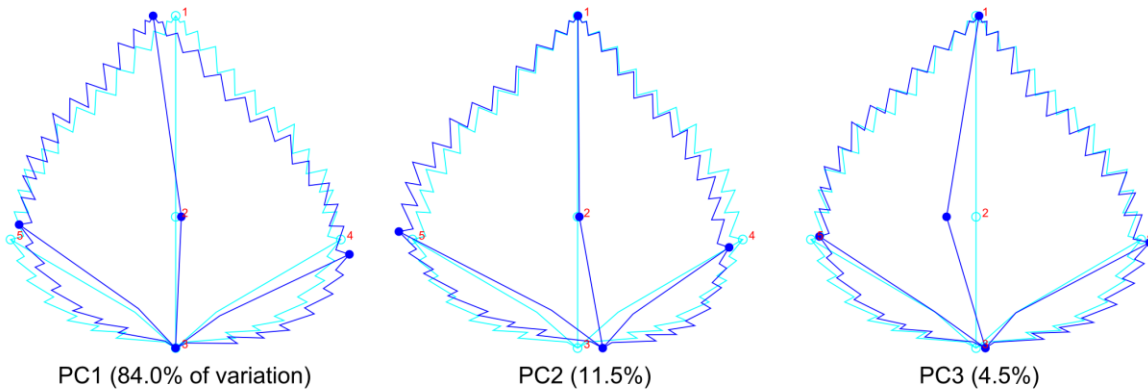
#### *3.1. Asymmetry in leaf shape*

The asymmetric component of the leaf shape defined by five landmarks can be visualised by the three principal components of a PCA. In both sets of leaves (i.e. collected from mature birches in natural populations and from birch seedlings under experimental stress), PC1 (which explained more than 60% of variation in asymmetry) was associated with an upwards or downwards shift in the tips of the first lateral veins, combined with a bent leaf tip (Fig. 2). PC2 explained less than one third of the total variation and corresponded to the differences in width between leaf halves (more or less similar to the commonly used measure of leaf FA), whereas PC3 explained less than 8% of variation in asymmetry and was related to a curved midrib (Fig. 2).

(a) Leaves of plants from the pollution gradient



(b) Leaves of plants from the stress experiment

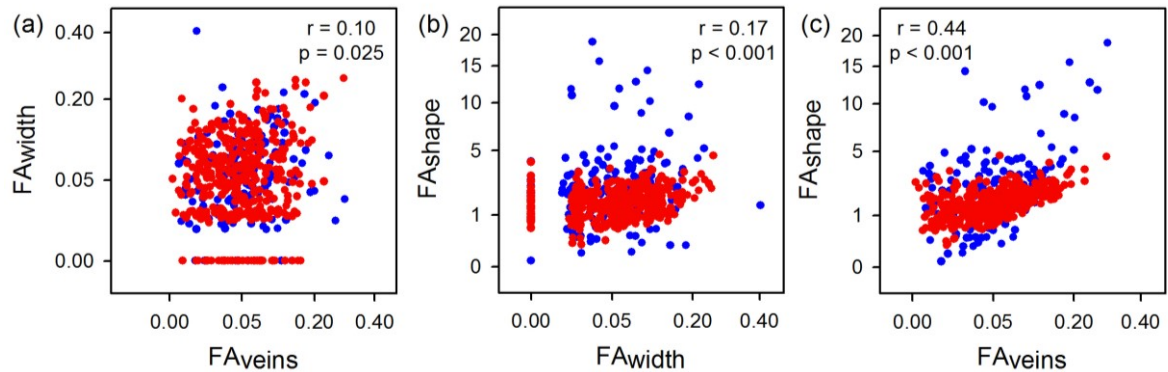


**Fig. 2.** Principal components (PC1–PC3) of the asymmetry of the shape of birch leaves sampled (a) from mature trees in different natural populations along a strong pollution gradient and (b) from seedlings grown in a greenhouse under experimental stress conditions. Light blue lines show the average shape of all leaves, whereas dark blue lines show the shape of the leaves for 0.1 higher values of the corresponding PC, which is close to the most extreme values of asymmetry found for PC1 (0.13 and 0.12 in leaves from the pollution gradient and from the stress experiment, respectively) and PC2 (0.10 and 0.07) and is exaggerated for PC3 (0.03 and 0.05).

### 3.2. Correlation between measures of FA

The three measures of FA were poorly but positively correlated (Fig. 3a-c). The highest correlation was found between  $FA_{veins}$  and  $FA_{shape}$  (Fig. 3c).  $FA_{shape}$  is based on three PCs of leaf asymmetry (Fig. 2), and the high correlation of  $FA_{veins}$  with  $FA_{shape}$  was due to a high correlation of  $FA_{veins}$  with  $|PC2|$  and a low correlation with  $|PC1|$  (Table S5). By contrast, the weak correlation of the traditional measure  $FA_{width}$  with  $FA_{shape}$  (Fig. 3b) was due to weak

correlations with |PC2| and |PC3| (Table S5). Many leaves identified as symmetric based on  $FA_{width}$  and  $FA_{veins}$  had high values of  $FA_{shape}$ , whereas leaves identified as symmetric by  $FA_{shape}$  were mostly also symmetric based on  $FA_{width}$  and  $FA_{veins}$  (see the triangular pattern in Fig. 3b and 3c). This suggests that  $FA_{shape}$  has the highest sensitivity for detection of asymmetry in a leaf. Examples of leaves that were classified as most symmetric or most asymmetric, based on the three FA indices, are shown in Fig. S3.

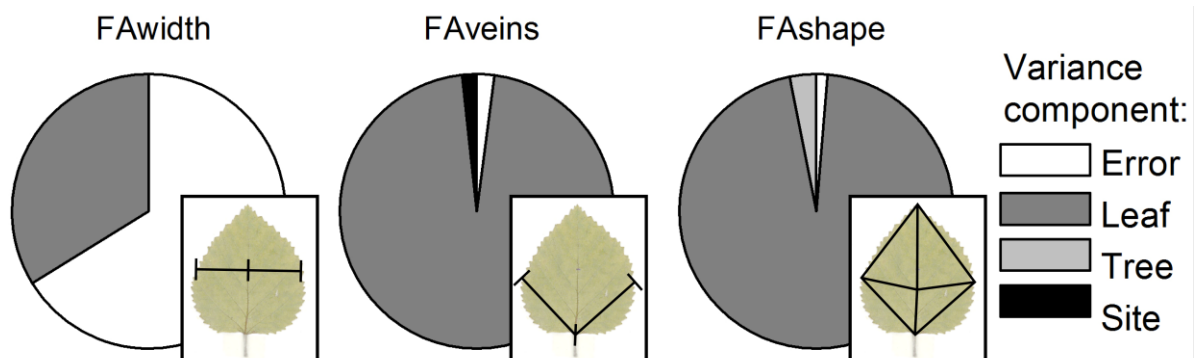


**Fig. 3.** Correlations between the three FA measures in birch leaves. Colours indicate the 395 leaves sampled from 40 mature birches growing in eight sites along a pollution gradient (red) and the 150 leaves of birch seedlings in growing in a greenhouse under experimental stress (blue). Note square-root scale of the axes.

### 3.3. Variance components of FA measures

More than 60% of the variation in  $FA_{width}$  (based on the width of leaf halves measured to the nearest 0.5 mm) was due to ME (Fig. 4). By contrast, ME explained only negligible parts of the variation in landmark-based FA measures, whereas more than 90% of the variation in these measures was due to differences among the leaves of individual trees (Fig. 4).

Differences among trees were responsible for only a small part (3.7%) of the variation in  $FA_{shape}$ , and only 1.7% of the variation in  $FA_{veins}$  was due to different sites of origin.



**Fig. 4.** Variance components of the three FA measures compared in this study ( $FA_{width}$ ,  $FA_{veins}$ ,  $FA_{shape}$ ), based on 200 leaves sampled from 20 mature birch trees growing in four sites along a pollution gradient.

### 3.4. Effects of industrial pollution and experimental stress on FA measures

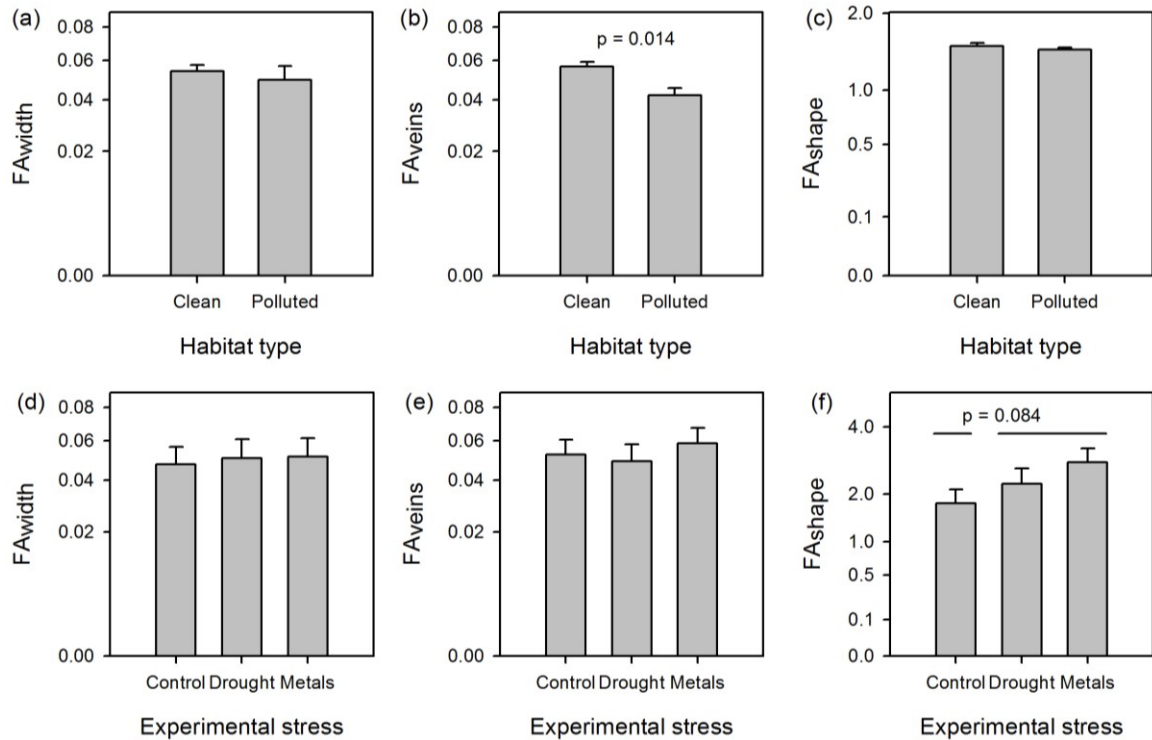
$FA_{veins}$  was the only measure of FA that differed between leaves of trees from polluted and unpolluted sites ( $p = 0.014$ , Table 1, Fig. 5a-c). However, contrary to the expectation, this measure of FA was higher in leaves from unpolluted sites than in leaves from polluted sites (Fig. 5b).

Experimental stress did not influence the distance-based FA measures (Fig. 5d,e), but weakly influenced the multivariate FA measure:  $FA_{shape}$  tended to be higher in leaves of seedlings grown under stress than under control conditions (Table 2, Fig. 5f). The ordered heterogeneity test of the directional hypothesis that FA is highest under metal stress, intermediate under drought and lowest in the control treatment was significant for  $FA_{shape}$  ( $r_sP_c = 0.872$ ,  $p = 0.02$ ), but not for the two other FA measures ( $r_sP_c = 0.051$ ,  $p > 0.4$  for  $FA_{width}$ ;  $r_sP_c = 0.144$ ,  $p > 0.3$  for  $FA_{veins}$ ). The effect of stress on  $FA_{shape}$  was related to marginally significant increases in  $|PC3|$  of asymmetry under stress compared to the control ( $F_{1,90} = 3.32$ ,  $p = 0.072$ ) and in  $|PC2|$  under metal stress compared to drought ( $F_{1,90} = 2.80$ ,  $p = 0.098$ ), whereas  $|PC1|$  was not significantly influenced by the stress treatments ( $p > 0.50$ ).

**Table 1**

Analyses of variance of the effects of pollution (high vs. low), site (four at each pollution level) and tree (five per site) on three measures of fluctuating asymmetry in leaves sampled from mature birches growing at different levels of environmental pollution. Significant ( $p < 0.05$ ) F-value is marked with an asterisk.

Source of variation	$FA_{width}$				$FA_{veins}$		$FA_{shape}$	
	df	denom. df	MS	F	MS	F	MS	F
Pollution	1	6	0.008	0.28	0.103	11.92 *	0.036	0.89
Site	6	32	0.028	1.80	0.009	1.22	0.040	0.39
Tree	32	355	0.016	1.22	0.007	0.74	0.103	1.37
Residual	355		0.013		0.010		0.075	



**Fig. 5.** Effects of industrial pollution in natural habitats (a-c) and of experimental stress (d-f) on three measures of fluctuating asymmetry in birch leaves (means + 1 SE): a traditional width-based measure (a, d), a landmark-based measure describing differences vein length (b, e) and a landmark-based measure describing asymmetry of shape (c, f). Note square-root scale for FA.

**Table 2**

Analyses of variance of the effects of site of origin and stress treatment (control, heavy metals and drought) on three measures of fluctuating asymmetry in leaves of seedlings grown in a greenhouse. The treatment effect was split into two orthogonal contrasts: control vs. stress and drought vs. metals. Marginally significant ( $0.05 < p < 0.10$ ) F-value is marked with a cross (+).

Source of variation	df	FA <sub>width</sub>		FA <sub>veins</sub>		FA <sub>shape</sub>	
		MS	F	MS	F	MS	F
Site of origin	16	0.010	0.83	0.007	0.73	0.52	1.25
Treatment	2	0.001	0.05	0.003	0.34	0.88	2.10
__Control vs. stress	1	0.001	0.10	0.0002	0.02	1.28	3.06 +
__Drought vs. metal	1	0.000	0.01	0.006	0.66	0.48	1.15
Residual	90	0.012		0.009		0.42	



## 4. Discussion

### 4.1. Differences and similarities between the three measures of FA

The three measures of FA in birch leaves compared in this study were only poorly (although positively) correlated. This is not surprising, as many studies have reported low correlations between FA in different traits of the same individuals (Kryazheva et al. 1996, Sherry & Lord 1996, Waldmann 1999, Leung et al. 2000, Llorens et al. 2002, Freeman et al. 2005, Sandner & Matthies 2017, Zverev et al. 2018). However, this finding emphasises that a crucial methodological problem exists in FA studies, namely that the conclusions drawn by a study may differ depending on the trait selected for FA measurements, as demonstrated by Llorens et al. (2002) and Ivanov et al. (2015). The majority of plant studies report only the FA of a single trait (Kozlov 2017), and those that study several traits often report only the results of the trait showing significant responses of FA, which results in a presentation bias. To avoid wrong conclusions, establishment of a standardized protocol, like that proposed by Kozlov et al. (2017a), is preferable. However, this may not be possible with landmark-based indices. In particular, many plant species lack some of our landmarks, and results based on different sets of landmarks cannot easily be compared among species.

Low correlations between different measures of FA in the same individual may arise from differences in FA between organs that develop at different times. For example, in *Sinapis alba* plants grown under five different experimental stresses, the trait that showed increased FA differed among stress types (Roy & Stanton 1999). The authors suggested that effects on asymmetry may not be general; instead, different stresses may affect the development of different organs at specific ontogenetic stages (Roy & Stanton 1999). However, the inability to make clear predictions regarding which particular stress should affect FA in which particular trait precludes the use of trait-specific FA to indicate stress. This problem may be partially overcome by combining FA in different traits into one composite FA index (Leung et al. 2000, Palmer & Strobeck 2003); for example, many Russian researchers average the FA values calculated for five leaf traits, following the practice established by Kryazheva et al. (1996). Alternatively, multiple leaf landmarks do not have to be regarded as representing different traits, but can be analysed simultaneously by geometric morphometrics.

Geometric morphometrics offer a promising alternative to the traditional methods used in FA studies, as variations in shape are analysed with multivariate methods (Rohlf & Marcus 1993, Klingenberg & McIntyre 1998, Palmer & Strobeck 2003, Klingenberg 2015). Although distances can be calculated from landmarks, the shape cannot be reconstructed from the distances alone (Rohlf & Marcus 1993). Consequently, distance-based measures of FA reflect only a part of the variation in shape-based FA, even when these distances are calculated from landmarks instead of traditional arbitrary positions on the lamina. Of the total  $FA_{\text{shape}}$  of a birch leaf described by three principal components, the traditional distance-based measure  $FA_{\text{width}}$  weakly reflected PC2 (differences in leaf width on both sides) and PC3 (curvature of the midrib). However, the quality of this FA measure was reduced by its very high ME, which may result predominantly from the serrated leaf margins, as small deviations from the midpoint of the leaf or from perpendicularity of the measurement line to the midrib will lead to large differences in the obtained FA values (Kozlov 2017, Kozlov et al. 2017a).

By contrast, the measure  $FA_{\text{veins}}$  calculated from the distances between landmarks had a much lower ME when compared with  $FA_{\text{width}}$ , because landmarks, by definition, can be precisely located (Klingenberg 2015). This measure ( $FA_{\text{veins}}$ ) was strongly correlated with PC2, which explained 11–30% of total variation in leaf shape asymmetry. Nevertheless, the largest part of the variation in asymmetry (associated with PC1) was only weakly reflected by  $FA_{\text{veins}}$ . This variation corresponded primarily to longitudinal shifts of the tips of the first lateral veins, combined with a bent leaf tip. Geometric morphometrics thus can quantify variation in FA that is not covered by distance-based methods.

Traditionally, the use of width measurements to quantify deviations from bilateral symmetry was a pragmatic approach, because it is easy to apply, can be standardized among studies and its analysis does not require much computational power. However, all scientists studying FA realise that these measures only roughly reflect asymmetry. Two of the authors of the present study (VZ and MVK) were particularly dissatisfied with a traditional measure of FA ( $FA_{\text{width}}$ ) while experimentally exploring the relationships between leaf FA and insect herbivory (Kozlov et al. 2018). For these experiments, the ten most symmetric and ten least symmetric leaves were collected from the same individual plants. During this sampling, we recognized that the leaves that were sampled because they looked the most symmetric often had considerable differences in their half-widths. Conversely, the leaves with equal widths of left and right halves of the lamina often looked rather asymmetric (e.g. due to a curved midrib). The use of the multivariate index  $FA_{\text{shape}}$  solves this problem to a large extent: the birch leaves

with the lowest  $FA_{\text{shape}}$  look much more symmetric when compared to birch leaves with the lowest values of  $FA_{\text{width}}$  and  $FA_{\text{veins}}$  (Figure S3).

#### 4.2. Effects of industrial pollution and experimental stress on FA

The multivariate measure  $FA_{\text{shape}}$  was the only measure that tended to increase under experimental stress. Different types of stress may have different effects on FA, and particular stresses that stop plant growth, like salinity or drought, may not increase FA (Graham et al. 2010). Previous studies have shown that FA of plant leaves increases much more strongly in response to heavy metal stress than to drought stress (reviewed by Zverev et al. 2018). This expected increase in FA (control < drought < metals) was shown in our study by  $FA_{\text{shape}}$  – an indication that geometric morphometrics may be suitable for detecting subtle environmental effects on FA that are overlooked by distance-based FA measures. The FA measure based on distances calculated from landmarks was no better in this respect than the traditional FA measure based on leaf half width. Similarly, the FA in vein length of *Vitis vinifera* was not increased by salinity stress (Sinclair & Hoffmann 2003), and nutrient deficiency did not increase the FA in the length of *Acer platanoides* veins (Black-Samuelsson & Andersson 2003). However, even  $FA_{\text{shape}}$  does not qualify as an indicator of stress, because the effect was so weak that the sample sizes would need to be much larger than those commonly used in indicatory studies, and false interpretations of the results would still be likely.

None of our three FA measures showed the theoretically predicted increase in birches experiencing severe industrial pollution at multiple sites in the natural environment. Similarly, a study of FA in several woody plant species in the surroundings of different industrial factories found no effect of environmental pollution on FA, although this effect was reported as strong in the published literature (Kozlov et al. 2009). In their meta-analysis, the authors concluded that methodological problems in studies conducted before the year 2000 led to overestimations of pollution effects on FA, as did object selection bias (i.e. the preferential investigation of study systems that are known to respond to the stress in a predictable manner) and publication bias (Kozlov et al. 2009). Furthermore, non-blind measurements have a potential to yield a pattern that is consistent with the researcher's expectations ('confirmation bias'), so that higher FA values are found in polluted or generally more stressful environments (Kozlov & Zvereva 2015).

When these methodological problems are avoided and FA is not higher in leaves from polluted than in leaves from clean sites, this may suggest that FA does not generally increase

with stress. Instead, FA responses to stress may be idiosyncratic (i.e. they are stress-, trait- and species-specific). This hypothesis is supported by an increasing number of studies showing that different types of stress can increase or decrease FA (reviews by Kozlov et al. 2009 and Graham et al. 2010, Sandner & Matthies 2017, Zverev et al. 2018). Even in studies using landmarks, the FA in natural populations was not consistently higher under conditions regarded as stressful. The FA in vein length of the perennial herb *Cnidioscolus stimulosus* was higher in disturbed than in undisturbed sites, but only in the absence of burning, while other leaf traits showed different patterns (Freeman et al. 2005). In the tropical tree *Heliocarpus pallidus*, the multivariate FA of leaf shape was higher in the more favourable riparian habitats than in deciduous habitats (Cuevas-Reyes et al. 2011), which is in line with our finding that even FA<sub>shape</sub> was not higher in birches growing in polluted sites than in those from pristine natural sites.

#### 4.3. Within-individual variation in FA

In the studied birch leaves, more than 90% of the variation in FA<sub>veins</sub> and FA<sub>shape</sub> was due to differences among the leaves of individual trees, whereas differences among trees or populations were small or absent. Although some studies report differences in FA among populations of a species (e.g. Sherry & Lord 1996, Siikamäki & Lammi 1998), others have not (Vaupel & Matthies 2012), and differences in FA found among natural populations may disappear when studied under common garden conditions (Siikamäki & Lammi 1998). As in our study, the FA in leaves of *Erythroxylum tortuosum* varied predominantly among the leaves of a plant, and not among shoots, branches or even plants (De Sibio & Rossi 2012).

Previous studies have associated some of the within-individual variation in FA with plant structure or environmental factors. For example, the FA of individual leaves in *Ulmus glabra* trees changed during ontogeny (Møller & van Dongen 2003), needle FA in *Pinus sylvestris* increased with increasing branching order (Kozlov & Niemelä 1999), and FA was higher in leaves from the outside top of a crown in *Ficus carica* trees than in inner leaves, which was ascribed to stress by higher temperature, light or drought (Cowart & Graham 1999).

Differences in light availability may lead to asymmetric growth of leaves, as has been shown by experimentally shading parts of leaves with aluminium foil (Freeman et al. 2003). Flowers of *Iris pumila* showed directional asymmetry related to compass direction, which was interpreted as a plastic response related to differences in light intensity (Tucić et al. 2017). Furthermore, gnawing by herbivores or infestation with gall-inducing midges during

development may lead to compensatory growth and increase FA (Alves-Silva & del Claro 2016, but see Alves-Silva 2012). However, a large part of the variation in FA among leaves may not be due to micro-environmental differences or leaf position, but may arise due to random developmental variation (Coward & Graham 1999) caused, for example, by the position of leaf primordia in the buds (Chitwood et al. 2012). This would raise questions regarding the ecological and evolutionary importance of the symmetry of leaf shape.

Generally, within-individual variation is an often overlooked part of ecology that can be substantial and evolutionarily important (Herrera 2017), and variation in FA among individual leaves may have consequences across trophic levels. For example, high-FA leaves of *Quercus laevis* and *Q. geminata* contained significantly lower levels of tannins and more nitrogen when compared with low-FA leaves, and high-FA leaves were preferred over low-FA leaves by leaf-mining insects in natural environments (Cornelissen & Stiling 2005). Similarly, defoliating insects distinguished between low-FA and high-FA leaves of the same plant individual in controlled experiments, but did not distinguish between disks cut from these leaves (Kozlov et al. 2018).

#### 4.4. The nature of FA in birch leaves

The leaves of mountain birch are partially consistent objects, because the topology is similar in all leaves, whereas the number of lateral veins differs between leaves (Graham et al. 2010). The fact that the three principal components of variation in asymmetry illustrated similar changes in leaf shape, both in the leaves of mature plants from natural populations and in leaves of seedlings under experimental conditions, suggests a common nature of asymmetry in leaves from different populations and from different age classes of mountain birch. However, since the lateral veins of birch leaves are often not opposite but alternate, the question arises whether symmetry really is the normal and optimal state of these leaves. Even in leaves which have high base levels of FA, environmental or genetic stresses may further increase FA (Roy & Stanton 1999), as shown in our study by the increase in FA<sub>shape</sub> with experimental stress. Nevertheless, the suitability of FA to indicate stress is strongly hampered by the extremely high within-plant variation, so that the low predictive power of FA does not justify the high workload associated with sampling, measurement and analysis of leaves from a sufficient number of plants (Freeman et al. 2005, Zverev et al. 2018).

## 5. Conclusions

We showed that a multivariate landmark-based FA measure ( $FA_{\text{shape}}$ ) is more suitable for describing FA in birch leaves than are either a traditional distance-based FA measure ( $FA_{\text{width}}$ ) or a distance-based FA measure calculated from landmarks ( $FA_{\text{veins}}$ ).  $FA_{\text{shape}}$  also tended to show the expected increase in FA from control conditions to drought and especially in response to heavy metal stress. However, even this shape-based FA measure was not increased in birches growing in industrially polluted sites. This suggests that FA in birch leaves, which has often been used as an indicator of environmental stress, is not reliable for this purpose, even when quantified with modern methods. All FA measures were characterised by extremely high levels of within-plant variation, but geometric morphometrics show promise in determining the causes of this variation and its possible ecological consequences.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/\\*\\*\\*](http://dx.doi.org/***)

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