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Reproducibility of fluctuating asymmetry measurements in plants: Sources of variation and implications for study design

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ABSTRACT

Fluctuating asymmetry (FA), i.e. small, non-directional deviations from perfect symmetry in morphological characters, increases under genetic and/or environmental stress. Ecological and evolutionary studies addressing FA became popular in past decades; however, their outcomes remain controversial. The discrepancies might be at least partly explained by inconsistent and non-standardised methodology. Our aim was to improve the methodology of these studies by identifying factors that affect the reproducibility of FA measurements in plant leaves. Six observers used a highly standardised measurement protocol to measure FA using the width, area and weight of the same set of leaves of 10 plant species that differed in leaf size, shape of the leaf margin and other leaf traits. On average, 24% of the total variation in the data was due to measurement error. Reproducibility of measurements varied with the shape of leaf margin, leaf size, the measured character and the experience of the observer. The lowest reproducibility of the width of leaf halves was found for simple leaves with serrate margins and the highest for simple leaves with entire margins and for compound pinnate leaves. The reproducibility was significantly lower for the weight of leaf halves than for either their width or area, especially for plants with small leaves. The reproducibility was also lower for measurements made by experienced observers than by naïve observers. The size of press-dried leaves decreased slightly but significantly relative to fresh leaves, but the FA of press-dried leaves adequately reflected the FA of fresh leaves. In contrast, preservation in 60% ethanol did not affect leaf size, but it decreased the width-based values of FA to 89.3% of the values measured from fresh leaves. We suggest that although reproducibility of leaf FA measurements depends upon many factors, the shape of the leaf margin is the most important source of variation. We recommend, whenever possible, choosing large-leaved plants with entire leaf margins as model objects for studies involving measurements of FA using the width of leaf halves. These measurements should be conducted with high accuracy from images of fresh or press-dried leaves.

1. Introduction

A key challenge for ecologists is the development of reliable and standardised methods that can improve our understanding of ecological patterns and processes (Bocedi et al., 2012). Methodological issues become increasingly critical when studies addressing a problem of both theoretical and applied importance yield inconsistent or equivocal results, and especially when an inadequate or non-standardised methodology is suspected to contribute to this inconsistency. The studies addressing fluctuating asymmetry (FA) of living beings represent a striking example of such inconsistency.

FA is defined as small, non-directional deviations from perfect symmetry in morphological characters that arise when an individual is unable to control development because of genetic and/or environmental stress (Møller and Swaddle, 1997). Once considered a universal stress indicator (Zakharov, 1990; Clarke, 1992;

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Table 1
Leaf traits of the studied plant species[a].

Plant species	Leaf type	Width (mm)	Area (mm^2)	Weight (mg)	SLW (mg/cm^2)	Leaf margin	Com-pact-ness	Teeth/lobe number
Betula pubescens	Simple	31.6	540	20.6	5.96	Double serrate	21.5	42.4
Populus tremula	Simple	39.8	580	40.1	9.26	Sinuate	16.8	20.4
Prunus avium	Simple	31.8	649	20.3	4.25	Serrate	34.8	70.2
Quercus robur	Simple	27.2	759	45.9	6.98	Entire/Lobate	26.3	7.6
Rubus idaea	Compound	142.4	1858	77.8	4.44	Serrate	25.2	52.8
Salix caprea	Simple	35.6	950	51.6	7.46	Entire	17.1	0
Sorbus aucuparia	Compound	95.6	560	20.9	4.44	Double serrate	67.1	39.6
Spiraea chamaedryfolia	Simple	24.4	484	16.8	4.42	Double serrate	40.1	40.8
Vaccinium myrtillus	Simple	13.0	103	4.0	5.96	Serrate	19.6	57.2
V. uliginosum	Simple	15.2	160	7.4	5.92	Entire	13.4	0

[a] Weight was measured from press-dried leaves; all other characteristics–from images of fresh leaves.

Graham et al., 1993), FA was a popular target of past ecological and evolutionary studies addressing multiple taxa of plants and animals. However, an accumulation of negative or inconclusive results dampened the initial optimism regarding FA (Lajus et al., 2009), and sceptical reviews (Palmer, 1996; Clarke, 1998; Bjorksten et al., 2000; Rasmuson, 2002) began to point out a general inconsistency in the relationships between FA, stress and fitness. For example, stressors that clearly suppressed growth and increased mortality often had no effect on FA (Bjorksten et al., 2000; Cárcamo et al., 2008; Kozlov et al., 2009). This inconsistency remains unresolved (Van Dongen, 2001; Lens et al., 2002; Hendrickx et al., 2003), raising the question of whether the variation in the outcomes of individual studies reflects differential responses of study species to stressors or results from incoherent and non-standardised research methodology.

The ISI Web of Science (assessed on 3 May 2016) shows FA mentioned in 343 titles published from 2006 to 2015 and in abstracts or key words of an additional 1088 papers. The recent publications cover a wide range of biological research areas;, including sexual selection (Polak et al., 2015);, human attractiveness (Van Dongen, 2014);, susceptibility to infectious diseases (Thomas et al., 2015);, consequences of inbreeding (Wiig and Bachmann, 2014) and monitoring of pollution (Lajus et al., 2015). Importantly;, most studies employed FA for evaluation of the relative strength of a stress experienced by various organisms (humans; in particular) in different environments. The conclusions drawn therefore can have far-reaching consequences for policy decisions on human health and quality of life issues; therefore;, the data must be incontestable.

Historically, FA studies have focussed on animals (Zakharov, 1990; Clarke, 1992; Møller and Swaddle, 1997); however, plants have also become favoured subjects of FA studies owing to their modular structure that allows repeated measurements of the same character within an individual. Frequently, these studies relate the FA of leaves (or, less frequently, flowers) to certain environmental characteristics, using the absolute or relative difference in width (and/or some other character) of the left and right leaf halves as the measure of FA (Kozlov et al., 1996; Chudzinska et al., 2014; Erofeeva, 2014; Klisarić et al., 2014; Shadrina et al., 2014; Telhado et al., 2016). Other FA calculations are based on either the areas or the weights of the leaf halves (Vaupel and Matthies, 2012; Wuytack et al., 2013). These various measurements naturally require different approaches and use different measuring instruments; nevertheless, we are not aware of any study that has compared the reproducibility (i.e. the closeness between independent results obtained with the same method on the same objects by different observers) between FA values calculated from measurements of widths, areas and weights of the same objects. A further complication is that plant FA has been measured using fresh leaves (Erofeeva, 2014; Ivanov et al., 2015), press-dried leaves (Kozlov et al., 1996; Telhado et al., 2016) or leaves fixed in ethanol (Heard et al., 1999; Shadrina et al., 2014), but the effects of leaf preservation on either the absolute FA values or their reproducibility remain unknown. The reproducibility may also depend on the measurement methods, on the characteristics of the study object and the experience of the observer (Yezerinac et al., 1992).

The methods and techniques used for measurements of FA have advanced significantly over the past decade; nevertheless, the quality of some published estimates of FA remains problematic. The measured FA is often smaller than measurement error (Palmer and Strobeck, 2003; Lajus et al., 2009), leaving substantial potential for the actual FA to be confounded or masked by the measurement error (Goodenough et al., 2012), but many researchers pay little attention to the accuracy of the measurements used for FA calculations. For example, of the 31 scientists who published at least one paper reporting FA in plants, one third measured the half-width of birch leaves, which varies from 10–25 mm, using a ruler having 1 mm accuracy (Kozlov, 2015). Consequently, the reproducibility of the measurements among these 31 scientists was an unacceptably low 7.4% (Kozlov, 2015). Measurements of FA are also prone to confirmation bias: we demonstrated that the results obtained from the same set of test images differed significantly when the observers were told that the samples originated from either 'stressful' or 'benign' environments (Kozlov and Zvereva, 2015). These findings emphasise an urgent need to develop protocols that assure sufficient reproducibility of the measurements used to calculate FA.

The aim of the present study was therefore to explore the sources of variation in the reproducibility of measurements of leaf FA, in order to advance the research methodology for studies addressing the FA of plants. Here, we asked how the reproducibility of FA measurements, used as an index of data quality, is affected by the species-specific leaf traits (size, thickness and shape of leaf margin), the mode of leaf preservation (fresh, press-dried, or in ethanol), the measured character (width, area, weight) and the experience of the observer. We tested the following predictions: (1) the reproducibility of the half-width measurements is lower in simple leaves with serrate margins than in leaves with entire margins, because minor changes in the position of the landmark (i.e., conspicuous and distinct morphological feature serving as the end point in conducting measurements) at the margin of a serrate leaf can result in substantial changes in the measured leaf width; (2) the reproducibility of the length measurements of leaflets in pinnate compound leaves (an equivalent of leaf half-width in simple leaves) is higher than that of the half-width of simple leaves due to a more objective selection of landmarks; (3) the reproducibility of FA based on the area and weight of leaf halves is higher than based on leaf half-width of leaves due to the lower subjectivity of computerised measurements relative to manual methods; (4) leaf preservation in ethanol causes smaller impacts on leaf FA than does press-drying; and (5) reproducibility increases with the experience of the observer.

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