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How many leaves are enough? The influence of sample size on estimates of plant developmental instability and leaf asymmetry

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ABSTRACT

One issue in studies of leaf asymmetry is the lack of standardization of sample sizes for estimating the different types of leaf asymmetry, namely fluctuating asymmetry (FA –, differences in leaf sides around zero, normally distributed data), directional asymmetry (DA – one leaf side is substantially larger than the other) and antisymmetry (AS – both right-and left-sidedness; bimodal data). FA is the only parameter regarded as a biomarker of stress and it is used consistently for population monitoring. Here we investigated whether leaf samples of different sizes showed different asymmetries (FA, DA or AS) in three unrelated plant species (*Miconia fallax*, *Solanum lycocarpum* and *Bauhinia brevipes*). We hypothesized that larger samples would show FA, as substantial side differences would be buffered and so that the sample would display differences in leaf sides around zero (i.e., FA). The results were highly dependent on both sample size and plant species. Larger samples did not consistently display FA, but they did provide leaf asymmetry values close to zero, which is a prerequisite of FA. AS was pervasive, but detailed exploration of histograms revealed that none of the data sets had a bimodal distribution and most curves were leptokurtic. On this basis, we suggest that kurtosis tests in combination with histograms should be given preference over normality tests as a method to evaluate potential AS. We conclude that at this time it is still not possible to give a rule-of-thumb for sample sizes for this type of investigation, and that graphical representations of data should be used to classify the asymmetry types more accurately. We suggest that the statistical tests used to examine FA should be reconsidered and that graphs such as histograms can be more informative than *p*-values when it comes to distinguishing between types of leaf asymmetry.

1. Introduction

Developmental instability (Box 1) occurs when a genotype fails to correct for random disturbances during development, resulting in a phenotype that deviates from the presumed ideal (Møller and Swaddle, 1997). In the case of plants, it has been assumed that deviations from the perfect bilateral pattern of leaf growth (i.e. leaf asymmetry) reflect developmental instability, which can be evaluated statistically via analysis of fluctuating asymmetry (FA) (Alados et al., 2002; Graham et al., 2010). The term FA is used to refer to small, non-directional differences between leaf sides (a leaf side is defined as the length between the midrib and one leaf edge – Kozlov and Zvereva, 2015) and reflects the plant's inability to buffer stresses (Box 1) experienced during development. In the past ten years or so there has been a renewed interest in developmental instability and the response of populations to several alleged stressors, namely biotic stressors (e.g. herbivory, parasitism and competition, see Cuevas-Reyes et al., 2013; Komac and Alados, 2012; Santos et al., 2013; Alves-Silva and Del-Claro, 2016a) and environmental stressors (e.g. pollutants, sun-shade, see Raz et al., 2011; Wuytack et al., 2011). Under optimal conditions, both sides of a leaf should follow the same developmental pattern, but the aforementioned variables tend to increase leaf asymmetry, which is an indicator of population stress and low habitat or micro-habitat quality (Alados et al., 2002; Andalo et al., 2000).

In addition to FA, two other types of bilateral asymmetry are found in populations, namely directional asymmetry (DA: one side of the character is consistently larger than the other) and antisymmetry (AS: the population contains examples with both larger right sides and larger left sides) (see Leary and Allendorf, 1989; Palmer and Strobeck, 1992) (Box 1, Fig. 1). Both DA and AS are defined as 'broken symmetries'

Abbreviations: *Rs*, right side of leaf; *Ls*, left side of leaf; FA, fluctuating asymmetry; DA, directional asymmetry; AS, antisymmetry; d_{signed}, difference between leaf sides, i.e. *Rs* minus *Ls*; d_{abs}, absolute difference between leaf sides, i.e. |*Rs* minus *Ls*

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Box 1
Brief definition of some parameters used to investigate developmental instability (DI) and different types of asymmetry. *Rs* and *Ls* refer to the right and left side of leaves, respectively.

Parameters: **Brief definition**

Developmental instability (DI): The inability to buffer factors that affect the stability of normal patterns of growth

Stress: Any factor, biotic or abiotic that is detrimental to an organism performance, development and net energy utilization

Fluctuating asymmetry (FA): Subtle departures from bilateral symmetry; average of d_{signed} not different from zero, and data normally distributed

Directional asymmetry (DA): One side of the character is greater than the other side; mean of d_{signed} significantly different from zero; data either right or left sided/skewed

Antisymmetry (AS): d_{signed} with bimodal or platykurtic distribution; population with both right and left sidedness

Signed *Rs minus Ls* measures, i.e. (d_{signed}): Leaf asymmetry; the signed difference between leaf sides, usually taken from the midrib to the edge of leaf blades; it is used to categorize leaf measurements into FA, DA or AS

Absolute *Rs minus Ls* measures, i.e. $|d_{abs}|$: The absolute values of *Rs minus Ls* measures; used for analyses of comparison and/or relationships, e.g., relationship between leaf asymmetry and pollutants, herbivory, fertilizers, etc.

For further information, see Koehn and Bayne (1989); Palmer (1994); Palmer and Strobeck (1992); McKenzie and O'Farrell (1993); Møller (1995); Møller and Shykoff (1999); Rowe et al. (1997); Graham et al. (1998); Graham et al. (2010); Alves-Silva and Del-Claro (2013); Cornelissen and Stiling (2005); Kozlov and Zvereva (2015).

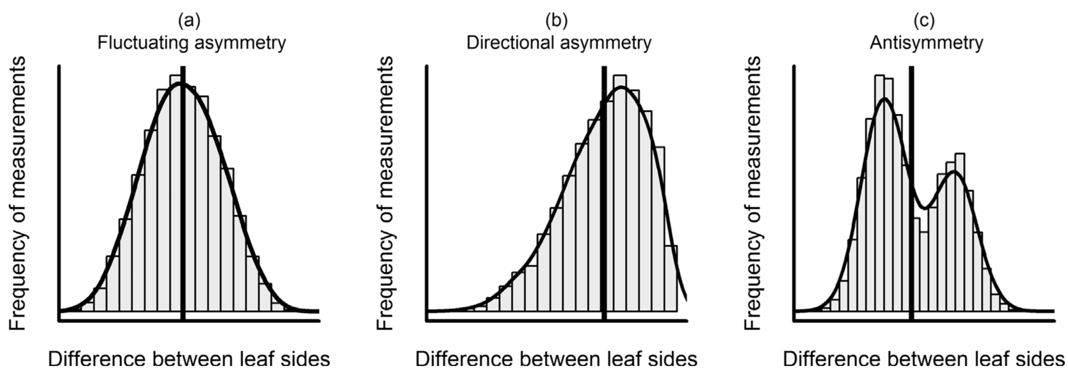

Fig. 1. Three types of asymmetry found in leaves. Asymmetry is evaluated as the difference between leaf sides from the leaf edge to the midrib. (a) Fluctuating asymmetry has mean equal to zero and a bell-shaped distribution. (b) In directional asymmetry, data are either left or right skewed, and the mean is different from zero. (c) Antisymmetry presents bimodal distribution. Curves represent the adjusted distribution of data; vertical lines display the mean.

(Graham et al., 2010) as the differences between sides are substantially different from zero (DA) or bimodally distributed (AS) (Palmer and Strobeck, 1992; Rowe et al., 1997). In addition, and unlike FA, both DA and AS are genetically determined (Graham et al., 2010; Pie et al., 2007; Pratt and Mclain, 2002) and not considered suitable indicators for population monitoring, management and conservation (Cornelissen and Stiling, 2010; Ivanov et al., 2015; Leary and Allendorf, 1989; Møller, 1998; Palmer and Strobeck, 1992; Raz et al., 2011; but see Lens and Van Dongen, 2000; McKenzie and O'Farrell, 1993). Most published studies on developmental instability have found that FA is pervasive in plants (Graham et al., 1998; Møller and Shykoff, 1999; Santos et al., 2016), and DA and AS are less commonly found (Pie et al., 2007; Sakai and Shimamoto, 1965; Venâncio et al., 2016a).

Plants are ideal models for investigations of the effects of a given stressor on population development, as they are generally easy to sample, tag and produce several leaves that can be used as within-individual replicates, thus providing a valuable index of individual-based FA (Alves-Silva and Del-Claro, 2016a; Cornelissen and Stiling, 2011). A wide range of sample sizes have been used in research on plant FA, ranging from less than five leaves (Alados et al., 2002; Black-Samuelsson and Andersson, 2003; Venâncio et al., 2016b) to tens (Costa et al., 2013; Møller, 1995) or even hundreds of leaves (Cuevas-Reyes et al., 2011) per individual plant. Furthermore, there is also variability in the number of individual plants sampled, from five (Cowart and Graham, 1999), to tens (Martel et al., 1999), hundreds (Andalo et al., 2000) or over a thousand (Klisarić et al., 2014).

One widely recognized advantage of using FA as an index of developmental instability is the rapid estimation of a population's response to environmental factors (Møller and Lope, 1998). Nonetheless, large sample sizes require extensive field and laboratory work as well as increasing costs. It is worth asking whether there is an ideal sample size that would provide consistent and reliable estimates of FA (Klisarić et al., 2014). The literature suggests that there is no rule-of-thumb for determining sample size in studies of leaf asymmetry, and the choice of sample size may be *(i)* arbitrary, as in the case of trees that produce abundant leaves; *(ii)* restricted by the number of leaves available, as in seedlings; *(iii)* or constrained by the resources (personnel, funds, time) available.

Wuytack et al. (2011) noted that large sample sizes might be necessary to provide accurate estimates of FA and to detect the influence of a given stressor on leaf development, but to the best of our knowledge there has been no systematic investigation of the effects of sample size on *(i)* the manifestation of different types of leaf asymmetry (FA, DA and AS); *(ii)* the magnitude of leaf asymmetries, i.e. whether asymmetry increases or decreases according to sample size; *(iii)* whether the relationship between leaf asymmetry and a stressing variable changes with sample size. The aim of this study was, therefore, to carry out such investigation.

We first investigated how the representation of different leaf asymmetries (FA, DA and AS) varied according to leaf sample sizes, using three plant species as model systems (objective *i*). *Miconia fallax* DC. (Melastomataceae), *Solanum lycocarpum* St. Hil. (Solanaceae) and *Bauhinia brevipes* Vogel. (Fabaceae) are tropical species that show leaf FA (Alves-Silva, 2012; Alves-Silva and Del-Claro, 2016b; Santos et al.,