Original Articles

Fluctuating asymmetry of leaves is a poor indicator of environmental stress and genetic stress by inbreeding in *Silene vulgaris*

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A B S T R A C T

Fluctuating asymmetry (FA) is often used as a measure of developmental instability and has been proposed as an indicator of both environmental and genetic stress. However, the empirical evidence for the effects of stress on FA in plants is inconsistent, and there are few controlled experimental studies. We analyzed different distance- and shape-based measures of the fluctuating asymmetry of the leaves of clonally replicated self- and cross-pollinated lineages of *Silene vulgaris* (Moench) Garcke (Caryophyllaceae) grown under a control and seven different stress treatments (drought, copper, simulated herbivory, and two levels of nutrient deficiency and of shade). Overall, FA differed among stress treatments, but was not generally higher under stress and even reduced in some treatments. Different measures of FA were only weakly correlated. Inbreeding increased only one measure of leaf FA, and only under high stress intensities. Our findings suggest that in *S. vulgaris* leaf fluctuating asymmetry does not serve as an indicator of environmental stress or of genetic stress by inbreeding.

1. Introduction

Fluctuating asymmetry (FA) is the amount of random deviations from bilateral symmetry (Palmer and Strobeck, 1986) or perfect symmetry of any other form (Graham et al., 2010). FA is often used as a measure of developmental instability, which is the inability of a genotype to buffer its development against random noise (Møller and Shykoff, 1999; van Dongen, 2006). However, differences in FA may also reflect random but repeatable effects of the environment on form (Palmer and Strobeck, 2003). In plants, developmental instability has been shown to increase both with environmental and genetic stress, though not consistently (Palmer and Strobeck, 1986; Freeman et al., 1993; Møller and Shykoff, 1999; Graham et al., 2010). FA has been proposed as a non-destructive measure of stress intensity (e.g. Graham et al., 1993; Leung et al., 2000; Mal et al., 2002; but see Hochwender and Fritz, 1999; Anne et al., 1998). As an indication of genetic stress, FA was increased after inbreeding in flower traits of *Silene diclinis* (Waldmann, 1999) and *Scabiosa canescens* (Waldmann, 2001), as well as in small populations of *Lychnis viscaria* (Siikamäki and Lammi, 1998). However, in many studies inbreeding or increased homozygosity did not increase FA in leaf traits (Sherry and Lord, 1996; Waldmann, 1999) and hybridization may even lead to an increase of FA with hetero-zygosity (Hochwender and Fritz, 1999), so that the consistency of the effects of homozygosity on FA has been questioned (van Dongen, 2006).

Many studies of FA in plants have compared different populations in natural environments (e.g. Freeman et al., 1993; Hódar, 2002; Kozlov et al., 2009; references in Graham et al., 2010), which makes it difficult to clearly separate the effects of the studied stress from that of other environmental influences, e.g. herbivory, and from genetic effects on FA (Siikamäki and Lammi, 1998; Kozlov et al., 2009). Møller and Shykoff (1999) called for controlled experimental studies of environmental and genetic effects on developmental instability of plants. In particular, studies that compare genetically identical plants under different stresses would help to separate the effects of genetic variation and stress (Leamy and Klingenberg, 2005). If FA is increased by stress, a stronger increase of FA with stress is expected under laboratory conditions due to fewer confounding sources of variation, and when there are clear negative effects of the stressor on fitness (Beasley et al., 2013). In a controlled experiment in which *Sinapis arvensis* was grown under six different conditions, stress influenced FA, but different stress treatments increased FA in different organs (leaves, fruits, petals; Roy and Stanton, 1999). In another experiment, different genotypes of *Lotus corniculatus* were grown under four experimental conditions, and FA differed among treatments, but did not correlate well with individual fitness (Andalo et al., 2000).

We used a large experiment in which the biomass and flower production of clonally replicated self- and cross-pollinated *Silene vulgaris* plants was strongly affected by eight different environmental conditions (Sandner and Matthies, 2016) to study the effects of inbreeding and environmental stress on fluctuating asymmetry. To

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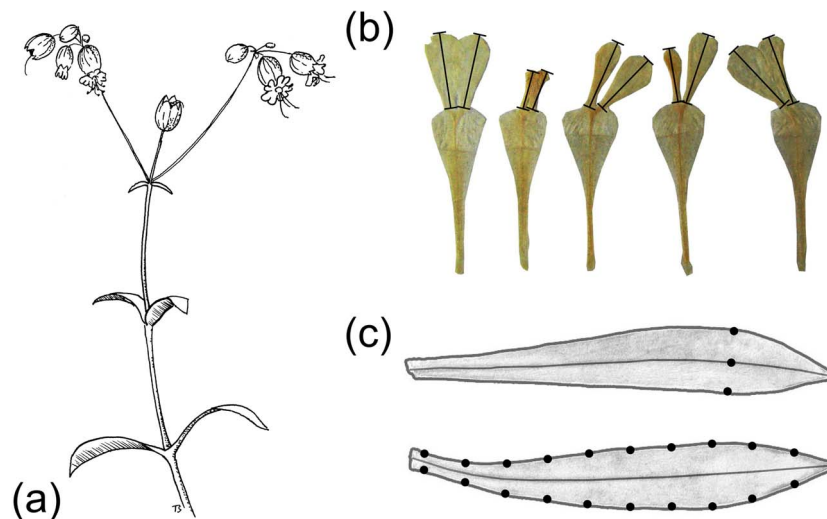
Fig. 1. Study species and measurement of FA. (a) Habitus of *Silene vulgaris*. (b) Pressed petals of a flower of *S. vulgaris* showing how lobe length was measured for the calculation of petal and flower FA. (c) Leaves of *S. vulgaris* showing the three manually set landmarks for calculation of width asymmetry (upper leaf) and the 21 regularly spaced landmarks used for Procrustes analysis (lower leaf). Each method was applied to both leaves of a pair.

our knowledge, this is the first study comparing the effects of many different controlled abiotic stress treatments and inbreeding on various measures of FA. Our hypothesis was that both environmental stress and inbreeding would increase FA as a measure of developmental instability.

2. Methods

2.1. Study species

Silene vulgaris (Moench) Garcke (Fig. 1) is a perennial herbaceous species from the Caryophyllaceae family that naturally occurs throughout Europe and has been introduced into North- and South-America, Australia and Japan (Randall, 2012). *S. vulgaris* is a species of nutrient-poor to moderately nutrient-rich grasslands and roadsides. It is known to grow under a wide range of conditions, including habitats contaminated by heavy metals (Schat and Ten Bookum, 1992). *S. vulgaris* has simple, opposite leaves and radially symmetric flowers consisting of five petals with a deeply bifid blade (Clapham et al., 1987). We chose *S. vulgaris* as a study species because it is self compatible, grows quickly and could be clonally replicated in a pilot study. It was not our aim to test the suitability of *S. vulgaris* as an indicator species, but to use it as a model plant to test if FA is increased by inbreeding and various stresses that reduce the fitness of the plant.

2.2. Stress and inbreeding experiment

The experimental design (pollination of the mother plants, germination and clonal propagation and experimental conditions) is described in detail by Sandner and Matthies (2016). Briefly, seeds from six different mother plants from a natural population in Northern Hesse, Germany, were grown in a greenhouse, and flowers on each plant were either self- or cross pollinated with a pollen mix from the other plants in the experiment. Offspring from self and cross pollinations was then germinated, and individual seedlings (=genotypes) were clonally replicated in-vitro using $2 \, mg \, l^{-1}$ BAP to induce the formation of multiple shoots. Two clonal replicates of each of 29 genotypes were grown under each of eight experimental conditions in a greenhouse. We selected stress treatments which required very different physiological responses of the plants. Based on a pilot study, the intensity of each stress was chosen to be strong without causing mortality. The treatments included (1) control conditions with abundant light, water and weekly fertilization; (2) a drought treatment, in which plants were put

on a balance every 2–3 days and watered to a constant soil water content of 6%; (3) a simulated herbivory treatment, in which plants were cut 2 cm above ground after 5 weeks of growth; (4), a copper treatment, in which pots were added 132.6 mg copper per kg soil as $CuSo_4$ solution during the 3rd week of growth; two nutrient deficiency treatments, in which plants received only (5) 25% or (6) 6.25% of the fertilizer concentration in the control; and two shade treatments, with only (7) 37% or (8) 14% of the light intensity in the control.

After eight weeks of growth, one healthy (i.e. not wilting or senesced) pair of opposite leaves per plant was cut at its base and pressed between two sheets of paper to later analyze fluctuating asymmetry. To reduce variation in leaf shape due to position on the plant, always the third to fifth pair of leaves from the top was chosen. Pressed leaf pairs were scanned at a resolution of 800 dpi. Curved leaves of a pair were arranged on the scanner to face each other (Fig. 1c). To avoid confirmation bias in the estimates of FA (Kozlov and Zvereva, 2015), leaves were scanned in a random order and identified only by a number, which only during statistical analysis was related to the corresponding treatments. After nine weeks of growth the plants were harvested. Although the stresses strongly reduced plant size, only very few plants died. Plant size and number of flowers are important components of the fitness of a plant. Biomass was correlated to flowering probability and the number of flowers, but some plants, especially those grown under shade, did not flower during the experiment (Sandner and Matthies, 2016). For perennial plants like *S. vulgaris*, biomass may be a better estimate of fitness than reproductive traits. From a small subset of plants (n = 40), one flower was sampled and the five petals were individually spread on filter paper, pressed and dried at room temperature.

2.3. Calculation of fluctuating asymmetry

Leaves of *S. vulgaris* are simple, and veins are often not visible except for the midrib. Thus, in the scanned images of the leaves, three landmarks were set manually for every leaf with ImageJ (Rasband 2016) marking the midrib at the widest point of the leaf and the distance to each leaf margin perpendicular to the midrib (upper leaf in Fig. 1c). To estimate and reduce measurement error, three independent measurements were performed on each scan (see Supplement 1 for details). For the analysis of shape-based FA 21 landmarks were positioned regularly along the margin of each leaf (lower leaf in Fig. 1c) with the software LeafAnalyser (Weight et al., 2008). The first landmark marked the leaf tip; the other twenty were arranged counter-