

Methods

Geometric morphometrics of corolla shape: dissecting components of symmetric and asymmetric variation in *Erysimum mediohispanicum* (Brassicaceae)

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Summary

- Symmetry is an important feature of floral structure, and floral symmetries are diverse and often complex. We use a new morphometric approach for analysing shapes with complex types of symmetry, which partitions shape variation into a component of symmetric variation and different components of asymmetry. This approach, based on the mathematical theory of symmetry groups, can be used for landmark configurations with any type of symmetry and is therefore promising as a general framework for morphometric analyses of floral symmetry and asymmetry.
- We demonstrate this approach by quantifying floral shape variation in a wild population of *Erysimum mediohispanicum* (Brassicaceae). Flowers of this species are disymmetric, so that the symmetry in the left–right and adaxial–abaxial directions can be considered separately and in combination.
- Both principal component analysis and Procrustes ANOVA indicate that symmetric variation accounts for most of the total variance and that adaxial–abaxial asymmetry is the dominant component of fluctuating asymmetry. Each component is associated with specific patterns of shape variation.
- These results illustrate the potential of the new method and suggest new areas for future research. The new morphometric approach is promising for further analyses of floral symmetry and asymmetry in evolutionary and developmental contexts.

Introduction

Floral symmetry and its evolution have attracted much attention in plant evolutionary and developmental biology (Coen, 1996; Endress, 2001; Citerne *et al.*, 2010). Evolution of floral symmetry and its developmental basis have been investigated both on a microevolutionary scale (Kim *et al.*, 2008) and across large clades (Zhang *et al.*, 2010; Bartlett & Specht, 2011; Howarth *et al.*, 2011; Busch *et al.*, 2012). Traditionally, flower morphology has been assessed in a qualitative manner, but increasingly investigators are quantifying floral shape and symmetry with the methods of geometric morphometrics (Shipunov & Bateman, 2005; Gómez *et al.*, 2006; Frey *et al.*, 2007; Benitez-Vieyra *et al.*, 2009; Gómez & Perfectti, 2010; Nattero *et al.*, 2010; van der Niet *et al.*, 2010; Kaczorowski *et al.*, 2012). Because floral symmetry is important as a potential target of selection and because it is key to understanding the development and evolution of flowers, morphometric analyses

of floral shape need to identify the patterns of symmetric variation and asymmetry and quantify them. Many flowers show complex symmetry, with multiple components of asymmetry (e.g. left–right, adaxial–abaxial, or asymmetry under rotation), which are challenging for morphometric analysis but offer opportunities for innovative studies in different biological contexts. So far, however, no morphometric study of floral shape has used methods that explicitly take symmetry into account and can separate the different components of symmetric variation and asymmetry.

Morphometric analyses of floral symmetry and asymmetry for zygomorphic flowers are relatively straightforward, because methods for analysing objects with bilateral symmetry are firmly established (Auffray *et al.*, 1999; Mardia *et al.*, 2000; Kent & Mardia, 2001; Klingenberg *et al.*, 2002). Nevertheless, these methods have not yet been used for the study of floral symmetry (but for applications to leaf asymmetry, see Albarrán-Lara *et al.*, 2010; Klingenberg *et al.*, 2012). For other types of floral

symmetry, morphometric analyses are more difficult. Frey *et al.* (2007) proposed a measure of rotational symmetry based on how close a single set of corresponding landmarks is to a regular polygon. Symmetry and asymmetry of algal cells with two perpendicular axes of reflection symmetry were studied with a generalization of the approach for bilateral symmetry (Potapova & Hamilton, 2007; Savriama *et al.*, 2010). Savriama & Klingenberg (2011) offered a further generalization that is applicable to any type of symmetry, which constitutes a general framework for morphometric studies of floral symmetry and asymmetry. This approach is based on the theory of symmetry groups, which offers a rigorous and flexible mathematical foundation for the analysis of symmetric shapes (Weyl, 1952; Armstrong, 1988; Savriama & Klingenberg, 2011). The method is a generalization of the geometric morphometric methods devised for the study of bilateral symmetry (Klingenberg & McIntyre, 1998; Mardia *et al.*, 2000; Kent & Mardia, 2001; Klingenberg *et al.*, 2002). Depending on the type of symmetry under study, this method can separate a component of symmetric variation from one or more components of asymmetric shape changes and it can further resolve the asymmetric components into directional and fluctuating asymmetry (Savriama & Klingenberg, 2011). This paper aims to bring this new method to the attention of plant biologists and to demonstrate it in a first application to the study of floral shape.

Fluctuating asymmetry has been widely used as an indicator of stress or individual quality, with variable results (Møller & Swaddle, 1997; Møller & Shykoff, 1999; Perfectti & Camacho, 1999; Roy & Stanton, 1999; Freeman *et al.*, 2003b; Raz *et al.*, 2011). Fluctuating asymmetry is usually thought to originate from random variation in the developmental processes that produce the structures of interest (Palmer & Strobeck, 1986; Klingenberg, 2003). It is therefore a component of within-individual variation (Herrera, 2009) and is of nongenetic origin, although genetic factors may modulate the expression of fluctuating asymmetry (Queitsch *et al.*, 2002; Klingenberg, 2003; Leamy & Klingenberg, 2005; Takahashi *et al.*, 2011). Because fluctuating asymmetry arises from variation in developmental processes, it is patterned by those processes and the analysis of the patterns of fluctuating asymmetry offers an opportunity to investigate the developmental origin of phenotypic variation (Klingenberg, 2010; Klingenberg *et al.*, 2012).

The developmental processes that are responsible for establishing floral symmetry and asymmetry have been investigated with the tools of developmental genetics and are known in increasing detail (Coen, 1996; Endress, 2001; Citerne *et al.*, 2010). These studies have revealed regulatory networks that play a major role in conferring adaxial or abaxial identities to floral organs (e.g. Coen, 1996; Almeida & Galego, 2005; Busch & Zachgo, 2007; Preston & Hileman, 2009; Citerne *et al.*, 2010). Mutations of genes belonging to these networks can disrupt adaxial–abaxial polarity, but do not affect left–right symmetry (e.g. Preston & Hileman, 2009). By contrast, flowers with left–right asymmetry are relatively rare and no specific developmental pathways are known that establish this type of symmetry (Endress, 2001; Jesson & Barrett, 2002a; Marazzi & Endress, 2008). From this information, it appears that adaxial–abaxial and left–right asymmetries are the result of distinct sets of developmental processes, and therefore it is

important to distinguish and characterize the patterns of fluctuating asymmetry for both components of asymmetry. The new morphometric methods for complex symmetry provide the tools for quantifying these components of fluctuating asymmetry and dissecting their patterns of variation.

We illustrate this approach by analysing symmetric and asymmetric components of floral shape variation in a population of *Erysimum mediohispanicum* grown under natural conditions (Gómez *et al.*, 2006). As usual for the Brassicaceae, flowers of this species have four petals separated by two perpendicular axes of symmetry: one axis divides the petals of the left side from the right side and the other axis bisects the flower into adaxial and abaxial parts. Closer examination, however, reveals that there are many deviations from this ground plan (Fig. 1). A range of different symmetries occur even within single populations, there is a genetic basis for floral shape and symmetry, and different pollinators favour different floral symmetries (Gómez *et al.*, 2006, 2008, 2009a,b,c; Gómez & Perfectti, 2010; Ortigosa & Gómez, 2010). Therefore, *Erysimum* flowers are a particularly interesting and relevant model for studying shape variation and the evolution of floral symmetry. Here we demonstrate the new morphometric approach to quantify the different components of symmetry and asymmetry and to investigate the patterns of variation associated with them. The case study demonstrates the utility of specifically quantifying symmetry and asymmetry for studies of floral morphology in evolutionary, functional and developmental perspectives (Breuker *et al.*, 2006a; Klingenberg, 2010; Murren, 2012).

Materials and Methods

Data

We analysed the landmark data for flowers from a wild population of *Erysimum mediohispanicum* in the mountains of Sierra Nevada (southeast Spain; 37°4.8'N, 3°27.9'W; 1830 m elevation). The same data were previously used in the study of Gómez *et al.* (2006). This data set is derived from photographs of 193 flowers, each flower from a different plant. Because the distances between plants greatly exceeded the very small dispersal distances in this population (Gómez, 2007), the plants can be considered as a random sample from the population. For each flower, 32 landmarks were digitized, either at the points of intersection of primary and secondary veins with the petal margin or at the base of the petals (Fig. 1a; Gómez *et al.*, 2006; Gómez & Perfectti, 2010).

All analyses were carried out using MORPHOJ (Klingenberg, 2011) and SAS/IML software (SAS Institute Inc, 2004).

Shape analysis for complex symmetry

We applied a new morphometric method for the analysis of landmark configurations with any type of symmetry, which combines the theory of symmetry groups with the methods of geometric morphometrics (Savriama & Klingenberg, 2011). Here, we briefly summarize this method in the context of the current study; for additional details, readers should consult the paper by Savriama & Klingenberg (2011).



Fig. 1 Floral shape diversity in a wild population of *Erysimum mediohispanicum* (Brassicaceae). (a) Example of a flower that is almost perfectly symmetric about both the left–right and adaxial–abaxial axes. The configuration of landmarks used in the analysis is also shown. (b) Example of a flower with adaxial–abaxial asymmetry that is symmetric in the left–right direction (zygomorphy). (c) Example of a flower with left–right asymmetry, but with little adaxial–abaxial asymmetry. (d) Example of a completely asymmetric flower, both in the adaxial–abaxial and left–right directions.

In geometry, symmetry is defined as invariance under specific transformations (Weyl, 1952; Armstrong, 1988). Examples of transformations of this kind are reflection about a line or plane (from an object to its mirror image) or rotation by an angle that is an integer fraction of 360° (e.g. 90° or 180°). Another example of a transformation is the identity, which leaves the object unchanged (it is equivalent to two successive reflections about the same axis or a rotation by 360°). An object is considered symmetric if it remains the same after a transformation has been applied to it. The object's symmetry can be characterized by the set of transformations that leave it unchanged in this manner. These transformations are called symmetry transformations. For instance, zygomorphic flowers are symmetric under two transformations: reflection about the median plane and the identity. Disymmetric flowers are symmetric under reflections about two perpendicular axes, their combination (a rotation by 180°) and the identity. Equivalently, disymmetry can be defined by a reflection about one axis and a rotation by 180° , their combination (a reflection about the perpendicular axis) and the identity (Fig. 2).

Every type of symmetry is characterized by a set of symmetry transformations. Because this set fulfils the criteria for a special kind of set, termed a group, it is called the symmetry group for that type

of symmetry, and the mathematical tools of group theory can be used (Weyl, 1952; Armstrong, 1988). For instance, every combination of symmetry transformations is itself a symmetry transformation (e.g. applying reflection about the same axis twice yields the identity) and is therefore included in the symmetry group.

To incorporate the information on symmetry and the structure of the symmetry group in the framework of geometric morphometrics, the original landmark configurations are copied and one transformation from the symmetry group is applied to each copy. The landmarks of the transformed copies need to be relabelled so that the original and transformed configurations are compatible (e.g. exchanging the identities of corresponding landmarks on the left and right sides of a zygomorphic flower after the reflection about the midline). For instance, two copies of the landmark configurations of zygomorphic flowers are made and one is reflected and relabelled (the other copy is left unchanged, as the identity is applied to it), or for a disymmetric flower, four transformed and relabelled copies are made (Fig. 2; the count of four copies includes the copy that is left unchanged).

All the transformed and relabelled copies are then entered together in a generalized Procrustes superimposition (e.g. Dryden & Mardia, 1998). The consensus shape from this Procrustes fit is

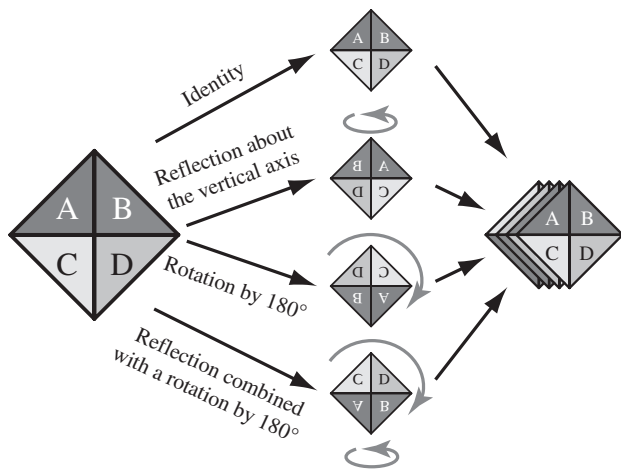


Fig. 2 Shape analysis of a symmetric structure with two perpendicular axes of reflection symmetry, for example a disymmetric flower. The object is symmetric under a set of four symmetry transformations: reflection about the vertical axis, rotation by 180° (which is equivalent to two successive reflections about the vertical and horizontal axes), the identity (equivalent to a rotation by 360°) and a combination of reflection with rotation by 180° (equivalent to a reflection about the horizontal axis). All four symmetry transformations are applied to separate copies of each landmark configuration and shape variation is extracted from the combined data by a Procrustes fit (for details, see the section on 'Shape analysis for complex symmetry' in this article, and Savriama & Klingenberg, 2011).

guaranteed to be perfectly symmetric (i.e. it is invariant under all transformations in the symmetry group; Savriama & Klingenberg, 2011). This procedure of copying, transforming and relabelling configurations and then subjecting them to a generalized Procrustes fit is a direct extension of the established method for bilateral symmetry (Mardia *et al.*, 2000; Klingenberg *et al.*, 2002) and has been applied to reflection symmetry about two perpendicular axes before (Potapova & Hamilton, 2007; Savriama *et al.*, 2010; Savriama & Klingenberg, 2011). The key advantage of the perspective based on symmetry groups is that it is completely general and can be applied to any type of symmetry (Savriama & Klingenberg, 2011).

The deviations of the Procrustes coordinates from the consensus configuration can be decomposed into a component of symmetric variation and one or more components of asymmetry by computing the averages or differences of the copies. The component of symmetric variation represents the variation among individuals that affects all parts equally and is obtained by averaging all transformed and relabelled copies for each individual. One or more components of asymmetry can be obtained by calculating differences among the transformed and relabelled copies of each individual. For disymmetric flowers, there are three components of asymmetry: a component of variation that is asymmetric under reflection in the adaxial–abaxial direction but is left–right symmetric, a component that is asymmetric in the left–right direction but has adaxial–abaxial symmetry and, finally, a component of variation that is asymmetric concerning reflection about both axes but is symmetric under rotations by 180° . Note that, in this case, there is no component of shape variation that is totally asymmetric because of the constraints imposed by the Procrustes fit; completely asymmetric patterns result from combining multiple components of asymmetry (for more details see Savriama & Klingenberg, 2011).

Principal component analysis

The different components of symmetric and asymmetric shape variation occupy orthogonal subspaces of shape tangent space (Savriama & Klingenberg, 2011). The structure of these subspaces, including the amount of variation that is apportioned to each of them and the associated shape changes, can be explored by a principal component analysis (PCA) of the variation of Procrustes coordinates in the complete sample of all transformed and relabelled copies of landmark configurations (Savriama *et al.*, 2010; Savriama & Klingenberg, 2011). Each principal component (PC) unambiguously belongs to one of the subspaces, and the associated shape change shows the corresponding type of symmetry or asymmetry. The amount of variation in each subspace can be obtained by summing up the eigenvalues associated with the PCs that have the respective type of symmetry or asymmetry. Note that this approach does not distinguish between fluctuating and directional asymmetry, but quantifies the overall amount of asymmetry in each of the subspaces.

Procrustes ANOVA

An alternative approach for quantifying the different components of variation is Procrustes ANOVA (Klingenberg & McIntyre, 1998; Klingenberg *et al.*, 2002; Savriama & Klingenberg, 2011). The ANOVA approach was originally developed for linear measurements of bilaterally symmetric structures and was a two-factor, mixed-model ANOVA design containing individuals and sides as the factors (Leamy, 1984; Palmer & Strobeck, 1986). In this context, directional asymmetry is the main effect of side, whereas fluctuating asymmetry is the interaction between individuals and sides. This interpretation implies that directional asymmetry is defined as the average asymmetry and fluctuating asymmetry as the individual variation of asymmetry. The approach was adapted for shape analyses with Procrustes methods (Klingenberg & McIntyre, 1998; Klingenberg *et al.*, 2002) and was recently generalized to any type of symmetry (Savriama & Klingenberg, 2011).

The analysis includes all the transformed and relabelled copies of the landmark configurations in the sample. The factors in the ANOVA model are the individual and one or more ‘asymmetry factors’ designating the transformations of the copies (e.g. reflections or rotations). For complex types of symmetry, more than one asymmetry factor may be needed in the Procrustes ANOVA to reflect the structure of the symmetry group (Savriama & Klingenberg, 2011). For the data in our study, two reflections are included in addition to the factor of individuals: the adaxial–abaxial and the left–right reflections. This model is equivalent to a model with one reflection and a rotation by 180° (Savriama & Klingenberg, 2011). In either version, there are three components of directional asymmetry: one for adaxial–abaxial differences, one for left–right differences, and one of ‘diagonal’ differences of the upper-left and lower-right petals vs the lower-left and upper-right petals (in the ANOVA, these are the main effects of adaxial–abaxial and left–right reflections and the interaction between the adaxial–abaxial and left–right reflections). Similarly, there are three corresponding

components of fluctuating asymmetry, the two-way interactions of individuals with the two reflections and the three-way interaction of individuals with both reflections, which stand for the individual variation in the respective components of asymmetry.

The statistical significance test of the main effect of individuals faces a difficulty, because fluctuating asymmetry, which is used as the error effect in that test, does not occupy the same subspace of shape tangent space (this problem also exists for bilateral symmetry; Klingenberg *et al.*, 2002). To address this problem, we adopted an approach akin to Goodall's *F* test (Goodall, 1991), which is based on a null model assuming isotropic variation throughout the entire shape tangent space. We computed a combined estimate of fluctuating asymmetry by pooling sums of squares across all three subspaces of asymmetric variation (Savriama & Klingenberg, 2011). For each of the three components of directional asymmetry, the fluctuating asymmetry for the respective subspace was used as the error effect. Statistical tests use Goodall's *F* (Goodall, 1991) as it has been extended for Procrustes ANOVA (Klingenberg & McIntyre, 1998; Klingenberg *et al.*, 2002; Savriama & Klingenberg, 2011). The Procrustes ANOVA provides sums of squares and mean squares for all effects, which can be used to quantify the variation associated with the different components of symmetry and asymmetry.

Results

Principal component analysis

The PCA of the combined data consisting of all transformed and relabelled copies of landmark configurations yields 60 PCs with nonzero eigenvalues (i.e. PCs that account for some variation). This outcome reflects the fact that the shape tangent space has 60 dimensions—the number of landmark coordinates (twice the total number of landmarks, 32) minus four dimensions due to the constraints imposed by the Procrustes fit (two for translation and one each for scaling and rotation). These 60 PCs can be partitioned into four different categories according to the symmetry of the associated shape changes (Fig. 3). Each category encompasses 15 PCs that span one of the four subspaces of the shape tangent space, which correspond to different components of symmetric or asymmetric shape variation. For each of the four components of variation, the shape changes of the five PCs that account for the most variation are shown in Fig. 3.

The first category of PCs is the component of completely symmetric shape variation, which subsumes shape changes that are symmetric under reflections in both the left–right and adaxial–abaxial directions and also under rotation by 180° (Fig. 3a). For instance, the shape changes associated with PC1 are mainly rotations of the petals, so that the whole flower varies between a more square and a more rectangular outline, whereas PC4 is associated with changes in the relative widths of the four petals. For all the PCs in this category, there are identical changes of all four petals (all four quadrants of the flower). Together, the 15 PCs associated with the completely symmetric component of shape variation account for 49.2% of the total variance.

The second class consists of shape changes that are asymmetric under reflection in the adaxial–abaxial direction but show left–right

symmetry (Fig. 3b). The shape changes for this component of variation show opposite modifications in the adaxial and abaxial petals, but the changes are the same in the left and right halves of the flower. For instance, for a positive change in PC2 the adaxial petals turn upwards and towards each other, whereas abaxial petals diverge from each other laterally. For PC7, the changes are in the relative sizes of the adaxial and abaxial petals. The 15 PCs that span this subspace of adaxial–abaxial asymmetry account for 22.8% of the total variance.

The third component is left–right asymmetry combined with symmetry in the adaxial–abaxial direction (Fig. 3c). The shape changes of this class have opposite effects on the left and right sides, but affect the adaxial and abaxial halves in the same way. For example, for a change of PC3, all four petals bend towards the left or the right side, or the relative sizes of the left and right petals change for PC8. The 15 PCs associated with this component of shape variation account for 18.2% of the total variance.

Finally, the fourth class of shape changes is asymmetric under reflection about either of the two axes but symmetric under rotations by 180° (Fig. 3d). This means that there are corresponding changes in the petals that are diagonally opposite each other, and opposite changes in the petals that are next to each other in the left–right or adaxial–abaxial direction. For instance, the shape change in the positive direction for PC5 is an expansion of the right adaxial and left abaxial petals relative to the left adaxial and right abaxial petals, whereas PC10 is associated with relative rotations of petals and relative shifts of the petal bases (Fig. 3d). The 15 PCs that occupy this subspace account for 9.8% of the total variance.

Procrustes ANOVA

The Procrustes ANOVA differs from the PCA in that it separates directional from fluctuating asymmetry, but is otherwise largely consistent with the PCA. The sum of squares for the main effect of individuals is the same as the total of the eigenvalues of the symmetric component of variation in the PCA, and accounts for just under half of the total sum of squares (Table 1). For each of the three components of asymmetry, the Procrustes ANOVA separates directional asymmetry, the main effects of the two reflections and their interaction, from fluctuating asymmetry, the two- and three-way interactions of the reflections with individuals. For all three components of asymmetry, directional asymmetry is highly significant statistically, but accounts for only a small proportion of the total variation.

The amount of adaxial–abaxial asymmetry exceeds the amount of left–right asymmetry both for directional and fluctuating asymmetry, although the difference is not dramatic (Table 1). In addition, there appears to be more fluctuating asymmetry concerning the adaxial–abaxial reflection or the left–right reflection (interactions of the respective reflections with individuals; Table 1) than there is fluctuating asymmetry about both axes (the three-way interaction of the adaxial–abaxial and left–right reflections and individuals).

The patterns of the three components of fluctuating asymmetry, as they can be obtained from a PCA of the respective matrices of sums of squares and cross-products (not shown), are almost

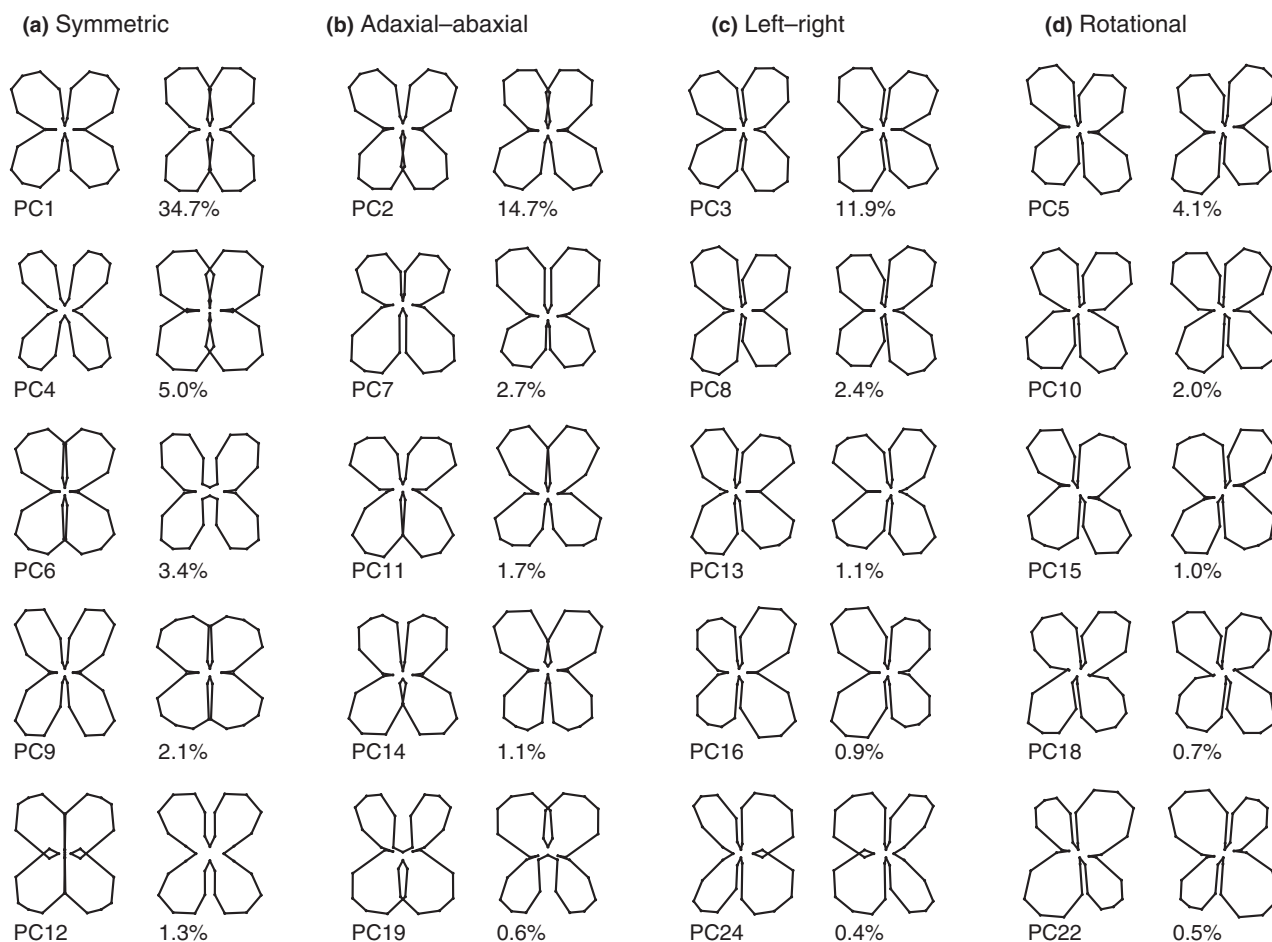


Fig. 3 Decomposition of shape variation in *Erysimum mediohispanicum* (Brassicaceae) flowers with two perpendicular axes of symmetry. The first five principal components (PCs) for each category of symmetry or asymmetry are shown. For each PC, the diagrams show the shapes that correspond to scores of -0.1 (left diagram) or $+0.1$ (right diagram) for the respective PC and scores of 0.0 for all other PCs. The percentages represent the part of the total shape variation for which each PC accounts. (a) Symmetric component. (b) Adaxial–abaxial asymmetry. (c) Left–right asymmetry. (d) Symmetry under rotations by 180° , with asymmetry under adaxial–abaxial and left–right reflection.

Table 1 Procrustes ANOVA for *Erysimum mediohispanicum* flowers

Source	Degrees of freedom	Sums of squares	Mean squares	<i>F</i>	<i>P</i>
Individual	2880	17.822	0.00619	3.01	< 0.000001
Ad–Ab reflection	15	0.244	0.01627	5.86	< 0.000001
L–R reflection	15	0.167	0.01114	5.01	< 0.000001
Ad–Ab reflection × L–R reflection	15	0.222	0.01478	12.74	< 0.000001
Ad–Ab reflection × individual	2880	8.001	0.00278		
L–R reflection × individual	2880	6.406	0.00222		
Ad–Ab × L–R × individual	2880	3.342	0.00116		
[Total FA]	8640	17.7497	0.00205		

The main effect of individuals is tested against the mean square for the total fluctuating asymmetry (Total FA, pooling sums of squares and degrees of freedom across all three subspaces with asymmetric variation: Ad–Ab reflection × individual, L–R reflection × individual and Ad–Ab × L–R × individual). This total asymmetry is not used otherwise in the analysis. Ab, abaxial; Ad, adaxial; L, left; R, right.

identical to those obtained in the PCA of deviations from the symmetric consensus shape (Fig. 3b–d). The reason for this close agreement of the two types of PCAs is that directional asymmetry accounts for very little of the total asymmetry.

Discussion

In this example, we have demonstrated the new morphometric approach for investigating floral shapes with complex symmetry. The results of this case study are useful to highlight some of the possibilities of this method, but they also raise some issues of general relevance for morphometric studies of symmetry and asymmetry in plants.

PCA and Procrustes ANOVA indicate that just under half of the total corolla shape variation consists of completely symmetric shape changes. This component represents the variation among flowers that affects the shapes and relative positions of all four petals equally (Fig. 3a). The other half of the total variation is asymmetry, mostly fluctuating asymmetry and a relatively small amount of directional asymmetry.

Directional asymmetry is statistically highly significant in all three components of asymmetry, but it is very subtle (Table 1). This result indicates that there are systematic differences among the four quadrants of the flowers. For the adaxial–abaxial component, this is a differentiation as it is characteristic for zygomorphic flowers, and corresponds to a tendency reported for this population before (Gómez *et al.*, 2006). In contrast, directional asymmetry in the other two components of asymmetry is much harder to interpret. Directional asymmetry in all three components is quite subtle and, as a contribution to the asymmetries of individual flowers, is negligible by comparison to the magnitude of fluctuating asymmetry. This result corresponds to findings from animals, including humans, where most studies using geometric morphometrics have observed subtle but statistically significant directional asymmetry (Klingenberg *et al.*, 1998, 2002, 2010; Debat *et al.*, 2000; Schaefer *et al.*, 2006; Ercan *et al.*, 2008; Savriama & Klingenberg, 2011). Whether such subtle directional asymmetry of flower shape is widespread—clearly relevant for topics such as the evolution of zygomorphy and completely asymmetric flowers—needs to be studied further.

Among the components of asymmetric shape variation, adaxial–abaxial asymmetry accounts for the biggest share of variation (Table 1, Fig. 3b). This feature corresponds to variation in the degree of zygomorphy of the flowers, which has been found to be a prime aspect of phenotypic and genetic variation of flower shape in this species (Gómez *et al.*, 2006, 2008, 2009b). This type of asymmetry also has been implicated in natural selection on *Erysimum* flower shape (Gómez *et al.*, 2006); indeed, it is an important aspect of floral evolution in Brassicaceae and throughout angiosperms (Busch & Zachgo, 2009; Citerne *et al.*, 2010; Knapp, 2010; Busch *et al.*, 2012). Developmental genetic studies have demonstrated a network of regulatory genes that establish adaxial and abaxial identities of flower organs, and changes in the expression of the respective genes have been associated with evolutionary transitions to and from zygomorphy (Busch & Zachgo, 2007; Kim *et al.*, 2008; Citerne *et al.*, 2010; Zhang *et al.*, 2010; Bartlett & Specht, 2011; Howarth *et al.*, 2011; Busch *et al.*, 2012). Variation in the activities of these regulatory networks may contribute to the observed level of variation in adaxial–abaxial asymmetry.

A further component of asymmetric shape variation is left–right asymmetry (Table 1, Fig. 3c). Left–right asymmetry is somewhat intriguing because the developmental mechanisms that are responsible for it are unknown (note, however, that the fact that no mechanism is known does not imply that no mechanism exists). Examples of such asymmetry include enantiostyly, where the style points to the left or right side of the flower, and asymmetries that involve the entire flower (Tucker, 1999; Jesson *et al.*, 2003; Etcheverry *et al.*, 2008; Marazzi & Endress, 2008). There is evidence that left- or right-sidedness in enantiostyly has a genetic basis in some instances (Jesson & Barrett, 2002a,b), but no specific genes or molecular pathways have been discovered that control any of these asymmetries.

The last component of asymmetry consists of shape changes that are asymmetric under reflection about both the left–right and adaxial–abaxial axes, but symmetric under rotation by 180°. This

component includes some variation that is symmetric under rotation by 90°, but does not appear in the PC patterns, and includes phenomena such as flower contortion (Endress, 1999). The corresponding shape changes are twisting or diagonal deformations of the flower (Fig. 3d). The amount of fluctuating asymmetry for this component is distinctly smaller than those for adaxial–abaxial or left–right asymmetry (Table 1). There are known mechanisms that can produce this type of asymmetry. In *Arabidopsis thaliana*, mutations in α -tubulin genes have been shown to cause twisted growth of the petals in response to cytoskeletal defects, which led to rotational symmetry of flowers and reflection asymmetry (Furutani *et al.*, 2000; Thitamadee *et al.*, 2002). Twisting of petals and other floral organs is also involved in the development of completely asymmetric flowers (e.g. Etcheverry *et al.*, 2008; Marazzi & Endress, 2008).

The three components of floral asymmetry appear to relate to different biological processes. Because these components occupy orthogonal subspaces in shape tangent space, separating and quantifying the phenotypic outputs of these processes are straightforward. Combination of such morphometric analyses of asymmetry with developmental genetic and comparative study designs will be a powerful and promising strategy for investigating the evolution and development of floral shape.

Fluctuating asymmetry is generally considered to originate from developmental noise, that is, from random perturbations of the developmental processes involved in producing a structure. Such perturbations produce differences between repeated parts, for instance different petals or left and right sides, even if parts are genetically identical and develop in the same environment (e.g. Klingenberg, 2003). Environmental stresses and genetic factors can affect the predisposition to fluctuations in developmental processes or influence how such variation is expressed in the phenotype (Klingenberg & Nijhout, 1999; Roy & Stanton, 1999; Queitsch *et al.*, 2002). Even though these external factors can mediate the expression of developmental noise, the actual asymmetries arise from random differences among repeated parts in the activities of developmental processes. It may therefore be possible to relate the patterns and amounts of fluctuating asymmetry, as well as shape variation among flowers, to the mechanisms of flower development. Several studies of this kind have been conducted in animal models such as *Drosophila* (Breuker *et al.*, 2006b; Debat *et al.*, 2006, 2011) or mice (Klingenberg *et al.*, 2003; Willmore *et al.*, 2006; Jamniczky & Hallgrímsson, 2009). In flowers, analyses of the different components of fluctuating asymmetry can provide additional information that is unique to structures with complex symmetry. This is an application of morphometric approaches that is so far unexplored, but holds considerable potential.

This discussion has focussed on the traditional view that fluctuating asymmetry of flowers is the expression of intrinsic instability in development, so that it is present even in a completely homogeneous environment (Møller & Shykoff, 1999; Freeman *et al.*, 2003b). Another possible source of fluctuating asymmetry, however, is plasticity in response to localized variation in the immediate surroundings of developing flowers. This mechanism is well established for variation among whole flowers, leaves or fruits within individual plants (reviewed by Herrera, 2009), but might

also apply to asymmetry within these structures. Asymmetry can arise from plastic responses to heterogeneity, for instance, in the solar irradiation or in the flow of sap to different parts of the flower bud. Because plants are sessile, their parts are thus exposed to heterogeneity in their microenvironment constantly throughout development and plasticity is a mechanism that can produce fluctuating asymmetry, in addition to developmental instability. This is different from fluctuating asymmetry in motile animals, which move through their environment so that, over the entire period of development, differences between sides cancel out and both sides experience effectively the same environment (Klingenberg, 2003). There is experimental evidence that plasticity in response to light can produce asymmetry in leaves (Freeman *et al.*, 2003a) and can therefore contribute to the substantial levels of fluctuating asymmetry often observed in leaf shape (Klingenberg *et al.*, 2012). If this reasoning also applies to asymmetry of flower shape, the patterns of asymmetry induced by such plasticity would reflect the spatial distribution of heterogeneity of the relevant microenvironmental factors. Unfortunately, it is not possible to separate or quantify the contributions of plasticity and developmental noise to the fluctuating asymmetry observed in our data. The contribution of plasticity to fluctuating asymmetry in plants needs to be demonstrated and quantified in studies designed for this specifically; the morphometric methods for complex symmetries are promising tools for this purpose.

Analyses in *Erysimum* (Gómez *et al.*, 2006, 2008, 2009a,c; Gómez & Perfectti, 2010) and other plants (Herrera, 1993; Benitez-Vieyra *et al.*, 2009; Nattero *et al.*, 2010; Gaskett, 2012; Kaczorowski *et al.*, 2012) suggest that pollinators exert selection on floral shape. Symmetry is important in plant–pollinator interactions because insects can perceive, and appear to prefer, left–right symmetry (Møller, 1995; Giurfa *et al.*, 1999; Rodríguez *et al.*, 2004; but see Plowright *et al.*, 2011). Also, some plant–pollinator systems favour flowers where adaxial and abaxial petals are differentiated and may perform different functions, for instance if abaxial petals function as a landing platform or to guide pollinating insects. These aspects of floral asymmetry concern two different components of shape asymmetry and occupy two separate subspaces of shape tangent space. Accordingly, analyses of selection on these two aspects of asymmetry can be conducted separately, using the appropriate component of asymmetry. The new morphometric methodology allows investigators to conduct analyses that specifically target particular components of variation.

The results presented here resemble those from earlier analyses of overall corolla shape without *a priori* separation into components of variation according to symmetry and asymmetry. The shape features captured in the first few PCs in our analysis (Fig. 3) resemble those of the corresponding PCs in the same data (Gómez *et al.*, 2006), for other data sets for this species (Gómez *et al.*, 2008, 2009b) and even for data from other species of *Erysimum* (Ortigosa & Gómez, 2010; our unpublished analyses of the data of Abdelaziz *et al.*, 2011). The resemblance of shape features among corresponding PCs is particularly close for the analyses with the largest sample sizes (Gómez *et al.*, 2009b; Fig. 2). This consistency of the main patterns of shape variation, obtained by applying different morphometric methods to several independent data sets, indicates

that symmetry and asymmetry are fundamental for floral shape variation in *Erysimum*. This outcome is not universal, however, as is evident from another study where the shape changes associated with PCs of overall floral shape do not show identifiable types of symmetry or asymmetry (Nattero *et al.*, 2010). In such cases, morphometric methods that specifically take into account floral symmetry are the only way to separate the different components of variation.

The limitations of the approach are that the user needs to specify the type of symmetry and the correspondence of landmarks. Because the type of symmetry is clear for most flowers, the requirement to choose a specific type of symmetry is not likely to cause difficulties in practice. By contrast, finding landmarks that clearly correspond across the different parts of each flower and among the flowers included in a study can be challenging. It may seem tempting to use outline methods to avoid this difficulty, but each algorithm used for analysing outline data also makes assumptions about the correspondence of points along the outline, even though they may not always be apparent to the user (Klingenberg, 2008). In principle, the approach of using copies that are transformed and relabelled according to the symmetry group is also applicable for semilandmarks and similar approaches (Bookstein, 1997; McCane & Kean, 2011), although there may be difficulties in practice. In many instances, however, landmarks can be found, as is clear from the growing number of studies using landmark methods for investigating floral shape.

In conclusion, the new morphometric method for complex symmetries is promising for studies of floral shape. It can quantify biologically meaningful components of symmetric and asymmetric shape variation and analyse the patterns of variation associated with each component. Different components of symmetric variation and asymmetry are of interest for investigating selection by pollinators, developmental instability and plasticity, as well as for taxonomic studies. We have demonstrated this type of analysis for one example, but we stress that it can accommodate any possible type of symmetry (Savriama & Klingenberg, 2011). The morphometric tools demonstrated here are opening a range of new possibilities for studying floral shape in evolutionary, developmental and ecological contexts.

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