

GEOMETRIC MORPHOMETRICS OF FUNCTIONALLY DISTINCT FLORAL ORGANS IN *IRIS PUMILA*: ANALYZING PATTERNS OF SYMMETRIC AND ASYMMETRIC SHAPE VARIATIONS

Sanja Radović*, Aleksandar Urošević, Katarina Hočevar, Ana Vuleta, Sanja Manitašević Jovanović and Branka Tucić

Department of Evolutionary Biology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia

*Corresponding author: sanja.radovic@ibiss.bg.ac.rs

Received: Revised: September 12, 2016; **Accepted:** September 21, 2016

Abstract: The *Iris* flower is a complex morphological structure composed of two trimerous whorls of functionally distinct petaloid organs (the falls and the standards), one whorl of the stamens and one tricarpeal gynoecium. The petal-like style arms of the carpels are banded over the basal part of the falls, forming three pollination tunnels, each of which is perceived by the *Iris* pollinators as a single bilaterally symmetrical flower. Apart from the stamens, all petaloid floral organs are preferentially involved in advertising rewards to potential pollinators. Here we used the methods of geometric morphometrics to explore the shape variation in falls, standards and style arms of the *Iris pumila* flowers and to disentangle the symmetric and the asymmetric component of the total shape variance. Our results show that symmetric variation contributes mostly to the total shape variance in each of the three floral organs. Fluctuating asymmetry (FA) was the dominant component of the asymmetric shape variation in the falls and the standards, but appeared to be marginally significant in the style arms. The values of FA indexes for the shape of falls (insects' landing platforms) and for the shape of standards (long-distance reward signals) were found to be two orders of magnitude greater compared to that of the style arms. Directional asymmetry appeared to be very low, but highly statistically significant for all analyzed floral organs. Because floral symmetry can reliably indicate the presence of floral rewards, an almost perfect symmetry recorded for the style arm shape might be the outcome of pollinator preferences for symmetrical pollination units.

Key words: directional asymmetry; geometric morphometrics; floral organ shape; fluctuating asymmetry; *Iris pumila*

INTRODUCTION

Angiosperm flowers are complex phenotypic structures, which are composed of four functionally distinct organs arranged in concentric floral whorls [1-3]. The outer two whorls constitute the perianth, with sepals in the first and petals in the second whorl, while the inner two whorls consist of reproductive structures: the male-functioning stamens in the third and the female-functioning carpels in the fourth whorl. An immense morphological diversity of the floral organ shapes in the extant angiosperm taxa is thought to be the outcome of different selective pressures, affecting each floral organ separately [4,5].

The essential function of angiosperm flowers is sexual reproduction, a phenomenon which involves two separate processes: the transfer of the male gametes to the female ovules (pollination) and the fusion of sperm and egg cells (fertilization) [5-8]. In plants that use foraging insects for pollen transfer, floral organs are greatly modified in order to attract pollinators and to facilitate the movement of pollen from one flower to the insect and from

the insect back to the next flower. Such modifications (or floral signals) include flower shape, color, scent, heat and even floral electric fields [9,10]. Because visiting insects are “paid” for their pollination services by means of nectar carbohydrate and edible pollen, floral displays advertise, in fact, the plants’ floral rewards. It has been pointed out that “pollinator perception and its associated behavior constitute a key selective environment for floral traits because it mediates the relationships between floral signals and pollen receipt and export” [11]. The pollinator-mediated selection can be very strong, favoring floral signals that exploit the sensory preferences of pollinators that are locally abundant [11]. The shape of flower corollas is considered to be an important advertising signal that can attract the pollinators. A number of studies have shown that the levels of corolla shape variation within and among conspecific populations or species reflect the differences in the preference patterns of their local pollinators [5,12-17].

The innate sensory preferences have been reported to enhance the foraging efficiency of bumblebees, one of the most common pollinators in many plant taxa [17]. For example, the flowers of *Epilobium angustifolium* that were visited by bumblebees in a natural habitat were larger and more symmetrical than those of their nearest neighbors. Nectar production was also greater in symmetrical flowers, explaining the bumblebee preference for flower symmetry. In that context, fluctuating asymmetry, defined as small random deviations from otherwise bilateral symmetry, would be a measure of the phenotypic quality of individuals, indicating their capability to control development under given genetic and environmental conditions [18].

Geometric morphometrics (GM) have been increasingly used as a powerful tool for quantifying the shape variation of structures with complex types of symmetry [19-22]. These methods can partition the shape variation into a symmetric and asymmetric component [20]. GM analyses have been widely used in zoological studies, however, botanical investigations based on GM are still very scarce [5,16,21-24].

In this study, we used the methods of GM [22,25,26] to identify patterns of symmetric and asymmetric shape variation in three functionally distinct floral organs (fall, standard and style arm) of an insect-pollinated monocotyledonous plant, *Iris pumila*. Similarly to other congeneric species, the *Iris* flower consists of four trimerous floral whorls, whose elements are symmetrically arranged around the central axis [27-29] (Fig. 1A). The outer two whorls are composed of petaloid structures called tepals. The tepals are fused at the base, forming a floral tube. The outer tepals, called falls, are bent downward, serving as a landing platform for pollinating insects. The upper surface of falls, ranging from the base to the center, are equipped with a beard consisting of short fine hairs, which functions as a foothold for pollinating insects when they enter the flower in search of rewards. The vertically oriented inner tepals, called standards, are involved in the long-distance pollinator attraction. The reproductive organs occupy the two inner floral whorls: the male sex organs, the stamens, are positioned in the third whorl, while the female sex organs, the petaloid style arms with the stigmatic lips at the top, occupy the fourth whorl, in the center of the flower. A conspicuous feature of *I. pumila* flowers are their pollination tunnels, each of which is made up of the haft of the fall (the dorsal side) and the faced style arm (the ventral side), bending over a single stamen [29]. Due to such architecture, each pollination tunnel is perceived by pollinating insects as a zygomorphic flower [29].

The genus *Iris* is comprised of about 250 species [27], which are exclusively pollinated by Hymenoptera, mostly bumblebees [30]. A number of studies on pollination success in different *Iris* species provided evidence that the large-sized flower, which is characteristic for these taxa [31-35], might have been shaped by pollinator-mediated selection, either because a larger floral display implies more floral rewards or it can be better detected from a distance [31-35]. Since the primary pollinators in *I. pumila* are also bumblebees and because the

higher ramets with larger flowers are more often pollinated than the shorter ones (A. Vuleta, personal observation), we suppose that the floral organ symmetry may be equally attractive for its pollinators as the large flower display. If this supposition is correct, we expect a higher bilateral symmetry in the shape of floral organs involved in pollen transfer, such as the petaloid style arms, compared to the falls and standards, which are involved in attracting pollinating insects in *Iris* species. If the symmetry of floral organs is irrelevant for their pollinators, all three floral organs should exhibit similar patterns of shape variation.

MATERIALS AND METHODS

Study species and experimental set-up

Iris pumila is a rhizomatous perennial herb naturally growing at the exposed dune sites in the Deliblato Sands (44°47'N/21°20'E), Serbia [36]. Natural populations of *I. pumila* are polymorphic for flower color, which can be attributed to the segregation at several gene loci [37]. Consequently, each of the flower color morphs commonly found in a population is thought to be a unique clonal genotype [38].

The *Iris* plants used in this study were pot-grown in an experimental garden in the yard of the Institute for Biological Research "Siniša Stanković" in Belgrade (44°49'2.94"N/20°29'15.51"E). The plants originate from a reciprocal factorial experiment conducted at the Deliblato Sands in April 1996 during the blooming phase of *I. pumila*. Seeds obtained from these crosses were germinated singly in plastic pots in a growth-room and the developed seedlings, after repotting into clay pots, were transferred at the age of six months to the experimental garden, where they still grow under common garden conditions as 20-year-old adult clones [39,40].

Collection of samples

Flowers were collected each day from March 21 to April 1, 2008, starting at 11:00 and finishing at about 15:00. From each of 101 pots, two flowers were harvested and put immediately in 50-ml bottles filled with 70% ethanol. The conserved flowers were stored at room temperature until dissection. During dissection, each flower was cut at the end of the floral tube to separate the floral organs, which were then spread over a glass plate coated with 50% glycerol. Digital images (600 dpi resolution) of three floral organs, the fall, standard and style arm, were recorded using an optical scanner (CanoScan 5600F).

Morphometric data

The shape variation of floral organs in *I. pumila* was quantified by the methods of geometric morphometrics [22,41,42]. Each organ was represented by the relative positions of a set of morphological landmarks, taking into consideration the bilateral object symmetry of each of them [43] (Fig. 1B). Landmarks were digitized using tpsDig software (Rohlf, 2006). For the fall, a set of 18 landmarks was used (7 pairs and 4 median landmarks). The standard was represented by 19 landmarks (8 pairs and 3 median landmarks), and for the style arm, 18 landmarks were used (8 pairs and 2 median landmarks).

Morphometric analysis

To extract shape information from samples of multiple landmark coordinates on the floral organs, a generalized Procrustes superimposition was employed, which removes the variation in size, position and orientation in each dataset [41]. Variation in size was eliminated by scaling every landmark configuration to have a centroid size of 1.0. Centroid size is defined as the square root of the sum of squared distances of all the landmarks to their center of gravity, and is used as a measure of size for landmark configurations. Variation in position was removed by translating the configurations to have the same center of gravity (the centroid, the point in the Procrustes coordinate system whose coordinates are the means of the respective coordinates of all the landmarks). Finally, the variation in orientation was removed by rotating the configurations so that the sum of squared distances between

corresponding landmarks of each configuration and the common target configuration is minimal. The remaining variation in landmark positions is due to variation of shape.

Given that falls, standards and styles are bilaterally symmetric structures, the method of shape analysis for object symmetry, which uses the landmark configuration and the reflected and relabeled copies, was applied [43,44]. This method separates the shape variation into two components: a symmetric component, obtained by an averaging of the original and reflected and relabeled copies, and an asymmetric component calculated from differences between them [43]. While the symmetric component represents the shape variation among individuals, the asymmetric component can be used as a measure of developmental instability [45-47].

To quantify measurement error (ME) relative to the other effects, all floral elements were digitized twice and analyzed by Procrustes analysis of variance (ANOVA) [43,48]. As a descriptor of ME we used the SD of repeated measurements (ME2, according to Palmer and Strobeck [49]). $ME2 = \sqrt{MS_m}$, where MS_m is the error MS from a side's x individual effects in Procrustes ANOVA.

To test whether the fluctuating asymmetry of floral organ shape might be an allometric consequence of the fluctuating asymmetry of size, a multivariate regression of shape FA scores onto log centroid size was applied [44]. A statistically significant regression would indicate that the level of FA was influenced by the asymmetry of size. To eliminate the influence of allometry on the amount of shape FA, for each floral organ, all specimens were standardized to the mean centroid size [22] using the Standard 6b program (Sheets, 2001). Thereafter, a Procrustes ANOVA was run on the standardized data [50].

The amount of fluctuating asymmetry was estimated using a FA index (FA10a, according to [49]). $FA10a = 0.798 \sqrt{2(MS_{sj} - MS_m)/M}$, where MS_{sj} is the interaction MS and MS_m the error MS from a side's x individuals Procrustes ANOVA using the standardized data. This index describes the magnitude of fluctuating asymmetry of shape for a structure after ME and the influence of allometry has been partitioned out.

The main feature of floral organ shape variation was examined by principal component analysis (PCA). PCAs were carried out at two levels: the covariance matrix of the symmetric component of variation and the covariance matrix of the asymmetric component of variation [43,48]. All GM analyses were conducted using MorphoJ software [42].

RESULTS

Principal components analyses

PCA of shape variation for three floral organs, the fall, standard and style, revealed that more than 80% of total variance was concentrated in relatively few PCs for both the symmetrical and asymmetric variation component (Table 1). In the PCA of the symmetric component of the shape variation for the fall, PC1 accounted for the largest proportion of the total variation (55.8%) and was therefore the dominant pattern of variation in fall shape (Table 1). PC1 concerns a concerted change in the shape of the falls with regard to their relative length vs. width (the aspect ratio), and is directed at narrowing of the entire fall area (Fig. 2A(A)). The shape change associated with PC2 is a contrast between the relative width of blade vs. haft of the same fall, and is reflected in compressing of the blade (from the end-point of beard to the tip of blade) and broadening of the half of falls (Fig. 2A(A)). In contrast to PC2, PC3 corresponds to the broadening of the blade and narrowing of the haft of fall (Fig. 2A(A)). All shape changes described are those related to the positive end of PC axes.

PCA of the asymmetric component of shape variation showed that the largest proportion of the total variance was concentrated in PCA1 (51.2 %) (Table 1) as well. Regarding shape changes toward the positive end of PC axes, PC1 primarily reflects a slight torsion of the whole fall in a left direction relative to the main fall axis (Fig. 2A(B)). PC2 is associated with the downward bending of the right fall area with concomitant the upward

change of the left fall side (Fig. 2A(B)). PC3 revealed a more pronounced bending of the distal fall blade to the left side and the haft of fall to the right side relative to the main axis (Fig. 2A(B)).

In the PCA of the symmetric component of shape variation for the standard, PC1 accounts for 53.3% (Table 1.) of the total variance and is associated with a change in the aspect ratio of this floral organ, with a narrow blade of the standard at the positive end of the PC axis (Fig. 2B(A)). PC2 is related to the elongation of the standard blade and shortening of the haft of the standard, whereas, PC3 corresponds to a shape change vis-à-vis the broadening of distal part of the standard blade (Fig. 2B(A)).

PCA of the asymmetric component of standard shape variation revealed that the first three PCs account for more than 70% of the total variance (Table 1.). Considering the shape changes related to the positive end of PC axes only, PC1 represents a concerted banding of the tip of standard blade and the base of its haft to the left side (Fig. 2B(B)). PC2 represents an opposite movement of the standard parts, the tip to the right side and the haft to the left side relative to the main organ axis (Fig. 2B(B)). Conversely, PC3 is associated with torsion of the whole standard blade to the left and the standard haft to the right side relative to the main axis (Fig. 2B(B)).

PCA of the style arm shape change showed that a vast majority of the symmetric variation in this floral organ was associated with the first two PCs (81.2%) (Table 1). PC1 is associated with a shape change related to the width of the entire style arm, ranging from a relatively wide style arm at the negative end of the PC axis to a relatively narrow style arm at its positive end (Fig. 2C(A)). PC2 represents a shape change in the width of the style arms' base, which increases from the negative to the positive end of the PC axis (Fig. 2C(A)). PC3 is associated with a concerted change in the shape of the lip areas, i.e. their relative width vs. length (Fig. 2C(A)).

PCA of the asymmetric component of style arm shape variation revealed that PC1 accounted for less than half of the total variance (44.2%) and was related to a bending of the lip area relative to the main axis of the style arm (Table 1). PC2 is associated with an up-lifting of the right and down-lifting of the left side of the lip area at the positive end of the PC axis (Fig. 2C(B)). PC3 concerns a shift of the style arm base relative to the main axis (Fig. 2C(B)).

Procrustes ANOVA

Procrustes ANOVA of shape variation revealed that all causal effects were highly statistically significant for each of the three floral organs analyzed (all $P < 0.0001$), with the exception of the individual-side interaction for the style arm ($P > 0.05$) (Table 2). A significant effect of the individuals indicates the existence of individual phenotypic differences in the shape of a particular floral organ, while a significant effect of the reflection suggests that one side of a floral organ is systematically larger than the other (directional asymmetry). A significant individual x reflection interaction indicates the presence of individual variations among individuals in a particular floral organ in the left-right difference (i.e. fluctuating asymmetry). The amount of ME for all analyzed floral organs was found to be similar ($ME_{\text{fall}} = 0.0071$, $ME_{\text{standard}} = 0.0063$ and $ME_{\text{style arm}} = 0.0045$). The level of FA in the shape of floral organs in *I. pumila* was estimated for the fall ($FA10a_{\text{fall}} = 1.43 \times 10^{-5}$), the standard ($FA10a_{\text{standard}} = 2.65 \times 10^{-5}$) and the style arm ($FA10a_{\text{stylearm}} = 4.3 \times 10^{-7}$).

Multivariate regression of the asymmetric component (FA) of shape variation onto log centroid size appeared to be highly statistically significant ($P < 0.0001$) for the fall and the style arm, but non-significant for the standard ($P = 0.343$). The impact of allometry on the asymmetric component (FA) of shape FA was found to be only 2.4% for the fall and 3.6% for the style arm.

Table 3 gives the results of an allometry-corrected Procrustes ANOVA for the shape of fall, standard and style arm. The individual x reflection interaction MS from these ANOVAs were used for the calculation of an allometry-free shape FA index. The level of shape FA was revealed to be two-fold greater for the fall and standard compared to the style arm ($FA10a_{fall} = 1.49 \times 10^{-5}$, $FA10a_{standard} = 1.43 \times 10^{-5}$ and $FA10a_{stylearm} = 5.8 \times 10^{-7}$).

DISCUSSION

This study provided evidence that functionally distinct floral organs of *I. pumila*, including falls, standards and style arms, differed remarkably in the degree of shape variation. Specifically, the shape of floral organs involved in pollen transfer, such as petaloid style arms, appeared to be more bilaterally symmetrical relative to that of falls and standards, which function in as long-distance environmental signals.

Regarding the symmetric component of the shape variation, PC1 uncovered the aspect ratios of each of the three floral organs, exhibiting continuous variation, from a wide to a narrow organ type. PC2 and PC3 indicated the aspect ratios of the floral organ haft and the floral organ blade, respectively. The hafts of falls and style arms varied in shape from a narrow to a wide, particularly at the base of each of these structures, while the haft of standards changed from an elongated to a shortened type. In contrast to their hafts, the blades of falls and standards changed from a contracted to an expanded type relative to their mean shapes. Because in an *Iris* flower the fall and the faced style arm form the pollination tunnel, the shape variation in the proximal part of these two floral organs may impact the size of the entrance of pollination tunnels, with possible consequences for the insect visitation rate [17].

The Procrustes ANOVAs revealed that the asymmetric component of floral organ shape variation in *I. pumila* consisted of both fluctuating and directional asymmetry, as well as that fluctuating asymmetry contributes to the total shape for the fall and the standard, with the exception of the style arm shape, where the impact of FA was non-significant. Given that the effect of directional asymmetry on the total shape variation was highly significant for all three floral organs of *I. pumila* (all $P < .0001$), the obtained results clearly suggested that the shape of their left and right sides differed systematically from each other. The existence of a subtle directional asymmetry has been already detected in a number of biological species, including plants, animals and humans [20,21,25,51].

Fluctuating asymmetry (FA) is another component of asymmetric shape change that was detected in this study. FA is generally thought to originate from small random irregularities in the developmental processes or “developmental noise” [52] that occur independently on each side of a bilateral structure [44,53-56]. The propensity of the developmental system to produce FA as a consequence of random developmental perturbations is called developmental instability [54,55]. The principal presumption for interpreting FA as the phenotypic consequence of developmental instability is that the left and the right sides of an organism or a structure share the same genome and similar environmental conditions [55,57]. However, this hypothesis is acceptable for motile organisms that move through their environment, averaging out the possible environmental variation, but is unrealistic for sessile organisms such as plants, because their parts are exposed to the heterogeneity in their immediate surroundings in a constant manner. For example, even in full sunlight, the intensity of solar radiation impinging horizontally on floral organs of an actinomorphic *Iris* flower can vary significantly depending on their orientation. In this particular case, the resulting asymmetry would stem from FA that is not only due to developmental instability but also due to a phenotypic plasticity to microenvironmental heterogeneity [21,23,44].

In *I. pumila*, the contribution of FA to the total shape variation of the analyzed floral organs appeared to be highly significant for all analyzed floral organs; however, the

magnitude of shape FA quantified by the FA10a index was found to be markedly smaller for the style arm as compared to the fall or standard. Because the contribution of directional asymmetry to the total shape variance of the style arm was extremely low, the obtained results have suggested that the development of this reproductive structure of *I. pumila* flowers was more canalized than that of the two sterile floral organs – the fall and the standard.

Flower shape is an important trait of entomophilous angiosperms because it serves as a cue that attracts their pollinators. A behavioral study of the ability of pollinating insects to discriminate the shape of artificial *Primula sieboldii* flowers revealed that bumblebees exhibited a strong preference for flowers with extremely narrow petals, even after training using flowers with extremely wide petals [58]. Very recently, Gómez et al. [5] provided evidence that the evolution of corolla shape variation, integration and disparity in a highly diversified plant family, the Brassicaceae, was likely driven by pollinator-mediated selection. The most outstanding result of that study was that “the type of pollinator visiting the flower, rather than diversity, has the major effect on the evolution of floral integration and disparity” [5]. Moreover, “the effect of pollinators on phenotypic integration is expected to be more intense for those traits related to the efficiency of pollen transfer (anthers, pistils, stigmas) than for traits related to the attraction of pollinators” [5].

I. pumila is an entomophilous plant, the primary pollinators of which are bumblebees. The floral organs of this species are specialized for insect pollination. The style arm of *Iris* flowers is a highly modified pistil, which together with the haft of an opposed fall forms the pollination tunnel. PCAs provided evidence that the increasing values of PC2 for the shape variation of both the falls and the style arms were associated with a greater size of the pollination tunnels. However, the Procrustes ANOVA results have shown that the asymmetry of these two organs differed significantly from each other. While the style arm was almost perfectly bilaterally symmetric, the fall and, especially, the standard exhibited a notably greater level of asymmetry. Flowers with a wide and bilaterally symmetrical pollination tunnel could attract pollinators and allow those that enter the tunnel to move forward only, as do bumblebees, leading to an efficient pollen deposition from their thorax to the sticky stigmatic lip. Hence, a comparatively greater developmental robustness of the style arm than that of other floral organs might be the outcome of a strong prepollination selection that endowed *Iris* flowers with a wide and bilaterally symmetrical pollination unit [17,58-61].

Acknowledgments: We are very grateful to V. Jojić and C.P. Klingenberg for their advice on various statistical questions. We also thank two anonymous reviewers for their stimulating criticism. This work was supported by the Ministry for Education, Science and Technological Development of Serbia, Grant No. 173007.

REFERENCES

1. Coen ES, Meyerowitz EM. The war of the whorls: genetic interactions controlling flower development. *Nature*. 1991;353(6339):31-7.
2. Weigel D, Meyerowitz EM. The ABCs of floral homeotic genes: Review *Cell*. 1994;78:203-9.
3. Rijpkema AS, Vandenbussche M, Koes R, Heijmans K, Gerats T. Variations on a theme: changes in the floral ABCs in angiosperms. *Semin Cell Dev Biol*. 2010;21(1):100-7.
4. Sauret-Güeto S, Schiessl K, Bangham A, Sablowski R, Coen E. JAGGED controls *Arabidopsis* petal growth and shape by interacting with a divergent polarity field. *PLoS Biol*. 2013;11(4):e1001550.
5. Gómez JM, Torices R, Lorite J, Klingenberg CP, Perfectti F. The role of pollinators in the evolution of corolla shape variation, disparity and integration in a highly diversified plant family with a conserved floral bauplan. *Ann Bot*. 2016;117(5):889-904.
6. Harder LD, Barrett SCH. *Ecology and evolution of flowers*. Oxford: Oxford University Press; 2006. 370 p.
7. Glover BJ. *Understanding flowers and flowering: an integrated approach*. Oxford, UK: Oxford University Press; 2007. 227 p.
8. Córdoba SA, Cocucci AA. Flower power: its association with bee power and floral functional morphology in papilionate legumes. *Ann Bot*. 2011;108(5):919-31.

9. Willmer P. Pollination and floral ecology. Princeton: Princeton University Press; 2011. 778 p.
10. Clarke D, Whitney H, Sutton G, Robert D. Detection and learning of floral electric fields by bumblebees. *Science*. 2013;340(6128):66-9.
11. Schiestl FP, Johnson SD. Pollinator-mediated evolution of floral signals. *Trends Ecol Evol*. 2013;28(5):307-15.
12. Gómez JM, Bosch J, Perfectti F, Fernández JD, Abdelaziz M, Camacho JPM. Association between floral traits and rewards in *Erysimum mediohispanicum* (Brassicaceae). *Ann Bot*. 2008;101(9):1413-20.
13. Gomez JM, Munoz-Pajares AJ, Abdelaziz M, Lorite J, Perfectti F. Evolution of pollination niches and floral divergence in the generalist plant *Erysimum mediohispanicum*. *Ann Bot*. 2014;113(2):237-49.
14. Gómez JM, Perfectti F, Camacho JPM. Natural selection on *Erysimum mediohispanicum* flower shape: insights into the evolution of zygomorphy. *Am Nat*. 2006;168(4):531-45.
15. Gómez JM, Verdú M, Perfectti F. Ecological interactions are evolutionarily conserved across the entire tree of life. *Nature*. 2010;465(7300):918-21.
16. Gómez JM, Perfectti F. Evolution of complex traits: the case of *Erysimum* corolla shape. *Int J Plant Sci*. 2010;171(9):987-98.
17. Møller AP. Bumblebee preference for symmetrical flowers. *Proc Nat Acad Sci USA*. 1995;92(6):2288-92.
18. Parsons PA. Fluctuating asymmetry: an epigenetic measure of stress. *Biol Rev*. 1990;65(2):131-45.
19. Lawing AM, Polly PD. Geometric morphometrics: recent applications to the study of evolution and development. *J Zool*. 2010;280(1):1-7.
20. Savriama Y, Klingenberg C. Beyond bilateral symmetry: geometric morphometric methods for any type of symmetry. *BMC Evol Biol*. 2011;11(1):280.
21. Savriama Y, Gómez JM, Perfectti F, Klingenberg CP. Geometric morphometrics of corolla shape: dissecting components of symmetric and asymmetric variation in *Erysimum mediohispanicum* (Brassicaceae). *New Phytol*. 2012;196(3):945-54.
22. Zelditch ML, Swiderski DL, Sheets HD. Geometric morphometrics for biologists: a primer. Amsterdam: Academic Press; 2012. 478 p.
23. Klingenberg CP, Duttke S, Whelan S, Kim M. Developmental plasticity, morphological variation and evolvability: A multilevel analysis of morphometric integration in the shape of compound leaves. *J Evol Biol*. 2012;25(1):115-29.
24. Gardner AG, Gerald JNF, Menz J, Shepherd KA, Howarth DG, Jabaily RS. Characterizing floral symmetry in the Core Goodeniaceae with geometric morphometrics. *PLoS One*. 2016;11(5):e0154736.
25. Klingenberg CP. Evolution and development of shape: integrating quantitative approaches. *Nat Rev Genet*. 2010;11(9):623-35.
26. Adams DC, Rohlf FJ, Slice DE. A field comes of age: geometric morphometrics in the 21st century. *Hystrix*. 2013;24(1):7-14.
27. Mathew B. *The Iris*. Portland, OR: Timber Press; 1981.
28. Pande PC, Singh V. Floral development of *Iris decora* Wall. (Iridaceae). *Bot J Linn Soc*. 1981;83:41-56.
29. Proctor M, Yeo P. *The pollination of flowers*. New York: Taplinger Pub. Co.; 1973. 418 p.
30. Goldblatt P, Manning JC, Bernhardt P. Pollination biology of *Lapeirousia* subgenus *Lapeirousia* (Iridaceae) in southern Africa; floral divergence and adaptation for long-tongued fly pollination. *Ann Missouri Bot Gard*. 1995;517-34.
31. Sapir Y, Shmida A, Ne'eman G. Pollination of *Oncocyclus* irises (Iris: Iridaceae) by night-sheltering male bees. *Plant Biol*. 2005;7(04):417-24.
32. Sapir Y, Shmida A, Ne'eman G. Morning floral heat as a reward to the pollinators of the *Oncocyclus* irises. *Oecologia*. 2006;147(1):53-9.
33. Segal B, Sapir Y, Carmel Y. Fragmentation and pollination crisis in the self-incompatible *Iris bismarckiana* (Iridaceae), with implications for conservation. *Isr J Ecol Evol*. 2006;52(2):111-22.
34. Imbert E, Wang H, Anderson B, Hervouet B, Talavera M, Schatz B. Reproductive biology and colour polymorphism in the food-deceptive *Iris lutescens* (Iridaceae). *Acta Bot Gall*. 2014;161(2):117-27.
35. Lavi R, Sapir Y. Are pollinators the agents of selection for the extreme large size and dark color in *Oncocyclus* irises? *New Phytol* 2015;205(1):369-77.
36. Gajić M. *The Flora of the Deliblato Sand*. Novi Sad, Serbia: Fac Nat Sci Inst Biol Univ Novi Sad. 1983.
37. F. K. *Iris*. Stuttgart: Verlag Eugen Ulmer; 1981.
38. Tucić B, Milojković S, Vujčić S, Tarasjev A. Clonal diversity and dispersion in *Iris pumila*. *Acta oecologica*. 1988;9(2):211-9.
39. Manitašević Jovanović S, Tucić B, Matić G. Differential expression of heat-shock proteins Hsp70 and Hsp90 in vegetative and reproductive tissues of *Iris pumila*. *Acta Physiol Plant*. 2011;33(1):233-40.

40. Tucić B, Vuleta A, Manitašević Jovanović S. Exploring phenotypic floral integration in *Iris pumila* L.: a common-garden experiment. *Arch Biol Sci.* 2013;65:781-93.
41. Dryden IL, Mardia K V. *Statistical shape analysis.* Chichester, New York: John Wiley & Sons; 1998. 347 p.
42. Klingenberg CP. MorphoJ: an integrated software package for geometric morphometrics. *Mol Ecol Resour.* 2011;11(2):353-7.
43. Klingenberg CP, Barluenga M, Meyer A. Shape analysis of symmetric structures: quantifying variation among individuals and asymmetry. *Evolution.* 2002;56(10):1909-20.
44. Klingenberg CP. Analyzing fluctuating asymmetry with geometric morphometrics: Concepts, methods, and applications. *Symmetry.* 2015;7(2):843-934.
45. Leamy LJ, Klingenberg CP. The genetics and evolution of fluctuating asymmetry. *Annu Rev Ecol Evol Syst.* 2005;1-21.
46. Debat V, David P. Mapping phenotypes: canalization, plasticity and developmental stability. *Trends Ecol Evol.* 2001;16(10):555-61.
47. Palmer AR, Strobeck C. Fluctuating asymmetry: measurement, analysis, patterns. *Annu Rev Ecol Syst.* 1986;391-421.
48. Klingenberg CP, McIntyre GS. Geometric morphometrics of developmental instability: analyzing patterns of fluctuating asymmetry with Procrustes methods. *Evolution.* 1998;52(5):1363-75.
49. Palmer AR, Strobeck C. Fluctuating asymmetry analyses revisited. In: Polak M, editor. *Developmental instability. Causes and consequences.* Oxford: Oxford Univ Press; 2003. p. 279-319.
50. Jojić V, Blagojević J, Vujošević M. B chromosomes and cranial variability in yellow-necked field mice (*Apodemus flavicollis*). *J Mammal.* 2011;92(2):396-406.
51. Debat V, Alibert P, David P, Paradis E, Auffray JC. Independence between developmental stability and canalization in the skull of the house mouse. *Proc R Soc London B Biol Sci.* 2000;267(1442):423-30.
52. Waddington CH. *The strategy of the genes. A discussion of some aspects of theoretical biology.* London: Allen & Unwin; 1957.
53. Palmer AR. Waltzing with asymmetry. *Bioscience.* 1996;46(7):518-32.
54. Klingenberg CP, Nijhout HF. Genetics of fluctuating asymmetry: a developmental model of developmental instability. *Evolution.* 1999;53(2):358-75.
55. Klingenberg CP. A developmental perspective on developmental instability: theory, models and mechanisms. In: Polak M, ed. *Developmental instability: causes consequences.* Oxford: Oxford University Press; 2003. p. 14-34.
56. Merlin F. Developmental noise: Explaining the specific heterogeneity of individual organisms. In: Braillard PA, Malaterre C, editors. *Explanation in Biology.* Netherlands: Springer; 2015. p. 91-110.
57. Nijhout HF, Davidowitz G. Developmental perspectives on phenotypic variation, canalization, and fluctuating asymmetry. In: Polak M, ed. *Developmental instability: causes and consequences.* Oxford: Oxford University Press; 2003. p. 3-13.
58. Yoshioka Y, Ohashi K, Konuma A, Iwata H, Ohsawa R, Ninomiya S. Ability of bumblebees to discriminate differences in the shape of artificial flowers of *Primula sieboldii* (Primulaceae). *Ann Bot.* 2007;99(6):1175-82.
59. Wilson P. Variation in the intensity of pollination in *Drosera tracyi*: Selection is strongest when resources are intermediate. *Ecol Evol.* 1995;9(4):382-96.
60. Møller AP, Sorci G. Insect preference for symmetrical artificial flowers. *Oecologia.* 1998;114(1):37-42.
61. West EL, Lavery TM. Effect of floral symmetry on flower choice and foraging behaviour of bumble bees. *Can J Zool.* 1998;76(4):730-9.

Table 1. Eigenvalues (λ) and contributions of principal components (PC) in the symmetric and asymmetric shape variation component of floral organs (fall, standard and style) in *Iris pumila*.

	Symmetric			Asymmetric		
	λ (10^{-5})	Variance%	Cumulative%	λ (10^{-5})	Variance%	Cumulative%
<i>Fall</i>						
PC1	69.681	55.831	55.831	13.330	51.186	51.186
PC2	22.339	17.899	73.730	5.417	20.802	71.988
PC3	12.528	10.038	83.768	2.787	10.702	82.690
Total variance (10^{-5})	124.808			26.041		
<i>Standard</i>						
PC1	114.062	53.348	53.348	12.205	36.363	36.363
PC2	57.333	26.815	80.163	9.267	27.609	63.972
PC3	15.954	7.462	87.625	3.442	10.256	74.229
Total variance (10^{-5})	213.808			33.564		
<i>Style arm</i>						
PC1	34.127	65.906	65.906	4.337	44.208	44.208
PC2	7.896	15.248	81.154	2.763	28.167	72.375
PC3	3.442	9.021	90.175	1.030	10.497	82.872
Total variance (10^{-5})	51.782			9.810		

Table 2. Allometry included Procrustes ANOVA of floral organ (fall, standard and style arm) shape in *Iris pumila*.

Effect	SS	MS	df	F	P
<i>Fall</i>					
Individual	2.10982	0.00022	9680	3.28	<.0001
Side	0.12418	0.00776	16	116.92	<.0001
Individual*Side	0.64258	0.00006	9680	1.37	<.0001
Error	0.93866	0.00005	19392		
<i>Standard</i>					
Individual	2.95056	0.00030	9775	5.42	<.0001
Side	0.06746	0.00397	17	71.31	<.0001
Individual*Side	0.54397	0.00006	9775	1.53	<.0001
Error	0.68439	0.00004	18768		
<i>Style arm</i>					
Individual	0.93348	0.00010	9680	4.65	<.0001
Side	0.00770	0.00048	16	23.20	<.0001
Individual*Side	0.20084	0.00002	9680	1.03	0.0666
Error	0.39188	0.00002	19392		

Table 3. Procrustes ANOVA on allometry-corrected data of floral organ (fall, standard and style arm) shape in *Iris pumila*.

Effect	SS	MS	df	F	P
<i>Fall</i>					
Individual	2.07516	0.00021	9680	3.24	<.0001
Side	0.12419	0.00776	16	117.20	<.0001
Individual*Side	0.64109	0.00007	9680	1.39	<.0001
Error	0.92309	0.00005	19392		
<i>Standard</i>					
Individual	2.14908	0.00020	9775	3.74	<.0001
Side	0.07325	0.00430	17	77.22	<.0001
Individual*Side	0.57389	0.00005	9775	1.47	<.0001
Error	0.78239	0.00004	18768		
<i>Style arm</i>					
Individual	0.90788	0.00009	9680	4.52	<.0001
Side	0.00771	0.00048	16	23.25	<.0001
Individual*Side	0.20069	0.00002	9680	1.04	0.0203
Error	0.38786	0.00002	19392		

Figure Legends

Fig. 1. *Iris pumila*, images of a flower. **A** – Side-view photo-image of an *I. pumila* flower with the names of its floral organs. **B** – Top-view scanned-images of three dissected floral organs (fall, standard and style arm) of an *I. pumila* flower, with the location of corresponding landmarks used in the GM analyses.

Fig. 2A-C. PCA for individual variation (A) and fluctuating asymmetry (B) in the fall shape (A), standard shape (B) and style arm (C) of *Iris pumila*. PCs explaining more than 10% of the total variation in shape (PC1 – PC3) are presented. The fall shapes corresponding to the two extreme values (negative: dotted line with open circles and positive: full lines with field circles) of the distribution along each shape PC, as well as the consensus shape (grey line with field circles) are depicted.

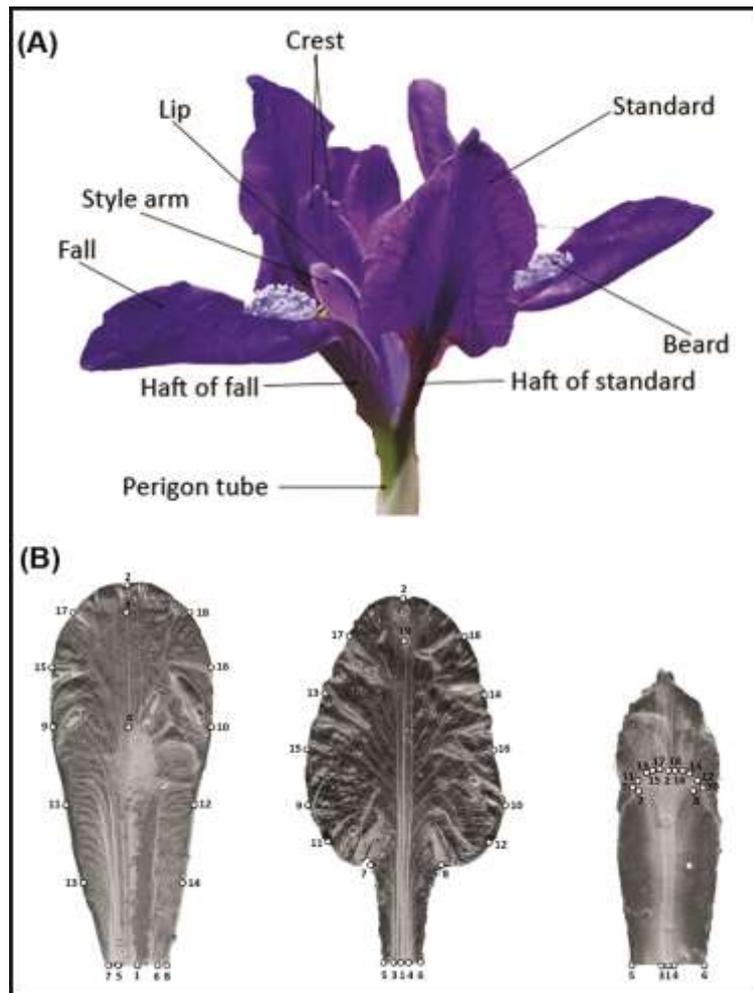
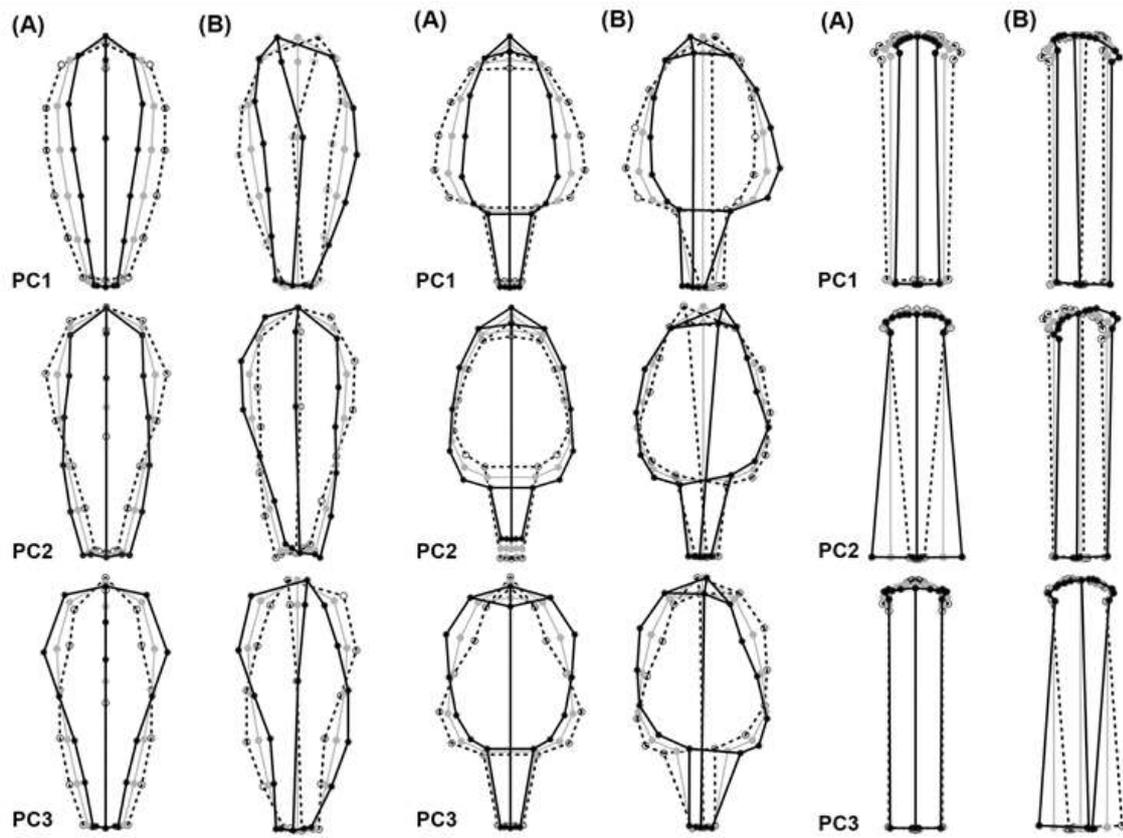


Fig. 1



A

B

C

Fig. 2.