Supplementary material for: Plant-pollinator specialization: Origin and measurement of curvature

#### Measuring *Epimedium* flowers and defining developmental stages

Flower size of *E. koreanum* and *E. violaceum* was measured daily from April 9 to May 2, 2019 at the UBC Botanical Garden (Supp Mat 1). Size was defined as the distance between the apex of the two outer sepals lying on the major axis of the flower (aestivation is imbricate). Width was measured to the nearest 0.1 mm using an SPI Polymid Dial Caliper. In *E. violaceum* the distance sepal distance was measured until the width of the inner sepals exceeded the width of the outer sepals (“G” Stage). From this point onwards, the inner sepal distance was measured. In *E. koreanum*, the inner sepals lack pigmentation and adhere closely to the petals, making them difficult to measure accurately. For this reason, the outer sepals were measured until they abscised (“T” stage).

Changes in flower size were punctuated by developmental landmarks (defined in Table S3). We tested for the validity of these developmental stages by fitting a linear mixed effects model using the R package lmerTest (Kuznetsova et al., [2017](#ref-kuznetsova_2017)):

Where is the intercept, is a constant, and is the individual-specific random effect.

We then tested for pairwise differences of estimated marginal means using the R package emmeans (Lenth et al., [2018](#ref-lenth_2018)). By correlating changes in flower size to developmental landmarks, we were able to define 4 discrete stages in *E. koreanum* and *E. violaceum* (Figure 5, Table S3). In the first stage (“C”) the petals are shorter in length than the sepals that envelop them - the following stage begins when the petals overtake the surrounding sepals in length. The “G” stage includes continued growth of the bud until the petals begin to separate. At the “T” stage nectar begins accumilating in the spurs. Anthesis takes place during the “A” stage at which point the flower opening may increase in size and anthers dehisce.

Flowering stage data was staggered because each flower developed independently. The data was also fragmented because some samples abscissed prematurely due to herbivory or weather. The flower development data was therefore a ‘censored’ dataset i.e. some subjects left the study before reaching maturity. Although manually aligning the stage data is possible, for convenience we used a multiple sequence alignment protocol to automate the process. To do this, we ran the stage data through ClustalW implemented in the R package msa v.3.9 (Bodenhofer et al., [2015](#ref-bodenhofer_2015)) with a neutral (identity) substitution matrix. Gap opening was prohibited. By aligning phenological data within species, a consensus (mean) stage sequence was calculated and used to estimate flower age where observations were censored.

We tested for differences in developmental time by fitting a linear mixed effects model using the R package lmerTest (Kuznetsova et al., [2017](#ref-kuznetsova_2017)):

Where is the intercept, is a constant, and is the individual-specific random effect.

We then tested for pairwise differences of estimated marginal means using the R package emmeans (Lenth et al., [2018](#ref-lenth_2018)). Results are presented in Table S4.

#### Landmarking, morphometrics, and curve-fitting

To assign landmarks and semi-landmarks, we adhered to the following protocol:

1. Rotate the photographs so that the opening of the corolla tube is parallel to the y-axis.
2. Build .tps file (a file listing all specimens) using *tpsUtil* (Rohlf, [2015](#ref-rohlf_2015)). This .tps file is used by *tpsdig* to add landmarks to.
3. Landmark specimens from .tps file using tpsDig (steps 1 and 2 will soon be possible in MomX (Bonhomme et al., [2014](#ref-bonhomme_2014)) and could be done in geomorph (Adams and Otárola-Castillo, [2013](#ref-adams_2013))). Landmarks used to measure the dorsal arc are 1) the farthest point on the apex of the spur before the inflection point where either the spur diminishes to a tip (*E. violaceum*) or widens into a saccate reservoir (*E. koreanum*), and 2) the dorsal point at which the spur widens to become an attachment point for the petal to the stem. 13 semi-landmarks (sensu Webster and Sheets, [2010](#ref-webster_2010)) were placed between them (15 points total). These landmarks were used for curve-fitting and analysis of curvature. Two additional landmarks were placed on the ventral arc, 1) the farthest point on the apex of the spur before the spur diminshes to a tip, and 2) the ventral point where the nectar tube transitions into an opening, characterized by a fold in the petal tissue. 13 semi-landmarks were placed between them. These cumulative 30 landmarks were used in morphometric analysis of shape.
4. Curve points are drawn in tpsDig using the “pencil tool”, from landmark 1 (see above) to landmark 2. Following the placement of points, a curve is automatically drawn that connects them. We used the *Resample Curve* function and selected to space the points evenly *by length*. We then manually adjusted the re-sampled points back onto the specimen and used the *Resample Curve* function until all landmarks were aligned and evenly spaced. This does not usually need to be repeated more than twice.
5. We set the scale by navigating *Options* -> *Image Tools* and typing in desired length and units. We selected *Set scale* and selected both ends of the scale bar (included in our images). We then navigated to back to the *Image options* dialogue box to confirm ‘OK’.
6. Semi-landmarks need to be treated like landmarks for curve-fitting. To do this, we used the *Append tps curve to landmarks* function in tpsUtil.
7. Import appended .tps files into R using from\_tps() function from Momit (Bonhomme et al., [2014](#ref-bonhomme_2014)).

We used the R package geomorph (Adams and Otárola-Castillo, [2013](#ref-adams_2013)) to analyse morphological development. First, landmark configurations (shapes) were aligned using a Generalized Procrustes Analysis (see: main text). We then fit a linear model to correlate changes in shape with developmental stage:

Where is the intercept and is a constant. Results are presented in Table S5. We then visualized the developmental trajectory for each taxon by implementing the trajectory.analysis function in geomorph (Figure 4).

To investigate whether variation in shape can be attributed to curvature, we regressed polynomial functions to the landmark coordinates for each specimen using Momocs - we chose polynomials of the third degree based on the recommendations of Rohlf ([1990](#ref-rohlf_1990)). Arc length was calculated from bounded polynomial functions using pracma v.2.2.5 (Borchers, [2019](#ref-borchers_2019)). Curvature, as defined in the previous section, was computed using custom functions modified from the maxcurv() function of the soilphysics package v.3.1 (Silva and Lima, [2017](#ref-silva_2017)). All custom functions used in this analysis are available as an R package curvr hosted at github.com/mannfred/curvr. We tested for differences in the development of curvature by fitting a linear mixed effects model using the R package lmerTest (Kuznetsova et al., [2017](#ref-kuznetsova_2017)):

Where is the intercept, is a constant, and is the individual-specific random effect.

We then tested for pairwise differences of estimated marginal means using the R package emmeans (Lenth et al., [2018](#ref-lenth_2018)). Results are presented in Table S6.

#### Supplementary Figures

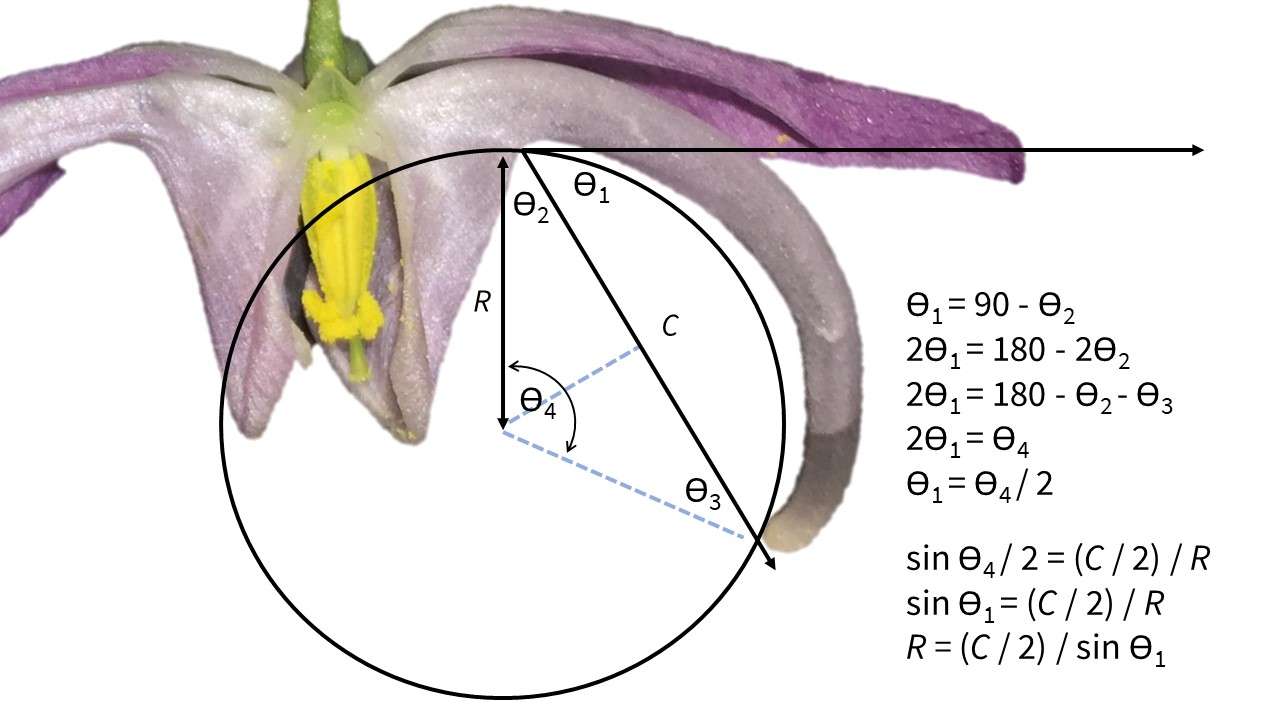


Figure S1. Demonstration that the angle of deflection and inverse radius methods are interchangeable

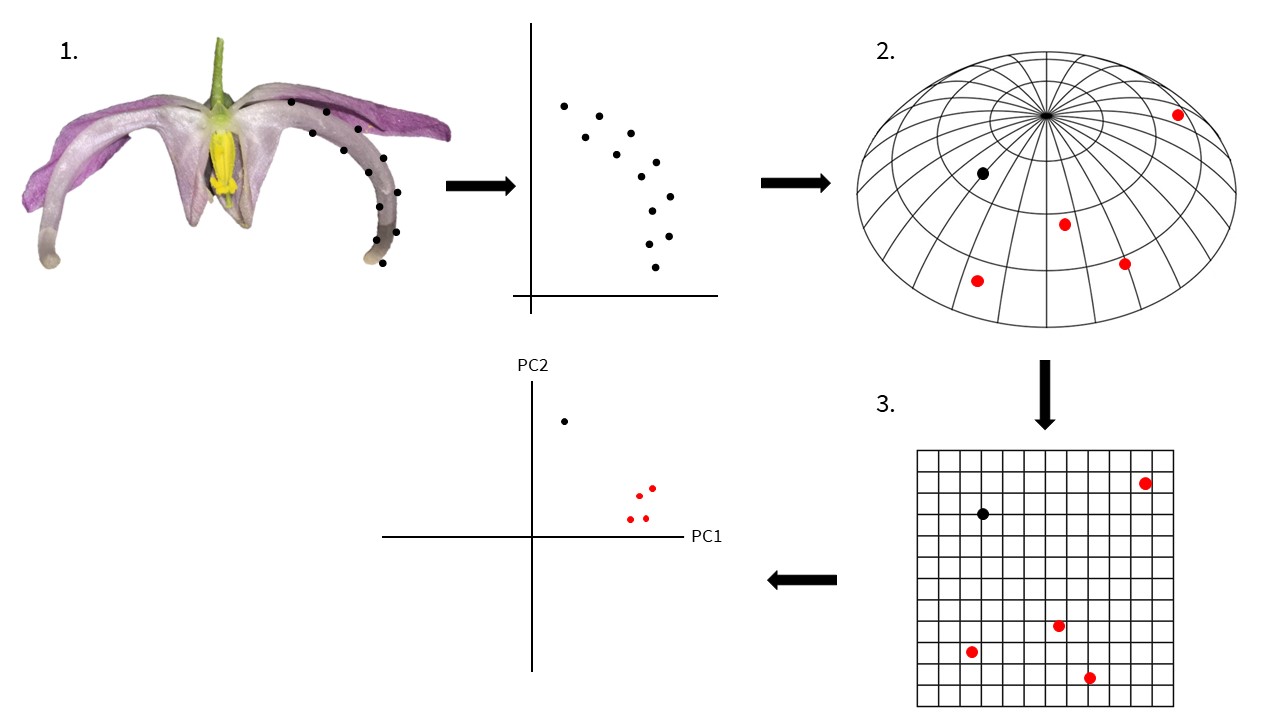


Figure S2. Overview of a geometric morphometrics protocol. 1. Landmarks and semi-landmarks are assigned to a specimen. Each landmark is assigned an xy coordinate. 2. For each specimen a configuration of landmarks exists as a single point in a non-Euclidian shape space (abstracted here as a sphere segment). Red points represent landmark configurations from other specimens. 3. Shape data is projected onto a Euclidian plane – a tangent space approximation. This allows statistical analyses of shape variation (e.g. principal components analysis).



Figure S3. Landmarks (red) and semi-landmarks (white) used to compare curvature between *E. koreanum* (left) and *E. violaceum* (right). Petals sampled at anthesis.

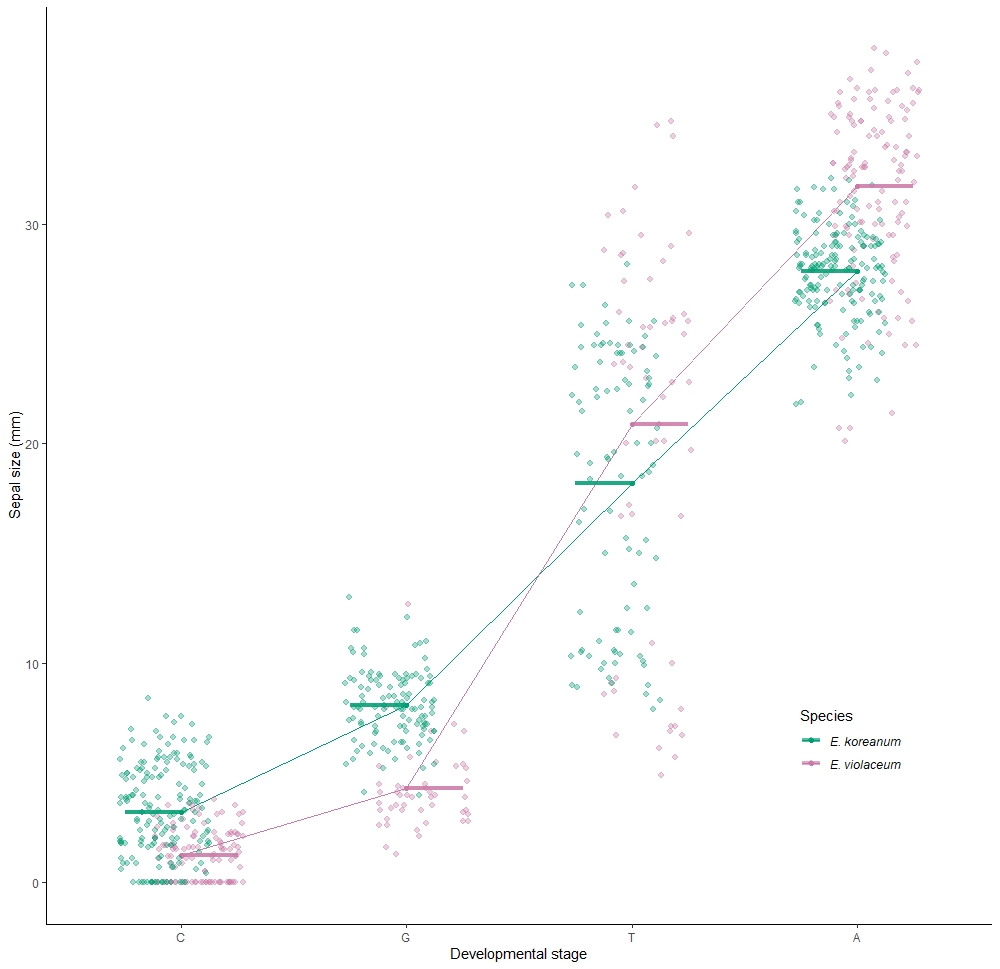


Figure S4: Comparison of developmental stages and size in *Epimedium*. Tukey’s HSD: p<0.01 for all within-species comparisons.

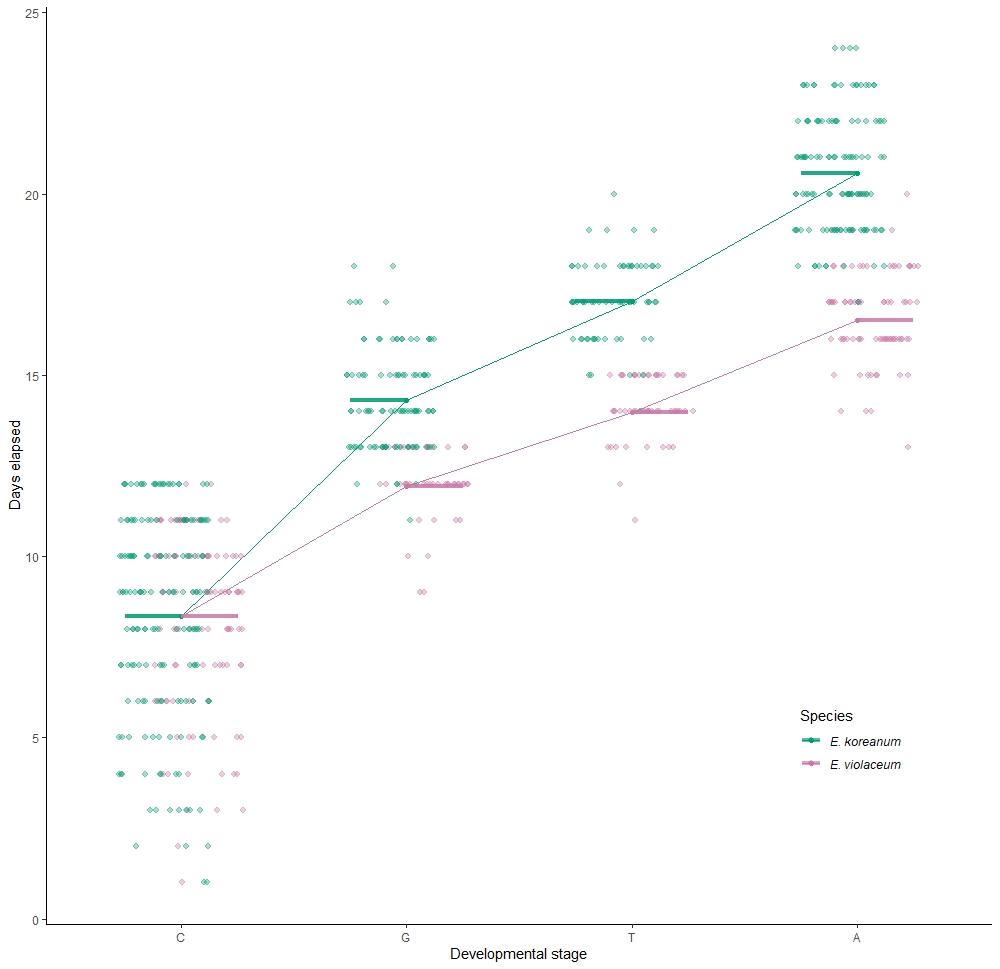


Figure S5: Comparison of timing of developmental stages in *Epimedium*.

#### Supplementary Tables

Table S1: Additional literature reviewed for the role of curvature in plant-pollinator systems.

|  |  |  |
| --- | --- | --- |
| Citation | System | Stated or Inferred Method |
| Baldwin et al. ([1931](#ref-baldwin_1931)) | Measurements of birds | inverse radius |
| Hamilton ([1975](#ref-hamilton_1975)) | Comparative behavior of the American Avocet and the Black-necked Stilt (Recurvirostridae) | inverse radius |
| Buttrose et al. ([1977](#ref-buttrose_1977)) | Significance of curvature of style branches in *Hibiscus trionum* (Malvaceae) for pollination | qualitative |
| Mountainspring ([1987](#ref-mountainspring_1987)) | sexual dimorphism and foraging preferences of the Hawaiian honeycreeper (*Pseudonestor xanthophrys*) | mandibular index |
| Muller ([1995](#ref-muller_1995)) | curved bristles on the proboscis on European bees for the extraction of pollen. | qualitative |
| Manning and Goldblatt ([1997](#ref-manning_1997)) | Tangle-veined fly pollination of South African Iridaceace, Geraniaceae, Orchidaceae | qualitative |
| Ortiz et al. ([2000](#ref-ortiz_2000)) | Pollination and breeding system of *Putoria calabrica* (Rubiaceae), a Mediterranean dwarf shrub | qualitative |
| Lindqvist et al. ([2003](#ref-lindqvist_2003)) | Cladogenesis and reticulation in the Hawaiian endemic mints (Lamiaceae) | qualitative |
| Ruan et al. ([2008](#ref-ruan_2008)) | The impact of pollen tube growth on stigma lobe curvature in *Kosteletzkya virginica* (Malvaceae) | qualitative |
| Kawabata et al. ([2009](#ref-kawabata_2009)) | Quantitative analysis of corolla shapes and petal contours in single-flower cultivars of *Lisianthus* (Gentianaceae) | fourier analysis |
| Dalayap et al. ([2011](#ref-dalayap_2011)) | Petal, sepal, and labellum shapes in Mokara orchids (Orchidaceae) | geometric morphometrics |
| Nii and Kawabata ([2011](#ref-nii_2011)) | Assessment of the association between the three-dimensional shape of the corolla and two-dimensional shapes of petals using Fourier descriptors and principal component analysis in *Eustoma grandiflorum* (Gentianaceae) | fourier analysis |
| Campos et al. ([2015](#ref-campos_2015)) | Generating 3D printed flowers to test efficacy of moth pollination | curve decay parameter |
| Berger et al. ([2017](#ref-berger_2017)) | Quantifying morphological modifications to floral form in gene knockdowns in *Fedia graciliflora* (Caprifoliaceae) | geometric morphometrics |
| Pour et al. ([2018](#ref-pour_2018)) | Curvature-based pattern recognition for cultivar classification of *Anthurium* (Araceae) flowers | point-wise curvature |
| Song et al. ([2018](#ref-song_2018)) | Quantitative classification of the morphological traits of ray florets in large-flowered *Chrysanthemum* (Asteraceae) | angle of deflection |
| Peng et al. ([2019](#ref-peng_2019)) | Morphospace exploration reveals divergent fitness optima between plants and pollinators | curve decay parameter |

Table S2: Sample sizes.

|  |  |  |  |
| --- | --- | --- | --- |
| Species | n (Individuals) | n (total panicles) | n (total flowers) |
| E. koreanum | 2 | 11 | 50 |
| E. violaceum | 2 | 3 | 33 |

Table S3: Stages of *Epimedium* flower development.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Stage | Defintion | Species | Estimated margin of means (Sepal size in mm) | Standard error | Degrees of freedom | Lower CL | Upper CL |
| C | Petals do not exceed the length of the inner and outer sepals. | E. koreanum | 3.21 | 0.479 | 3.18 | 0.128 | 6.30 |
| NA | NA | E. violaceum | 1.24 | 0.520 | 4.40 | 0.000 | 3.78 |
| G | Petals exceed the length of the inner and outer sepals. | E. koreanum | 8.09 | 0.511 | 4.14 | 5.493 | 10.70 |
| NA | NA | E. violaceum | 4.31 | 0.657 | 11.16 | 2.113 | 6.51 |
| T | Opening and separation of the petals. At least one petal is free from touching adjacent petals. Outer sepals begin to abscise. Nectar is visibly collecting in spurs. | E. koreanum | 18.19 | 0.542 | 5.22 | 15.812 | 20.57 |
| NA | NA | E. violaceum | 20.88 | 0.625 | 8.86 | 18.659 | 23.10 |
| A | Initiated by partial anther dehiscence, followed by complete dehiscence, and finally flower abscisson | E. koreanum | 27.84 | 0.476 | 3.10 | 24.694 | 30.99 |
| NA | NA | E. violaceum | 31.75 | 0.511 | 3.98 | 29.067 | 34.43 |

Table S4: Timing of flower development in *Epimedium*.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Developmental stage | Species | Estimated margin of means (Elapsed days) | Standard error | Degrees of freedom | Lower CL | Upper CL |
| C | E. koreanum | 8.347212 | 0.3299095 | 2.507147 | 5.515885 | 11.17854 |
| NA | E. violaceum | 8.357769 | 0.3467908 | 3.058148 | 6.035077 | 10.68046 |
| G | E. koreanum | 14.313171 | 0.3512249 | 3.229466 | 12.092260 | 16.53409 |
| NA | E. violaceum | 11.941145 | 0.4080819 | 5.868882 | 10.252550 | 13.62974 |
| T | E. koreanum | 17.027220 | 0.3763897 | 4.227753 | 15.138820 | 18.91562 |
| NA | E. violaceum | 13.964860 | 0.3962915 | 5.145850 | 12.209970 | 15.71975 |
| A | E. koreanum | 20.559784 | 0.3422595 | 2.871271 | 18.096690 | 23.02288 |
| NA | E. violaceum | 16.498930 | 0.3657876 | 3.724022 | 14.472770 | 18.52509 |

Table S5: Pairwise comparisons of shape through flower development in *Epimedium*.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Species\*Developmental stage | d | UCL (95%) | Z | Pr > d |
| E. koreanum.A:E. violaceum.A | 0.0888750 | 0.0983998 | 1.228653 | 0.125 |
| E. koreanum.A:E. koreanum.C | 0.3925122 | 0.3516376 | 2.692997 | 0.005 |
| E. koreanum.A:E. violaceum.C | 0.2380103 | 0.3600152 | -1.569970 | 0.940 |
| E. koreanum.A:E. koreanum.G | 0.3082453 | 0.3229295 | 1.335232 | 0.110 |
| E. koreanum.A:E. violaceum.G | 0.2644044 | 0.3550981 | -0.138090 | 0.555 |
| E. koreanum.A:E. koreanum.T | 0.0898843 | 0.1134182 | 0.873026 | 0.225 |
| E. koreanum.A:E. violaceum.T | 0.1233314 | 0.1281155 | 1.574370 | 0.080 |
| E. violaceum.A:E. koreanum.C | 0.3328026 | 0.3273555 | 1.750913 | 0.050 |
| E. violaceum.A:E. violaceum.C | 0.1762778 | 0.3320400 | -2.772690 | 1.000 |
| E. violaceum.A:E. koreanum.G | 0.2333255 | 0.2983086 | -0.143690 | 0.595 |
| E. violaceum.A:E. violaceum.G | 0.1877704 | 0.3308074 | -1.421170 | 0.935 |
| E. violaceum.A:E. koreanum.T | 0.0593581 | 0.1073827 | -0.673030 | 0.705 |
| E. violaceum.A:E. violaceum.T | 0.0507369 | 0.1045217 | -0.544380 | 0.650 |
| E. koreanum.C:E. violaceum.C | 0.1657957 | 0.1274803 | 3.206805 | 0.010 |
| E. koreanum.C:E. koreanum.G | 0.2191675 | 0.2496843 | 0.906959 | 0.205 |
| E. koreanum.C:E. violaceum.G | 0.2414010 | 0.2554716 | 1.455593 | 0.085 |
| E. koreanum.C:E. koreanum.T | 0.3644213 | 0.3161567 | 3.152697 | 0.005 |
| E. koreanum.C:E. violaceum.T | 0.2848581 | 0.2849447 | 1.441634 | 0.055 |
| E. violaceum.C:E. koreanum.G | 0.1722894 | 0.2508224 | -0.756400 | 0.795 |
| E. violaceum.C:E. violaceum.G | 0.1574016 | 0.2554303 | -0.657200 | 0.715 |
| E. violaceum.C:E. koreanum.T | 0.2129351 | 0.3267940 | -1.856080 | 0.990 |
| E. violaceum.C:E. violaceum.T | 0.1322136 | 0.2974996 | -3.333250 | 1.000 |
| E. koreanum.G:E. violaceum.G | 0.0602157 | 0.1378790 | -0.723240 | 0.755 |
| E. koreanum.G:E. koreanum.T | 0.2433230 | 0.2601762 | 1.142904 | 0.160 |
| E. koreanum.G:E. violaceum.T | 0.1965025 | 0.2480414 | 0.099278 | 0.485 |
| E. violaceum.G:E. koreanum.T | 0.2009112 | 0.2948823 | -0.643290 | 0.765 |
| E. violaceum.G:E. violaceum.T | 0.1532193 | 0.2803604 | -1.246380 | 0.920 |
| E. koreanum.T:E. violaceum.T | 0.0910085 | 0.0790522 | 2.540634 | 0.010 |

Table S6: Estimated marginal means of total adjusted curvature through flower development in *Epimedium*.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| new\_stage | species | Estimated margin of means (curvature in degrees/mm) | SE | df | lower.CL | upper.CL |
| A | E. koreanum | 0.2032339 | 0.378570 | 11.630850 | -1.052520 | 1.458989 |
| C | E. koreanum | 4.5952827 | 0.479897 | 10.900900 | 2.980692 | 6.209873 |
| G | E. koreanum | 3.1315309 | 0.356897 | 8.564115 | 1.852474 | 4.410588 |
| T | E. koreanum | 0.4198968 | 0.285760 | 2.115997 | -2.788720 | 3.628516 |
| A | E. violaceum | 0.2538409 | 0.331157 | 6.482586 | -1.056890 | 1.564569 |
| C | E. violaceum | 0.9015970 | 0.493325 | 10.387200 | -0.776970 | 2.580161 |
| G | E. violaceum | 0.5574859 | 0.492260 | 27.051270 | -0.897630 | 2.012602 |
| T | E. violaceum | 0.2948611 | 0.328828 | 3.082219 | -1.888830 | 2.478547 |

# References

Adams, D.C., and Otárola-Castillo, E. (2013). Geomorph: An R package for the collection and analysis of geometric morphometric shape data. Methods in Ecology and Evolution *4*, 393–399.

Baldwin, S.P., Oberholser, H.C., and Worley, L.G. (1931). Measurements of birds (Cleveland Museum of Natural History).

Berger, B.A., Ricigliano, V.A., Savriama, Y., Lim, A., Thompson, V., and Howarth, D.G. (2017). Geometric morphometrics reveals shifts in flower shape symmetry and size following gene knockdown of *CYCLOIDEA* and *ANTHOCYANIDIN SYNTHASE*. BMC Plant Biology *17*, 205–214.

Bodenhofer, U., Bonatesta, E., Horejš-Kainrath, C., and Hochreiter, S. (2015). Msa: An r package for multiple sequence alignment. Bioinformatics *31*, 3997–3999.

Bonhomme, V., Picq, S., Gaucherel, C., and Claude, J. (2014). Momocs: Outline analysis using R. Journal of Statistical Software *56*, 1–24.

Borchers, H.W. (2019). Pracma: Practical numerical math functions. R package version 2.2.5.

Buttrose, M., Grant, W., and Lott, J. (1977). Reversible curvature of style branches of *Hibiscus trionum* L., a pollination mechanism. Australian Journal of Botany *25*, 567–570.

Campos, E.O., Bradshaw, H.D., and Daniel, T.L. (2015). Shape matters: Corolla curvature improves nectar discovery in the hawkmoth *Manduca sexta*. Functional Ecology *29*, 462–468.

Dalayap, R.M., Torres, M.A.J., and Demayo, C.G. (2011). Landmark and outline methods in describing petal, sepal and labellum shapes of the flower of mokara orchid varieties. International Journal of Agriculture and Biology *13*, 652–658.

Hamilton, R.B. (1975). Comparative behavior of the American Avocet and the Black-necked Stilt (Recurvirostridae). Ornithological Monographs iii–98.

Kawabata, S., Yokoo, M., and Nii, K. (2009). Quantitative analysis of corolla shapes and petal contours in single-flower cultivars of *Lisianthus*. Scientia Horticulturae *121*, 206–212.

Kuznetsova, A., Brockhoff, P.B., and Christensen, R.H.B. (2017). lmerTest package: Tests in linear mixed effects models. Journal of Statistical Software *82*, 1–26.

Lenth, R., Singmann, H., Love, J., and others (2018). Emmeans: Estimated marginal means, aka least-squares means. R Package Version *1*.

Lindqvist, C., Motley, T.J., Jeffrey, J.J., and Albert, V.A. (2003). Cladogenesis and reticulation in the Hawaiian endemic mints (Lamiaceae). Cladistics *19*, 480–495.

Manning, J.C., and Goldblatt, P. (1997). The *Moegistorhynchus longirostris* (diptera: Nemestrinidae) pollination guild: Long-tubed flowers and a specialized long-proboscid fly pollination system in southern Africa. Plant Systematics and Evolution *206*, 51–69.

Mountainspring, S. (1987). Ecology, behavior, and conservation of the Maui Parrotbill. The Condor *89*, 24–39.

Muller, A. (1995). Morphological specializations in Central European bees for the uptake of pollen from flowers with anthers hidden in narrow corolla tubes (Hymenoptera: Apoidea). Entomologia Generalis *20*, 43–57.

Nii, K., and Kawabata, S. (2011). Assessment of the association between the three-dimensional shape of the corolla and two-dimensional shapes of petals using Fourier descriptors and principal component analysis in *Eustoma grandiflorum*. Journal of the Japanese Society for Horticultural Science *80*, 200–205.

Ortiz, P., Arista, M., and Talavera, S. (2000). Pollination and breeding system of *Putoria calabrica* (Rubiaceae), a Mediterranean dwarf shrub. Plant Biology *2*, 325–330.

Peng, F., Campos, E.O., Sullivan, J.G., Berry, N., Song, B.B., Daniel, T.L., and Bradshaw Jr, H. (2019). Morphospace exploration reveals divergent fitness optima between plants and pollinators. PloS One *14*, e0213029.

Pour, A.S., Chegini, G., Zarafshan, P., and Massah, J. (2018). Curvature-based pattern recognition for cultivar classification of *Anthurium* flowers. Postharvest Biology and Technology *139*, 67–74.

Rohlf, F.J. (1990). Fitting Curves to Outlines. In Proceedings of the Michigan Morphometrics Workshop, F.J. Rolhf, and F.L. Bookstein, eds. (Ann Arbor, MI: University of Michigan Museum of Zoology), pp. 177–188.

Rohlf, F.J. (2015). The tps series of software. Hystrix *26*.

Ruan, C., Li, H., and Mopper, S. (2008). The impact of pollen tube growth on stigma lobe curvature in *Kosteletzkya virginica*: The best of both worlds. South African Journal of Botany *74*, 65–70.

Silva, A.R. da, and Lima, R.P. de (2017). Determination of maximum curvature point with the R package soilphysics. International Journal of Current Research *9*, 45241–45245.

Song, X., Gao, K., Fan, G., Zhao, X., Liu, Z., and Dai, S. (2018). Quantitative classification of the morphological traits of ray florets in large-flowered Chrysanthemum. HortScience *53*, 1258–1265.

Webster, M., and Sheets, H.D. (2010). A practical introduction to landmark-based geometric morphometrics. The Paleontological Society Papers *16*, 163–188.