Relaxed selection in the chasmogamous flowers of *Impatiens capensis* (Balsaminaceae): A natural mutation accumulation experiment

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Abstract:

When an organismal structure experiences relaxed selection, mutation and drift can become major forces that drive its evolution. Spontaneous mutations tend to accumulate at a steady rate under relaxed selection, but their effects are less well documented, especially in the case of the angiosperm flower. *Impatiens capensis* is an annual herb. Individual plants have the capacity to produce open (chasmogamous) flowers and closed (cleistogamous) flowers. Based on divergent vegetative characteristics, sun and shade-ecotypes of this species have been described in previous research on this species. Typically, in their natural habitats, sun-ecotype plants produce both types of flowers, while shade-ecotype plants produce only cleistogamous flowers—though they retain the ability to produce chasmogamous flowers if transplanted to sunny places. Therefore, under natural (shady) habitat condition, the flowers of shade-ecotype plants experience relaxed selection and are expected to accumulate mutations that affect their shape and size. We grew population samples from shade and sun-ecotypes under sunny common garden conditions which induced chasmogamous flowers in all plants. We analysed shape and size variation of the modified sepal (the central attractive organ in chasmogamous flowers). In shade-ecotype plants, we found that the accumulated mutations both increased shape variance in the sepal body and reduced sepal size (compared to the sun ecotype). Using evidence from artificial inbreeding experiments, we infer that the mutations affecting sepal shape tend to be partially recessive while those affecting sepal size tend to be additive. While spontaneous mutation is likely to be deleterious at the individual population level, we suggest that the chasmogamous/cleistogamous dual reproductive strategy may allow chasmogamous flowers to accumulate mutations of various effect under relaxed selection and possibly lead to adaptations to new pollinator species, which in turn may facilitate speciation.

Résumé:

Lorsqu'une structure organique subit une sélection relâchée, la mutation et la dérive peuvent devenir des forces majeures qui conduisent son évolution. Les mutations spontanées ont tendance à s'accumuler à un rythme constant sous sélection relâchée, mais leurs effets sont moins bien documentés, en particulier dans le cas de la fleur d'angiosperme. L'Impatiens capensis est une plante herbacée annuelle. Les plantes individuelles ont la capacité de produire des fleurs ouvertes (chasmogames) et des fleurs fermées (cléistogames). Sur la base de caractéristiques végétatives divergentes, les écotypes de soleil et d'ombre de cette espèce ont été décrits dans des recherches antérieures sur cette espèce. En règle générale, dans leurs habitats naturels, les plantes à écotype solaire produisent les deux types de fleurs, tandis que les plantes à écotype à l'ombre ne produisent que des fleurs cléistogames, bien qu'elles conservent la capacité de produire des fleurs chasmogames si elles sont transplantées dans des endroits ensoleillés. Par conséquent, dans un habitat naturel (ombragé) conditionné, les fleurs des plantes à écotype d'ombre subissent une sélection relâchée et devraient accumuler des mutations qui affectent leur forme et leur taille. Nous avons cultivé des échantillons de population à partir d'écotypes d'ombre et de soleil dans des conditions de jardin commun ensoleillé qui ont induit des fleurs chasmogames dans toutes les plantes. Nous avons analysé la variation de forme et de taille du sépale modifié (l'organe central d'attraction des fleurs chasmogames). Chez les plantes à écotype d'ombre, nous avons constaté que les mutations accumulées augmentaient à la fois la variance de forme dans le corps du sépale et réduisaient la taille du sépale (par rapport à l'écotype soleil). En utilisant des preuves d'expériences de consanguinité artificielle, nous déduisons que les mutations affectant la forme des sépales ont tendance à être partiellement récessives tandis que celles affectant la taille des sépales ont tendance à être additives. Alors que la mutation spontanée est susceptible d'être délétère au niveau de la

population individuelle, nous suggérons que la double stratégie de reproduction chasmogame/cleistogame peut permettre aux fleurs chasmogames d'accumuler des mutations d'effets divers sous une sélection relâchée et éventuellement conduire à des adaptations à de nouvelles espèces de pollinisateurs, qui à leur tour peut faciliter la spéciation.

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This thesis is dedicated to my parents and my two odd-tempered cats.

Contributions of Authors:

This thesis is presented in the manuscript style to be submitted to the journal *Evolution.* Yi Zhao and Daniel Schoen conceived of the project together, and Yi Zhao conducted the research, data analysis, interpretation of results, and writing of the manuscript. Dan Schoen assisted with stylistic and grammatical editing of the manuscript.

Introduction:

The major forces of evolutionary change include mutation, migration, drift, and selection (Endler, 1986; Fong et al., 1995; Lahti et al., 2009). While mutation is ever-present, evolutionary biologist have long argued about the relative importance of drift versus selection to trait evolution, for example, as typified by the long running debate between Wright and Fisher (reviewed in Lande, 1976). Nevertheless, in some cases, when a specific source of selection is present, it is possible to predict how traits or structures will evolve. Classical examples include beak evolution in Darwin's finches in response to different ecological niches (Grant, 1976; Grant 1999), as well as 'industrial melanisation'(Kettlewell, 1955). In the case of plants, one set of classic examples include 'pollinator ecotypes', where flowers evolve different colours or corolla shape in response to colour preference or tongue length to various pollinators (Harder and Johnson, 2009; Newman et al., 2014).

On the other hand, when an important source of selection is removed or reduced (relaxed selection), it is more difficult to predict how traits or structures will evolve. In these instances, the remaining sources of selection, as well as mutation, migration, and drift can interact and produce various outcomes (e.g., persistence, vestigialisation, or complete loss of the trait in question) (Lahti et al., 2009). An example is the Mexican tetra fish *Astyanax mexicanus*. In some cave-dwelling populations, eyes have been lost completely, while in other cave-dwelling populations, eyes have evolved to become more variable in size (Jeffery, 2005; Wilkens, 1988).

One of the possible consequences of relaxed selection is that it can unmask the effects of the remaining evolutionary forces, especially mutation (Lahti et al., 2009). Under relaxed selection, many kinds of mutations can accumulate freely in separate linages with the help of genetic drift. Over the years, evolutionary biologists have studied the effects of accumulated mutations under

relaxed selection mainly in the lab. Many concluded that most accumulated mutations are mildly deleterious as they impair reproductive performance and survivorship (Lynch et al., 1999), although there is debate on this issue (Bataillon, 2003). These studies mainly used the two approaches to relax selection and allow mutations to accumulate: (1) different linages within a population are forced, generation after generation, through extreme bottlenecks; (2) populations are maintained in benign environments under conditions where each individual contributes equally to the next generation (e.g., Fry et al., 1996; Mukai, 1964; Shabalina et al., 1997). These types of lab experiments generally have found that accumulated mutations gradually reduced organismal fitness (e.g., survival, fecundity, growth rate). However, some studies have shown that the accumulated mutations increased only the variances of fitness traits, while the mutations were roughly evenly distributed in their positive and negative effects (Shaw et al., 2000). While debate continues over the effects of such accumulated mutations accumulated under lab conditions, there is even less information and about how mutations affect the fitness consequences of traits and their evolution in nature.

Flowers are the angiosperm structure that harbour the male and female gametes, and thus are thought to be strongly shaped by selection (Barrett, 2002). Many plants require pollinators (wind or animals) to disperse and receive pollen, and pollinators are one of the most important selection agents of floral traits, such as shape, size, colour and scent (e.g., Galen and Kevan, 1980; Galen, 1989; Menz et al., 2015; Miller, 1981; Stelleman 1984). There is widespread evidence that pollinators facilitate the adaptive divergence or convergence of flowers in many plant species (Fenster et al., 2004; Grant and Grant, 1965; Jabbour and Renner, 2012). On the other hand, when plants lose their pollinators, the fate of floral traits is less certain. In some cases, the loss of pollinators led to the reduction of flower size and pollinator rewards, presumably a response to

selection to reduce costly floral energy investment or promote self-fertilisation for reproductive assurance (Lekberg et al., 2012; Sicard and Lenhard, 2011; Steiner, 1998). In a few cases, loss of pollinators led to the evolution of asexual reproduction, which presumably is tantamount to completely relaxed selection on the flower (Eckert et al., 1999). Because the insect pollinators are no longer required for seed set and flower production is not energy-costly for these plants, mutation and drift have likely become the main evolutionary forces acting on the flower (Eckert et al., 1999; Ally et al., 2010). These cases are akin to natural mutation accumulation experiments and have the potential to reveal the effects of *de novo* mutations on floral traits.

Impatiens capensis (Balsaminaceae) is an annual herb that grows in damp places from shady forest understories to sunny riverbanks and marshes (Waller, 1980). All plants have the capacity to produce large open (chasmogamous, CH) flowers that attract pollinators for outcrossing, as well as tiny closed (cleistogamous, CL) flowers that self-pollinate in bud without pollinators (Waller, 1979; Lord, 1981). In sunny habitats, both types of flowers are produced, while in shady habitats, only CL flowers are produced due to insufficient light intensity (Waller, 1980). Many populations of this species inhabit stable, shady forest floors, and have likely experienced many sequential generations of relaxed selection of the CH flower. Indeed, in this species, there are vegetative changes that accompany shady and sunny conditions. For instance, Dudley and Schmitt (1995) reported that 'shade ecotypes' of this species show a reduced internode response to low ratios of red: far-red light compared with their 'sun ecotype' counterparts. This growth response provides a convenient metric for locating populations that have inhabited shady or sunny conditions for many generations.

The CH flower of *Impatiens capensis* has a large, sack-like modified sepal with an opening in the front and a recurved spur at the back which together form the principal attractive and reward

structure of the flower (Rust, 1977; Waller, 1979; Fig. 1a). This modified sepal resembles the flower corolla due to its large size, bright orange colour, and dotted scarlet nectar guides. The shape and size of the modified sepal is critical for successful pollen removal and deposition (Rust, 1979; Travers et al., 2003). When CH flowers are produced, pollinator-mediated selection is expected to constrain morphology of the modified sepal.

In this study, we relied on the internode growth response feature described by Dudley and Schmitt (1995) to locate populations of stable sun and shade ecotypes of *Impatiens capensis*. These populations could serve as seed sources for a common garden experiment. Specifically, we grew plants from both ecotypes under full sunlight to force CH flower production. We then compared shape and size variation of the modified sepal between shade and sun ecotypes. Given that CH flowers of shade-ecotype plants are not produced in the wild, they are expected to be shielded from selection (i.e. relaxed selection). Therefore mutations affecting the size, shape, etc. of these flowers should be free to accumulate within population through lineages that are under drift—since shade-ecotype plants reproduce mainly through CL seeds, propagation of each generation is by lineages of reduced effective population size (i.e., strong drift or bottleneck within lineages so that different mutations easily fix within different lineages). In this way, the work described here is akin to a natural mutation accumulation experiment for mutations affecting the CH flower, and thus allowed us to reveal the phenotypic effect of these mutations. The results of this study are also important for understanding how populations may evolve when they migrate beyond the range limits of their pollinators.

Materials and Methods:

STUDY POPULATIONS, SEED COLLECTION, ECOTYPE CLASSIFACTION

We collected seeds of *Impatiens capensis* from ten natural populations located across the south-eastern portion of Quebec, Canada from August through October 2018 (GSN, MSH, STR, VAR, RIG) and 2019 (RVY, PET, SAC, VOY, PHB) (Table 1). One population, RIG was only used only to test methods and was not otherwise part of the main study.

We noted the habitat of each population (e.g., shady forest, sunny riverbank), whether CH flowers were observed at the site (Table 1). To classify populations into ecotypes, we examined the response of plants to the red: far-red (R: FR) light ratio. Dudley and Schmitt (1995) have previously shown that sun-ecotype plants exhibit pronounced elongation of the first internode (internode 1) under low R: FR ratios, a response that is thought to be adaptive to avoid shading by neighbouring plants in high-density populations. Since the response of the first internode to R: FR is an evolved characteristic that reflects the history of the growth conditions of the population (Schmitt and Wulff, 1993; Dudley and Schmitt, 1995), it is reliable for classifying the ecotypes. For example, a population in a shady habitat should still exhibit the R: FR internode elongation response, even if this population was established a few generations ago from seeds originating in a stable sunny habitat.

Internode elongation growth response to R: FR ratio

On an early, sunny afternoon in mid-August 2019, we estimated light intensity and R: FR ratio under a fully grown forest canopy (shady habitat) and an open area (sunny habitat) using MQ-200 quantum separate sensor with hand-held meter (Apogee Instruments, USA) and a SKR 110 red/far-red sensor (Skye Instruments Ltd, UK) in Mount Royal Park, Montreal, where *I.*

capensis grows naturally. The R: FR ratios for shade and sun were 0.4-0.5 and 0.95-1.0, respectively. These values correspond closely to the R: FR ratios used in past growth experiments that studied ecotypic differentiation in *I. capensis* (Dudley and Schmitt, 1995), and were used in our experimental studies.

We conducted R: FR growth response experiments in two Conviron PGW 36 growth chambers (Controlled Environment Ltd, Winnepeg, Canada) in the McGill University Phytotron. In one chamber, we adjusted the R: FR ratio to 0.4 (low R: FR) using a Heliospectra LX601C lamp (Heliospectra Canada, Toronto) mounted in the centre of chamber. This lamp was programmed for a given R: FR ratio by adjusting the number and power of the light emitting diodes that differ in wavelength output. In the other chamber, we adjusted R: FR ratio to 0.95-1.0 (high R: FR) using a combination of fluorescent and incandescent bulbs. R: FR values were monitored with the same SKR 110 red/far-red sensor used for field measurements. We set illumination levels in both chambers to 70μmol m^{-2} s^{-1}.

To break dormancy, we kept seeds on moistened filter paper at 4°C in the dark for four months. The young seedlings (at the stage when radicles first appeared) were then moved to the chambers and grown in 10 x 10 cm pot in a 1:1 mixture of black earth and Promix^{TM}. Each seedling was randomly assigned to either the low R: FR ratio or to the high R: FR ratio conditions ($N \approx 15$ per population per treatment). Within each chamber, seedlings were assigned to random positions and watered daily. To minimize a chamber and position effect, we switched the lights and the seedlings between chambers once a week, and re-randomised seedling positions. When the seedlings were 5 weeks old (as determined from the transplant date), we measured the length of their first internode with a 150cm soft ruler.

We used one-way ANOVA and *a posteriori* tests (Tukey's HSD) in R (version 4.0.2) to

test for differences in growth response of internode 1 to R: FR ratios. In experiments conducted with the 2018 collections (GSN, MSH, STR, VAR), seeds were moved from the dark to the illuminated growth chambers when radicles were 1.5-3cm long, whereas in experiments conducted with the 2019 collections (RVY, PET, SAC, VOY, PHB), seedling were moved to the chambers when radicles were less than 1cm in length. Because this difference in timing affected subsequent growth measurements, we analysed the data separately for the 2018 and 2019 experiments.

SHAPE AND SIZE ANALYSIS FOR THE CHASMOGAMOUS (CH) FLOWERS

Plant growth conditions and photography of CH flowers

We grew field-collected seeds to full-sized plants under sufficient light intensity so that they produced CH flowers. Dormancy was broken as described above for the R: FR growth response experiments. We grew all plants in the McGill Phytotron greenhouse. GSN, MSH, STR, VAR, RIG plants were grown from January-June of 2019, and RVY, PET, SAC, VOY, PHB plants from February-July of 2020. We watered the plants each day and fertilised them once every two weeks with a 10-10-10 nitrogen-phosphorus-potassium salts (NPK) fertiliser and a mixture of black earth and water.

We selected two CH flowers per plant at random from the main stem and photographed each in the side view. To illuminate the flowers, 500 lumen LED light systems were positioned 15cm from both sides and the top of the flower. We used a Nikon DL7300 digital camera with AF-S DX Micro-NIKKOR 40mm f/2.8G lens. F-stop and shutter speed were adjusted so that the depth of field encompassed the entire flower. We placed a 10 cm ruler immediately behind the flower for size calibration of each photo. These side view photos capture the large, modified sepal with its nectar spur. This portion of the flower contains the androecium, gynoecium, and

nectar (Fig. 1a).

Landmarking the modified CH sepals for shape and size analysis

To conduct shape and size analyses of the modified sepal (hereafter, simply 'sepal') we recorded the positions of 'landmarks' and 'semi-landmarks' from the flower photographs (Table S2 and Fig. 1a). Landmarks identify different points on the sepal and are defined by a set of repeatable rules based on clear anatomical criteria, for example, the tip of the spur, the attachment point of pedicel to the sepal (Klingenburg, 2010). Semi-landmarks are located along the outline of the sepal and capture additional shape information. They are allowed to slide along the sepal's outline when conducting geometric morphometric analysis (Klingenburg, 2010).

We digitised landmarks and semi-landmarks for shape and size analysis using the software package, tps.dig (https://life.bio.sunysb.edu/ee/rohlf/software.html). All photos included a centimetre scale ruler.

Sepal shape variation among plants

We analysed shape variation of all landmarked sepals using the Geomorph R-Package 3.3.1 (Adams et al., 2020). Before proceeding with the full shape analysis, we first determined whether the sepal could be considered as an integrated organ or as two separated substructures, i.e., the sack-like sepal body and the curved nectar spur (Wagner and Altenburg, 1996; Klingenburg and Zaklan, 2000; Klingenburg 2010). We split the landmarks into two groups, one describing the spur and the other describing the sepal body (Fig. 1b). These substructures were Procrustes transformed to eliminate variation due to size and orientation (Klingenburg, 2010). We performed a morphological integration test on the transformed substructures to determine whether the two

substructures significantly co-vary using two-block partial least squares (PLS) (Adams and Otárola-Castillo, 2013). The two substructures did not significantly co-vary (Table S4) suggesting that they evolve independently. Accordingly, we analysed the shape variation of the two substructures (the spur and the sepal body) separately.

For the main analysis of shape variation, we Procrustes-transformed the landmark and semi-landmark configurations for each substructure. These transformed landmark configurations were then analysed by Principal Component Analysis (PCA). The principal components (PCs) represent a set of fewer, uncorrelated linear combinations of the landmark and semi-landmark Procrustes-transformed coordinates (Zelditch et al., 2004). We used the first two PCs to visualise the major axes of shape variation of each substructure (e.g., 95% confidence ellipses). Finally, to measure shape variation within each population, we calculated the weighted Euclidean distance between each sepal (spur) and the population mean sepal (spur) shape (i.e., distance to centroid) described by the coordinates PC 1 through n (n = total number of PCs) with the following formula:

$$d_i = \sqrt{\sum_{j=1}^{n} wt_j \times (x_{ij} - \bar{x}_j)^2}$$

where d_i denotes the weighted Euclidean distance of the i^{th} sepal body/spur in the population to the population centroid, wt_j is the overall shape variance captured by the j^{th} PC (i.e., eigenvalue of the j^{th} PC), x_{ij} is the position of the i^{th} sepal body/spur along the j^{th} PC, and $\bar{x}_j$ is the mean position of the population along the j^{th} PC. We compared the distances to centroid among the ecotypes with a nested ANOVA (after cube root transformation) in R (4.0.2) and with a Tukey test at the ecotype level (lme package in R 4.0.2) to test whether sepal shape is more variable in the shade ecotype than sun ecotype. This was predicted by the hypothesis that relaxed selection on the CH flower should lead to less developmental stability in the structure.

Sepal shape variation within plants (developmental stability)

To further analyse shape variation among the ecotypes, we calculated the weighted Euclidean distances between two sepal bodies/spurs from the same plant (i.e., paired flower distance) for each substructure. The paired flower distance is another measure of developmental stability (how much on average to the shapes of the two flowers differ from one another). The previous formula was modified as follows:

$$d_{pk} = \sqrt{\sum_{j=1}^{n} wt_j \times (x_{k1} - x_{k2})^2}$$

where d_{pk} denotes the weighted Euclidean distance between the two sampled sepal bodies/spurs of the k^{th} plant, wt_j is overall shape variance captured by the j^{th} PC (i.e., eigenvalue of the j^{th} PC), x_{k1} of is the position of the 1st sepal (spur) along the j^{th} PC in the k^{th} plant, and x_{k2} is the position of the 2nd sepal (spur) along the j^{th} PC in the k^{th} plant. We compared the paired flower distances among the ecotypes with a nested ANOVA (with cube root transformed data) in R (4.0.2) and a Tukey test at the ecotype level (lme package in R 4.0.2) to test whether sepal shape is more variable in the shade ecotype than sun ecotype.

Sepal size variation

We estimated and compared sepal sizes by adding up the area of seven triangles (Fig. 1c) based on nine pre-defined landmarks. To verify the accuracy of this size estimation method, we randomly chose twenty flower photos and estimated sepal size using this triangle method, as well as by directly measuring the areas enclosed by sepal outlines using the entire photographed sepal body and ruler using software package ImageJ (https://imagej.nih.gov/ij/docs/guide/). The latter

method requires significantly more effort. The two sets of estimated areas did not differ significantly from one another based on the results of a Model II regression (smatr package in R 4.0.2), as the slope of the regression line did not differ significantly from 1 (Table S5, $p = 0.835$). We thus used the triangle method to capture sepal size variation for all flowers in the study. We tested whether sepal size differed significantly among ecotypes, among populations within an ecotype and among plants within a population by a nested ANOVA in R (4.0.2) and Tukey test on the ecotype level (lme package in R 4.0.2).

RESPONSE OF SEPAL SHAPE AND SIZE TO INBREEDING

Seed sources and crosses

We used two populations (RVY and PHB) for the inbreeding depression experiment. The inbred seeds came directly from CL flowers (RVY self, N = 45; PHB self, N = 55; ~20 seeds per maternal parent). The outcrossed seeds came from hand-pollination of CH flowers using pollen from a different, randomly selected plant. All seeds were then labelled by their pollination treatment (i.e., self or cross) and their maternal and paternal parents. The seeds were kept on moist filter paper in petri dishes at 4°C for three and a half months before planting.

Shape and size analysis of CH flowers for inbreeding depression

We grew the seedlings to full size in three separate Conviron PGW 36 growth chambers. Lights were sufficient such that all plants produced CH flowers. Plants were watered daily and fertilised once a week with 10-10-10 NPK. Once every two weeks, we randomised the position of plants within and among chambers to minimise chamber and position effect. When the plants began to produce CH flowers, we randomly chose two flowers per plant from the main stem and

photographed the side view of the flower for shape and size analysis of the sepal within population between pollination treatments as described above.

Results:

ECOTYPE CLASSIFICATION

Based on the response of internode 1 to red: far-red (R: FR), we classified populations GSN, MSH, RVY as the shade ecotype; populations PHB, STR, VAR as the sun ecotype; and populations PET, SAC, VOY as the intermediate ecotype. The R: FR experiment showed that for the 2018 populations, under the low R: FR treatment, plants from STR and VAR had significantly longer first internode than plants from GSN and MSH ($p = 0.0014$, Fig. 2a; Table S7). There were no significant differences between plants from GSN and MSH or between plants from STR and VAR (Fig. 2a; Table S7a). In contrast, under the high R: FR treatment, the first internode length did not differ significantly among the populations ($p = 0.569$, Fig. S1a; Table S7b). Accordingly, we classified populations STR and VAR as the sun ecotype and GSN and MSH as the shade ecotype.

For 2019 populations, under the low R: FR treatment, PHB plants had significantly longer first internode than RVY plants, while the first internode length of PET, SAC, VOY did not differ significantly from either RVY or PHB ($p < 0.0001$, Fig. 2b; Table S7a). Under high R: FR treatment, no significant difference existed between the first internode, except for RVY and PHB. In addition, the mean length difference for RVY and PHB (1.5cm) under high R: FR treatment was smaller than that of the low R: FR treatment (2.9cm; Fig. S1b). We classified PHB as the sun ecotype, RVY as the shade ecotype, PET, SAC, VOY as the intermediate ecotype based on these results.

SHAPE AND SIZE VARIATION OF THE MODIFIED SEPAL

Sepal shape variation among plants

The first two principal components captured about 57% of the total shape variation (Fig. 3). Along the PCs, the sepal body's main shape changed from a round pocket to a narrow funnel, while the sepal opening changed from narrow to wide along PC 2 (Fig. 3, wire diagrams). For the spur, the first two PCs captured about 70% of the total shape variation and its shape changed from nearly straight to highly curved along both PCs (Fig. 4, wire diagrams).

Sepal body shape, but not spur shape was more variable in the shade ecotype than in the sun ecotype, based on results from weighted Euclidean distances between individual sepals and their respective population mean shape (i.e., distance to centroid). For the sepal body, the shade ecotype had significantly larger distance to centroid (~28.0% larger for untransformed data) than the sun ecotype, based on a nested ANOVA and a Tukey test on the ecotype level ($p = 0.049$, Fig. 5; Table 2a). The shade-ecotype populations also had relatively larger 95% confidence ellipses than sun-ecotype populations on the first two PCs (i.e., the sepals scattered more on the PC1-PC2 coordinate plane for shade-ecotype populations) (Fig. 3, green and orange panel). For the spur, there were no significant differences among ecotypes or populations in the distance to centroid (Fig. 6; Table 2b). There were no specific trends on 95% confident ellipses between shade and sun ecotypes (Fig. 4).

Sepal shape variation within plants (developmental stability)

Plants of the shade ecotype did not show significantly more within plant variation than plants of the sun ecotype for either the sepal body or spur, based on results from weighted paired flower Euclidean distances. Although shade ecotype plants had larger mean paired flower distance than sun ecotype for sepal body (~19.6% larger for untransformed data), the result was not significant, as based on a nested ANOVA (cube-root-transformed data) and a Tukey test on the

ecotype level (Fig. S2; Table S8a). The mean paired flower distance for spur was nearly the same for both shade and sun ecotypes (Fig. S3; Table S8b).

Sepal size variation

The shade ecotype had significantly smaller sepal size than the sun ecotype (~23.2% smaller for original data), while 'intermediate' ecotype had flowers whose sizes fell between those of the sun and shade ecotypes, as based on a nested ANOVA and a Tukey test at the ecotype level ($p = 0.013$, Table 3; Fig. 7).

RESPONSE OF SEPAL SHAPE AND SIZE TO INBREEDING

Sepal shape variation in progeny from outcrossing and selfing

For both RVY and PHB populations, the progeny from selfing were more variable than progeny from outcrossing, as based on results of weighted Euclidean distance from individual sepal to centroid. For both populations, progeny from selfing had significantly larger mean distance to centroid than progeny from outcrossing (for untransformed data, sepal body: ~32.0% larger for RVY, ~18.8% larger for PHB; spur: ~66.4% larger for RVY, ~31.8% larger for PHB), based on a nested ANOVA (cube-root-transformed data) and a Tukey test on pollination treatment level (all p-value < 0.01, Figs 8 and 9; Table 4). The self-fertilised groups (i.e., RVY self and PHB self) also had relatively larger 95% confidence ellipses on the first two PCs for both substructures (Figs S4 and S5).

Except for the spur in population RVY, progeny from selfing did not show significantly more variation in paired flower distance than progeny from outcrossing, as based on results of the weighted Euclidean distance analysis between paired sepal body/spur within plants. Although for

both populations, progeny from selfing always had relatively larger paired flower distance than progeny from outcrossing, the result was not significant except for the spur in population RVY, as based on an ANOVA and Tukey test on the pollination treatment ($p = 0.044$ for spur of RVY, Figs S6 and S7; Table S9).

Sepal size variation in progeny from outcrossing and selfing

For PHB, progeny from selfing did not have significantly smaller sepal size than progeny from outcrossing, based on a nested ANOVA (untransformed data) and a Tukey test on pollination treatment level ($p = 0.745$, Fig. S8; Table S9). For RVY, however, progeny from selfing from had significantly larger sepal size than progeny from outcrossing (~11.5% larger for original data) based on the same analysis ($p = 0.0079$, Fig. S8, Table S10).

Discussion:

ECOTYPIC VARIATION AND THE STRENGTH OF SELECTION ON CH FLOWERS

This study examined the evolution of chasmogamous (CH) flowers in *Impatiens capensis* under relaxed selection. It is possible to analyse the effects of relaxed selection because the vegetative growth, ecology and evolution of this species help us infer the environmental history of different populations. The first internode response to low red: far-red (R: FR) light ratio is key to inferring such history. Dudley and Schmitt (1995) interpreted first internode elongation under shady conditions (low R: FR) as an evolved, morphogenic response. Plants with a history of growing in sunny locations (i.e., sun-ecotype plants) show a more pronounced first internode elongation than plants with a history of growing in shady locations (i.e. shade-ecotype plants). Under sunny conditions, shading by neighbouring plants is far more detrimental to growth than it is under shady conditions. The pronounced first internode elongation gives the sun-ecotype plants an early growth advantage which leads to an overall fitness advantage (Dudley and Schmitt, 1995), while shade-ecotype plants lacks such advantage. Conveniently, this adaptive characteristic allowed us to infer with some level of certainty the level of CH flowering in a population's past evolutionary history.

Some of our studied populations (GSN, MSH, RVY) showed little internode elongation to low R: FR light ratios, while others (PHB, STR, VAR) exhibited a pronounced internode elongation (Fig. 2). For these six populations, the first internode response to low R: FR ratios matches what we expected from evolution in either shady or sunny habitat where the seeds from these habitats were collected (Table 1). This suggests that these six populations have persisted in either stable shady or sunny habitats for a sufficient number of generations for their vegetative traits to diverge. We referred to these as 'shade' and 'sun' ecotypes, respectively. A few

populations (PET, SAC, VOY) showed a response that was intermediate between these ecotypes ("intermediate ecotype" is used to refer to these below and in the results)—their first internode response to low R: FR did not differ significantly from either shade or sun-ecotype populations. These populations likely experienced some years of shady and some years of sunny conditions. For example, fire and storms may have changed their habitats from shady to sunny; or the plants may have established in their present habitat from recent seed dispersal that originated from plants in the opposite habitat type. It is more difficult to detect the degree of relaxed selection on the CH flower in these cases.

Under natural shady conditions *Impatiens capensis* seldom produces CH flowers (Waller, 1980). In other words, CH flowers do not experience selection in shady conditions and the populations are free to accumulate mutations that affect CH flowers. Moreover, these populations consist largely of inbred lines, propagating generation after generation by seeds arising from self-pollination in CL flowers. From the standpoint of mutations that influence the CH flower, the three shade-ecotype populations studied here can be viewed as evolving under relaxed selection, akin to natural 'mutation accumulation' experiment. The three sun-ecotype populations can be viewed as the natural 'control' group under selection, as CH flowers in the three sun-ecotype populations presumably experience pollinator-mediated selection.

SHAPE AND SIZE VARIATION OF THE MODIFIED SEPAL UNDER RELAXED SELECTION

Shape variation of sepal body and spur among plants under relaxed selection

In the shade-ecotype populations we observed increased shape variance in sepal body—variance in sepal shape is *ca*. 30% larger compared to that in the sun ecotype populations (Figs. 3

and 5; Table 2a). PC coordinates for the sepal body are scattered widely over the PC axes in the shade-ecotype populations, rather than clustering around the population mean shape as in the sun-ecotype populations, especially with respect to PC 1 (the PC that captures the largest amount of the shape variance, ~32%). Under sunny conditions where CH flowers are produced, purifying selection may normally constrain shape variance because successful pollination requires precise spatial interaction between animal pollinators and floral organs. In bilaterally symmetric flowers such as *Impatiens capensis*, many genes control floral shape, each gene interacts with one another in specific ways (e.g., *Antirrhinum majus*, reviewed in Kramer, 2019). Shape development in bilateral symmetric flowers may thus be especially prone to disruption by mutation.

Since CH flowers are rarely produced in shade ecotype, mutations that increase shape variance in sepal body are selectively neutral in the normal shady habitats of the populations, unless they have pleiotropic effects elsewhere in the lifecycle. However, it is less clear whether such mutations are advantageous or deleterious should the progeny of these plants establish in sunny habitats. On the surface, disrupting spatial relationship between attractive organs of the flower and the anthers and stigmas may normally reduce pollination success. On the other hand, sepal shape divergence could be adaptive if it leads to more effective pollination by new pollinators. In the three sun-ecotype populations, we note that mean shape of the sepal has diverged (Fig. 3). This divergence may result from divergent pollinator-mediated selection among populations which favoured different sepal shapes. It is well documented that floral phenotypic variation (e.g., shape, colour and scent) can lead to fitness differences mediated by the interaction of flowers and pollinators (e.g., Galen et al., 1987; Johnson and Steiner, 1997; Newman et al., 2012). These examples also indicate that the same floral trait value can have very different fitness in habitats with different pollinators. Therefore, lacking field studies on the pollination of shade-ecotype CH

flowers, we cannot determine what would be the effects of the accumulated sepal shape mutations.

In contrast to the sepal body, relaxed CH flower selection did not significantly increase shape variance in the spur (Fig. 4 and 6; Table 2b). Shape variances for the spur were roughly similar between shade and sun ecotypes. It is possible that the spur of sun-ecotype populations experiences temporally-varying selection, which maintains high shape variance within population. For instance, past field studies of *Impatiens capensis* found that highly curved spur increases pollination success by hummingbirds (Travers et al., 2003, Young, 1988). On the other hand, highly curved spurs also increase nectar robbery by some bee species (Pan 1999; Temeles and Pan, 2002), which may decrease fitness. If there is year-to-year variation in hummingbird and bee abundance, the optimal spur shape may also vary, which maintains high shape variance in the spur. This could make it more difficult to detect how accumulated mutations under relaxed selection affect spur shape.

Sepal size under relaxed selection

Sepal size in shade-ecotype flowers is 23% less compared with sun-ecotype flowers (Fig. 7; Table 3). As in the case of mutations affecting the shape of the sepal body, mutations that reduce sepal size are likely neutral in shady habitats. On the other hand, if shade-ecotype plants re-establish in sunny habitats, the smaller sepal size may be deleterious, given that the major function of CH flower is to attract pollinators for outcrossing (Rust 1977; Schemske, 1978). Previous studies generally found that plants with larger petals or inflorescences attract more pollinators (e.g. Bell, 1985; Thomson, 1988), although some exceptions exist (e.g. Galen et al., 1987). When researchers artificially reduced petal size (Bell, 1985) or removed flowers on inflorescences (Thomson, 1988), the pollinators often visit the plant less frequently. As for *Impatiens capensis*,

field observations found that when flowers of various size were present, pollinators tend to prefer larger flowers (Bell, 1985). A more recent study of *I. capensis* found that when researchers artificially reduce the abundance of pollinators, plants that produce larger CH flowers (and more CL flowers) are selectively favoured (Panique and Caruso, 2020). Therefore, based on the pollinator preference on CH flowers, mutations that reduce sepal size are likely to be deleterious.

Shape variation of sepal body and spur within plants under relaxed selection (developmental (in)stability)

While relaxed selection on the CH flower seems to increase between-plant shape variation, it did not significantly increase within plant sepal shape variation, although 'paired flower distance' of sepal body for shade ecotype was about 20% larger than the sun ecotype (Fig. S2; Table S8a).—i.e., the stability of floral development within individual plants is not significantly affected. One possible reason for the insignificant increase in developmental instability is insufficient time. The populations did not accumulate sufficient mutations to express significant developmental instability. One mutation accumulation experiment on *Raphanus raphanistrum* also showed that after about 10 generations, mean fitness declined in the mutation accumulation group but the result was not significant compared to the control group (Roles and Conner, 2008). Another study on male clones of *Populus tremuloides* showed that older clones had lower pollen quality than younger clones, as older clones has been under relaxed selection for pollen quality for longer period (Ally et al., 2010). Another possible reason is that such within-plant variation has only a weak genetic basis (Leamy and Klingenberg, 2005). For instance, in a *Drosophila melanogaster* mutation accumulation study, fluctuating-asymmetry for bristle number and wing length was unaffected by mutation (Monodero et al. 1997).

INBREEDING REVEALS GENETIC ASPECTS OF MUTATIONS THAT AFFECT SEPAL SHAPE AND SIZE

Our results show that selfing (i.e., extreme inbreeding) significantly increased shape variance for both sepal body and spur (Figs. 8 and 9; Table 4). Most studies revealed that most deleterious mutations tend to be partially recessive (reviewed in Lynch et al. 1999). The increased shape variation after inbreeding is consistent with a partially to completely recessive basis for the mutations that cause increased sepal shape variation—i.e., selfing increases homozygosity in the progeny, which brings out the phenotypic effect of such mutations.

On the other hand, inbreeding had no consistent effect on sepal size or within plant variation in sepal shape (Fig. S6-S8; Table S9 and S10). This is consistent with an additive genetic basis for sepal size, and a nongenetic basis for within plant sepal variation. Additivity for mutations that affect the size of plant organs has frequently been reported (Kelly and Arathi, 2003; Carouso, 2007)

The increased shape variance in sepal body and spur in selfing versus outcrossing treatment matches the increased shape variance in sepal body in the shade versus sun ecotypes from field-collected seeds in sunny common garden conditions. Under normal field conditions, shade-ecotype plants reproduce mostly through selfing, while sun-ecotype plants reproduce partially through outcrossing (CH and CL flowers). Therefore, it is possible that some of the increased shape variance when progeny are grown under sunny common garden conditions result from uncovering the effects of accumulated recessive sepal-shape mutations through normal selfing (and consequent increased homozygosity) in shade ecotypes. Quantitative genetic approaches could help to resolve this (Cockerham and Weir, 1984; Cornelius, 1988), but are outside the scope of the

current investigation.

RELATIONSHIP OF THIS STUDY TO OTHER STUDIES OF RELAXED SELECTION

Lahti et al. (2009) reviewed multiple outcomes of relaxed selection on structures or traits in nature and found that may persist, become vestigial or disappear depending on remaining sources of selection, mutation, migration, drift and time. When the remaining sources of selection are absent on the structure or trait, mutation, migration and drift become dominant forces. Such 'neutral' systems exist when the structure or trait is no longer expressed (such as in the case of CH flowers in the shade race populations) or when the expression of the structure or trait does not reduce the organism's fitness (e.g., Ally et al., 2010; Eckert et al., 1999; Wilkens, 1988). In these instances it has been found that the structure or trait in question evolved smaller size or lost their original functions. The sepal size reduction in our study is consistent with these findings, but in this case, we can be more certain that the effects observed reflect the pre- as opposed to post-selection phenotypic consequences of the underlying mutations.

On the other hand, few studies have reported that relaxed selection affects trait variance within populations. In order to reveal how mutations affect trait variance, a relatively large population sample is needed. Large population samples such as those used in the current study capture many mutations from different lineages.

Conclusion:

In conclusion, our study suggests that relaxed selection increases sepal body variance and decreases sepal size in *Impatiens capensis*, presumably due to mutation accumulation in different lineages through drift. It is interesting to speculate about the possible macroevolutionary

consequences of our findings. Many, though not all *Impatiens* species produce cleistogamous and chamogamous flowers, with some species exhibit environmentally-dependent production of CH flowers seen in *I. capensis* (e.g., *I. pallida*, Schemske, 1978). The macroevolutionary consequences of a dual CH and CL flower-based reproduction system is somehow similar to gene duplication (Taylor and Raes, 2004; Crow and Wagner, 2005). In other words, having a duplicate may free the counterpart gene (or structure) to evolve under mutation, drift, and new selection and thereby lead to new genes or forms (as in the case of the flower). The flower itself plays a central role in reproductive isolation and speciation (Harder and Johnson, 2009). *Impatiens* as a group is one of the most diverse plant genera in eudicots (Yu et al., 2016), and exhibits such a wide degree of variation in flower structure that it has been referred to as the 'orchids of the dicots'. The genus underwent adaptive radiation under climate change in the Pliocene and Pleistocene (Janssens et al., 2009). Whether in general, CH floral mutations accumulated under relaxed selection facilitates the adaptive radiation in *Impatiens* is an interesting question to pose. Emerging genomic and computational morphometric approaches (Xu and Bassel, 2020; Wessinger and Hileman, 2020) may assist in characterizing these mutations.

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mean (centroid). Ellipses around the dots are the 95% confidence ellipse on PC1 and PC2. The four wire diagrams at the bottom left and side represent shapes at the extremes of PC1 and PC2. Percentages are the amount of variation in shape accounted for by each PC axis. Sample sizes as in Fig. 3.

Fig. 5. Boxplots of weighted Euclidean distances for each sepal to its respective population centroid (untransformed data). Colours represent final ecotype classification as based on R: FR experiments. Letters above each ecotype represent statistically significant groups as based on a nested ANOVA and a Tukey test at the ecotype level. Sample sizes as in Fig. 3.

Fig. 6. Boxplots of weighted Euclidean distances for each spur to its respective population centroid (untransformed data). Colours represent final ecotype classification as based on R: FR experiments. Letters above each ecotype represent statistically significant groups as based on a nested ANOVA and a Tukey test at the ecotype level. Sample sizes as in Fig. 3.

Fig. 7. Boxplots of sepal size for each population (untransformed data). Colours represent final ecotype classification as based on R: FR experiments. Letters above each ecotype represent statistically significant groups based on a nested ANOVA (untransformed data) and a Tukey test on ecotype level. Sample sizes as in Fig. 3.

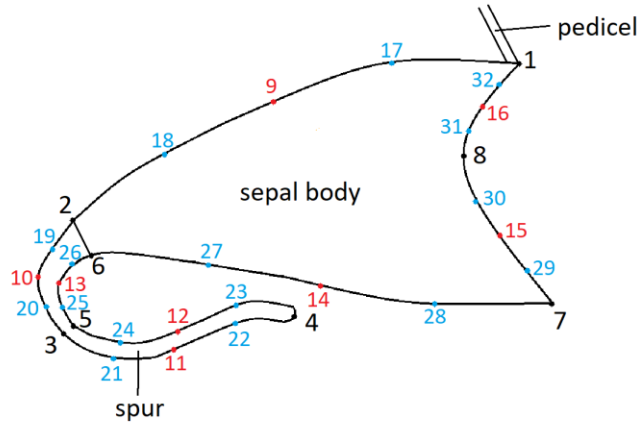
Fig. 8. Response of sepal body shape to inbreeding in populations RVY and PHB (sample sizes: total = 444; RVY cross = 32, RVY self = 90; PHB cross = 212, PHB self = 110). Boxplots show weighted Euclidean distance for each sepal body to its respective pollination treatment centroid

(untransformed data). Colours represent final ecotype classification as based on R: FR experiments. Letters above each pollination treatment represent statistically significant groups as based on a nested ANOVA (cube-root-transformed data) and a Tukey test on pollination treatment level for each population.

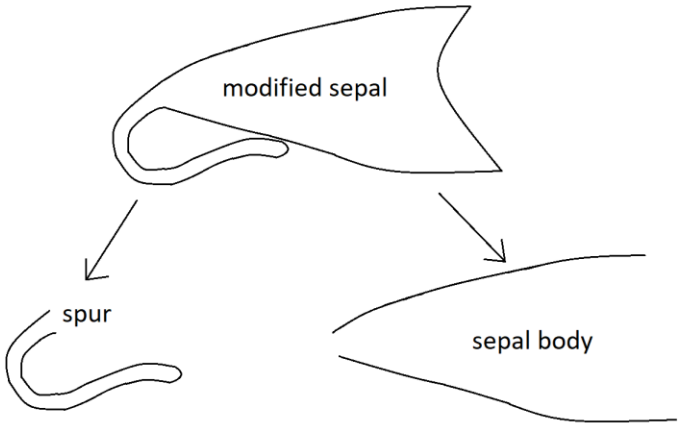
Fig. 9. Response of spur shape to inbreeding in populations RVY and PHB. Boxplots show weighted Euclidean distance for each spur to its respective pollination treatment centroid (untransformed data). Colours represent final ecotype classification as based on R: FR experiments. Letters above each pollination treatment represent statistically significant groups as based on a nested ANOVA (cube-root-transformed data) and a Tukey test on pollination treatment level for each population. Sample sizes as in Fig. 8.

Figures:

(a) Schematic diagram of the modified sepal



(b) The modified sepal (top) and its substructures (bottom)



(c) Seven trangles for estimating flower size

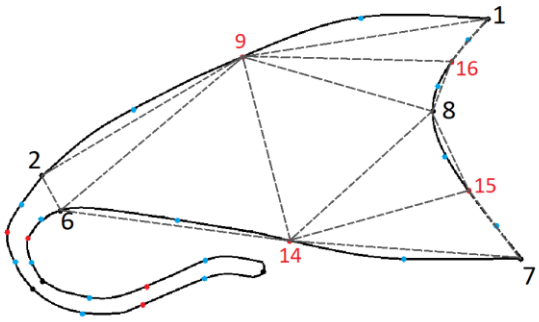


Fig. 1.

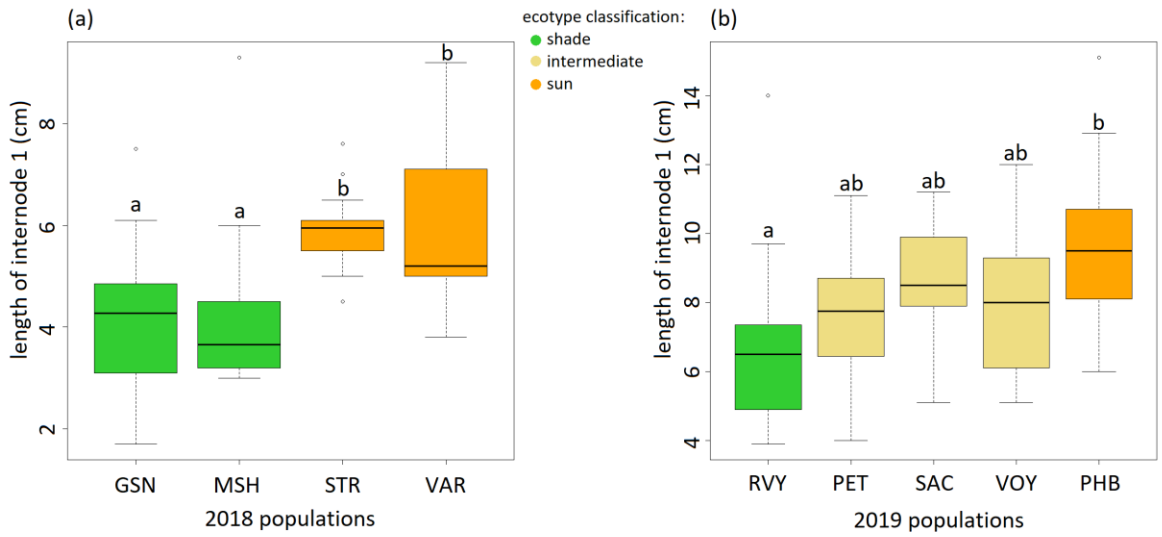
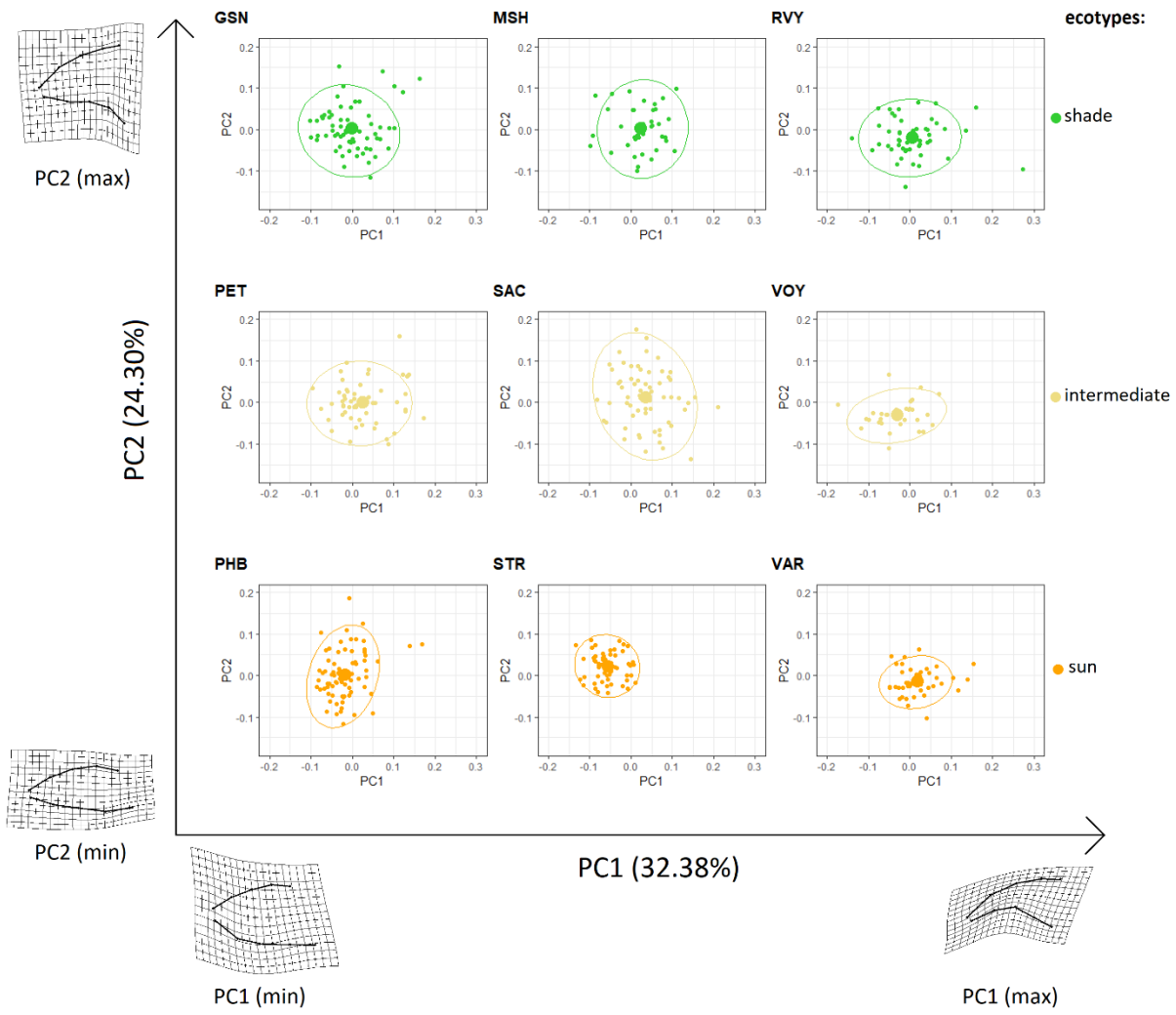

(a)
ecotype classification:
shade
intermediate
sun
(b)
length of internode 1 (cm)
2
4
6
8
a
a
b
b
GSN
MSH
STR
VAR
2018 populations
length of internode 1 (cm)
4
6
8
10
12
14
a
ab
ab
ab
b
RVY
PET
SAC
VOY
PHB
2019 populations

Fig. 2.


GSN
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ecotypes:
shade
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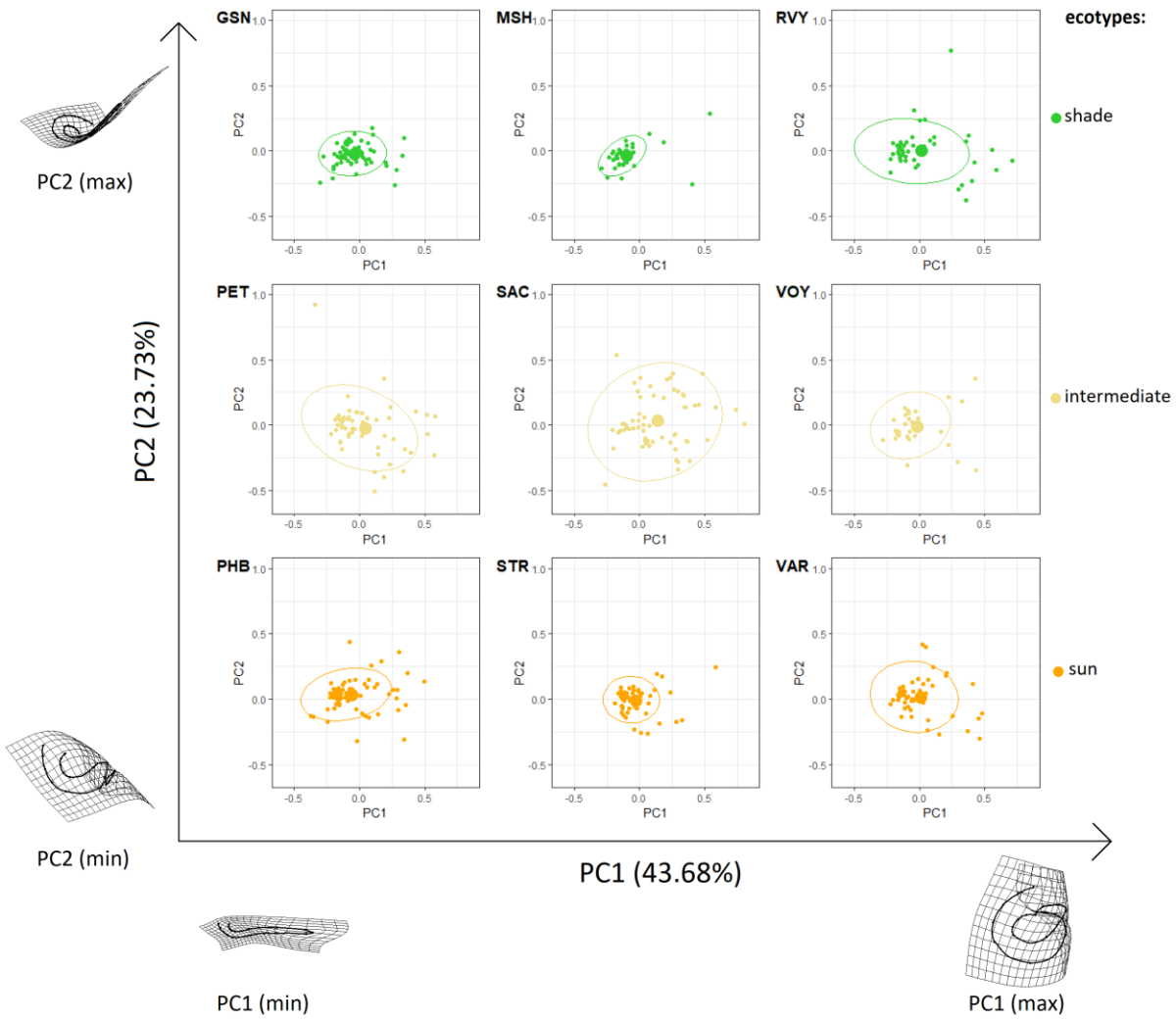
SAC
VOY
intermediate
PHB
STR
VAR
sun
PC1
PC2
PC2 (24.30%)
PC1 (32.38%)
PC2 (max)
PC2 (min)
PC1 (min)
PC1 (max)

Fig. 3.


GSN
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SAC
VOY
PHB
STR
VAR
PC1
PC2
ecotypes:
shade
intermediate
sun
PC2 (max)
PC2 (min)
PC2 (23.73%)
PC1 (43.68%)
PC1 (min)
PC1 (max)

Fig. 4.

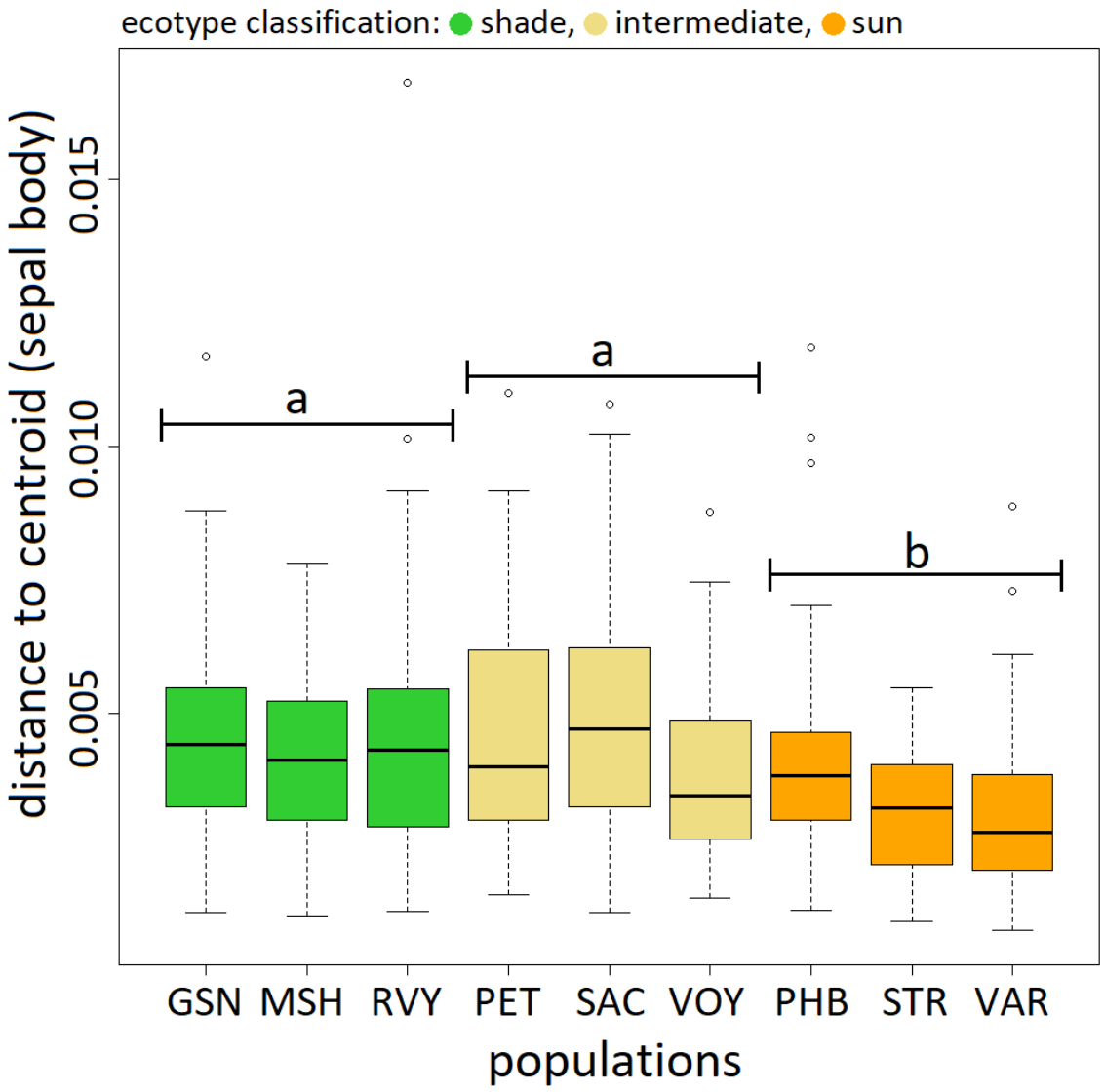
ecotype classification: shade, intermediate, sun
distance to centroid (sepal body)
0.005
0.010
0.015
a
a
b
GSN
MSH
RVY
PET
SAC
VOY
PHB
STR
VAR
populations

Fig. 5.

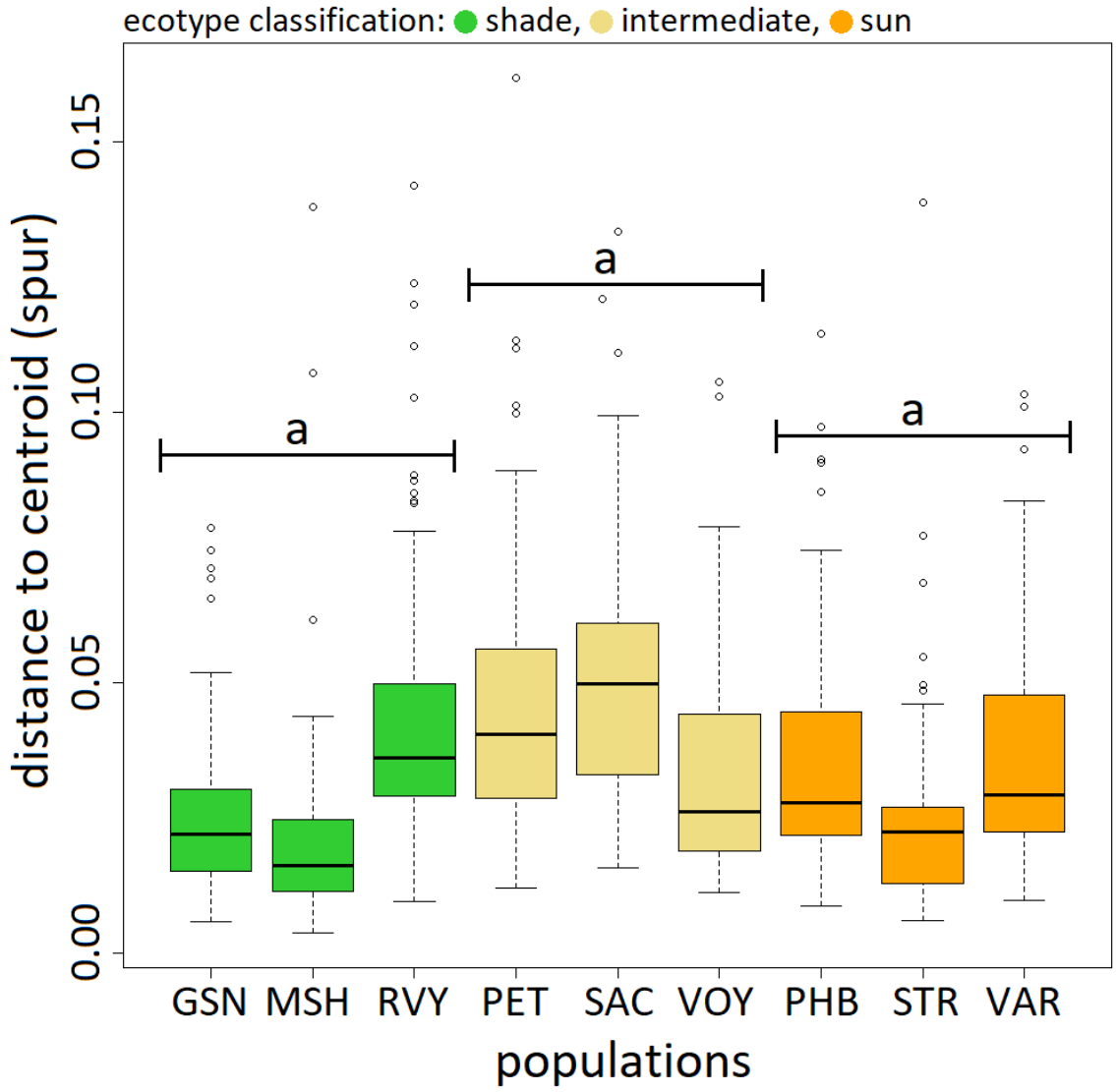
ecotype classification: shade, intermediate, sun
distance to centroid (spur)
0.00
0.05
0.10
0.15
a
a
a
GSN
MSH
RVY
PET
SAC
VOY
PHB
STR
VAR
populations

Fig. 6.

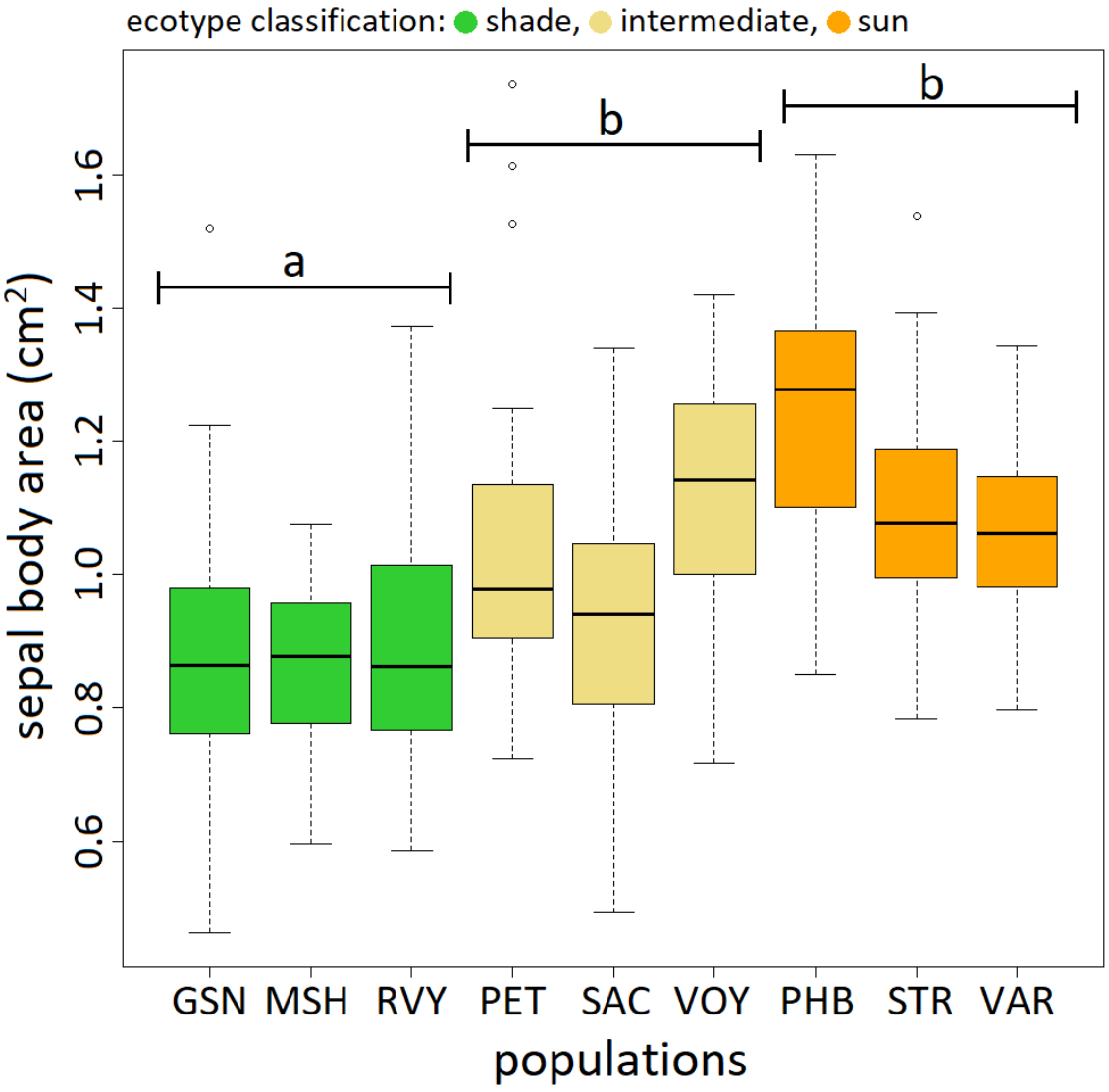
ecotype classification: shade, intermediate, sun
sepal body area (cm²)
0.6
0.8
1.0
1.2
1.4
1.6
a
b
b
GSN
MSH
RVY
PET
SAC
VOY
PHB
STR
VAR
populations

Fig. 7.

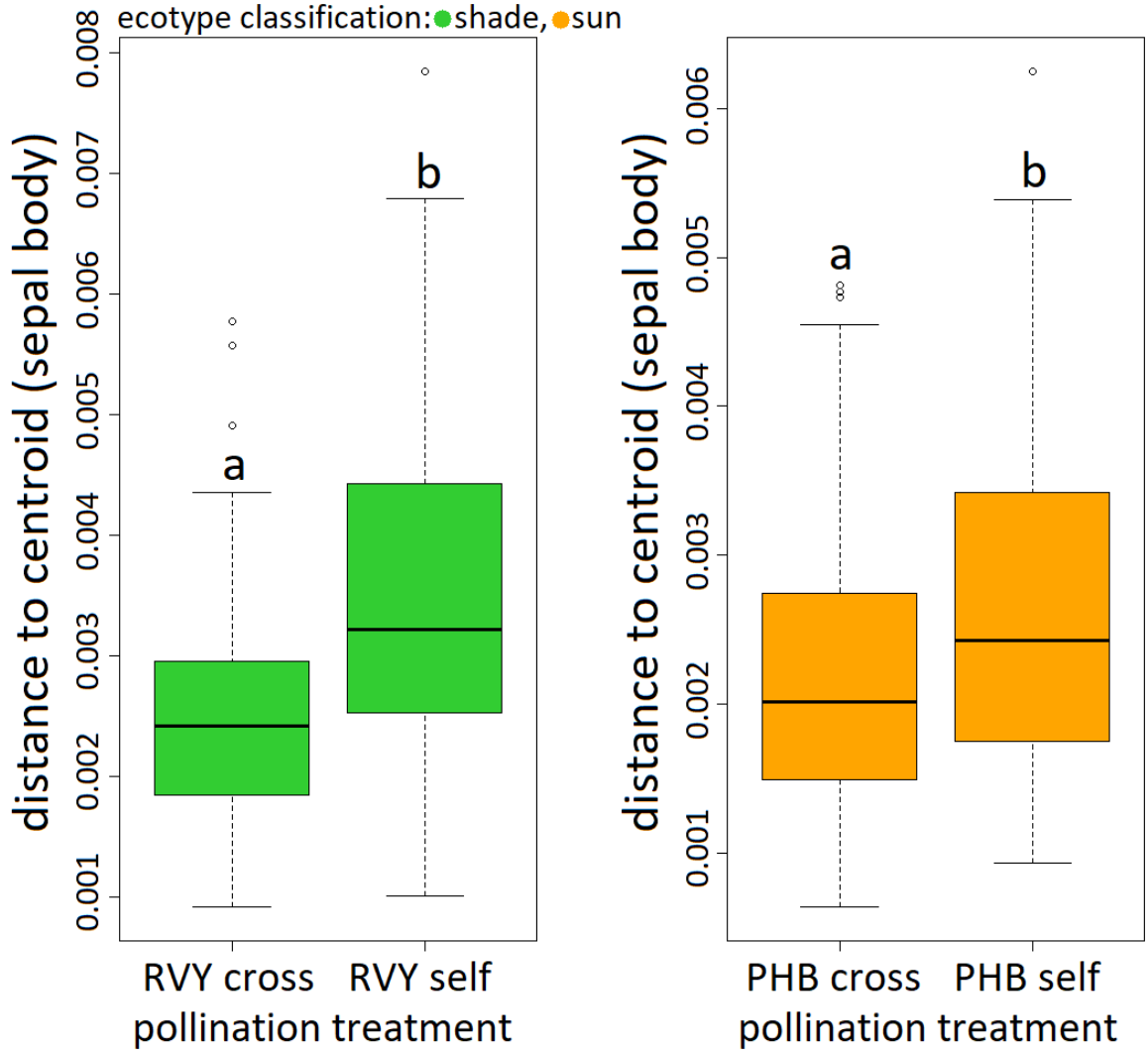
ecotype classification: shade, sun
distance to centroid (sepal body)
0.001
0.002
0.003
0.004
0.005
0.006
0.007
0.008
a
b
RVY cross
RVY self
pollination treatment
distance to centroid (sepal body)
0.001
0.002
0.003
0.004
0.005
0.006
a
b
PHB cross
PHB self
pollination treatment

Fig. 8.

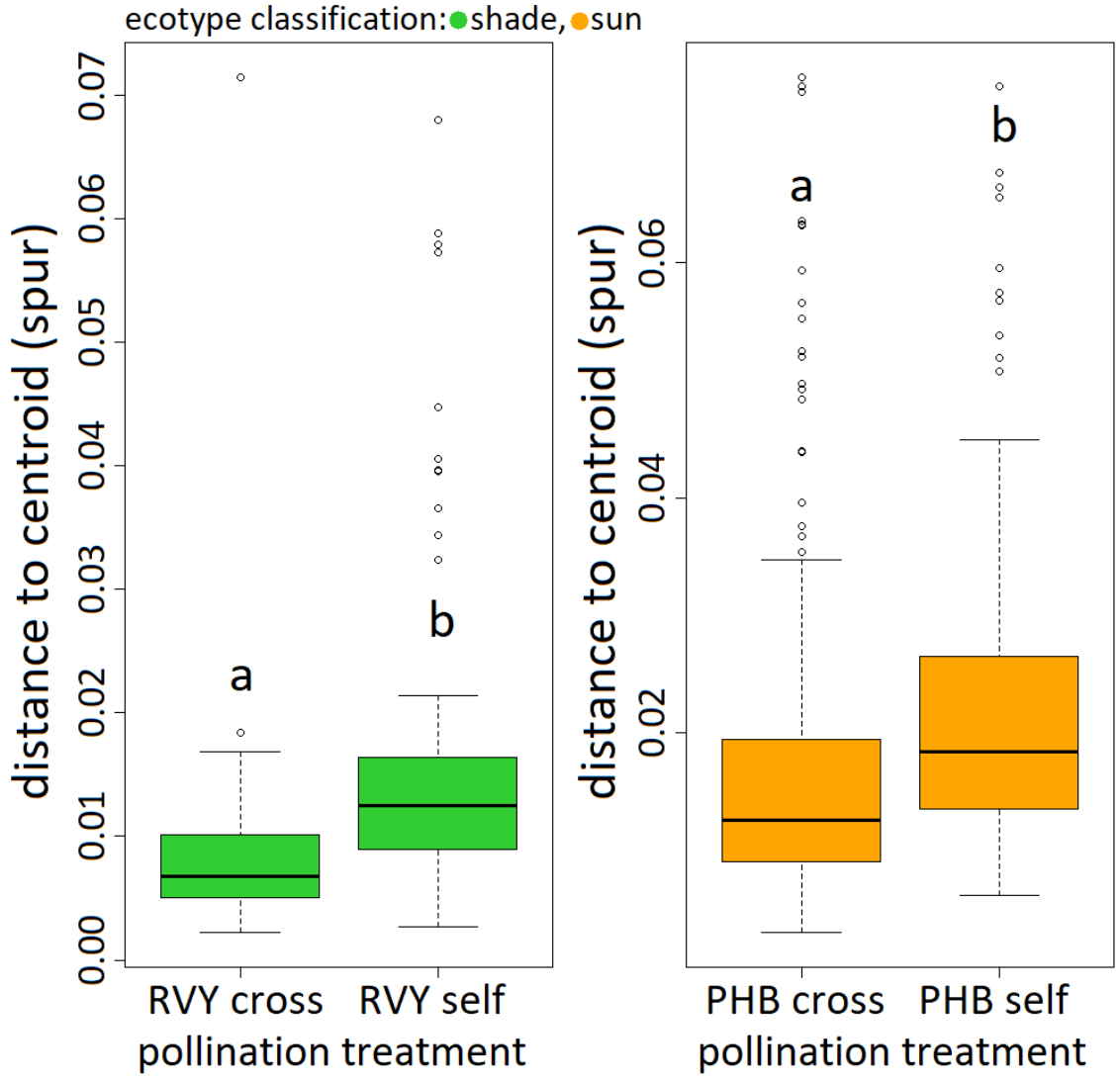
ecotype classification: shade, sun
distance to centroid (spur)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
a
b
RVY cross
RVY self
pollination treatment
distance to centroid (spur)
0.02
0.04
0.06
a
b
PHB cross
PHB self
pollination treatment

Fig. 9.

Tables:

Table 1. Habitat status and whether CH flowers were observed at site for each population.

Population	Habitat	CH flowers observed at site?
GSN	shade, hemlock forest	**no**
MSH	shade, beech-maple forest	**no**
STR	sun, riverbank with herbs	yes
VAR	sun, riverbank with herbs	yes
RIG†	mixed sun/shade, managed forest	yes
RVY	shade, beech-maple forest	**no**
PET	sun, riverbank with herbs	yes
SAC	mixed sun/shade, roadside	yes
VOY	sun, riverbank with herbs	yes
PHB	sun, *Typha* marsh	yes

†Only used for testing the repeatability of landmarking techniques and maternal effect.

Table 2. Nested ANOVA of weighted Euclidean distance between a sepal and its respective population mean (centroid) for sepal body and spur (cube-root-transformed data).

(a) Sepal body†

sources of variance	df	sum of squares	mean squares	F-ratio	p-value‡
ecotype	2	0.01919	0.009597	5.207271	0.048839*
ecotype:population	6	0.01106	0.001843	2.457989	0.050426
ecotype:population:plant	237	0.1777	0.0007498	1.990443	1.78E-07***
residual	234	0.08814	0.0003767	NA	NA
total	479	0.29609	NA	NA	NA

†mean and standard deviation for ecotypes (untransformed data): shade (0.004489; 0.002162); intermediate (0.004355; 0.002028); sun (0.003507; 0.001662).

‡one-tail test on ecotype level; two-tail test on all other level.

(b) Spur†

sources of variance	df	sum of squares	mean squares	F-ratio	p-value‡
ecotype	2	0.2118	0.1059	2.170527	0.195326
ecotype:population	6	0.2927	0.04879	11.19293	1.08E-10***
ecotype:population:plant	237	1.033	0.004359	1.160234	0.255052
residual	234	0.8792	0.003757	NA	NA
total	479	2.4167	NA	NA	NA

†mean and standard deviation for ecotypes (untransformed data): shade (0.03284; 0.02696); intermediate (0.04740; 0.02688); sun (0.03340; 0.02301).

‡one-tail test on ecotype level; two-tail test on all other level.

Table 3. Nested ANOVA of sepal size (untransformed data)†

sources of variance	Df	sum of squares	mean squares	F-ratio	p-value‡
ecotype	2	5.866	2.933	9.8809657	0.01265*
ecotype:population	6	1.781	0.296833333	6.3896004	5.95E-6***
ecotype:population:plant	237	11.01	0.046455696	3.1264403	0***
residual	234	3.477	0.014858974	NA	NA
total	479	22.134	NA	NA	NA

†mean and standard deviation for ecotypes (untransformed, cm^2): shade (0.8838; 0.1644); intermediate (1.0189; 0.2025); sun (1.1508; 0.1852).

‡one-tail test on ecotype level; two-tail test on all other level.

Table 4. Response of sepal shape to inbreeding. Nested ANOVA of weighted Euclidean distance between a sepal and its respective population mean (centroid) for sepal body and spur (cube-root-transformed data).

(a) Sepal body†

RVY

sources of variation	df	sum of squares	mean squares	F-ratio	p-value‡
pollination treatment	1	0.004248	0.004248	7.283951	0.009061**
pollination treatment:plant	59	0.03441	0.0005832	2.330935	0.001257**
residue (flowers)	61	0.01526	0.0002502	NA	NA
total	121	0.053918	NA	NA	NA

PHB

sources of variation	df	sum of squares	mean squares	F-ratio	p-value‡
pollination treatment	1	0.004128	0.004128	10.25335	0.001648**
pollination treatment:plant	159	0.06401	0.0004026	1.471491	0.014976*
residue (flowers)	161	0.04405	0.0002736	NA	NA
total	321	0.112188	NA	NA	NA

†mean and standard deviation for each pollination treatment (untransformed data): RVY cross (0.002600; 0.001190), RVY self (0.003431; 0.001365); PHB cross (0.002210; 0.0009190), PHB self (0.002625; 0.001117).

‡one-tail test on pollination treatment level; two-tail test on all other level.

(b) Spur†

RVY

sources of variation	df	sum of squares	mean squares	F-ratio	p-value‡
pollination treatment	1	0.04174	0.04174	14.03497	0.00025***
pollination treatment:plant	59	0.1754	0.002974	1.175494	0.307759
residue (flowers)	61	0.1543	0.00253	NA	NA
total	121	0.37144	NA	NA	NA

PHB

sources of variation	df	sum of squares	mean squares	F-ratio	p-value‡
pollination treatment	1	0.05483	0.05483	15.93432	9.99E-05***
pollination treatment:plant	159	0.5471	0.003441	1.263216	0.140352

residue (flowers)	161	0.4385	0.002724	NA	NA
total	321	1.04043	NA	NA	NA

†mean for each pollination treatment (results for untransformed data; results for cube-root-transformed data): RVY cross (0.009705; 0.01197), RVY self (0.01615; 0.01279); PHB cross (0.01738; 0.01399), PHB self (0.02290; 0.01481).

‡one-tail test on pollination treatment level; two-tail test on all other level.

Appendices:

Supplementary methods and results:

Sensitivity of shape and size variation results to landmark placement

To test the sensitivity of measurements of flower shape and size variation to investigator induced variation in the placement landmarks, we randomly chose ten plants from four populations (GSN, STR, VAR and RIG), each with two flower photos. Each photo was landmarked twice by the same person. We conducted Procrustes nested MANOVAs on sepal body and spur, and a nested ANOVA on flower size for variance component analysis (Geomorph R-package 3.3.1, Adams et al., 2020). Variance component analysis estimates the proportion of variance that results from variation in placing landmarks of single sepals (i.e., differences between the two individual sets of landmarks recorded separately for each sepal). The results showed that landmark placement variation contributed about 10.5% of the total variance for sepal body and spur, and 2% for flower size (Table S3).

Test whether sepal size variation is correlated with plant size variation (maternal effect)

We tested whether plant size affects sepal size (i.e., maternal effect) using plants grown from seed in the 2018 collections (GSN, MSH, STR, VAR, RIG). We harvested mature plants in the greenhouse and recorded their wet weight. The wet weight and sepal size data for each population was log base10-transformed when necessary. We then tested whether there is a significant relationship between plant weight and sepal size within each population by a Model II regression (smatr package in R 4.0.2). The results showed that there was no significant correlation between plant wet weight and sepal size, suggesting that maternal effect did not significantly affect flower size (Table S6).

List of supplementary figures:

Fig. S1. Boxplots of internode 1 length under high red: far-red treatment for each population (sample size: GSN = 13, MSH = 11, STR = 13, VAR = 18; RVY = 27, PET = 24, SAC = 28, VOY = 28, PHB = 26). The colours represent final ecotype classification on R: FR experiments (low R: FR treatment). The letters represent statistically significant groups based on ANOVA and *a posteriori* tests (TukeyHSD) within each year. Due to differences in the timing of seed germination, comparisons were made within years

Fig. S2. Boxplots of weighted Euclidean distances between the two sepal bodies within a plant (untransformed data) (sample size: total = 234; GSN = 30, MSH = 19, RVY = 24, PET = 27, SAC = 31, VOY = 14, PHB = 37, STR = 29, VAR = 23). Colours represent final ecotype classification as based on R: FR experiments. Letters above each ecotype represent statistically significant groups as based on a nested ANOVA and a Tukey test at the ecotype level.

Fig. S3. Boxplots of weighted Euclidean distances between the two spurs within a plant (untransformed data). Colours represent final ecotype classification as based on R: FR experiments. Letters above each ecotype represent statistically significant groups as based on a nested ANOVA and a Tukey test at the ecotype level. Sample size as in Fig. S2.

Fig. S4. Response of sepal body shape to inbreeding in populations RVY and PHB. Principal component analysis (PCA) plots of sepal body for inbred and outbred progeny. Each small dot represents a single sepal body and each large dot represents the mean of the pollination treatments (centroids). Ellipses around the dots are the 95% confidence ellipse on PC1 and PC2. Wire

diagrams at the bottom left and side represent shapes at the extremes of PC1 and PC2. Sample sizes as in Fig. 8.

Fig. S5. Response of spur shape to inbreeding in populations RVY and PHB. Principal component analysis (PCA) plots of spur for inbred and outbred progeny. Each small dot represents a single spur and each large dot represents the mean of the pollination treatments (centroids). Ellipses around the dots are the 95% confidence ellipse on PC1 and PC2. Wire diagrams at the bottom left and side represent shapes at the extremes of PC1 and PC2. Sample sizes as in Fig. 8.

Fig. S6. Response of paired flower distance (sepal body shape) to inbreeding in populations RVY and PHB (sample sizes: total = 222; RVY cross = 16, RVY self = 45; PHB cross = 106; PHB self = 55). Boxplots show weighted Euclidean distance for each sepal body pair to its respective pollination treatment centroid (untransformed data). Colours represent final ecotype classification as based on R: FR experiments. Letters above each pollination treatment represent statistically significant groups as based on a nested ANOVA (cube-root-transformed data) and a Tukey test on pollination treatment level for each population.

Fig. S7. Response of paired flower distance (spur shape) to inbreeding in populations RVY and PHB. Boxplots show weighted Euclidean distance for each spur pair to its respective pollination treatment centroid (untransformed data). Colours represent final ecotype classification as based on R: FR experiments. Letters above each pollination treatment represent statistically significant groups as based on a nested ANOVA (cube-root-transformed data) and a Tukey test on pollination treatment level for each population. Sample sizes as in Fig. S6.

Fig. S8. Response of sepal body area to inbreeding in populations RVY and PHB. Boxplots of sepal size for each population (untransformed data). Colours represent final ecotype classification as based on R: FR experiments. Letters above each ecotype represent statistically significant groups based on a nested ANOVA (untransformed data) and a Tukey test on ecotype level. Sample sizes as in Fig. 8.

Supplementary figures:

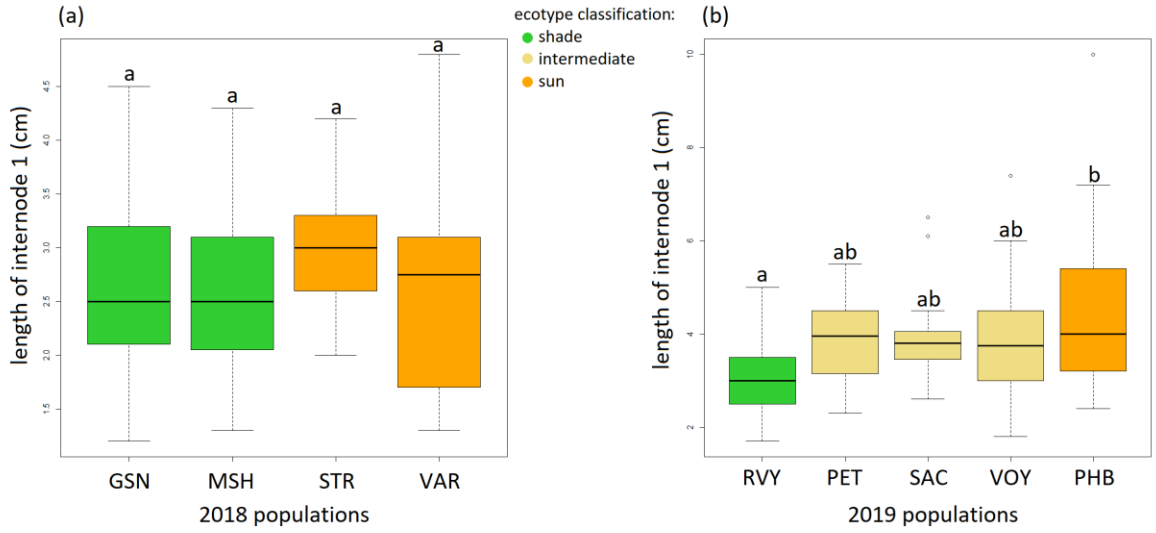

Fig. S1.

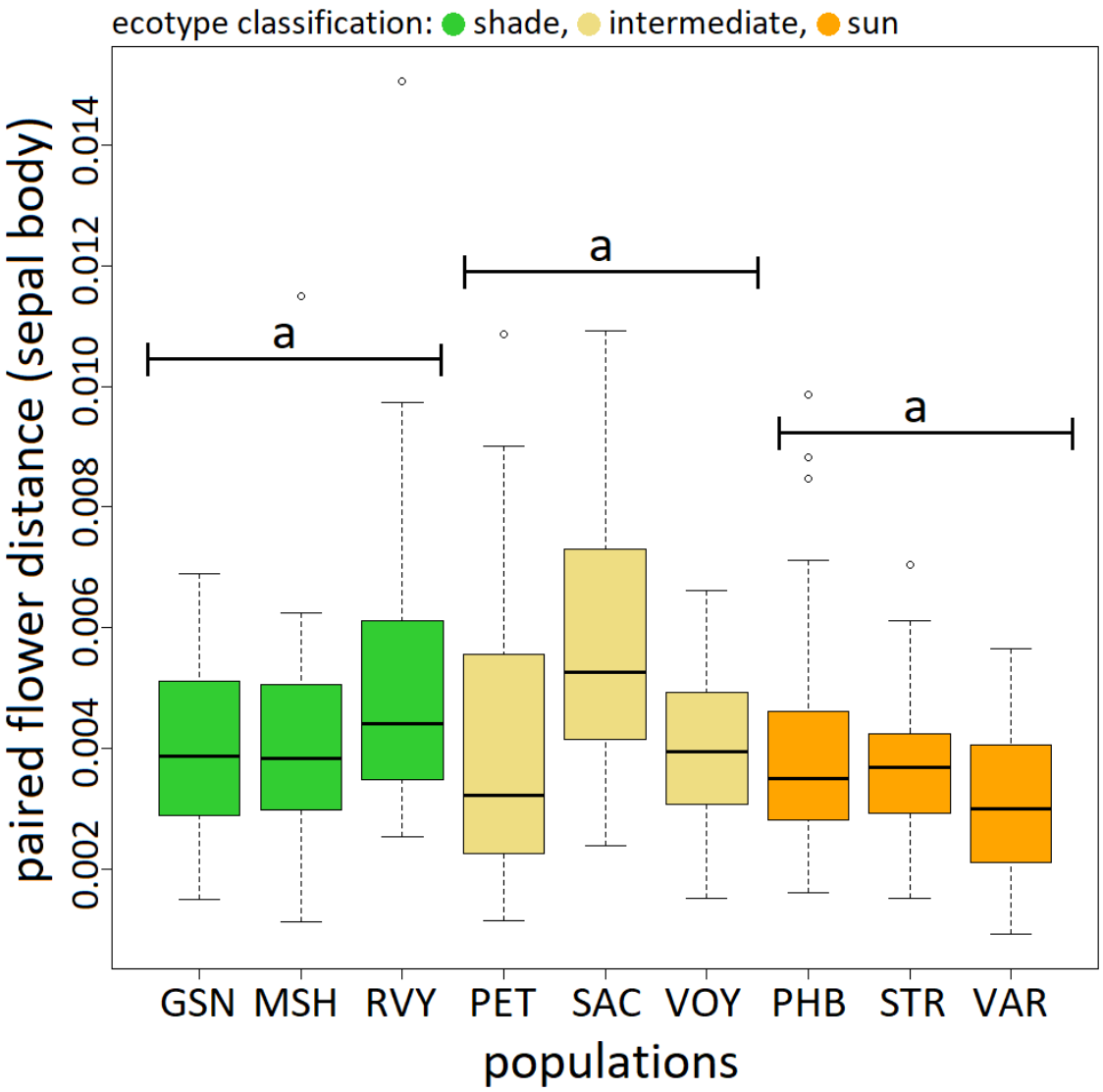
ecotype classification: shade, intermediate, sun
paired flower distance (sepal body)
0.002
0.004
0.006
0.008
0.010
0.012
0.014
a
a
a
GSN
MSH
RVY
PET
SAC
VOY
PHB
STR
VAR
populations

Fig. S2.

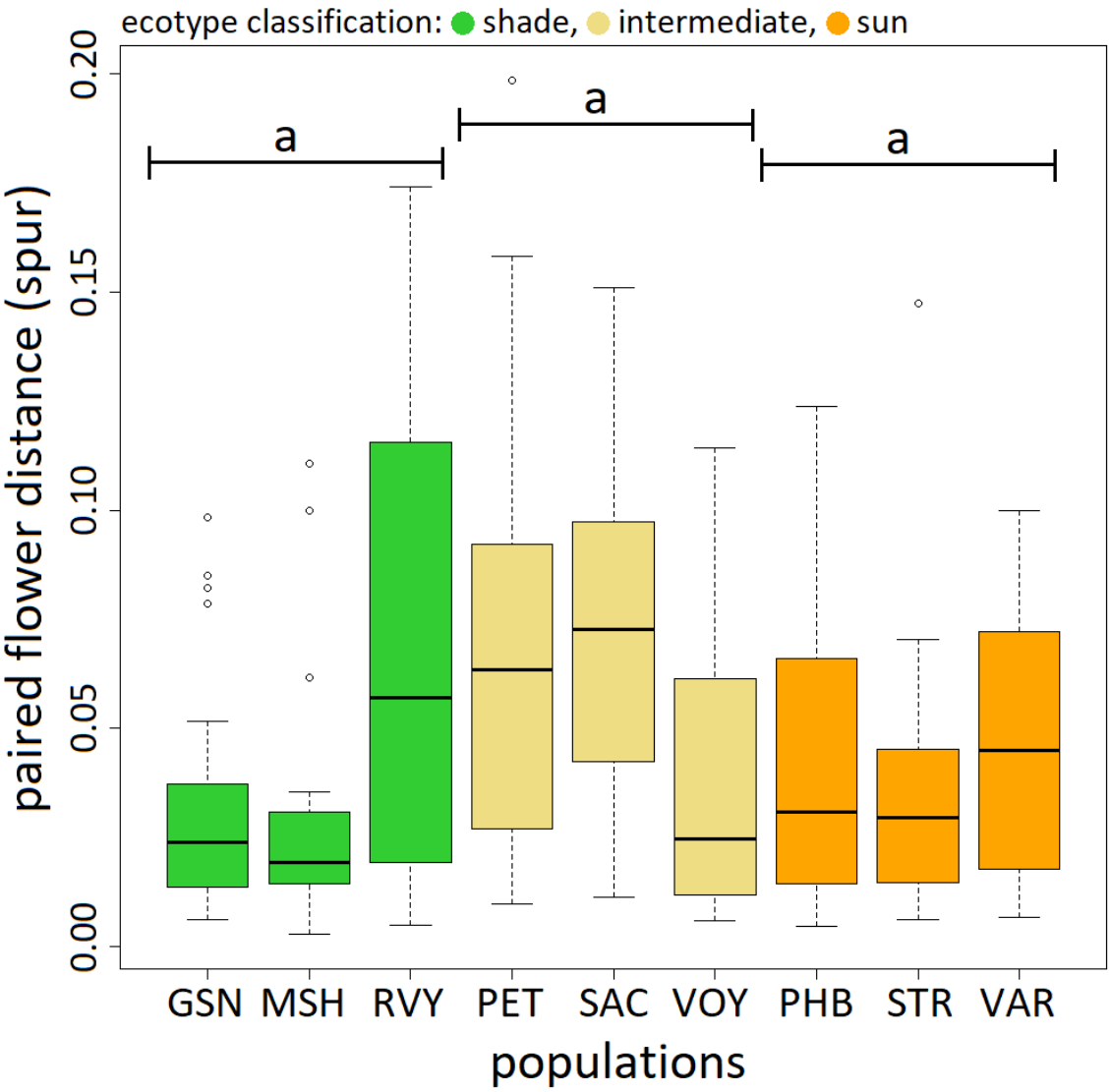
ecotype classification: shade, intermediate, sun
paired flower distance (spur)
0.00
0.05
0.10
0.15
0.20
a
a
a
GSN
MSH
RVY
PET
SAC
VOY
PHB
STR
VAR
populations

Fig. S3.

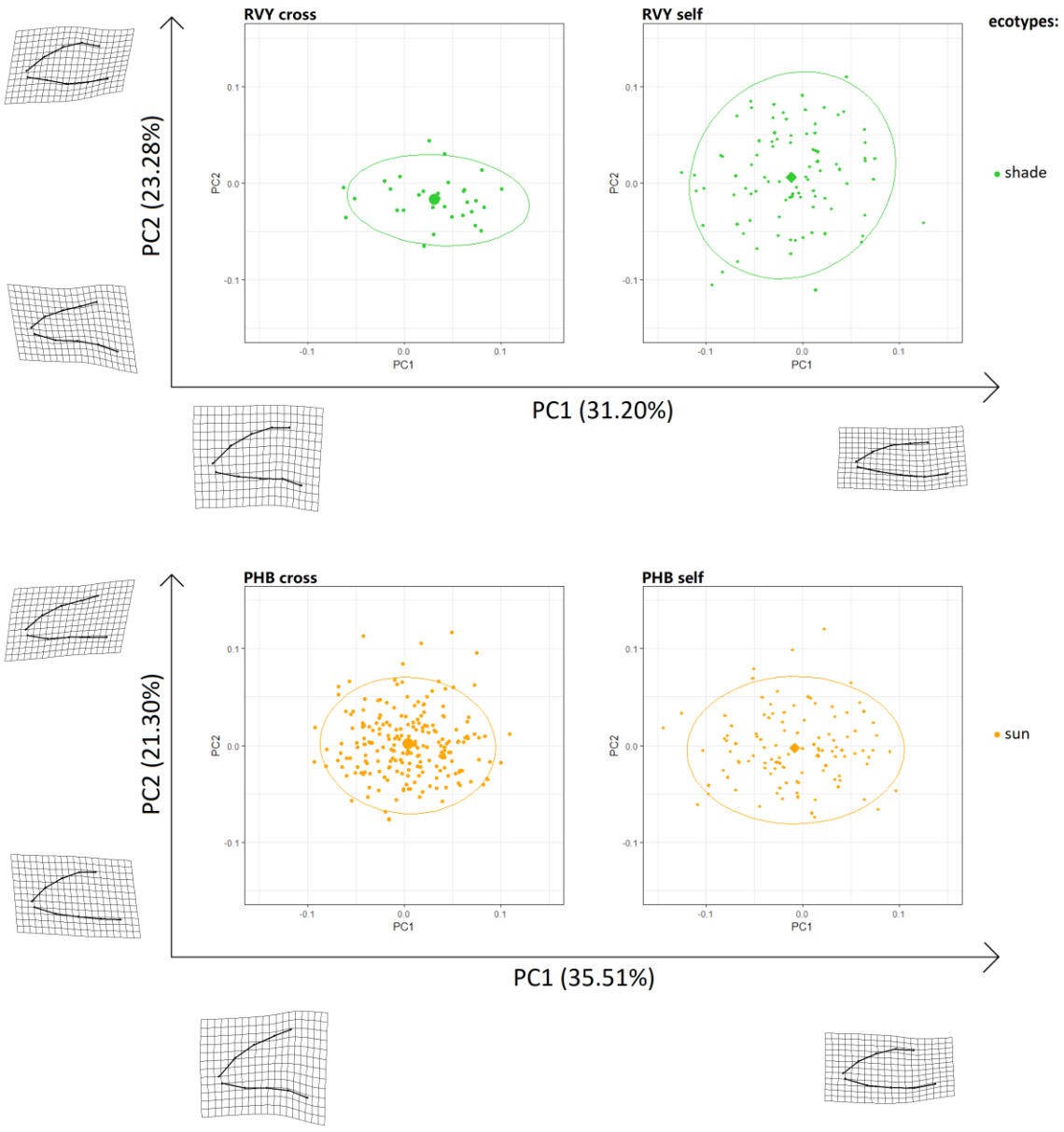
RVY cross
RVY self
ecotypes:
shade
PC2 (23.28%)
PC1 (31.20%)
PC2
PC1
PHB cross
PHB self
sun
PC2 (21.30%)
PC1 (35.51%)

Fig. S4.

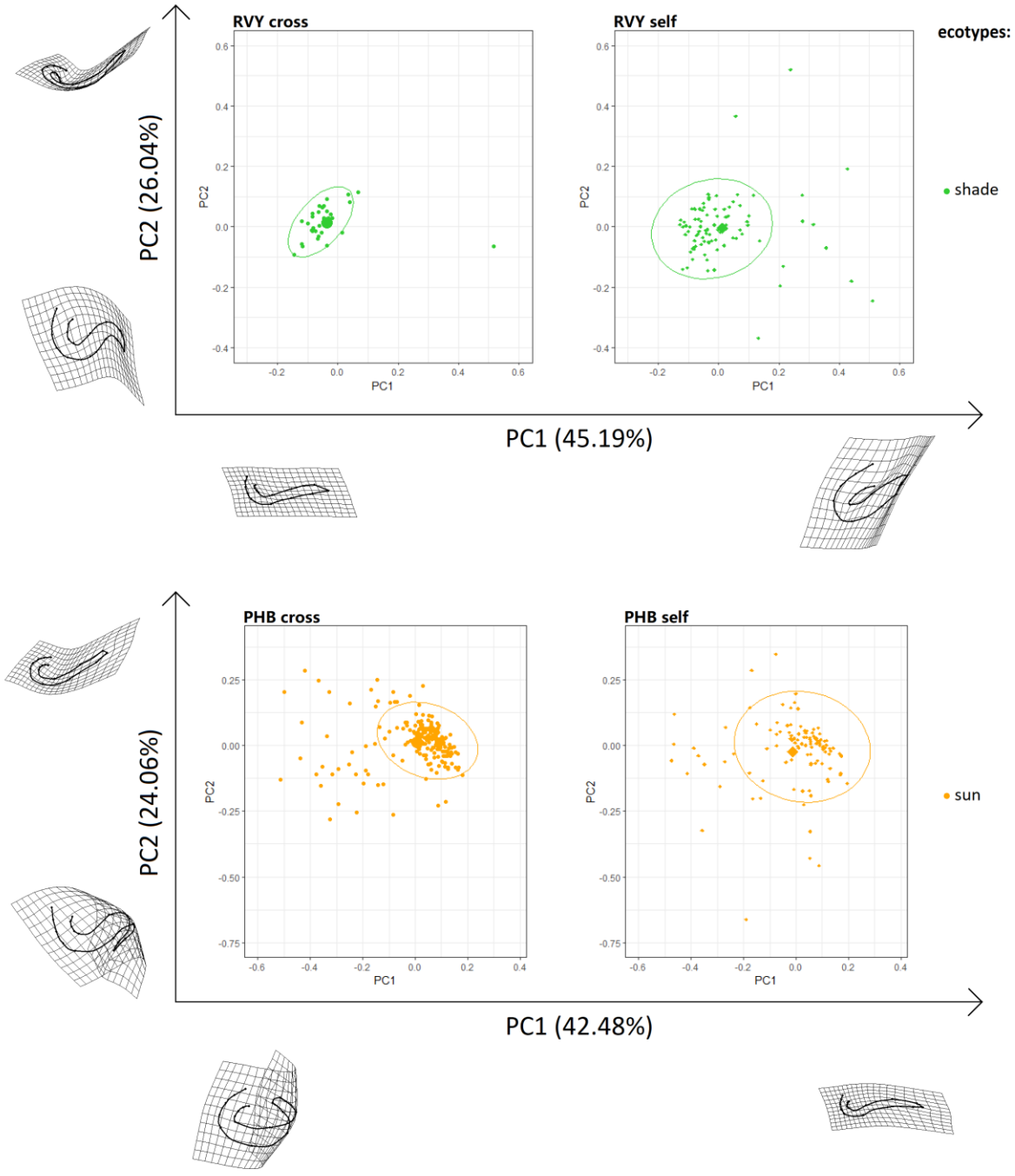
RVY cross
RVY self
ecotypes:
shade
PC2 (26.04%)
PC1 (45.19%)
PC1
PC2
PHB cross
PHB self
sun
PC2 (24.06%)
PC1 (42.48%)

Fig. S5.

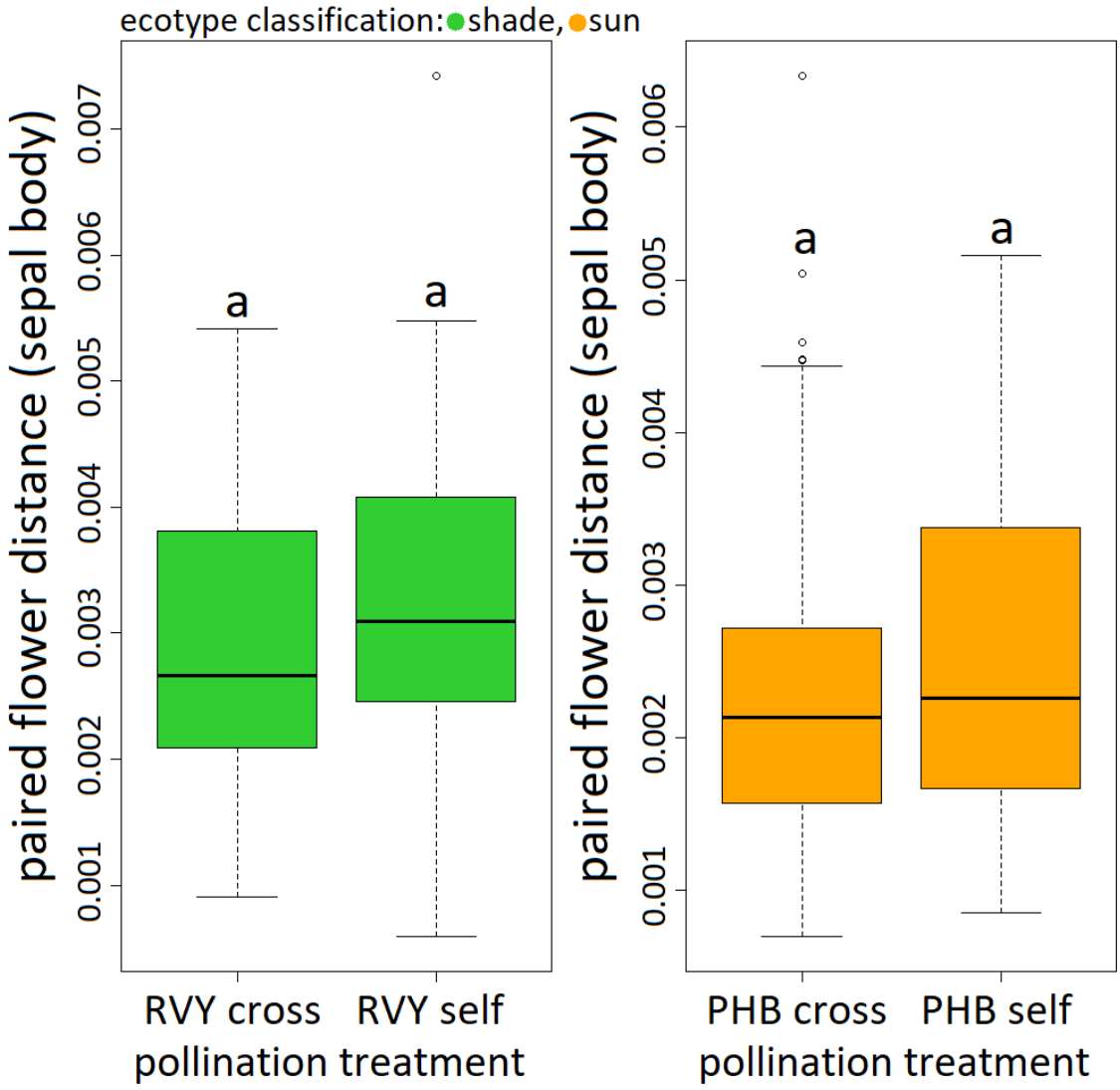
ecotype classification: shade, sun
paired flower distance (sepal body)
0.001
0.002
0.003
0.004
0.005
0.006
0.007
a
a
RVY cross
RVY self
pollination treatment
paired flower distance (sepal body)
0.001
0.002
0.003
0.004
0.005
0.006
a
a
PHB cross
PHB self
pollination treatment

Fig. S6.

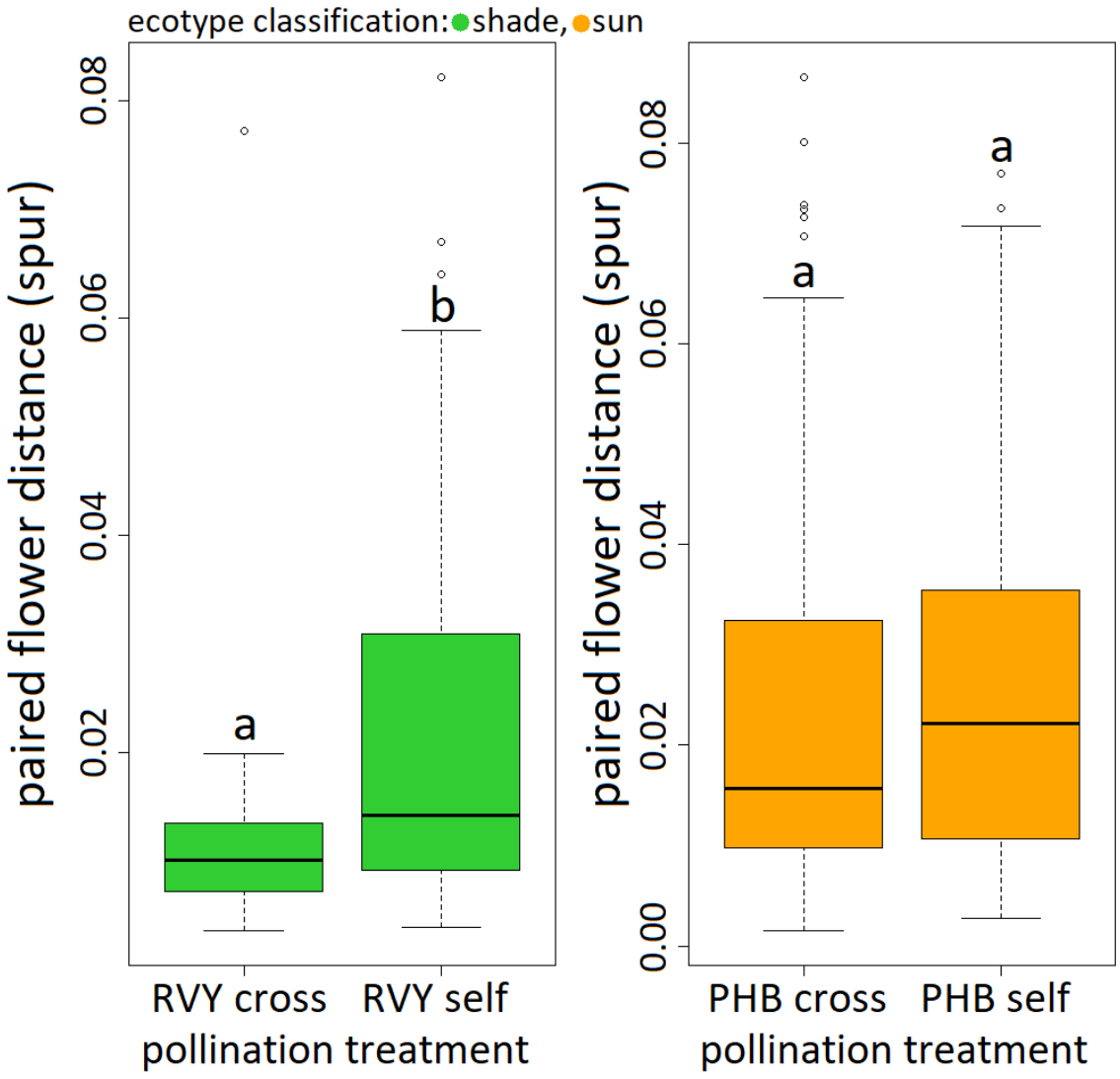
ecotype classification: shade, sun
paired flower distance (spur)
0.02
0.04
0.06
0.08
a
b
RVY cross
RVY self
pollination treatment
paired flower distance (spur)
0.00
0.02
0.04
0.06
0.08
a
a
PHB cross
PHB self
pollination treatment

Fig. S7.

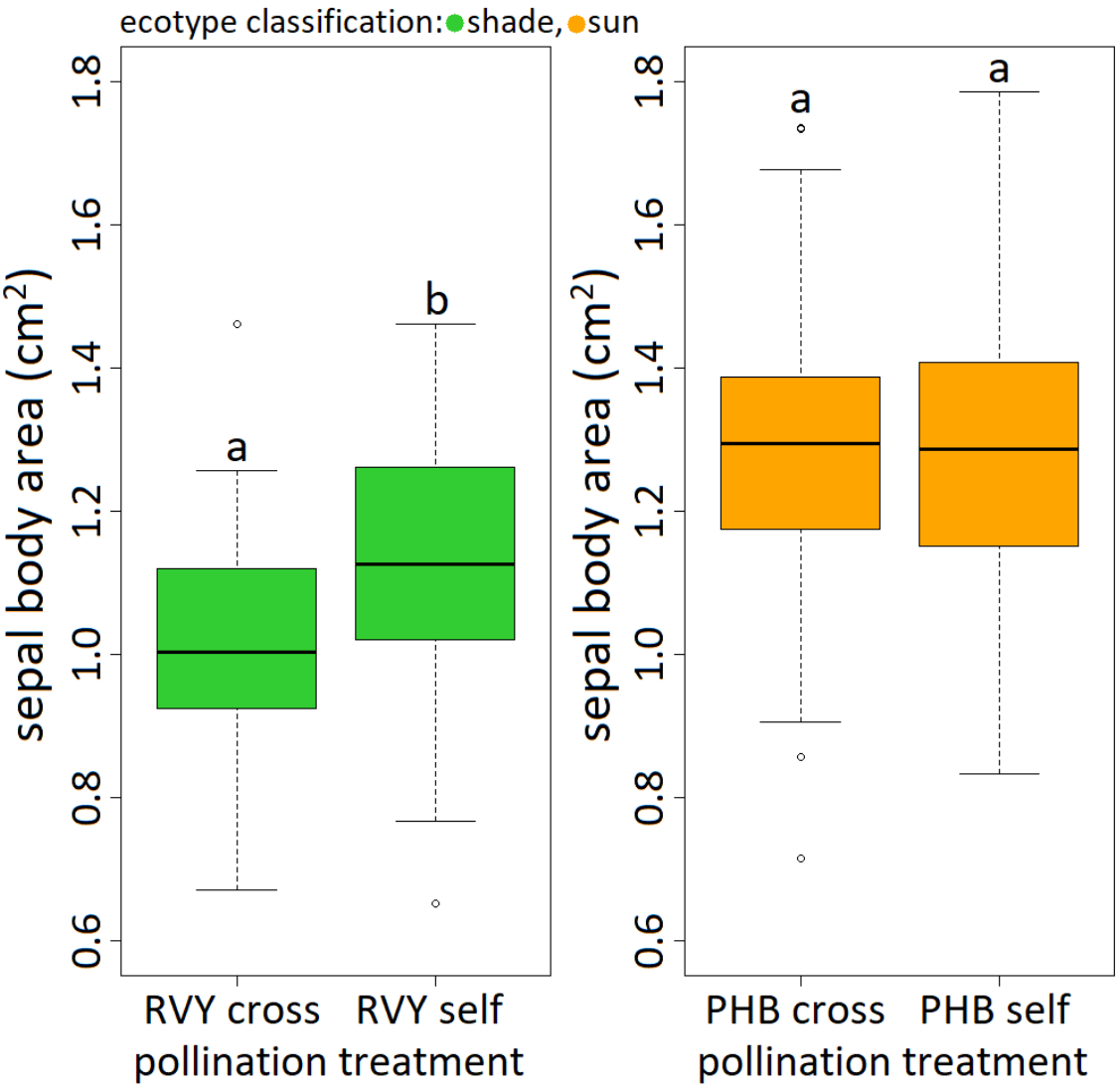
ecotype classification: shade, sun
sepal body area (cm²)
0.6
0.8
1.0
1.2
1.4
1.6
1.8
a
b
RVY cross
RVY self
pollination treatment
a
a
PHB cross
PHB self
pollination treatment

Fig. S8.

Supplementary tables:

Table S1. Locations of the ten study populations of *Impatiens capensis* in south-eastern Quebec, Canada.

Population (abbreviation)	Latitude (N)	Longitude (W)
Glen Sutton (GSN)	45°02’27.04”	72°33’01.54”
Mont Saint Hilaire (MSH)	45°32’37.22”	73°09’24.36”
Saint Roch (STR)	45°53’27.20”	73°09’16.14”
Varennes (VAR)	45°40’38.57”	73°26’29.67”
Rigaud (RIG)†	45°27’12.00”	74°18’23.24”
Ruiter Valley (RVY)	45°04’36.71”	72°26’10.42”
Petri Island (PET)	45°29’46.01”	75°30’24.69”
Sugar Loaf (SAC)	45°07’45.64”	72°19’53.45”
Voyageur (VOY)	45°33’38.20”	74°26’14.62”
Philipsburg (PHB)	45°01’39.28”	73°04’43.58”

†Only used for testing the repeatability of landmarking techniques and maternal effect.

Table S2. The landmarks and semi-landmarks used for shape (sepal body and spur) and size (sepal body size) analysis (see Fig. 1)

			Used for analysis of:		
		Description	Sepal body shape†	Spur shape	Sepal size‡
landmark	1	where the flower attaches to the pedicel	x		x
	2	start of the spur (upper)	x	x	x
	3	the point where the spur first changes direction (lower)		x	
	4	tip of the spur		x	
	5	the point where the spur first bends (inner)		x	
	6	start of the spur (lower round)	x	x	x
	7	lower tip of the sepal's opening	x		x
	8	lowest point of the sepal opening's parabola			x
semi-landmark	9	mid-point of 1 and 2	x		x
	10	mid-point of 2 and 3		x	
	11	mid-point of 3 and 4		x	
	12	mid-point of 4 and 5		x	
	13	mid-point of 5 and 6		x	
	14	mid-point of 6 and 7	x		x
	15	mid-point of 7 and 8			x
	16	mid-point of 8 and 1			x
	17	mid-point of 1 and 9	x		
	18	mid-point of 9 and 2	x		
	19	mid-point of 2 and 10		x	
	20	mid-point of 10 and 3		x	
	21	mid-point of 3 and 11		x	
	22	mid-point of 11 and 4		x	
	23	mid-point of 4 and 12		x	
	24	mid-point of 12 and 5		x	
	25	mid-point of 5 and 13		x	
	26	mid-point of 13 and 6		x	
	27	mid-point of 6 and 14	x		
	28	mid-point of 14 and 7	x		
	29	mid-point of 7 and 15§			
	30	mid-point of 15 and 8§			
	31	mid-point of 8 and 16§			
	32	mid-point of 16 and 1§			

†The opening of the sepal (landmarks 8, 15, 16, 29, 30, 31, 32) was not included, as in some photos the lateral sepal partially masked the opening.

‡Some landmarks describing the opening had to be included for sepal size analysis. We tested whether masking of lateral sepals significantly affect sepal size. The result shows that masking of lateral sepals do not significantly affect sepal size (Table S3d).

§These 4 landmarks aid in the placement of other landmarks.

Table S3. Sensitivity of shape and size variation results to placement of landmarks. Nested (M)ANOVA and variance component analysis (VCA) for sepal body, spur, and sepal body size. Model II regression for sepal body size with or without lateral sepals removed.

(a) Nested MANOVA and VCA for sepal body.

Source of variance	Df	SS	MS	Variance	VCA
Population (N=4)	3	0.392844	0.130948	0.002833	0.296136
Population:plant (N=10)	36	0.634588	0.017627	0.002604	0.272188
Population:plant:flower (N=2)	40	0.288473	0.007212	0.003082	0.322179
Residuals (N=2)	80	0.083801	0.001048	0.001048	0.109496
Total	159	1.399706	NA	0.009567	1

(b) Nested MANOVA and VCA for spur.

Source of variance	Df	SS	MS	Variance	VCA
Population (N=4)	3	1.482391	0.49413	0.009969	0.187758
Population:plant (N=10)	36	3.433217	0.095367	0.007379	0.138983
Population:plant:flower (N=2)	40	2.63399	0.06585	0.030103	0.566953
Residuals (N=2)	80	0.451552	0.005644	0.005644	0.106306
Total	159	8.00115	NA	0.053096	1

(c) Nested ANOVA and VCA for sepal body size.

Source of variance	Df	SS	MS	Variance	VCA
Population (N=4)	3	2.086	0.6953	0.0161	0.475994
Population:plant (N=10)	36	1.846	0.05129	0.008353	0.246937
Population:plant:flower (N=2)	40	0.7151	0.01788	0.008508	0.251543
Residuals (N=2)	80	0.06907	0.000863	0.000863	0.025526
Total	159	4.71617	NA	0.033824	1

(d) Model II regression for the relationship between the estimated sepal body size (triangle method) using sepals (i.e., modified sepals) with or without lateral sepals removed. Twenty randomly selected flowers were used. Null hypothesis: y (area with lateral sepal) = x (area without lateral sepal).

Mean of estimated regression line: $y = 1.010x - 0.054$ ($R^2 = 0.882$)	
H_0	P-value†
Variables uncorrelated	< 0.00001
Slope not different from 1	0.904
Elevation not different from 0	0.606

†For hypothesis test H_0

Table S4. Morphological integration test for Procrustes-transformed sepal body and spur for each population, using two-block partial least squares (PLS). The correlation coefficient (r-PLS) and p-values are reported. Null hypothesis: there is no morphological integration between spur and sepal body (r-PLS = 0).

Population	r-PLS	p-value†
GSN	0.458	0.028
MSH	0.291	0.945
RVY	0.56	0.011
PET	0.426	0.178
SAC	0.488	0.014
VOY	0.471	0.404
PHB	0.327	0.224
STR	0.427	0.172
VAR	0.398	0.519

†$\alpha = 0.05/9 = 0.00556$ after Bonfernoni correction.

Table S5. Model II regression for the relationship between the estimated sepal body size using the 'triangle method' and the estimated sepal body size using the program ImageJ. Twenty randomly selected flower photos were used. Null hypothesis: y (area of seven triangles) = x (area of ImageJ).

Mean of estimated regression line: $y = 1.013x - 0.079$ ($R^2 = 0.929$)	
H_0	P-value†
Variables uncorrelated	< 0.00001
Slope not different from 1	0.835
Elevation not different from 0	0.239

†For hypothesis test H_0

Table S6. Model II regression of sepal size (sepal area, cm^2) on plant wet weight (g). Null hypothesis: there is no significant correlation between sepal size and plant wet weight, i.e., the slope of the regression line does not differ significantly from 0.

Population	Estimated slope	R^2	P-value†
GSN	1.008	0.123	0.011
MSH	0.851	0.0046	0.736
RIG	−0.3596	0.0164	0.443
STR	−1.12	0.0027	0.706
VAR	0.0022	0.041	0.221

†significance threshold $\alpha = 0.05/5 = 0.01$ with Bonfernoni correction.

Table S7. ANOVA for response of internode 1 to red: far-red (R: FR) for 2018 and 2019 population samples, with means (cm) and standard deviations (sd, cm) included.

(a) low red: far-red
I. 2018 populations

sources of variance	df	sum of squares	mean squares	F-ratio	p-value[†]
population	3	41.52	13.84	5.977	0.0014**
residuals (plant)	52	120.41	2.315	NA	NA
total	55	161.93	NA	NA	NA

population	mean	sd
GSN	4.25	1.53
MSH	4.28	1.81
STR	5.91	0.79
VAR	4.07	1.72

II. 2019 populations

sources of variance	df	sum of squares	mean squares	F-ratio	p-value
population	4	124.9	31.23	8.176	7.02E-06***
residuals (plant)	123	469.8	3.82	NA	NA
total	127	594.7	NA	NA	NA

population	mean	sd
RVY	6.66	2.15
PET	7.58	1.68
SAC	8.66	1.72
VOY	7.92	1.90
PHB	9.61	2.25

(b) high red: far-red
I. 2018 populations

sources of variance	df	sum of squares	mean squares	F-ratio	p-value
population	3	1.69	0.5622	0.679	0.569
residuals (plant)	51	42.26	0.8286	NA	NA
total	54	43.95	NA	NA	NA

population	mean	sd
GSN	2.65	0.94
MSH	2.64	0.88
STR	3.07	0.65
VAR	2.68	1.06

II. 2019 populations

sources of variance	df	sum of squares	mean squares	F-ratio	p-value
population	4	30.16	7.539	5.634	0.000329***
residuals (plant)	128	171.29	1.338	NA	NA
total	132	201.45	NA	NA	NA

population	mean	sd
RVY	3.07	0.87
PET	3.89	0.83
SAC	3.91	0.81
VOY	3.83	1.19
PHB	4.57	1.78

†two-tail test for all levels

Table S8. Nested ANOVA of weighted Euclidean distance between two sepals within a plant for sepal body and spur (cube-root-transformed data).

(a) Sepal body†

sources of variance	df	sum of squares	mean squares	F-ratio	p-value‡
ecotype	2	0.006838	0.003419	1.494972	0.297292
ecotype:population	6	0.01372	0.002287	3.975317	0.001683**
residual (plant)	225	0.1295	0.000575	NA	NA
total	233	0.150058	NA	NA	NA

†mean and standard deviation for ecotypes (untransformed data): shade (0.004455; 0.002259); intermediate (0.004792; 0.002326); sun (0.003735; 0.001707).

‡one-tail test on ecotype level; two-tail test on all other level.

(b) Spur†

sources of variance	df	sum of squares	mean squares	F-ratio	p-value‡
ecotype	2	0.1695	0.08473	2.438975	0.167808
ecotype:population	6	0.2084	0.03474	4.078422	0.001327**
residual (plant)	225	1.917	0.008518	NA	NA
total	233	2.2949	NA	NA	NA

†mean and standard deviation for ecotypes (untransformed data): shade (0.04278; 0.04101); intermediate (0.06414; 0.04183); sun (0.04157; 0.03290).

‡one-tail test on ecotype level; two-tail test on all other level.

Table S9. Response of paired sepal distance to inbreeding. ANOVA of weighted Euclidean distance between two sepals within a plant for sepal body and spur (cube-root-transformed data).

(a) Sepal body†

RVY

sources of variance	df	sum of squares	mean squares	F-ratio	p-value‡
pollination treatment	1	0.000405	0.000405	0.986	0.325
residuals (plant)	59	0.024267	0.000411	NA	NA
total	60	0.024672	NA	NA	NA

PHB

sources of variance	df	sum of squares	mean squares	F-ratio	p-value‡
pollination treatment	1	0.00051	0.000507	1.368	0.244
residuals (plant)	159	0.05896	0.000371	NA	NA
total	160	0.05947	NA	NA	NA

†mean and standard deviation for each pollination treatment (untransformed data): RVY cross (0.002891; 0.001236), RVY self (0.003250; 0.001252); PHB cross (0.002319; 0.001047), PHB self (0.002526; 0.001109).

‡one-tail test on pollination treatment level; two-tail test on all other level.

(b) Spur†

RVY

sources of variance	df	sum of squares	mean squares	F-ratio	p-value‡
mating type	1	0.01993	0.019933	4.242	0.0438*
residuals (plants)	59	0.27721	0.004699	NA	NA
total	60	0.29714	NA	NA	NA

PHB

sources of variance	df	sum of squares	mean squares	F-ratio	p-value‡
mating type	1	0.0069	0.006852	1.178	0.279
residuals (plants)	159	0.9245	0.005814	NA	NA
total	160	0.9314	NA	NA	NA

†mean and standard deviation for each pollination treatment (untransformed data): RVY cross (0.01422; 0.01733), RVY self (0.02288; 0.01938); PHB cross (0.02332; 0.02011), PHB self (0.02685; 0.02093).

‡one-tail test on pollination treatment level; two-tail test on all other level.

Table S10. Response of sepal size to inbreeding: Nested ANOVA of sepal size (original data)†

RVY

sources of variation	df	sum of squares	mean squares	F-ratio	p-value‡
pollination treatment	1	0.3229	0.3229	7.556752	0.007921**
pollination treatment:plant	59	2.521	0.04273	3.686799	1.03E-06***
residuals (sepal)	61	0.7072	0.01159	NA	NA
total	121	3.5511	NA	NA	NA

PHB

sources of variation	df	sum of squares	mean squares	F-ratio	p-value‡
pollination treatment	1	0.004661	0.004661	0.106125	0.745028
pollination treatment:plant	159	6.983	0.04392	2.822622	1.36E-10***
residuals (sepal)	161	2.505	0.01556	NA	NA
total	321	9.492661	NA	NA	NA

†mean and standard deviation for pollination treatments (untransformed data, cm^2): RVY cross (1.0131; 0.1595), RVY self (1.1300; 0.1656); PHB cross (1.2848; 0.1649), PHB self (1.2768; 0.1856).

‡one-tail test on pollination treatment level; two-tail test on all other level.