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Linking Petal Cell Shape to Pollinator Mode in Mimulus

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Linking Petal Cell Shape to Pollinator Mode in *Mimulus*

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Contents

Abstract

Reproduction is essential for all organisms. In plants, reproduction relies on pollination. In addition to large-scale traits such as shape and color, flowers may use traits imperceptible to the naked eye in order to attract pollinators. One such trait may be conical cell shape of petal cells. Previous work has identified conical petal cells as providing a foothold for foraging bees, suggesting that conical cell shape is an adaptation to bee pollination. However, this hypothesis has not been fully tested and requires development of a rigorous methodology for quantifying cell shape. Here, we utilize natural variation in pollinator mode in pairs of *Mimulus* species to explore this hypothesis. We further developed a systematic and more robust methodology for measuring cell shape, Conical Value, *C.* We confirmed our *C* value in two wild-type *Mimulus* species and their flat-celled mutants. *C* also aligns more closely to qualitative observations than previously established metrics. Finally, we applied *C* to pairs of *Mimulus* species and found that petal cells were significantly more conical in bee-pollinated than self-pollinated species. Our findings suggest that pollination by bees either maintains or selects for petal cells with a conical shape.

Introduction

Plants rely on diverse pollinators for reproduction. In order to attract and accommodate diverse pollinators, plants have evolved pollination syndromes. These suites of floral traits, which are observed among many flowers that share a type pollinator, both invite specific pollinators to a flower and increase the ease of pollination. Pollination syndromes have a large impact on floral morphology. Bee pollinated flowers tend to be yellow, blue, or pink with a large landing platform. Hummingbird pollinated flowers are often red with a long calyx and large nectar rewards. Self-pollinated flowers, without need to attract a pollinator, are often small and duller in color (Abrol, 2012).

In addition to such macroscopic traits, there are also features of pollination syndromes that are not visible to the human eye. Bees can sense floral traits which humans cannot easily detect. These traits show a large range of complexity. For example, UV nectar guides on flower petals serve to point foraging bees to the flower's nectar reward, thus allowing the flower to be pollinated in the process (Abrol, 2012). Because bees must expend energy in order to maintain their body temperature, warmer flowers may also be considered as a reward. As such, bees have been shown to be able to predict floral temperature before landing (Dyer et al., 2007). In fact, invisible traits may be better predictors of pollinator syndrome than visible floral morphology. In five *Buddleja* species of flowers, floral scent was shown to more accurately correspond to a flower's pollinator as compared to floral shape (Bailes and Glover, 2018). Additionally, bees, like many insects, are sensitive to polarized light. They have demonstrated the ability to associate polarization with rewards on artificial flowers, suggesting the possibility that bees use polarization as a cue when searching for flowers (Foster et al., 2014).

Another microscopic trait that may be a part of the bee pollination syndrome is conical petal cell shape. Although the typical conception of a plant cell is a rigid, flat rectangle, approximately 79% of all plants have flowers with pointed, conical-shaped cells in the petals (Kay, Daoud, and Stirton, 1981). This prevalence begs the question: what purpose, if any, does conical petal cell shape have? Any definitive function of conical cells remains unknown, but they are frequently associated with bee pollination and there are many ways in which conical cell shape may contribute to the bee pollination syndrome. Conical cells refract and reflect visible and UV light differently from flat cells (Noda et al., 1994; Dyer et al., 2007). As a result, conical cells can produce epidermal surfaces with brighter spectral and UV color, though bees had some difficulty identifying differences between conical-celled flowers and mutant flat-celled flowers (Dyer et al., 2007). Polarization is caused by cell shape and micro/macropatterning of the petal surface, indicating that polarization may be yet another function of variation in petal cell shape (Moyroud and Glover, 2016). Gloss may also attract pollinators to a flower. However, the effect of gloss is greatest on smooth, flat-celled surfaces. Because most petals have conical cells, gloss frequently appears only on the tips of

such cells (Papioriek et al., 2014). However, whether or not gloss attracts pollinators to flowers remains uncertain (Moyroud and Glover, 2016). Though visual cues may be some of the most apparent purposes of petal cell shape, they are not the only benefit that conical cells may provide to pollinators. Conical cell shape may also influence petal wettability, or the amount of water that remains on a petal surface (Whitney et al., 2011a). Wettability can be a mechanism for controlling floral temperature, which could, in turn, attract bees, which prefer to visit warmer flowers (Dyer et al., 2006). Flat-celled mutant flowers tend to be colder than their conical-cell wild-type counterparts, indicating that conical cell shape could be attracting bees with heat. However, while conical cells do decrease petal wettability, petal wettability itself has not been shown to have any significant impact on floral temperature, though the cell shape may still attract bee pollinators with visual and tactile cues (Whitney et al., 2011a; Whitney et al., 2011b).

Additionally, conical cells could serve as foot grips for bees. Bees, using tactile cues alone, are able to distinguish between conical and flat celled floral surfaces. Bees prefer to visit conical-cell surfaces when the surface is more vertical, and thus more difficult to land on, but show no preference between conical and flat-celled surfaces when surfaces were presented horizontally. Finally, bees abort fewer landings on conical-cell surfaces than on flat-cell surfaces. Together, these observations indicate that conical cells could increase bees' grip on flowers during foraging. (Whitney et al., 2011b; Whitney et al., 2009). This was further supported by Bräuer et al. (2017), who found that both Carniolan honeybees and greenbottle flies could more easily walk on conical-celled surfaces than flat-celled surfaces. The textured surface provided by conical cells may be responsible for insects' increased ease of pollination on conical-celled petal surfaces.

In order to properly test for a relationship between cell shape and bee-pollination, cell shape must be measured in a systematic and robust way, which has proved challenging. There are a multitude of methods that have been used to quantify cell shape, though much of recent research has focused on measuring the angle of a cell's tip, a cell's dimensions, or ratios between the cell's tip and dimensions, but these measurements are typically considered independently (Ivakov and Persson, 2013; Bräuer et al., 2017; Ren et al., 2017; Dang et al., 2018). While a metric for evaluating cell shape with one comprehensive value, the Shape Parameter (*S*), has been proposed (Papiorek, Junker, and Lunau, 2014), this metric may not be the best way to capture cell shape. *S* is calculated by measuring the angles of the cell at the apical, lateral, and basal cell parts. These measurements are put into a formula to calculate a number on a scale from 0-1, where 1 represents a perfectly flat cell and 0 represents a perfectly conical cell (Papiorek, Junker, and Lunau, 2014; **Figure 1**). Papiorek, Junker, and Lunau (2014) developed *S* as a metric for measuring cells in order to compare shape with optical properties of the petal surface; thus, *S* was established though assumptions of the impact of cellular angles on light refraction. The formula, however, has major problems. For

example, it underestimates the conicality of cells with steep slopes while overestimating the conicality of cells with more gradual slopes. This is because the formula considers cells with lateral slopes closer to 90° to be flatter. However, this assumption does not always prove true; a cell with a straight lateral edge may be either flat or extremely pointed. Furthermore, the measurement ignores cellular dimensions and in Papiorek, Junker, and Lunau's 2014 study, the researchers did not take action to validate their cell shape measurements with qualitative observations. As such, developing a new metric to quantify cell shape is important to accurately understand the relationship between different petal cells' qualitative appearance and alternative metrics are explored in this paper.

An understanding of the genes underlying cell shape could provide additional insights into the nature of conical cell shape. One such candidate gene family is *MIXTA.* In snapdragons, the gene *MIXTA*, a MYB-related transcription factor, controls conical cell shape determination (Glover, Perez-Rodriguez, and Martin, 1998). In *Mimulus lewisii, MIXTA*-like R2R3 *MYB* controls petal cell development and pigmentation (Yuan et al., 2013), though there are numerous other *MIXTA*-like genes which may affect cell shape determination (Brockington et al., 2013). Evaluating *MIXTA* may help us to understand the evolutionary history of conical cell shape and thus provide information as to its function.

In this study, I investigate cell shape relationships within the genus *Mimulus*, an ancestrally bee pollinated genus with multiple independent derivations of bird- and self-pollination (Beardsley et al, 2004). The primary goal of this study is to investigate whether conical cell shape an adaptation to bee pollination. A secondary goal was to develop a methodology for robustly and simply determining cell shape using light microscopy. If conical cell shape is an adaptation to bee pollination, then I predict to observe 1) flatter cells in self-pollinated flowers than in bee-pollinated flowers, 2) differences in variance of cell shape in self-pollinated flowers with respect to the cell shape of bee-pollinated flowers, as self-pollinated flowers may not experience any selective pressure to maintain conical cell shape. Nonfunctional traits, such as conical cells in a self-pollinating flowers, often see either increases or decreases in variance (Royer et al., 2016). Additionally, we investigated whether any nonfunctional mutations were present in *MIXTA*-like genes of selfers. In order to assess if cell shape is an adaptation to bee pollination, I compared petal cell shape using this new metric in two ways: a broad-scale assessment of cell shape across the genus *Mimulus* and a fine-scale comparison of species pairs.

Methods

Study system

To fairly compare cell shape between flowers, cell shape must be quantified. Four key species pair comparisons from the genus *Mimulus* are considered in this study: bee-pollinated *M. guttatus* and self-pollinated *M. micranthus;* bee-pollinated *M. lewisii,* and self-pollinated *M. parishii; M. lewisii* (wild type) and *M. lewisii* (flat-celled *MIXTA* mutant); and *M. verbenaceus* (wild type) and *M. verbenaceus* (flat-celled mutant) (Ritland and Ritland, 1989; Bradshaw Jr et al., 1995; Yuan et al., 2013; Fishman et al., 2014).

Planting

In order to obtain flowers for analysis, plants were grown from seed. Seeds were planted in Edna's Best Potting Soil mixed with Perlite in 50 cell seedling starter trays. Seeds from *M. guttatus* (IM106), *M. parishii*, one wild-type *M. lewisii* line (LF10), one flat-celled mutant *M. lewisii* line (guideless), one wild-type *M. verbenaceous* line (MvBL), and one flat-celled mutant line of *M. verbenaceous* (MV00021) were stratified for 21 in a growth chamber at 2℃ and bottom watered. *M. micranthus* (EBR) seeds were stratified for 7 days in a growth chamber at 2℃ and bottom watered. After stratification, the plants remained in the growth chamber with 16 hour days at 22℃. After two months, *M. parishii*, LF10, guideless, MvBL, and MV00021 were transferred to four-inch pots while *M. guttatus* and *M. micranthus* were kept in the starter trays.

Confocal microscopy

Confocal microscopy was used to obtain a three-dimensional image of cell shape. One flower from *M. guttatus, M. lewisii* (wild-type), *M. lewisii* (flat-celled mutant), *M. parishii, M. verbenaceus* (wild-type), *and M. verbenaceus* (flat-celled mutant) each was picked. The center dorsal petal was removed and stained with 500nm Propidium Iodide for approximately 30 minutes (Molecular Probes, 1999). Petals were then washed with water and observed for surface cell shape using a Leica TSC SP Confocal Microscope.

Light microscopy

Light microscopy was used to obtain images for measurement. One ventral petal and the center dorsal petal were cut from flowers from each species or line for light microscope imaging. Flowers from 10 *M. guttatus,* 10 *M. micranthus,* 10 *M. parishii,* 5 *M. lewisii* (wild-type), 4 *M. lewisii* (mutant flat-celled), 3 *M. verbenaceus* (wild-type), and 3 *M. verbenaceus* (mutant, flat-celled) were analyzed with light microscopy. Differences in sample size were due to limited plant growth and floral blooming. Petals were folded in half along the cut edge with the epidermal side facing outwards to allow for clear viewing of the epidermal cells (Ren et al., 2017). Petals were mounted on water and photographed under the light microscope at 200x magnification. Pictures were taken along the natural edge and folded

edge of each petal. Six cells from the folded edge of the dorsal petal were measured in ImageJ (Rueden et al., 2017; Schindelin et al., 2012). Cell height and width were recorded, as were the apical angles 5 μm from the vertical bisection of the cell, lateral angles 5 μm from the horizontal bisection of the cell, and basal angles 5 μm from the vertical bisection of the cell (**Figure 1**). Finally, a numerical estimate of cell shape, on a scale of 1 to 5, where 1 is a perfectly conical cell with sharp side slopes and a narrow tip, and 5 is a perfectly flat, boxy cell, were recorded as estimate shape (**Figure 1).**

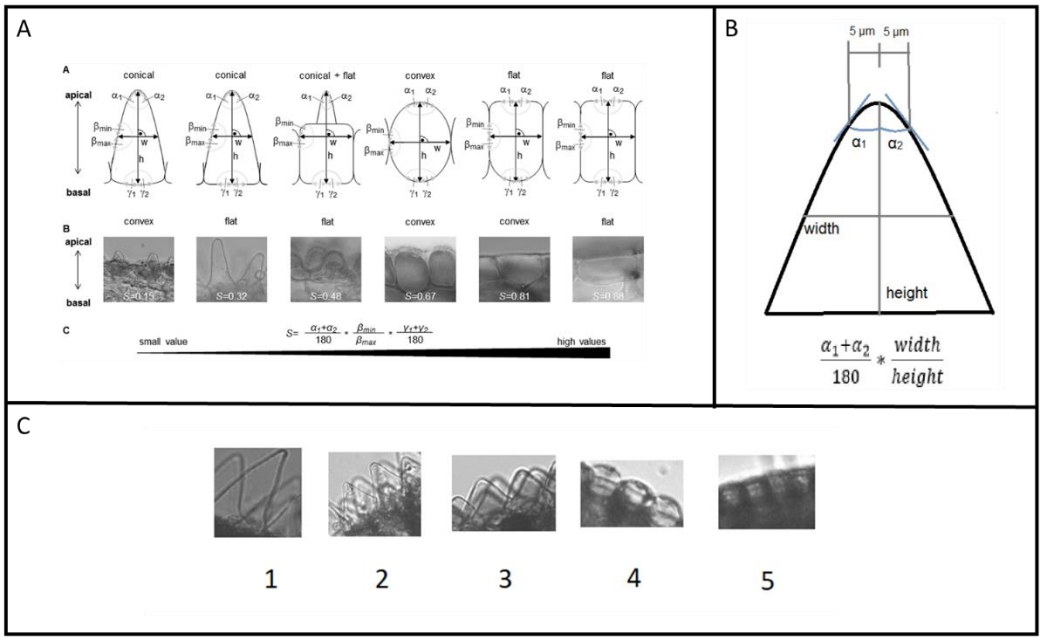

Figure 1. Methods of quantifying cell shape. A) The calculation of the shape parameter, *S*, a previously developed metric that has major issues (Papiorek, Junker, and Lunau, 2014). The shape parameter is a ratio measurement of angles at the cell's tip, side, and base. B) Measuring conicality to calculate C, the conical value. The conical vaule is a ratio measurement of the angles at the cell's tip and the ratio of the cell's height and width. C) Roughly estimating the shape of cells with Estimate Shape. Example cells are provided with assigned Estimate Shape values ranging on a scale of 1 to 5.

These measurements were used to calculate an *S* value (**Figure 1**) and a new metric, the Conical Value (*C*). *C* is calculated as $\frac{\alpha_1+\alpha_2}{180} * \frac{width}{height}$ where α_1 and α_2 are measured as outlined by Papiorek, Junker, and Lunau (2014); width is an individual cell's width measured at the midpoint, and height is an individual cell's height measured at the midpoint.

Bioinformatics

In this study, four candidate *MIXTA*-like genes were identified and analyzed. The genes Migut.E00076, Migut.K01307, and Migut.H00943 were selected for their high expression levels in *Mimulus* petals (Finley and Jammes, 2018). The gene Migut.B01718 was chosen as

it is the orthologue to the *MIXTA* gene discovered in *Arabidopsis* (Glover, Perez-Rodriguez, and Martin, 1998). These genes were evaluated in the *M. guttatus* complex due to *M. guttatus'* well-annotated genome.

Re-sequenced genome-wide Illumina data of self- and bee-pollinated species in the *M. guttatus* complex were aligned to the *M. guttatus* genome (*Mimulus guttatus* V2.0 annotation; www.Phytozome.org) with the Burrows-Wheeler Aligner (Li and Durbin, 2009). The chosen lines were AHQTN1.6_8 (*M. guttatus,* self-pollinated, sequenced by Fishman and Finseth), AHQT1.18 (*M. guttatus,* bee-pollinated, sequenced by Fishman and Finseth), BAG3 (*Mimulus tilingii,* bee-pollinated, Joint Genome Institute), BOG (*M. guttatus,* bee-pollinated, Brandvain et al., 2014), CACN9 (*M. nasutus,* self-pollinated, Brandvain et al., 2014), CP24 (*M. guttatus,* bee-pollinated, Brandvain et al., 2014), DENT (*M. dentilobus,* bee-pollinated, Brandvain et al., 2014), DPRN104 (*M. nasutus,* self-pollinated, Brandvain et al., 2014), DUN (*M. guttatus,* bee-pollinated), EBR10 (*M. micranthus,* self-pollinated, sequenced by Fishman and Finseth), IM109(*M. guttatus,* bee-pollinated, Sweigart and Flagel, 2015), IM62 (*M. guttatus,* bee-pollinated, Sweigart and Flagel, 2015), IM767 (*M. guttatus,* bee-pollinated, Sweigart and Flagel, 2015), INV (*M. guttatus,* bee-pollinated, sequenced by Fishman and Finseth), KOOT (*M. nasutus,* self-pollinated, Brandvain et al., 2014), LMC24 (*M. guttatus,* bee-pollinated, Brandvain et al., 2014), MAR3 *(M. guttatus,* bee-pollinated, Brandvain et al., 2014), NHN (*M. nasutus,* self-pollinated, Brandvain et al., 2014), NRM (*M. spp.,* self-pollinated, sequenced by Fishman and Finseth), PED5 (*M. guttatus,* bee-pollinated, Brandvain et al., 2014), RCT433 (*M. guttatus,* self-pollinated, sequenced by Fishman and Finseth), REM8 (*M. guttatus,* bee-pollinated, Brandvain et al., 2014), SF (*M. nasutus,* self-pollinated), Brandvain et al., 2014, SLP9 (*M. guttatus,* bee-pollinated, Brandvain et al., 2014), SWB-S3-1-8 (*M. guttatus,* bee-pollinated, Brandvain et al., 2014), and YJS6 (*M. guttatus,* bee-pollinated, Brandvain et al., 2014). The genomes were visualized in the Integrated Genome Viewer (Thorvaldsdóttir, Robinson, and Mesirov, 2013) at the genes Migut.E00076, Migut.K01307, Migut.H00943, and Migut.B01718. Genes were analyzed for frameshift or nonsense mutations using visual assessments of alignments in IGV.

Statistical analysis

Analysis of differences in conicality between flower species and lines were performed with two-tailed t-tests. Comparisons of *C* and *S* to estimate shape were made with Linear Regressions. The test assumption of normality was checked with Shaprio-Wilk tests. Differences in variance in conicality between bee- and self- pollinated flowers were tested with an F-test. All statistical tests were performed in Rstudio (Rstudio Team, 2015).

Results

Determination of a quantitative metric for cell shape determination

We first evaluated the *S* and *C* metrics by comparing their estimates with visual observations of cell shape on a categorical scale. Different methods for quantitatively analyzing cell shape had varying levels of efficacy. *S* did not correlate with the observed shape of petal cells of the flowers studied (r^2= 0.001712, p=0.6299). However, the first term of the *S* parameter equation, $\frac{\alpha_1 * \alpha_2}{180}$, was positively correlated with estimate shape (r^2= 0.6614, p<2.2e-16). An even greater correlation was observed between *C* and estimate shape (r^2=0.7715, p <2.2e-16, **Figure 2).**

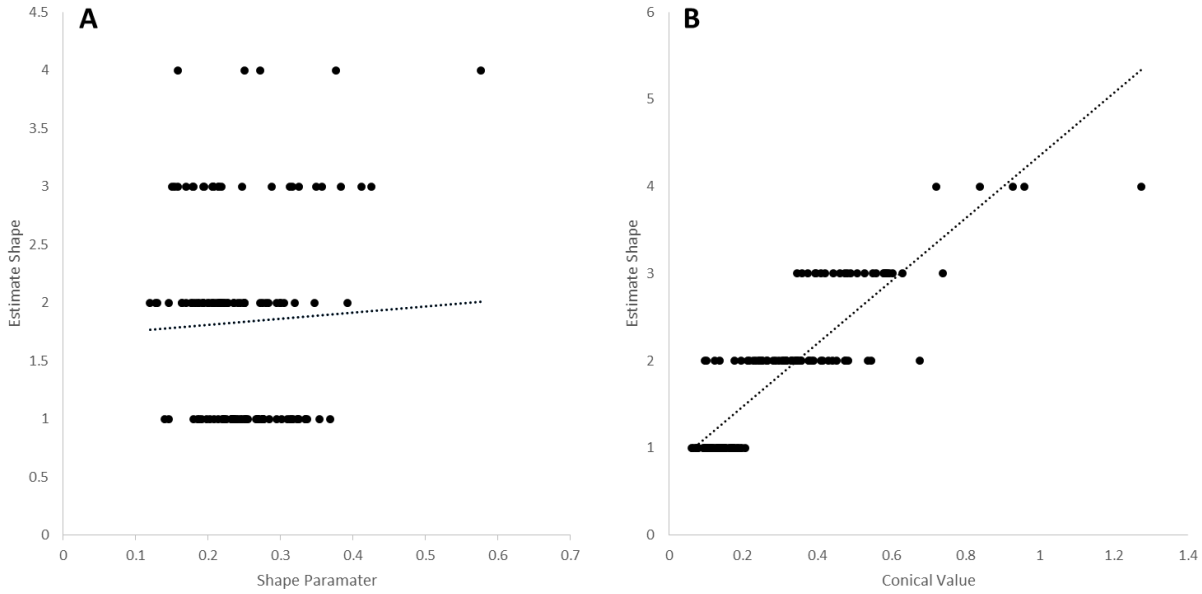

Figure 2. The relationship between two metrics for measuring cell shape and estimate shape. A) The Shape Parameter, *S*, has no significant relationship with estimate shape. B) The Conical Value, *C*, has a significant relationship with estimate shape.

We further confirmed our *C* value by comparing wild-type and mutant, flat-celled petals. When observed with both light and confocal microscopy, differences in cell shape between wild-type and flat-celled mutant flowers were apparent. Mutant flowers show the flat-celled phenotype as expected, and this phenotype is reflected in quantitative measurements of cell shape using *C* (**Figure 3**). Overall, wild-type flowers had more conical cells than their flat celled-mutant counterparts when conicality was measured with the *C* value (**Figure 4).** Larger *C*-values indicate a lower degree of conicality. Mutant *M. lewisii* had flatter cells than

wild-type *M. lewisii (*t =8.7392, p =0.00252) and mutant *M. verbenaceus* had flatter cells than wild-type *M. verbenaceus* (t =3.7306, p =0.03075).

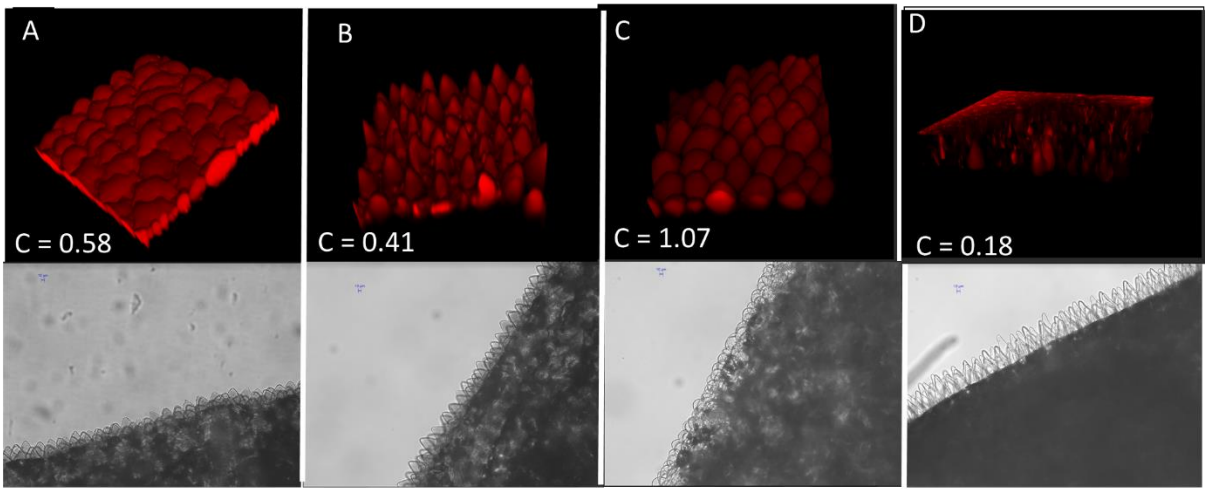

Figure 3. Confocal and light microscopy comparisons of petal cells from flat-celled mutant flowers and wild-type flowers. A) Images of mutant *M. verbenaceus* B) Images of *M. verbenaceus* (wild-type) C) Images of mutant *M. lewisii* D) Images of *M. lewisii* (wild-type)

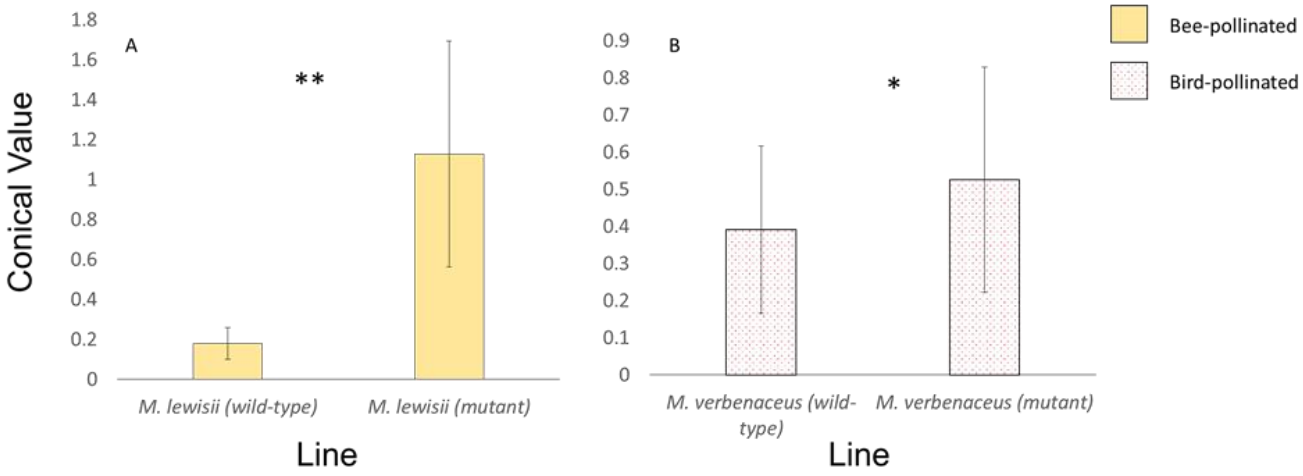

Figure 4. Differences in cell shape between wild-type and mutant, flat-celled flowers. A) Comparison of *M. lewisii* (wild-type) to its flat-celled mutant counterpart. B) Comparison of *M. verbenaceus* (wild-type) to its flat-celled mutant counterpart. Mean ± SEM. Asterisks indicate differences according to a t-test with significance levels of * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

Natural variation in cell shape and pollinator mode

Wild-type flowers had more conical cells than their flat celled-mutant counterparts when conicality was measured with the C value (**Figure 5).** Larger C-values indicate a lower degree of conicality. When bee pollinated flowers were compared to their self-pollinated

counterparts, differences were apparent. *M. micranthus* had flatter cells than *M. guttatus* (t=-7.5741, p=3.151e-06) and had more variance in cell shape than *M. guttatus* (F=3.6882, p=0.03258). Similarly, *M. parishii* had flatter cells than *M. lewisii* (t =4.8143, p=0.0005246) and had more variation in cell shape than *M. lewisii (*F=14.97, p=0.009614).

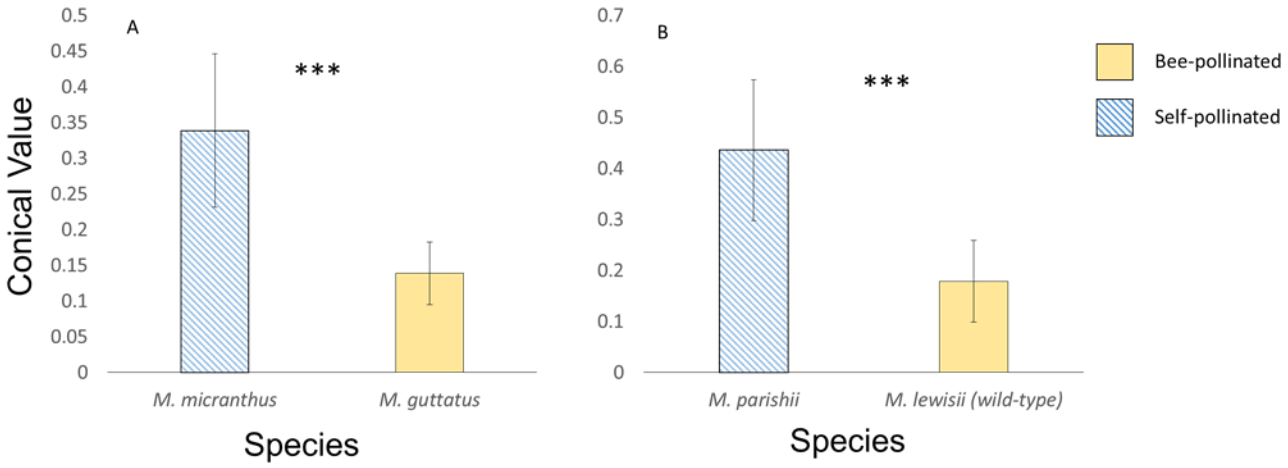

Figure 5. Differences in cell shape between bee-pollinated and closely related self-pollinated species. A) Comparison of self-pollinated *M. micranthus* to bee-pollinated *M. guttatus.* B) Comparison of self-pollinated *M. parishii* to bee-pollinated *M. lewisii*. C) Comparison of *M. lewisii* (wild-type) to its flat-celled mutant counterpart. D) Comparison of *M. verbenaceus* (wild-type) to its flat-celled mutant counterpart. Mean ± SEM. Asterisks indicate differences according to a t-test with significance levels of * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

Analysis of MIXTA genes

Analysis within IGV of the genes Migut.E00076, Migut.K01307, Migut.H00943, and Migut.B01718 within the *M. guttatus* complex showed no frameshift or nonsense mutations differentiating self-pollinated species from bee-pollinated species at these four genes.

Discussion

In this study, we aimed to develop a methodology for measuring cell shape, to quantify cell shape within two pairs of closely related bee-pollinated and self-pollinated species and two pairs of wild-type and mutant, flat-celled lines, and to investigate the genetic basis of conical cell shape. Our findings suggest that the Conical Value, *C*, can accurately quantify cell shape, and these findings were confirmed by *C*'s close relationship to qualitative cell shape observations and its ability to distinguish between the cell shape of wild-type and flat-celled flowers. Using *C* as a tool to measure cell shape, we found that in two separate instances within the genus *Mimulus*, the evolution of self-pollination was correlated with a loss of conicality and an increase in variance in cell shape. Finally, at the four *MIXTA*-like genes

analyzed in this study, we found no frameshift or nonsense mutations; therefore, the genetic basis of conicality in the *M. guttatus* complex remains uncertain.

Development of a robust methodology for quantifying cell shape

Imaging in both two and three dimensions is able to capture easily observable differences in cell shape. Making qualitative assessments of cell shape is possible with the Conical Value, *C*, which more accurately represents observations of cell shape as a quantitative metric than the previously established *S*-value does. This may be because *C* accounts for angle measurements, as *S* does, as well as ratio measurements. Ivakov and Persson (2013) assert that ratio measurements, such as a cell's width:height, benefit from increased accuracy because their allow for a normalization of the two dimensions, though they are often unable to account for complex shapes. As such, *C* benefits from accounting for the angles of the cell's tip and the ratio measurement of the cell width:height. Further verifying *C* as a reliable tool, flat-celled mutants of *M. lewisii* and *M. verbenaceus* had qualitatively flatter cells than their wild-type counterparts. As expected, *C* was able to distinguish between the cells of conical-celled wild-type flowers and flat-celled mutant flowers. The Conical Value remains an imperfect measure of cell shape due to its two-dimensional nature. As such, quantification of cell shape using three-dimensional microscopy, such as SEM or confocal microscopy, may more accurately represent cell shape; however, sample preparation with such techniques is more costly and time intensive than light microscopy. *C* may be improved by considering cells' relationship to neighboring cells rather than considering cells independently. Additionally, *C* may be improved with additional measurements of individual cells, such as measurements of a cell's approximate diameter and slope. Finally, *C* assumes cells to either have a conical, rounded, or boxy shape; however, many cells have an intermediate appearance with conical tips and boxy basal regions. Additional measurements accounting for intermediate cell shapes could further improve *C*.

Cell shape correlates with pollinator mode

Conical cell shape and reduced variation in cell shape are correlated with bee-pollination. In this study, we found that self-pollinated *M. micranthus* and *M. parishii* both had flatter cells than *M. guttatus* and *M. lewisii*, respectively. This is consistent with the results of Papiorek, Junker, and Lunau's (2014) findings that bird-pollinated flowers have flatter cells than bee-pollinated flowers, and the results of Whitney et al. (2011b), Whitney et al. (2009), and Bräuer et al. (2017) which indicated that bees were more capable walking and landing conical-celled surfaces and flowers as compared to flat celled-surfaces and flowers. Furthermore, self-pollinated flowers also had more variance in conicality than bee-pollinated flowers, as is expected since conical cells may be a nonfunctional trait in *M. parishii* and *M. micranthus* (Royer et al., 2016). This increased variance in self-pollinated flowers' cell shape suggests that there is decreased selective pressure for self-pollinated flowers to maintain

conical cell shape. Further directions for future study include examining cell shape and variation in cell shape across the genus *Mimulus* in order to gain a more complete understanding of how frequently a switch in pollinator mode to self-pollination is associated with losses of petal cell conicality.

Genetic basis of conical cell shape

We hypothesized that there would be major differences between the *MIXTA*-like genes of self-pollinated plants and bee-pollinated plants in the *M. guttatus* complex. Although no nonsense or frameshift mutations were identified in the identified *MIXTA* genes, *MIXTA* may still be important for cell shape determination. Missense mutations, which were not addressed within the scope of this study, may be responsible for differences in cell shape. Alternatively, the time of expression of genes may be of great importance to cell shape development. In *Lotus japonicas*, the gene *LjCYC2* is known to determine the presence of conical petal cells. Rather than the gene's presence, its temporal expression mattered for cell shape determination. In bee pollinated *Lotus* species, *LjCYC2* was expressed at the earliest stages of floral development. However, in bird pollinated species, *LjCYC2* was expressed only in later stages (Ojeda et al., 2017). It also may act as a downstream regulator for *MIXTA*-like genes (Feng et al., 2006).

In addition, there are a large number of alternate genes that have been identified as impacting cell shape determination in plants. The microtubule severing protein KTN1 is necessary for conical cell shape development, as is SPIKE1 and ROP GTPases. KTN1 promotes tip sharpening and cell height at late developmental stages. ROP GTPases activate KTN1 and SPIKE1 functions upstream. Both genes, when nonfunctional, produce swelling at the tip of conical cells, indicating that KTN1, SPIKE1, and ROP GTPases may all work together in a pathway which produces conical shaped cells (Ren et al., 2017). Further, mutations in *ANGUSTIFOLIA* (AN) are correlated with an accumulation of reactive oxygen species, which decrease the conicality of Arabidopsis petal cells. In AN mutants, mutations to KTN1 increased cell tip sharpness. An AN-ROS pathway that functions with KTN1 that is required for normal conical cell development (Dang et al., 2018).

Conclusions

This study demonstrates that the Conical Value may be a more effective quantitative measure of cell shape. With simple changes to established parameters, incorporating both cell angles and cell dimensions, light microscopy alone can reliably measure cell shape. Additionally, bee- and self- pollinated flower pairs from two separate clades within *Mimulus* display the same pattern of reduced conicality and increased variation in cell shape within the self-pollinated flowers, indicating at least two independent derivations of self-pollination correlated with changes to petal cell shape. These findings lend further support to the hypothesis that conical cell shape is an adaptation to bee pollination. The new technique for measuring cell shape developed in this study allows us to elucidate targets of selection within

the bee- and self-pollination syndromes and to unravel parts of pollinator modes that were previously undiscovered.

Acknowledgements

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