Examining Tegmina Polymorphism in Leaf-Masquerading Katydids (Orthoptera: Tettigoniidae)

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Editor: Katherine Damuth

Abstract: Insects employ a wide array of defensive mechanisms to avoid predation in the wild, evolving highly complex strategies for mimicking a wide range of organisms from other predators to surrounding foliage in the environment to handicap predators' ability to maintain a clear record of prey images to hunt. Cryptic insect species often evolve further mimicry strategies such as polymorphs and masquerade to provide a far more diverse range of forms, with even further variation of form seen through sexual dimorphism. Frequent and complex instances of polymorphism and cryptic masquerade can be found within the Orthopteran subfamily pterochrozinae (Orthoptera: Tettigoniidae), often posing taxonomic dilemmas as the hugely diverse and convincing forms are unable to be distinguished from eachother. This subfamily contains the genus *Mimetica* whose precision and accuracy in mimicking leaf appearance, as well as the wide diversity in shape and high color variation, make it an ideal system for observing of polymorphic variation of leaf forms. This experiment attempted to clarify the species relationships within *Mimetica*, in addition to developing a deeper understanding of the function and importance of polymorphs in insect populations and ecological phenomena. This was done to ascertain if the katydid species *incisa*, *crenulata*, *viridifolia*, *mortuifolia*, *tuberata*, and *simoni* within *Mimetica* display a distinct number of cryptically polymorphic forms versus continuous variation by utilizing digital landmark based geometric morphometric software to analyze the tegmina. Tps analyses provided strong support for the existence of sexually dimorphic forms for all species, and a lack of distinction in form within all *incisa* specimens, suggesting a more effective polymorph. In contrast, *simoni* and *viridifolia* specimens displayed the clearest distinction in form, supporting the possibility of the existence of discrete polymorphs in this genus but requiring further refined morphometric analyses.

Keywords: morphometrics, Orthoptera, polymorph, masquerade, crypsis, defense

In nature, insects have been observed to exhibit a wide variety of methods of defense against predators, ranging from passive strategies such as mimicry to more confrontational defenses like the use of modified ovipositors as stingers. Mimicry, a primary defensive method, can be considered more passive in that insects utilize it to circumvent any interaction with a potential predator and thus prevent any attack from even occurring (Edmunds and Edmunds 1974). Many insects practice similar

defensive strategies such as crypsis and masquerade, both of which are undertaken in an attempt to passively prevent interaction with predators (Olofsson et al. 2012). These strategies differ in that crypsis is characterized by an attempt at overall reduction in cognitive detection by predators, while masquerade is characterized by the ability of a predator to detect the insect's presence, but the inability to properly identify it as viable prey, instead viewing it as whatever it is masquerading as: something inedible or unappetizing. (Skelhorn et al. 2010b, Skelhorn and Rowe 2016).

Cryptic insect species, to more effectively avoid predator detection, are known to evolutionarily develop multiple colorful forms known as polymorphs. This strategy is particularly effective in that predators are not able to take stock of all existing forms of every polymorph of that species. This further propels polymorphic development as predators search for a singular polymorph while other polymorphic forms lie undetected (Endler 1981, Ruxton et al, 2004). Masquerading insect species also exhibit cryptic polymorphisms, which have been found to be so diverse and taxonomically problematic that in some cases multiple species have been found to all be members of the same species (Castner 1995, Castner & Nickle 1995a, Gutierrez-Valencia et al. 2017). This has been observed within the subfamily pterochrozinae (Orthoptera: Tettigoniidae), the katydids of which are dubbed true-leaf mimics, being known to display highly detailed and realistic leaf masquerades complete with imitation necrotic leaf lesions and even unique contouring of the tegmina to create the appearance of bite marks (Braun 2015, Mugleston et al 2016).

Beyond extreme leaf masquerading, pterochrozinae katydids have also been known to exhibit instances of sexual dimorphism in which male polymorphs differ from that of females, furthering the variation of leaf forms (Castner & Nickle 1995a). All of these factors, the precision and accuracy in mimicking leaf appearance, as well as the wide diversity in shape and high color variation, are what make the genus *Mimetica* an ideal system for observing cryptic polymorphism variation of leaf forms. In an attempt to clarify the species relationships within *Mimetica*, in addition to developing a deeper understanding of the function and importance of polymorphs in insect populations and ecological phenomena project all of these interactions were studied in order to ascertain if the katydid species within *Mimetica* display a distinct number of cryptically polymorphic forms versus continuous variation by utilizing digital landmark based geometric morphometric software to analyze the tegmina.

Materials and Methods

Mimetica (Pictet) specimens were loaned to the Song Laboratory of Insect Systematics and Taxonomy by The National Biodiversity Institue (INBio), Cornell University, University of Michigan, The Field Museum of Natural History, and the Philadelphia Academy of Science, for a total of 125 pinned adult specimens. Six species were present in total: *incisa*, *crenulata*, *viridifolia*, *mortuifolia*, *tuberata*, and *simoni*.

Relaxing and Spreading: To prevent molding or deterioration within the environment of the relaxing chamber, original loaning institution identifying, and locality labels were carefully removed from each pinned specimen, set aside, and numerically labelled to be replaced later. Correspondingly numbered pieces of paper were affixed to each pinned specimen, which were placed in a large glass relaxing chamber (Anchor Hocking Company, Lancaster, OH, USA) containing a layer of moth balls (Willert Home Products, St. Louis, MO, USA) paper towels (Proctor and Gamble, Cincinnati, OH) dampened with water, and a sheet of polyethylene foam (Bioquip Inc., CA, USA) to pin specimens on. Specimens were allowed to rehydrate for about a week until each was deemed appropriately flexible.

Once properly rehydrated, specimens were removed from the chamber and their original labels replaced. Each was then placed on an adjustable wooden spreading board (Bioquip Inc., CA, USA) and its left tegmen was carefully spread at a right angle by pinning small pieces of paper to cover the entire tegmen. Specimens were allowed to dry for approximately a week, and were then placed into collection trays organized by species and loaning institution.

Imaging: A small set-up comprised of a 3in x 2in x 2in block of polyethylene foam sandwiched between two layers of plastazote foam (Bioquip Inc., CA, USA), the bottom being 6in x 3in and the top being 4in x 3in, was prepared to hold the specimens for imaging. Each layer was fastened loosely with pins. Specimens were pinned to the bottom protruding layer of foam, and

positioned so that the outstretched tegmen of each specimen lay levelly on the top layer of the set-up.

Once positioned, a 2in x 3in piece of clear plastic cut from a page protector (Staples Inc. Framingham, MA, USA) was affixed firmly over the tegmen to allow a more level depth of field to photograph. This plastic layer was adjusted or replaced to accommodate larger tegmina as needed. A small hobby size two paint brush (Prang & Dixon, Lake Mary, FL, USA) was used to gently remove any dust or debris on the tegmen and overlying plastic layer before photographing. Specimens were also appraised for damage prior to photography, and those with considerable damage were removed from the original sample size of 125, with 90 *Mimetica* specimens remaining for photography.

High quality photos were taken using the customized camera set-up within the Song Laboratory. An SP 17-50mm F/2.8 XR Di II VC LD Aspherical lens (TAMRON USA, Inc., Saitama, Japan) and an EOS 6D DSLR Canon camera (Canon U.S.A., Tokyo, Japan) equipped with an HSA-PSU Universal Hot Shoe Adapter (Gradus Group LLC, Menlo Park, CA, USA) and a BL-HS2 Two-Axis Hot-Shoe Bubble Level (Gradus Group LLC, Menlo Park, CA, USA) affixed to a Bencher Copymate II Fluorescent Tabletop Producer (110-240V) (Bencher, USA) were used, along with a Dynalite MP800 800W/s Roadmax Power Pack (Philips-Dynalite, Sydney, Australia) and two Impact 32" Umbrellas, two Impact 8' light stands (Gradus Group LLC, KY, USA), and two single head E26/E27 lamp bases with two E27 45W 5500K bulbs (LimoStudio) and two

Ovation F 3.25" barndoors (CHAUVET Professional, FL, USA). The camera's settings were set to an aperture of F16, an ISO of 200, and 1/200 shutter speed. The Dynalite Roadmax Power Pack was set to half power, and Adobe Creative Cloud Photoshop and Lightroom (Adobe, San Jose, CA, USA) were used to process and capture images. Image files were named according to codes present on labels from original loaning institutions.

Geometric Morphometric Analysis: TpsUtil was first used to build a tps file of all 90 specimen photographs, and this file was then opened within TpsDig to place digitized landmarks on each imaged tegmen. A total of 42 landmarks were used, beginning at the origin of the tegmen from the notum and moving distally, placed at equidistance from each other around the outline of the tegmen. 20 were placed on the upper and lower margin, with a single landmark at the distal and proximal ends. A scaling tool was used to measure the scale present in each image, setting the standard for all images at one millimeter to ensure analysis on the same scale.

The digitized landmark file from TpsDig was then opened in TpsUtil where a silders file was created to allow for a more accurate relative warp analysis, hereafter referred to as RW. Due to the wide diversity in tegmen shape across the digitally landmarked area for all specimens, 40 sliding landmarks comprised of landmarks 2 through 21, and 23 through 42 were designated. Landmarks 1 and 22 located on the proximal and distal ends of the tegmen remained nonsliding due to their consistent placement across all specimens.

TpsRelw was then used to conduct a principal components analysis, hereafter referred to as PCA, of shape variation relative to spatial scale by inputting the slider file and tps digital landmark file. A consensus and RW ordination plot of the tegmina for all 90 specimens were generated. A slight modification was made after observation of this plot to further refine the specimen sample size in order to further

refine the morphometric analysis. The 90 Mimetica specimens were culled to 68, requiring the use of TpsUtil again for the creation of a sliders file for the new sample size. Tps Relw was then used once again to generate a new consensus, RW plot, and RW scores. The new total of 68 specimens were then separated by sex, and this process was repeated for 38 males and 30 females.

The generated RW scores were organized in Excel (Microsoft, Redmond, Washington, USA) in three separate plots: males and females, only females, and only males. Scatter plots were created for each set of scores by using only singular values 1 and 2 as the x and y axes, respectively. Data markers were labelled by species to allow for ease of identification of groupings.

Results

All Specimens: Singular value one generated in the Tps Relw RW analysis of all 68 *Mimetica* specimens (n = 68) was shown to account for 35.42% of the PCA, while singular value two accounted for 25.95% of the analysis. Of the 67 total singular values generated in this analysis, values one and two

combined represented over half of the generated analysis at 61.35%, while each of the remaining values individually comprised only 10% or less. This trend was consistent in the analysis of females only and males only as well, with female singular values one (49.34%) and two (19.58%) of the total 29 values making up 68.92% of the total analysis, and male singular values one (25.17%) and two (23.07%) of the total 37 values making up slightly less at 48.24% of the total. Because these values, compiled in **Table 1**, comprised the greatest total of the principal component analysis, they were utilized as x and y values, respectively, to create three excel plots.

The scatter plot of the singular values for males and females can be seen within **Figure 1**. The overall distribution is somewhat disorganized, but a tentative distinction can be seen on either side of the x-axis with only female specimens on the left and male specimens on the right, with the exception of

three *viridifolia* males that have crossed over to the left of the x-axis and one female *incisa* specimen that lies to the left among the male specimens. Species clusters appear less distinct: incisa is scattered seemingly without pattern aside from the distinction between the sexes, as are *tuberata*, *mortuifolia*, and *crenulata*. The most pronounced species groupings appear to be that of *simoni* and *viridifolia*, which are also the species that account for the fewest specimens, a total of 16. Overall, the most discrete specimen arrangement appears to be between females and males of all species across the x-axis.

The consensus plot, or the average tegmen shape of all specimens can be seen in **Figure 2,** and shows a leaf-like, ovate form that appears to taper slightly distally along the lower margin, while the upper margin exhibits an uneven, staggered contour along the distal half that may be intended to emulate a partially eaten leaf.

Percentage Variation of Relative Warps

| Relative Warp	Percent Variation		
	Males	Females	Males and Females
1	25.17%	49.34%	35.42%
2	23.07%	19.58%	25.95%
3	14.53%	10.15%	10.84%
4	9.86%	6.42%	8.11%
5	8.41%	3.80%	4.98%
6	5.05%	3.08%	3.31%

Table 1: The first six relative warps for each Tps Relw analysis conducted. Warps one and two for each analysis comprise no less than 45% of each test, and are plotted in Figure 1 to visually represent the variation in tegmen shape for all specimens. Only six initial values are shown; values after RW 6 account for less than 5% of each analysis.

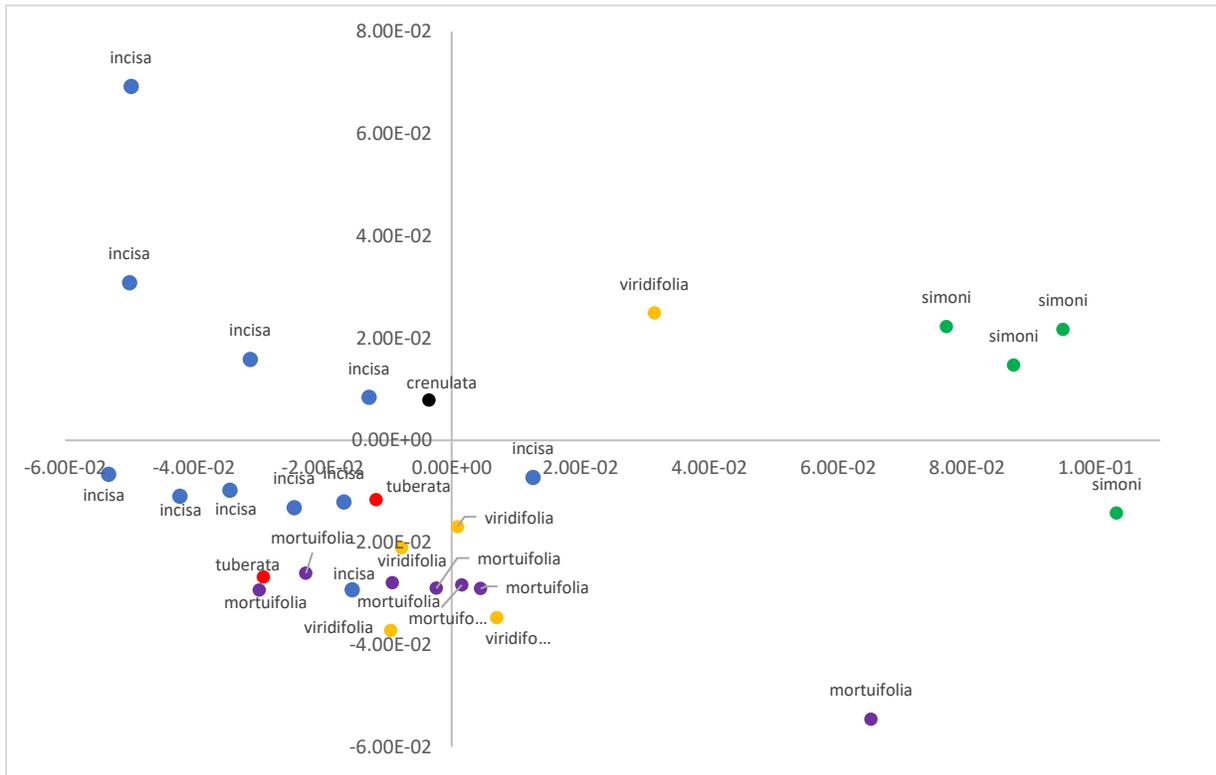# Relative Warp Analysis of Male and Female *Mimetica* Specimens
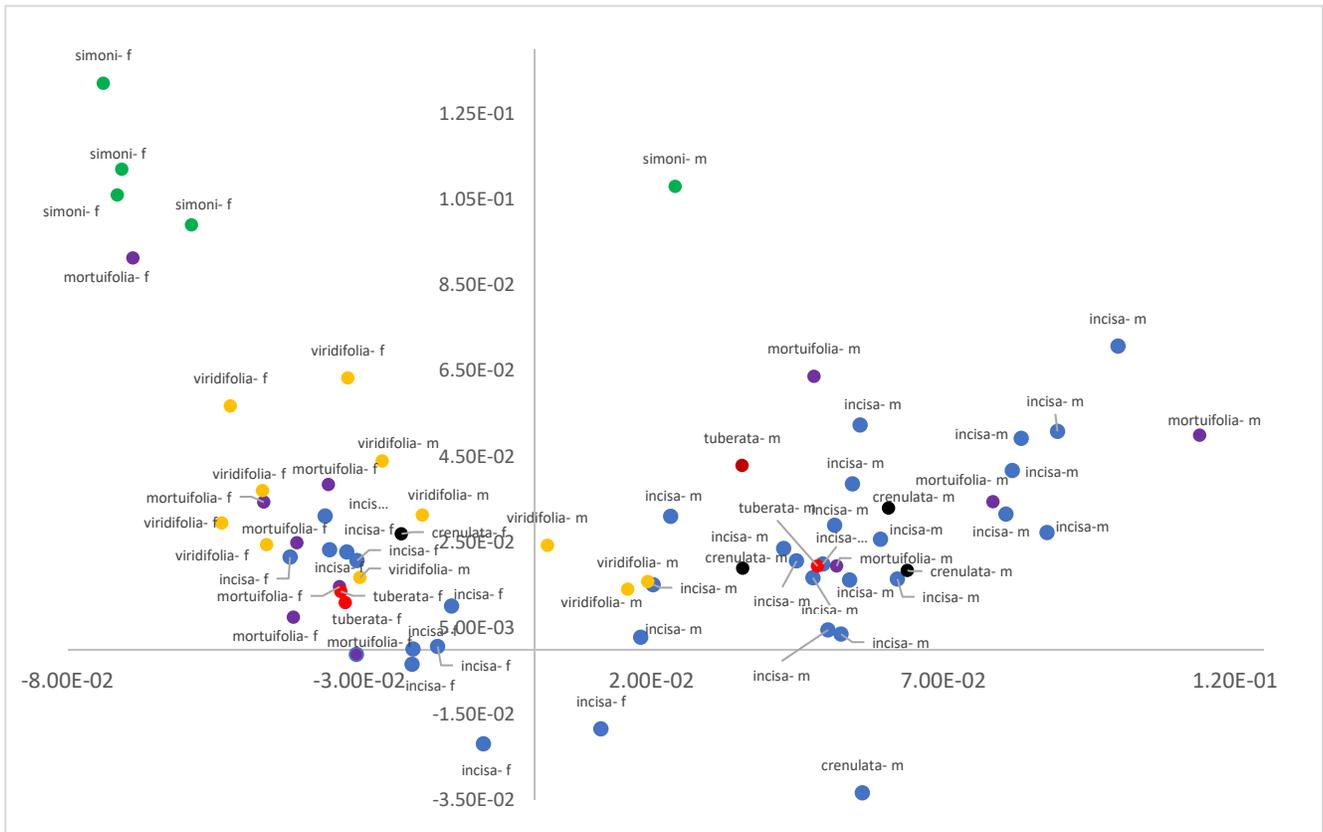
Figure 1. A scatter plot of RW singular values 1 and 2, x and y, respectively, generated from a tpsRelw analysis of all 68 specimens. Points are labelled according to species and sex, and are additionally color coded. *Simoni* = green, *mortuifolia* = purple, *viridifolia* = yellow, *crenulata* = black, *incisa* = blue, *tuberata* = red
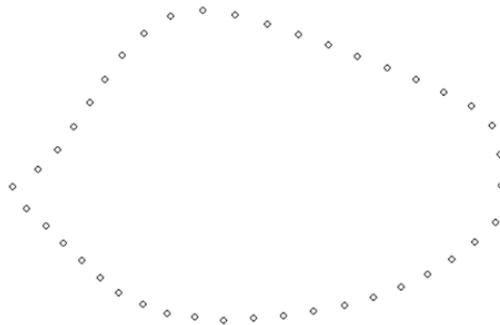
Total Consensus Plot of *Mimetica* Tegmina
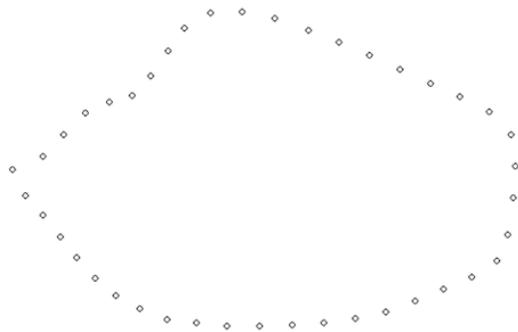
Figure 2. The consensus plot for all 68 specimens, male and female, generated by tpsRelw. The plot is made up of 42 digital markers, and appears to display an overall rounded, smooth shape, except for a slight, uneven dip on the top left distal margin

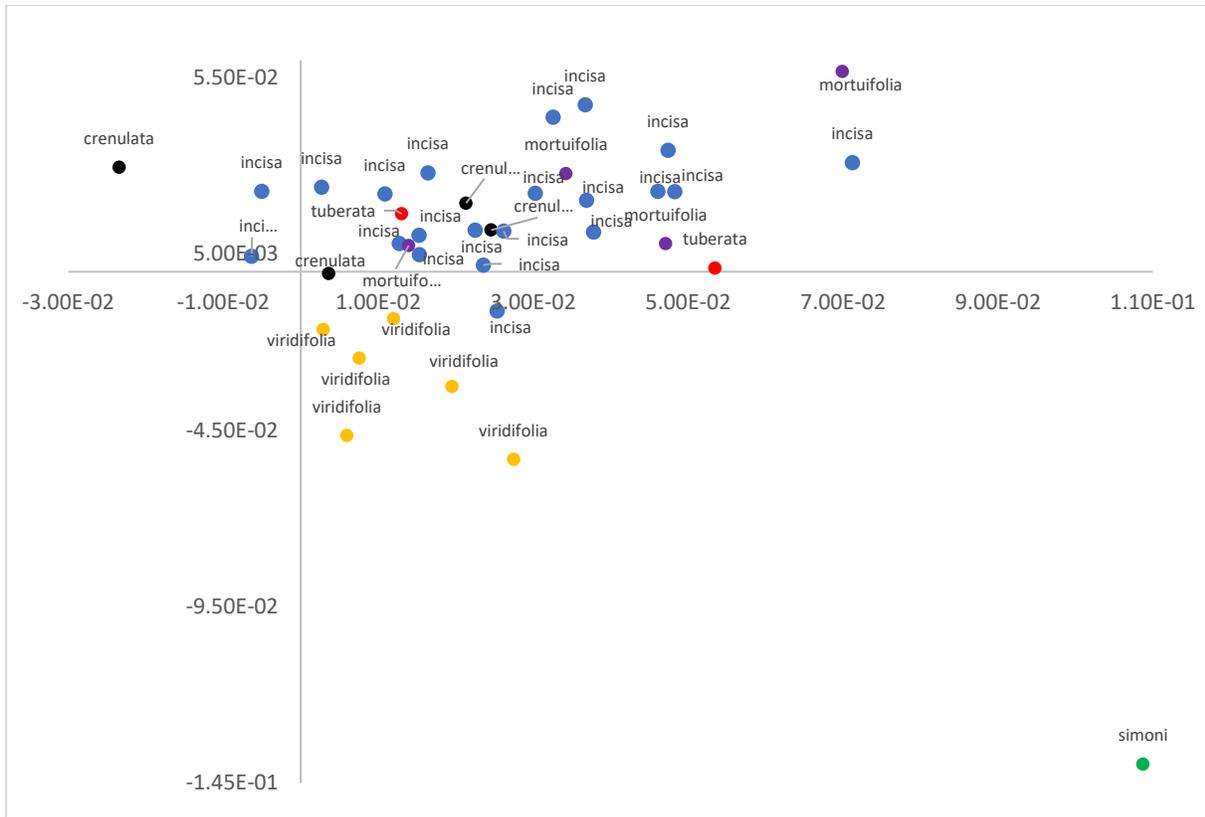## Relative Warp Analysis of Female *Mimetica* Specimens

Figure 3. A scatter plot of singular values 1 and 2, x and y, respectively, generated from a tpsRelw analysis of 30 female specimens. Values appear to be clustered most heavily within or near quadrant III, with small clusters of outliers. Points are labelled by species and are color coded. *Simoni* = green, *mortuifolia* = purple, *viridifolia* = yellow, *crenulata* = black, *incisa* = blue, *tuberata* = red.
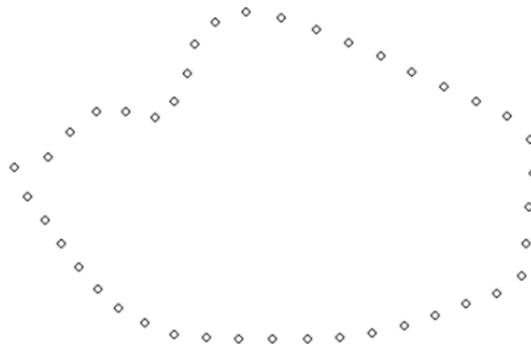
Female Consensus Plot of Mimetica Tegmina

Figure 4. The consensus plot for all 30 female *Mimetica* specimens generated by tpsRelw. The plot is made up of 42 digital markers, and appears to display an overall rounded, smooth shape that lacks the uneven divot in Figures 2 and 6.

## Relative Warp Analysis of Male Mimetica Specimens

Fig 5. A scatter plot of singular values 1 and 2, x and y, respectively, generated from a tpsRelw analysis of 38 male specimens. Values appear to be clustered heavily in or near quadrant I, with a cluster of *viridifolia* beneath in quadrant IV. Points are labelled by species and are color coded. *Simoni* = green, *mortuifolia* = purple, *viridifolia* = yellow, *crenulata* = black, *incisa* = blue, *tuberata* = red.

Male Consensus Plot of Mimetica Tegmina

Figure 6. The consensus plot for all 38 male *Mimetica* specimens generated by tpsRelw. The plot is made up of 42 digital markers, and appears to display a much more pronounced, uneven divot than is seen in Figures 2 and 4.

Relative Warping of Mimetica Tegmina
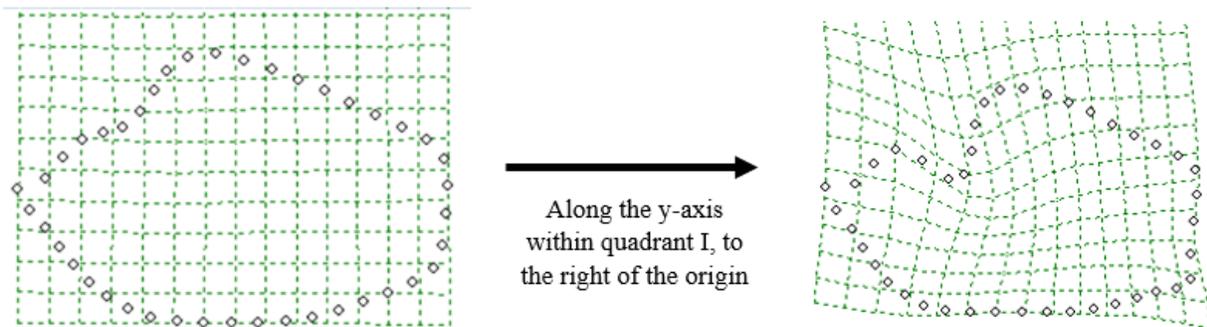
Figure 7: The RW visualization plots captured from quadrant I, from the RW ordination plot for all *Mimetica* specimens within figure 1. The first visual on the left was captured closest to the origin of the y-axis, and presents a less pronounced bite mark-like indentation. The second visual on the right at the farthest end from the origin of the y-axis, and displays a heavily pronounced divot on the upper margin.

Females: Figure 3 shows the resulting scatter plot for the TpsRelw generated scores for all 30 female Mimetica specimens (n = 30). Nearly half of all female specimens, 14, are concentrated within quadrant III, while the remaining 16 specimens are scattered within quadrants I, II, and III, with the distinct exception of *simoni* specimens which appear to lie in a loose cluster nearer to quadrant I, noticeably separate from the main concentration of values.

The consensus plot of the female tegmen shape can be seen in **Figure 4**, and like the consensus plot for all specimens, shows an ovate leaf-like shape. The female consensus differs however in that the entire plot displays an overall smooth shape, slightly tapered distally, tapering to a point, distinctly lacking the uneven, bite mark-like groove.

Males: Figure 5 shows the resulting scatterplot of RW scores for all 38 male specimens. Whereas the distribution of *simoni* specimens within the total RW plot and the female RW plot exhibited the most distinct species grouping, within the male plot *viridifolia* appears to cluster more prevalently than other species, concentrated within quadrant IV, just below the primary concentrated cluster of specimens in quadrant I.

The consensus plot of the male tegmen shape seen in **Figure 6**, like **Figures 2** and **4**, exhibits an ovate leaf-like shape. The male consensus clearly exhibits the most pronounced bite mark-like divot along the upper margin of the tegmen, along the distal half. This divot appears to extend deeper into the body of the tegmen than either previous plot, forming a near right angle before progressing into a less extreme ridge and tapering off smoothly into a pointed tip.

Discussion

Findings suggested the existence of multiple cryptically polymorphic forms, particularly within *incisa*, *viridifolia*, and *simoni*, and a clear indication of sexually dimorphic cryptic forms is seen within the data.

The overall distribution of specimens seen within **Figure 1**, while not presenting any immediately distinct patterns among species, does indeed show a clear distinction in placement between males and females. All females regardless of species can be seen to occupy quadrants II and III on the left side of the x-axis, with the exception of three *viridifolia* males that have crossed over among them, and a single *incisa* female that has cross over into quadrant IV. Other than these discrepancies, the x-axis perfectly bisects the distribution of females and males into respective halves of the plot. This is heavily suggestive of the presence of sexually dimorphic forms, as has already been observed within the pterochrozine subfamily (Castner and Nickle 1995).

This notion is further supported by **Figures 2**, **4**, and **6**, which show the consensus in tegmen shape for all specimens, females, and males. The slighter, less pronounced bite mark-like indentation, seen in the total consensus, when compared to its exaggerated presence in the male consensus and the distinct lack of any divot whatsoever within the female consensus, indicates that this natural tegmina contour designed to imitate a partially eaten leaf is likely a male-specific cryptic polymorphic form. This strong leaf resemblance, in addition to homochromy (the green-brown blending of color), and obliterative shading (irregular blotches and irregular color patterning), is described as essential to camouflage, and also presents itself as hyaline holes in the tegmina that one may expect to observe from the feeding of an insect on a leaf (Castner and Nickle 1995a). This phenomenon can be more easily observed along the y-axis in **Figure 1**, in

which, as values are placed further and further to the right of the origin of the y-axis, the exaggeration of the upper bite mark-like indentation becomes increasingly pronounced, as seen in **Figure 7** below.

Among female specimens on their respective half of the plot seen in **Figure 1**, the tegmina of which overall appear to lack males' imitation leaf bite mark in favor of a consistently smooth ovate shape, a great deal of indiscrimination can be seen in species placement. However, *simoni* specimens can be seen isolated from the central group of females in a clear cluster. This trend is additionally seen within **Figures 3** and **5** and could reasonably signify the presence of a distinct polymorph for this species. A similar trend, best present in **Figure 5**, may be present within the distribution of *viridifolia* specimens as well; while these specimens appear to be more loosely grouped than *simoni* specimens and are not as distinctly isolated from the main clusters within each plot, a semblance of a distinct grouping exists, and could again suggest the presence of a distinct polymorph for this species. However, because the distinction in form of *viridifolia* specimens is most pronounced within the male plot, this polymorph may be limited to only *viridifolia* males.

The species *viridifolia* and *simoni* present the most obvious distinction in tegmen shape of any species, regardless of sex, across all three RW plots, and yet also make up a very small portion of the total 68 specimens. Only five total *simoni* specimens exist within the total analysis, all of them being females except for one, while eleven total *viridifolia* specimens are present. In contrast to this, *incisa*

specimens across all three plots are far more indiscriminately scattered, lacking any clear distinction in form, and make up roughly half of all specimens present at a total of 32. The clear difference in discrete form between *incisa*, the largest species group present, versus *simoni* and *viridifolia*, which are very small groups within the total analysis, suggests that *simoni* and *viridifolia* take on a particularly obvious and highly distinct polymorphic form in comparison to all other species present. This more recognizable form, despite its function as a polymorph, may be a less successful polymorph that is more easily recognized than that of *incisa* or other species by predators due it's much more distinct appearance, perhaps explaining the low number of them accounted for within this analysis. This is partially supported by observations from Skelhorn and Ruxton (2011) and Castner and Nickle (1995a, 1995b), who saw that multiple polymorphic forms within a population serve to confuse predators by preventing them from orienting to a common search image, and that the more convincing mimicry and crypsis, the less likely an insect is to be found, thus, a clearly discernable form that sticks out is vulnerable to predators and may be successfully hunted.

Contrastingly, *incisa*, which exhibits little pattern in form other than the same sexually dimorphic characters seen across all species present, may in turn be a more successful in avoiding detection due to a wider variety of cryptic diversity, making it a more adept polymorph and allowing it to survive in greater numbers.

In summary, While the species' groupings across the created plots are by no means perfect or fully representative of the exact nature of the existence of a discrete number of cryptically polymorphic forms, the presence of sexually dimorphic cryptic forms appears to be highly likely among all of the species observed within the analysis. As supported by the relative warp analysis and resulting scores and consensus plots, within the male-specific form, the presence of a unique imitation leaf characteristic can be seen in the form of what may be a bite mark or other natural leaf formation, the prominence of which increases dramatically as specimens move rightward from the origin across the y-axis while females distinctly appear to lack this marking in favor of a smoother, fully ovate leaf shape. Additionally, when compared to the indiscriminate diversity of form and number of specimens seen in *incisa*, less successful polymorphs may exist within *viridifolia* and *simoni* due to their possible overt distinction in form from all other species regardless of sex and subsequent lack in number, causing them to be more identifiable to predators.

When all observations and gathered data are considered, the original hypothesis predicting a discrete number of cryptically polymorphic forms was not entirely disproved, but can undoubtedly be improved upon and more solidly supported. A few adjustments could be made to this study to correct discrepancies in data, as some shortcomings were evident such as the limits of sample size, as well as the lack of precision in tegmina shape analysis from limitations in placement and number of geometrical markers to outline the tegmen; an increase in markers could allow for a more descriptive and accurate analysis of shape. In addition, in multiple studies, the

importance and extent of intraspecific variation as related to color is studied and shown to play a role in the efficacy of polymorphic forms (Skellhorn and Ruxton 2011, Castner and Nickle 1995a), something that this experimental design did not account for.

Other inconsistencies may be partially due to slight errors in the identification of species due to the difficulty in accurate classification stemming from the diversity of polymorphic characters and sexual dimorphism. Further analysis, perhaps by utilizing DNA also could be undertaken to additionally refine this data and the implications of discrete polymorphs among species as well as sexes in order to further shed a light on the function and importance of polymorphs in insect populations and ecological phenomena, and provide a clearer understanding of the species relationships within the *Mimetica* genus.

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