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Why morphometrics: a short review and a case study on *Zygaena carniolica* (Scopoli, 1763)

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Summary: Morphometrics have had many uses in various fields of science. A modern trend of focusing on molecular data in biology might overlook the importance that morphometrics have had, and can have, in scientific experiments. The purpose of this paper is to illustrate the utility of geometric and linear morphometrics both in previous research and in a study we performed on four *Zygaena carniolica* (Scopoli, 1763) populations from Romania.

Key words: geometric morphometrics, linear morphometrics, fluctuating asymmetry, phenotypic plasticity, *Zygaena carniolica.*

Introduction

With modern molecular methods of analysis it is often easy to ignore "older" ways of data acquisition. However, some scientists are pushing towards the use of multiple methods of investigation during a scientific experiment of a biological nature (Will *et al.* 2005, Dincă *et al.* 2011, Dapporto *et al.*2014, Török *et al.* 2015). Morphometry (Blackith 1957) has had many uses in different fields over time, including medicine, geomorphology, anthropology, art, forensics, and, of course, biology (Elwa 2010) and can stand shoulder to shoulder to molecular studies in order to offer us a more coherent result, or it can offer interesting results on its own. In simple terms it is the analysis of variations in shape and size by different statistical methods (Reyment 2010).

Morphological characters are shaped by natural selection and are often the combined result of several genes (Will *et al.* 2005). Linear morphometrics can be used to examine these characters but only if the shape is relatively simple, even so it does not always offer compelling results about variations in shape, although it is highly valuable for measurements such as width and length (Krieger 2010). Geometric morphometrics, on the other hand, is very efficient in determining differences between shapes by use of different statistical and geometrical approaches. Some of these methods will be briefly explained below, but for a deeper understanding we recommend the work of Zelditch *et al.* (2004).

The basic tools for a morphological study are landmarks (Bookstein 1986).These are homologous points that can be easily identified and marked on all the specimens in the study, they must not change their position relative to other landmarks, must lie in the same plane and have an adequate coverage of form. These landmarks will be introduced into a coordinate system with X and Y axes. Here the mathematical approaches take over, starting with the calculation of the centroid, or the central point relative to the landmarks. One of the most basic operations in geometric morphometrics is the Procrustes Superimposition. This is done by overlaying the landmarks of all the specimens in the study, with the help of the centroid, and focusing only on shape by eliminating size and orientation. After the Procrustes superimposition is complete, a whole range of different forms of analysis can be used. Principal Component Analysis is often used to show a general direction of variation within a group. This can be done by using the Principal Component that accounts for most of the variation, most often this is PC1. The Thin-Plate Spline grid can also be used in tandem with PCA to better illustrate the results. If the purpose is to show the differences between groups then Canonical Variance Analysis is the tool for the job. This method constructs axes along the greatest distance between the landmarks of the studied groups (Zelditch *et al.* 2004).

These methods might seem slow and difficult to learn, however, modern software greatly reduces the time that would be taken by cumbersome mathematical calculations. The marking of landmarks can now be done on high resolution digital photographs, reducing the chances of errors and giving quick access for modifications. Examples of such software and the results that they offer will be described in the study on *Zygaena carniolica*.

Morphometrics in Ecology and Biology

By combining morphometric and genetic studies, several authors have come across some interesting results. One of the most eye-opening papers is that of Török *et al.* (2015), with a study on *Ptychoptera albamina*. Geometric measurements of wings and linear measurements of genitalia were used, combined with genomic DNA analysis. The result of this study was the description of a new species, *Ptychoptera incognita*, based solely on morphological differences, as the molecular analysis did not find significant differences between this new species and *P. albamina*. Contrasting this is the work of Dincă *et al.* (2011) on *Polyommatus icarus and Polyommatus celina*. For this study genomic DNA was again analyzed alongside genitalia shape, wing patterns and wing shape. Here the molecular results showed a deep divergence between the two species while the morphological differences were minor. In the end the paper served a greater understanding of the phylogeographical history of the two species. Dapporto *et al.* (2014) took matters a bit further by providing a new way of braiding together genetic and morphometric studies. In their work on *Maniola jurtina* they chose genetic and morphological markers and used a new recluster function which allowed population studies where the co-variation between the two different types of markers could be highlighted. Their method opens new paths for studies where heterogeneous traits can be used in tandem, with possible uses in ecology, systematics and many others. Monteiro *et al.* (1997) had a more focused approach in their paper on *Bicyclus anynana*. They bred three genetic lines of butterflies, each with a characteristic eyespot on the wings. Linear morphometrics were used to measure several variables, including eyespot size and wing length. Their results show the impact genetics have on the morphology of localized and general shape of the wing, with the selection affecting not only the form of the eyespot, but also the overall wing size.

As stated earlier, morphometrics can be used as a stand-alone method in scientific studies. Breuker *et al.* (2010) made a very descriptive paper on *Pararge aegeria,* where they sought to show the many uses geometric morphometry can have in ecological studies. Butterflies were captured from different landscapes in order to test if wing morphology was altered from one location to another. The results did not show significant differences between the butterflies from different landscapes, however the authors state that sample sizes were small and thus the experiment should be repeated for more accurate results. Even so, the paper is valuable for the methods employed. Linear morphometrics were used by Luebke *et al.* (1988) to measure the extent of hybridization between the two subspecies *Papilio glaucus glaucus* and *Papilio glaucus canadensis*, discovering a hybridization zone in South-Central Wisconsin. In a study on the phylogeography of the *Maniola jurtina* butterfly Dapporto *et al.* (2009) used geometric morphometric measurements of male genitalia. Besides classical landmarks, they also employed sliding semi-landmarks (Bookstein 1997), with results that show that the Mediterranean islands might function as refuge during glacial periods. A very similar study was performed by Dapporto and Bruschini (2011) in order to examine the distribution of two morphotypes of the same species. Dudley (1990) performed an interesting experiment, combining morphometrics and kinematics on 37 butterfly species from Panama with the purpose finding correlation between morphology and flight behavior. Butterflies were filmed in flight and measurements were made for wing length, wingspan and body length among others, with results that show correlation between flight speed and morphology.

One very interesting and controversial method that sometimes uses morphometrics is the study of random variations of the bilateral symmetry, or Fluctuating Asymmetry (van Valen 1962). Organisms that manifest this form of asymmetry are considered to have reduced metabolic efficiency and thus a reduced chance of procreation (Møller and Swaddle 1997), although this idea has been challenged (Martin and Hosken 2002). Martin and López (2001) performed such a study on the lizard *Psammodromus algirus* by measuring and comparing femur and crus length of the hindlimbs. They discovered that escape performance was negatively affected by the presence of Fluctuating Asymmetry, thus reducing the survival chances of asymmetric individuals. Polak (1993) performed an experiment on male *Drosophila nigrospiracula* to determine if parasites increase fluctuating asymmetry of the host. He purposely infected flies in the larval stage and also adults, to see if development stage was a factor in the appearance of asymmetry, while keeping a control group for comparison. His results showed increased asymmetry for individuals infected in the larval stage and also a reduction in the body size of the adults in that group. The group that was infected in the adult stage did not show a high degree of asymmetry while the control group developed normally.

Many other authors have approached similar methods to the ones presented above (Winding *et al.* 2001, Bartoš and Bahbouh 2006, Gibbs *et al.* 2011, Jorge *et al.* 2011, Cespedes *et al.* 2014), all highlighting the importance of morphometrics in Ecology and Biology.

A study on *Zygaena carniolica*

The species belongs to the burnet moth (Zygaenidae) family in the Lepidoptera order. The genus *Zygaena* holds approximately 100 species with an exclusively palearctic distribution (Guenin R. 1997). Their wings have specific patterns, aposematic coloration and a wingspan between 30-35 mm. It is widely spread in Europe and typical habitats are dry or steppe-like, extensively used calcareous grasslands (de Freina and Witt 2001, Naumann *et al.*1999). The hotspots of diversity for the genus are located in eastern Turkey and North Africa (Naumann *et al.*1984). Habitat fragmentation and abandonment of traditional agricultural practices threaten this species, therefore it is marked as endangered in Germany (Binzenhöfer *et al.* 2005). For our study we chose to analyze wing shape as a proxy for flight performance, which in turn affects individual fitness (Speight *et al.* 2008).

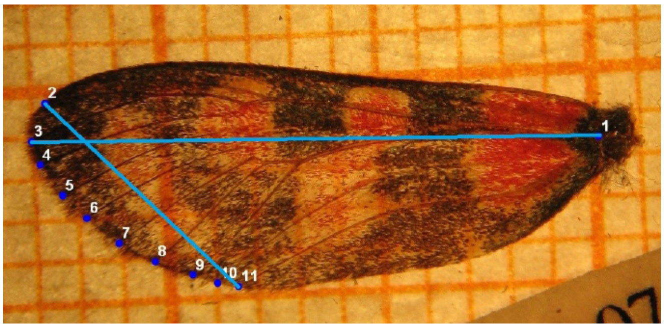

Fig. 1. Landmarks used in the analysis of the shape and size in four different *Zygaena carniolica* populations from Romania, the blue lines are the linear measurements for width and length.

Materials and methods

The wings of 199 *Z. carniolica* specimens (leg. *et* col. L. Rákosy, O. Făgărăşan) were stored at The Zoological Museum of the Babeș-Bolyai University, after the moths had been captured in 2010 from the following populations: Mărgăritești (Buzău County), Răscruci (Cluj County), Rimetea (Alba County) and Suatu (Cluj County). Although all wings were analyzed, not all were usable, as some of them were deteriorated or had been damaged during conservation. 127 individuals with a total of 254 usable wings were identified as follows: Mărgăritești 17, Răscruci 23, Rimetea 25, Suatu 62.

These were photographed through a stereo microscope while resting over milimetric paper. The images were then converted to the TPS format using the software TPSUtil, thus allowing them to be processed in a system of coordinates. Landmarks were placed with the program TPSDig2 (http://life.bio.sunysb.edu/morph/soft-utility.html), the placement was at the base of the wing and on the intersection of wing veins with the wing edge (fig1).The pigmentation of the wings made it difficult to mark the intersections at the anterior part of the wings, so in order to keep as many individuals as possible in the experiment, those points were not chosen as landmarks. Procrustes Superimposition, Principal Component Analysis, Canonical Variance Analysis and Permutation Test were calculated using the program MorphoJ (http://www.flywings.org.uk/morphoj_page.htm).

The linear measurements for the calculation of Fluctuating Asymmetry were made from the base of the wing to landmark 3 (Length) and from landmark 2 to 11 (Width). The milimetric paper was used to give the program (TPSDig2) a constant unit of measurement (fig1). The results were then placed in the equation |L-R| (left minus right) for each individual, based on the models offered by Palmer and Strobeck (1986). Differences among groups were analyzed with the ANOVA function of the program SALSTAT2 (http://sourceforge.net/projects/s2statistical/).

Results

ANOVA revealed no difference in Fluctuating Asymmetry among the four populations, $p=0.3025$. An interesting discovery was a meristic difference in one individual from Răscruci (fig2), the left wing having one extra vein.

Procrustes Superimposition for all individuals showed general variation around landmarks 1, 2, 3, 8, 9, 10 and 11 (fig 3)

Principal Component Analysis revealed the direction of variation for all individuals (fig 4), these are linked to the length of the wing, landmarks 1 and 2, and the curvature of the wing, landmarks 3 to 11.

Canonical Variance Analysis for all four groups showed greater difference at landmarks 9, 10 and 11 (fig 5), again pointing towards a difference in wing curvature.

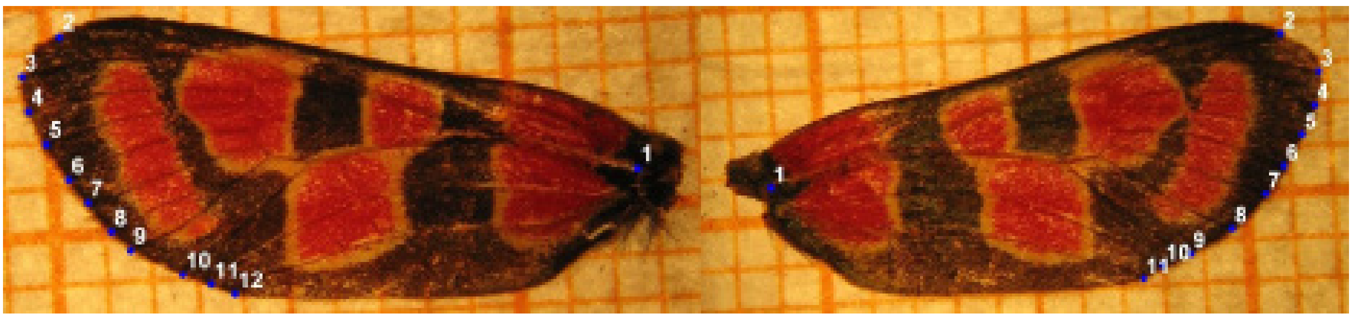

Fig. 2. A *Zygaena carniolica* individual from Răscruci (Cluj County, Romania) with one extra vein on the left wing.

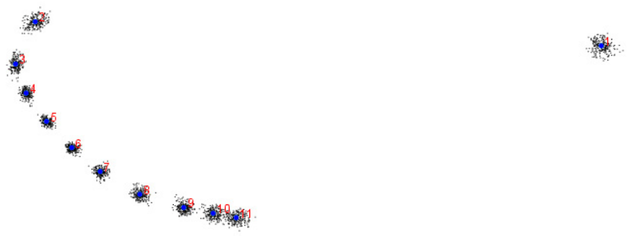

Fig. 3. Procrustes Superimposition for 127 *Zygaena carniolica* individuals from four Romanian populations (Mărgăritești, Răscruci, Rimetea, Suatu).

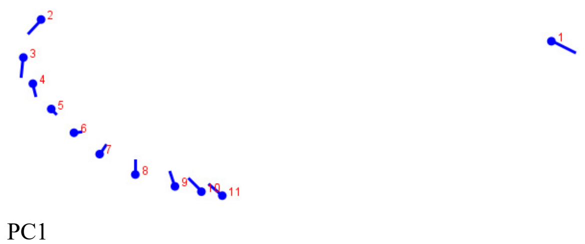

Fig. 4. Principal Component Analysis for 127 *Zygaena carniolica* individuals from four Romanian populations (Mărgăritești, Răscruci, Rimetea, Suatu), points correspond to landmarks and lines indicate the direction of variation.

Permutation Test for Procrustes Distance among the four groups revealed a significant difference between the groups from Suatu and Mărgăritești (table 1).

Canonical Variance Analysis on the populations from Suatu and Mărgăritești showed the greatest differences among the same landmarks, 9, 10 and 11 (fig 6).

Discussions

The value of morphometrics in research is easily brought to light by the many studies that have used it with fruitful results. Even so, it has to be underlined that the best results are shown when different methods of analysis are combined. With so many difficulties facing taxonomy and conservation we must not turn our backs to any of the resources at our disposal.

Our own study on *Zygaena carniolica* brings forth interesting results but also questions. As a start it can be noted that the methods we used are easy and quick to apply. Modern technology alleviates the hardship of sophisticated calculations and reduces the chances of error. The fact that there was no difference in Fluctuating Asymmetry suggests that the four populations are not under different levels of stress. We will state that the use of Fluctuating Asymmetry as an indicator for stress has been both challenged (Winding *et al.* 2001) and supported (Băncilă *et al.* 2010) and studies that focus on direct correlations with various stressors should be encouraged. Because our material was collected many years before our analysis, we were unable to compare various factors that could have induced asymmetry, this might not be necessary, however, because in this case no statistical difference between the four groups was found. Allenbach (2011) also points out the importance of using the correct methods in the analysis of Fluctuating Asymmetry, his focus being on meristic and morphometric measurements. In our case just one butterfly out of 127 displayed meristic differences, thus we conclude that the methods we used are correct.

Geometric morphometrics offer results that can be interpreted based on the analysis used. Procrustes superimposition can only give us a vague description of variability, but it is an excellent starting point.

Fig. 5. Canonical Variance Analysis for 127 *Zygaena carniolica* individuals from four Romanian populations (Mărgăritești, Răscruci, Rimetea, Suatu), points correspond to landmarks and lines indicate the greatest variation amongst groups.

Fig. 6. Canonical Variance Analysis for the *Zygaena carniolica* populations from Mărgăritești and Suatu, points correspond to landmarks and lines indicate the greatest variation amongst the two groups.

Table 1. *p*-values for the Permutation test (10000 permutations) among the four populations of *Zygaena carniolica*, variation is based on Procrustes Distance (Zelditch *et al.* 2004)

	Mărgăritești	Răscruci	Rimetea
Răscruci	0.405		
Rimetea	0.0557	0.0682	
Suatu	0.0231	0.483	0.0927

Through Principal Component Analysis we can observe the general pattern of variation within the studied group, in our case wing length and curvature. This information can be valuable for studies on adaptation and diversity. Canonical Variance Analysis reveals the exact points of these variations among populations.

The difference we discovered between the populations from Suatu and Mărgăritești is most likely a case of phenotypic plasticity (Pfennig *et al.* 2010), but we cannot confirm this without a thorough study of the habitats and the genetics of the two populations. One aspect that points towards this conclusion is the fact that there was no significant difference in Fluctuating Asymmetry. Thus, if we abide with the notion that this form of asymmetry is associated with stress, we can hypothesize that the populations are having a "healthy" response to differences in habitat.

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