



Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid



Outline-based morphometrics, an overlooked method in arthropod studies?

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ARTICLE INFO

Article history:

Received 9 April 2014

Received in revised form 25 July 2014

Accepted 31 July 2014

Available online xxx

Keywords:

Modern morphometrics

Landmarks

Outlines

Arthropods

Cryptic species

Population structure

ABSTRACT

Modern methods allow a geometric representation of forms, separating size and shape. In entomology, as well as in many other fields involving arthropod studies, shape variation has proved useful for species identification and population characterization. In medical entomology, it has been applied to very specific questions such as population structure, reinfestation of insecticide-treated areas and cryptic species recognition. For shape comparisons, great importance is given to the quality of landmarks in terms of comparability. Two conceptually and statistically separate approaches are: (i) landmark-based morphometrics, based on the relative position of a few anatomical “true” or “traditional” landmarks, and (ii) outline-based morphometrics, which captures the contour of forms through a sequence of close “pseudo-landmarks”.

Most of the studies on insects of medical, veterinary or economic importance make use of the landmark approach. The present survey makes a case for the outline method, here based on elliptic Fourier analysis. The collection of pseudo-landmarks may require the manual digitization of many points and, for this reason, might appear less attractive. It, however, has the ability to compare homologous organs or structures having no landmarks at all. This strength offers the possibility to study a wider range of anatomical structures and thus, a larger range of arthropods.

We present a few examples highlighting its interest for separating close or cryptic species, or characterizing conspecific geographic populations, in a series of different vector organisms. In this simple application, i.e. the recognition of close or cryptic forms, the outline approach provided similar scores as those obtained by the landmark-based approach.

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1. Introduction

Contrary to classical morphology, the main objective of morphometrics is not to describe organisms, but to compare them. Because of the geometric constraints of modern morphometrics, data are generally collected on a non-articulated part, often a single organ (but see David et al. (1996) and Adams (1999)). A few characters are enough, provided that they are homologous.

Insect species studies generally made use of the wings because these structures are almost bidimensional and relatively rigid, reducing digitizing error; also, importantly, because the wings of many groups of insects provide a large number of landmarks. Not only are they many, they also are of good quality, called “true”

(or “anatomical”, or “traditional”) landmarks. True landmarks are considered homologous. Homology here refers to the positional equivalence of a small biological structure, as small as a point at the requisite scale. The level of homology of a landmark is governed by the precision with which it can be localized from one organism to another. Thus, true, anatomical landmarks are homologous in the sense that they are relocatable points, and according to this criterion various levels of homology have been recognized (see type I, II and III landmarks, Bookstein (1991)). A special development of type III landmarks, called “semi-landmarks” (also “sliding semi-landmarks”) allows the description of curved lines between two classical landmarks (Bookstein, 1997).

Anatomical, true landmarks are opposed to “pseudo-landmarks” used in the outline-based approach. Pseudo-landmarks describing contours or boundary outlines do not depend on the presence of true anatomical landmarks, they can exist with no

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anatomical landmark at all, or can include one or more of them. Obviously, pseudo-landmarks are of another nature than true landmarks: comparability is not expected from them separately, but from the structure they describe. Thus, if carefully chosen, like the mandible of a beetle, the genital paramere of a sandfly or an internal cell of the wing, a contour represents an homologous structure allowing interindividual and/or intergroup comparisons.

It is quite possible to develop both approaches on the same organ (Baylac and Frieß, 2005). One can see the wings of an insect as a configuration of anatomical landmarks, but the wing can be seen also as an assemblage of closed contours, i.e. the internal cells designated by the intersecting veins (Francoy et al., 2012). Moreover, other anatomical parts, because they are generally deficient in true landmarks, are only amenable to an outline analysis. For instance, many insect species, including very close species, are separated by the shape of some genital pieces. Such parts often lack anatomical landmarks, like the paramere of sandflies, the pronotum of Coleoptera (Faille et al., 2007), the genital leaflet of *Culex neavei* (Boussès et al., 2013; Garros and Dujardin, 2013) or the mandible parts of beetles (Tatsuta et al., 2009). Eggs and juvenile instars which also provide important taxonomic characters generally lack acceptable configurations of true landmarks: they can be considered through outline analyzes. Also good or natural candidates for the outline approach are the many wingless forms of insects, as for instance fleas, lice, ants, many of them of great veterinary, medical or agronomic importance. Finally, arthropods other than insects may present poorly defined landmarks but a particular anatomy which can be submitted to outline analyzes, as for instance the rounded body of ticks. In sum, the outline approach offers the possibility to study a wider range of organs, along with a wider range of arthropods.

The question is not limited however to technical considerations about presence or absence of landmarks (Rohlf, 1986), it also concerns the biological relevance of outlines. For entomologists, the primary needs are to distinguish species, especially cryptic species, and to detect them where they are not expected. Conspecific, but geographically or ecologically separated populations, are of interest too, in order to design relevant control strategies (Kaba et al., in press) or to detect the origin of reinfestation after treatment (Felicangeli et al., 2007; Hernández et al., 2013; Gaspe et al., 2013). Does the outline approach distinguish species and populations as well as the landmark approach? We present here a few examples showing no significant difference between the two methods when it is about discriminating between close species or between conspecific populations.

2. Materials and methods

2.1. Material

2.1.1. *Rhodnius prolixus* and *Rhodnius robustus*

R. prolixus Stal, 1859 and *R. robustus* Larrousse, 1927 are morphologically very close species with a long history of controversy among taxonomists (Bargues et al., 2010). *R. prolixus* is a major vector of Chagas disease in the northern part of Latin America, recently eradicated from Central America (Hashimoto and Schofield, 2012) but still active in Venezuela and Colombia (Gorla et al., 2010). The two species generally are found in different ecological conditions: in domestic and peridomestic structures for *R. prolixus*, in the crown of palm trees for *R. robustus*. Because of its silvatic habit preferences, *R. robustus* is not considered as an important vector of Chagas disease, but it is involved in some local transmission (Felicangeli et al., 2002). The overall morphology of the two species is very similar, although striking size differences are often observed, with *R. robustus* being generally the larger species. In

the field, there is frequently some size overlap, so specimens collected from trees are assumed to be *R. robustus*, and those collected from houses are assumed to be *R. prolixus*. Our material contains three groups previously identified by molecular tools (Monteiro et al., 2003): one is *R. prolixus* from houses in the Pampanito village (State of Trujillo, Venezuela), the second one is *R. prolixus* from palm trees collected in another locality, San José Tiznados (State of Guárico, Venezuela), and the last one is *R. robustus* from two localities of Venezuela: Pampanito and Candelaria (State of Trujillo).

We performed analyzes both combining the two *R. prolixus* habitats, as well as the two *R. robustus* origins, into single samples and considering them as separated groups. A total of 7 landmarks could be used, thus excluding the eighth used in Felicangeli et al. (2007) because of the inconsistency of the clavum position on the pictures. The outline submitted to the elliptical Fourier analysis (EFA, see Section 2.2.2) contained 5 of them, as well as the external boundary of the membranous part of the wing (Fig. 1).

2.1.2. *Glossina palpalis palpalis* and *Glossina palpalis gambiensis*

G. p. palpalis (Robineau-Desvoidy), 1830 and *G. p. gambiensis* Vanderplank 1949 (Diptera: Glossinidae) are important vectors of sleeping sickness in West Africa. Both species hybridize readily in the laboratory but hybridized females produce fewer offspring and hybrid males are sterile (Gooding, 1988). These two subspecies are difficult to separate on morphological ground. Although males show consistent differences in the terminal dilatation of inferior claspers of their genitalia (Machado, 1954), morphological differentiation of female flies is not conclusive. The material we used here is from Ivory Coast and was collected in 2007. Male and female specimens of *G. p. palpalis* were collected in Aniassee, South of Ivory Coast. The *G. p. gambiensis* specimens came from Ganse, North of Ivory Coast. The subspecies identity of all the female and male specimens of the present study had no morphological nor molecular confirmation.

Ten landmarks were used as in Kaba et al. (in press). The EFA method was applied to the comparison of the central cell of the wing which includes five landmarks (Fig. 2). This cell is considered as having an important taxonomic significance for tsetse flies (De la Rocque et al., 2002).

2.1.3. *Anopheles strodei* and *Anopheles oswaldoi*

A. oswaldoi (Peryassú) has an unclear taxonomic and vectorial status in South America (Ruiz-Lopez et al., 2013), and *A. strodei* Root shows a high levels of morphological polymorphism (Bourke et al., 2013). Both species are difficult to distinguish based on existing dichotomous keys (Faran, 1980; Faran and Linthicum, 1981; Gonzales and Carrejo, 2007). Females were collected from the municipality of Puerto Asis, department of Putumayo, Colombia. They were induced to oviposit in the laboratory, where the male genitalia and associated immature stages of offspring were used for taxonomic identification. *A. oswaldoi* specimens were confirmed by PCR-RFLP of the rDNA marker ITS-2 (Ruiz et al., 2005). From *A. oswaldoi*, 30 adult F1 females and from *A. strodei*, 32 adult F1 females were randomly chosen for the morphometric studies.

Eleven anatomical landmarks were selected on the crossing of wing veins, while the EFA method used the contour of a small cell at the internal base of the wing, a contour which did not include any of the eleven landmarks (Fig. 3).

2.1.4. *Ornithodoros maritimus*

O. maritimus is a soft tick (Family Argasidae) and part of the widespread species complex, *Ornithodoros capensis* sensu lato, exploiting colonial seabirds in tropical and sub-tropical areas of the world (Dietrich et al., 2011). The different members of this complex are known vectors of several viruses and bacteria,

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J.-P. Dujardin et al. / Infection, Genetics and Evolution xxx (2014) xxx–xxx

3

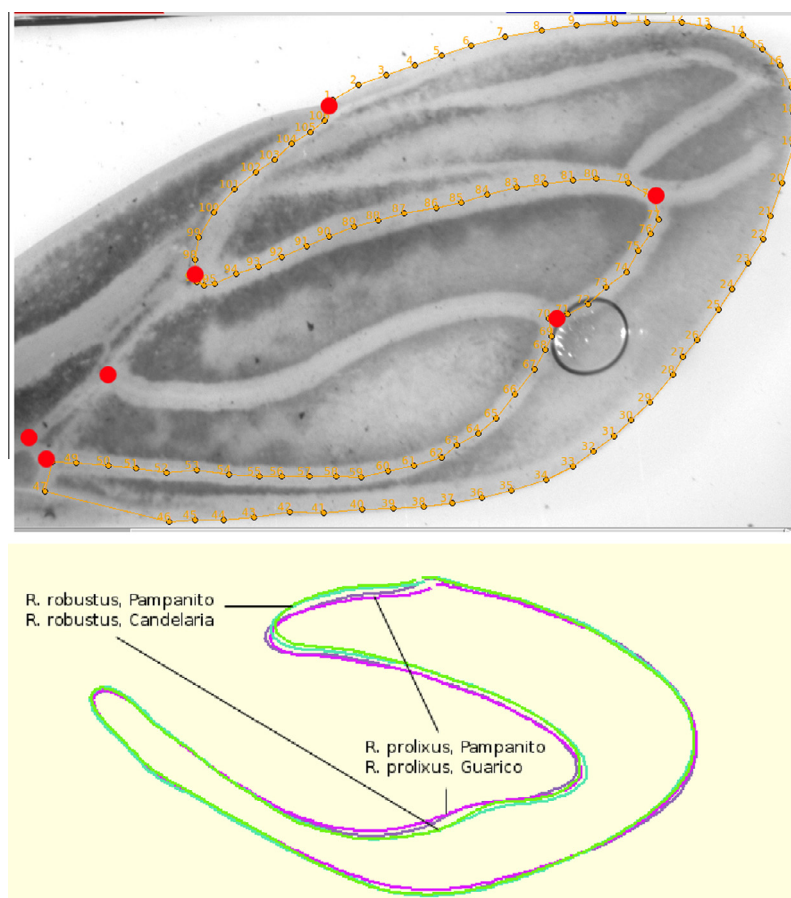


Fig. 1. Top: Outline as digitized for a *Rhodnius* sp. wing, and seven landmarks (red dots). Bottom: Shape differences between outlines of *R. prolixus* and *R. robustus*. It can be seen that the selected outline remains stable within each species. The two samples of *R. prolixus* are almost identical in spite of having different sizes (see Fig. 5), different geographic origins (Pampanito, Guarico) and habitats (domestic, silvatic). A similar observation can be made for the two samples of *R. robustus*.

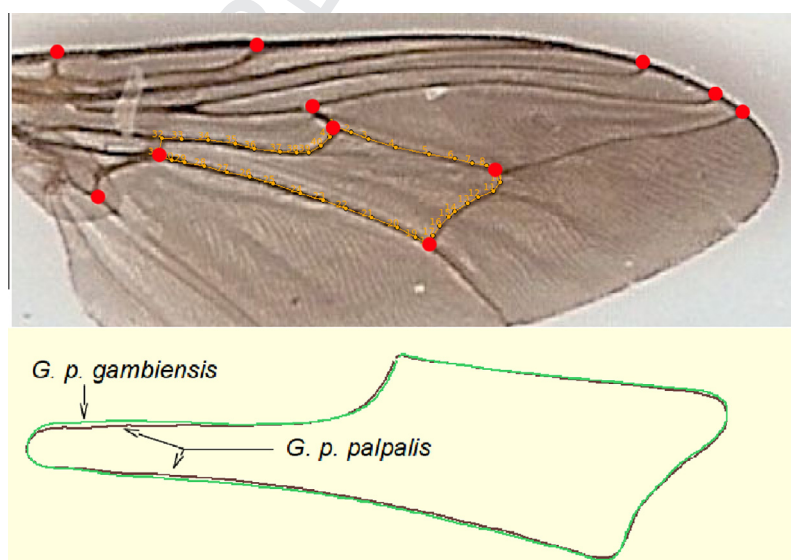


Fig. 2. Top: Outline as digitized for a *Glossina* sp. wing, and 10 landmarks (red dots). Bottom: Shape differences between outlines of female *Glossina palpalis palpalis* and *G. p. gambiensis*. The contours are very similar, with the elongated part of the cell thinner for *G. p. palpalis*.

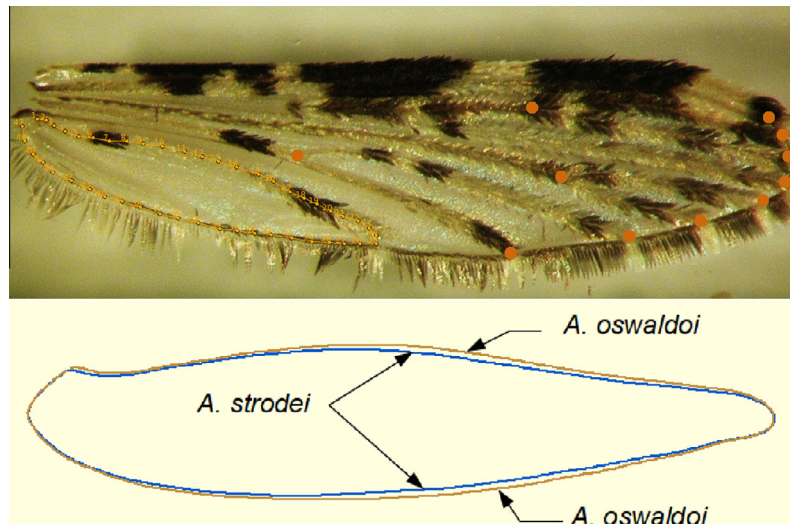


Fig. 3. Top: Outline as digitized for an *Anopheles* sp. wing, and 11 landmarks (red dots). Bottom: Shape differences between outlines of *A. strodei* and *A. oswaldoi*.

including the agents responsible for tick-borne relapsing fever and African tick bite fever (Takano et al., 2009; Dietrich et al., 2011, in press). Although the *O. capensis* complex currently includes eight species, the actual number of species and their geographical distribution have been recently called into question (Gómez-Díaz et al., 2012). Given the lack of reliable morphological characters for identifying soft tick species in the adult life stage (Estrada-Pena et al., 2010), alternative methods are greatly needed. In the present study, two distant French populations were analyzed, both within the described distribution of *O. maritimus*: one from Rouzic, one of the islands of the Sept-Iles archipelago off the northern coast of Brittany and one from Riou, a Mediterranean island lying near the city of Marseilles. In Rouzic, 18 adult ticks were collected from around the nests of the Northern gannet (*Morus bassanus*), whereas in Riou, the 20 adult ticks analyzed came from nests of the Yellow-legged gull (*Larus michahellis*).

Fifteen landmarks were used on the ventral face of the ticks. The EFA method used the contour of the external boundary of the dorsal face (Fig. 4).

2.2. Statistical approaches

2.2.1. Landmark-based analyzes

Shape variables were obtained through the generalized Procrustes analysis (GPA) superimposition algorithm and subsequent projection of the Procrustes residuals into an euclidean space (Rohlf, 1990b). Both non-uniform and uniform components were used as shape variables. The shape variables were produced separately for males and females. These variables – also called “partial warps” (Slice et al., 1996) – actually describe the differences in shape as deviations from an average configuration of landmarks, and their principal components or a subset of them (see Section 2.2.3) were used as input for the discriminant analysis.

2.2.2. Outline-based analyzes

For each organism, except for the ticks, a previous study was necessary to select the outline to be used in the comparisons. Various outlines are possible on the same wing, and their discriminating power can be different (Francoy et al., 2012). We tried to select outlines containing the fewest possible landmarks, since the justification for using the outline-based approach is just that: no landmarks, or not enough of them. In each comparison, we used the elliptic Fourier analysis (EFA) (Kuhl and Giardina, 1982). Briefly,

the observed contour is decomposed in terms of sine and cosine curves of successive frequencies called harmonics, and each harmonic is described by four coefficients. With this method (Kuhl and Giardina, 1982), the first harmonic ellipse parameters are used to standardize the Fourier coefficients so that they are invariant to size, rotation and the starting position of the outline trace. By doing this, the three first coefficients become constant (1, 0 and 0) and are not used in the remaining analyses. The fourth coefficient, the one related to the width-on-length ratio of the outline (Lestrel, 1989), has been used in our study.

As for the conventional Fourier analysis, the maximum number of harmonics is half the number of sample points (pseudo-landmarks). The optimal number of harmonics can be selected in various ways: by appraising the goodness-of fit using the sum of squared distances between the original data and reconstructed outline, by examining the spectrum of harmonic Fourier power, or simply by visualizing the reconstructed outlines (Lestrel, 1989, 1997; Claude, 2008). Ideally, more harmonics should be able to capture more shape parameters and produce a higher level of discrimination. However, more harmonics also inflate the digitization error and an optimal number should be selected (Crampton, 1995; Renaud et al., 1999; Firmat et al., 2010). In our study, the threshold regarding the number of harmonics was governed by the problem of high-dimensionality in the resulting data set (see next section).

The superiority of the elliptic Fourier algorithm over other methods (Rohlf, 1990a) is now commonly accepted: the algorithm does not require the points to be equidistant (as in eigenshape analysis (Lohmann, 1983)), and the use of the normalized coefficients “saves the investigator from having to worry about aligning the images in a standard fashion” (Rohlf and Archie, 1984).

2.2.3. Assigning individuals to their corresponding groups

Each individual was classified after a discriminant analysis of shape variables according to a validated classification procedure, also called the jackknife classification (Manly, 2004), where each individual is allocated to its closest group without being used to help determine a group center.

Because of statistical constraints (Sheets et al., 2006), the discriminant analysis did not use the original shape variables as input. The number of shape variables produced by a two-dimensional landmark approach is two times the number of landmarks less four (Rohlf, 1996), and can be larger than the number of individuals. To circumvent the problem of high-dimensionality in the data set, a

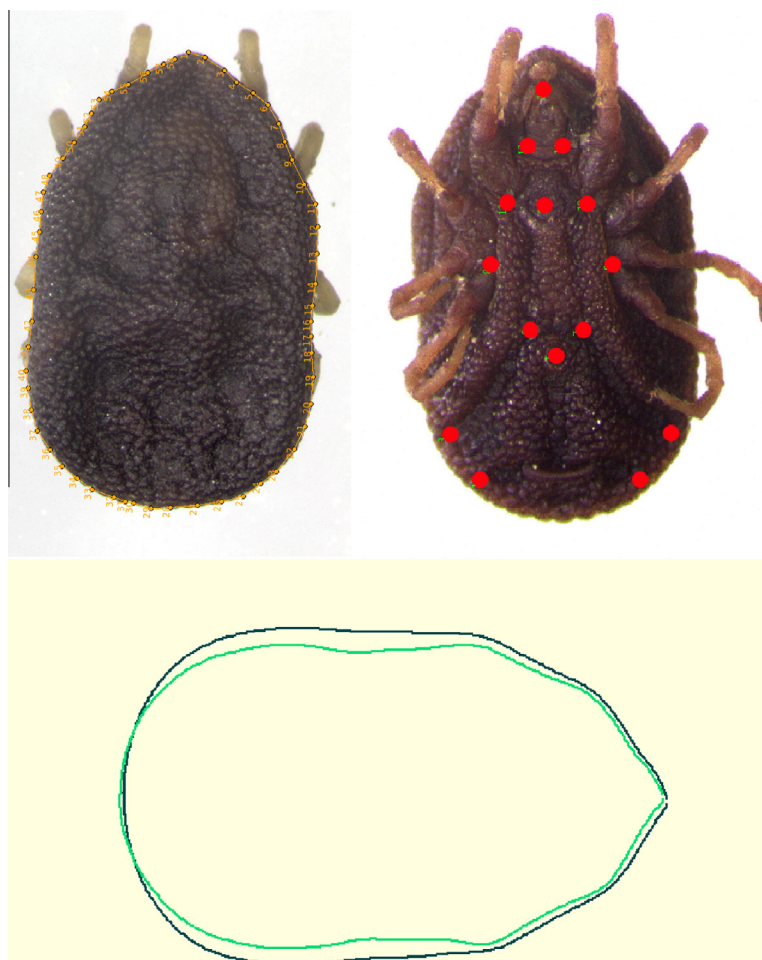


Fig. 4. Top, left: Outline as digitized for *Ornithodoros maritimus*. Top, right: 15 landmarks on the ventral face (red dots). Bottom: Riou and Rouzic localities have different outlines for *Ornithodoros maritimus*.

few first principal components (PC) of shape variables were used as input for the discriminant analysis and subsequent validated classification. The size of this subset of PC was simply the number of specimens of the smallest group less one. There are other, more sophisticated ways to select for an adequate morphological subspace, but most rules of selection are heuristic or ad hoc methods (Jolliffe, 1986; Krzanowski, 1987). One of the least subjective is to select the number of retained components in each analysis that minimize the total cross-validated misclassification percentages (Baylac et al., 2003; Baylac and Frieß, 2005; Sheets et al., 2006). We used here a much simpler approach by placing the two morphometric techniques in similar statistical conditions, even if these conditions were not optimal for obtaining the best scores.

The use of the outline method also poses difficulties for the discriminant analysis. To accurately represent a curve many harmonics may be needed, each with four coefficients, so that the analyzes would require the measurement of numerous samples (Iwata, 2011). In each of our examples, the PC of the (normalized) Fourier coefficients were not derived from the total number of coefficients because it was frequently larger than the total sample. Thus, only a part of the Fourier coefficients was submitted to the PCA, and only a part of the first PC was used as input for the DA, where the same rule as above ("the smallest group less one") was adopted.

2.2.4. Size estimation and its contribution to shape discrimination

Whichever way shape is computed, the estimate of size in modern morphometrics is restricted to the geometric area examined

and is contained in a single variable: the root-squared area of a contour (instead of its perimeter (Lestrel, 1997)), or the "centroid size" for a configuration of landmarks (Bookstein, 1991). The centroid size is defined as the square root of the sum of the squared distances between the center of the configuration of landmarks and each separate landmark (Bookstein, 1991). The root-squared area of a contour was actually the one of the starting ellipse in the Fourier decomposition (Rohlf, 1990a).

The estimate of size was then used to evaluate its contribution to species discrimination based on shape: in each specific study, the coefficient of determination was computed after regressing the first (and generally unique) discriminant function on size.

2.2.5. Measurement error

The precision of both techniques was estimated using the repeatability index. For each sample, we used an ANOVA design on repeated measurement performed on the totality the sample. The repeatability was measured using different variance components: the among-measurement (V_a) and the within-measurement (V_w) components. It was expressed in percentage, and computed as the ratio of $V_a/(V_a + V_w)$ (Arnqvist and Mårtensson, 1998), which is the reverse of the measurement error as suggested by Yezerinac et al. (1992). The input variables for the ANOVA were the four first principal components of shape. We only report the average and standard deviation of the repeatability across species and comparisons for each morphometric approach.

2.3. Software

All analyzes and graphical output were obtained through the various modules of the CLIC package (Dujardin and Slice, 2006; Dujardin et al., 2010): the COO module to digitize either landmarks or curves, the MOG and PAD modules for the landmark-based approach, the FOG module for EFA analyzes and subsequent validated classifications, and the VAR module for estimating the repeatability of size and shape.

3. Results

The quality of group discrimination was generally satisfactory in both methods (Table 1, see the “%” columns) (86% of correct assignation on average versus 78% for landmarks). On average, the repeatability was slightly higher for outlines ($85 \pm 11\%$) than for landmarks ($79 \pm 17\%$). The contribution of size to the discrimination appears in Table 1 as a percentage in the “r2%” column. On average, it was lower in the landmark-based approach ($23 \pm 18\%$ versus $33 \pm 23\%$).

3.1. Group distinction

3.1.1. *R. prolixus* and *R. robustus*

The landmark approach produced relatively satisfactory results: 15 (out of 17) *R. robustus* were correctly assigned, while 2 (out of 24) *R. prolixus* were wrongly attributed to *R. robustus*. More impressive results were obtained from outlines, where no *R. prolixus* was assigned to the *R. robustus* species, and only one *R. robustus* (out of 18) was confounded with *R. prolixus*. The *R. robustus* outline was not affected by the different geographic areas (Fig. 1) and the different sizes (Fig. 5). Similarly, the two samples of *R. prolixus*

showed a very close outline in spite of different habitats (Fig. 1) and very different sizes (Fig. 5).

3.1.2. *G. p. palpalis* and *G. p. gambiensis*

There was a notable difference in the morphometric discrimination of subspecies according to the sex. Both morphometric techniques produced comparable and unsatisfactory results for male specimens (67–70% versus 66–77%, for landmarks and outlines, respectively). For females, only the outline comparisons produced satisfactory results (80–85%, versus 70–73% for landmark comparisons). Thus, outlines could only discriminate the two forms for females.

3.1.3. *A. strodei* and *A. oswaldoi*

In this simple pairwise comparison of female mosquitoes, both landmarks and outlines performed satisfactorily (more than 83% were correctly classified). The scores obtained using landmarks (84–86%) were slightly inferior to the ones obtained using the contour of a single internal cell of the wing (87–94%).

3.1.4. *O. maritimus*

The geographic origin of the 39 specimens of *O. maritimus* was almost perfectly recognized by the body contour (94–100%), while imperfectly – but still consistently – by the landmark approach (80–83%).

4. Discussion

For analyzing curves containing anatomical landmarks, as is the case for most of the material used in this study, and in spite of some unresolved drawbacks (Sheets et al., 2004), the sliding semi-landmark approach becomes more frequently adopted in

Table 1
Validated classification based on shape. “n”, sample sizes; “%”, percentages of correct assignation of individuals to relevant species or group. “LM”, the number of true landmarks used; “H”, the number of harmonics corresponding to the number of normalized Fourier coefficients used as input for the principal component analysis; “r2%”, percent contribution of size to the first discriminant factor based on shape. The analyzes comparing *R. robustus* and *R. prolixus* were repeated with two different sampling distribution: either 14, 10 (both *R. prolixus*) and 18 *R. robustus* (mixing their geographic origins, i.e. Candelaria and Pampanito), or 14, 10, 8 *R. robustus* from Candelaria and 10 from Pampanito. 23/22, 30/22, 17/18 or 7/8 mean a different sample according to the approach: the first number refers to the sample size analyzed with landmarks, the second number to the one analyzed with outlines; F, females; M, males.

Species and groups	n	Validated classifications					
		Landmarks			Outlines		
		LM	%	r2%	H	%	r2%
Tsetse flies, females		10		1	11		0
<i>G. p. palpalis</i>	26		73			80	
<i>G. p. gambiensis</i>	20		70			85	
Tsetse flies, males		10		2	9		2
<i>G. p. palpalis</i>	15		67			66	
<i>G. p. gambiensis</i>	23/22		70			77	
Mosquitoes		11		27	13		59
<i>A. strodei</i>	32		87			87	
<i>A. oswaldoi</i>	30/22		93			94	
Kissing bugs, 2 groups		7		45	10		41
<i>R. prolixus</i> (2 habitats)	24		83			100	
<i>R. robustus</i>	17/18		94			94	
Kissing bugs, 3 groups		7		39	10		40
<i>R. prolixus</i> (domestic)	14		86			92	
<i>R. prolixus</i> (silvatic)	10		70			80	
<i>R. robustus</i>	17/18		88			94	
Kissing bugs, 4 groups		7		11	10		52
<i>R. prolixus</i> (domestic)	14		86			92	
<i>R. prolixus</i> (silvatic)	10		70			80	
<i>R. robustus</i> (Candelaria)	7/8		100			87	
<i>R. robustus</i> (Pampanito)	10		77			100	
Soft ticks		15		34	9		40
<i>O. maritimus</i> Rouzic	18		80			94	
<i>O. maritimus</i> Riou	20		83			100	

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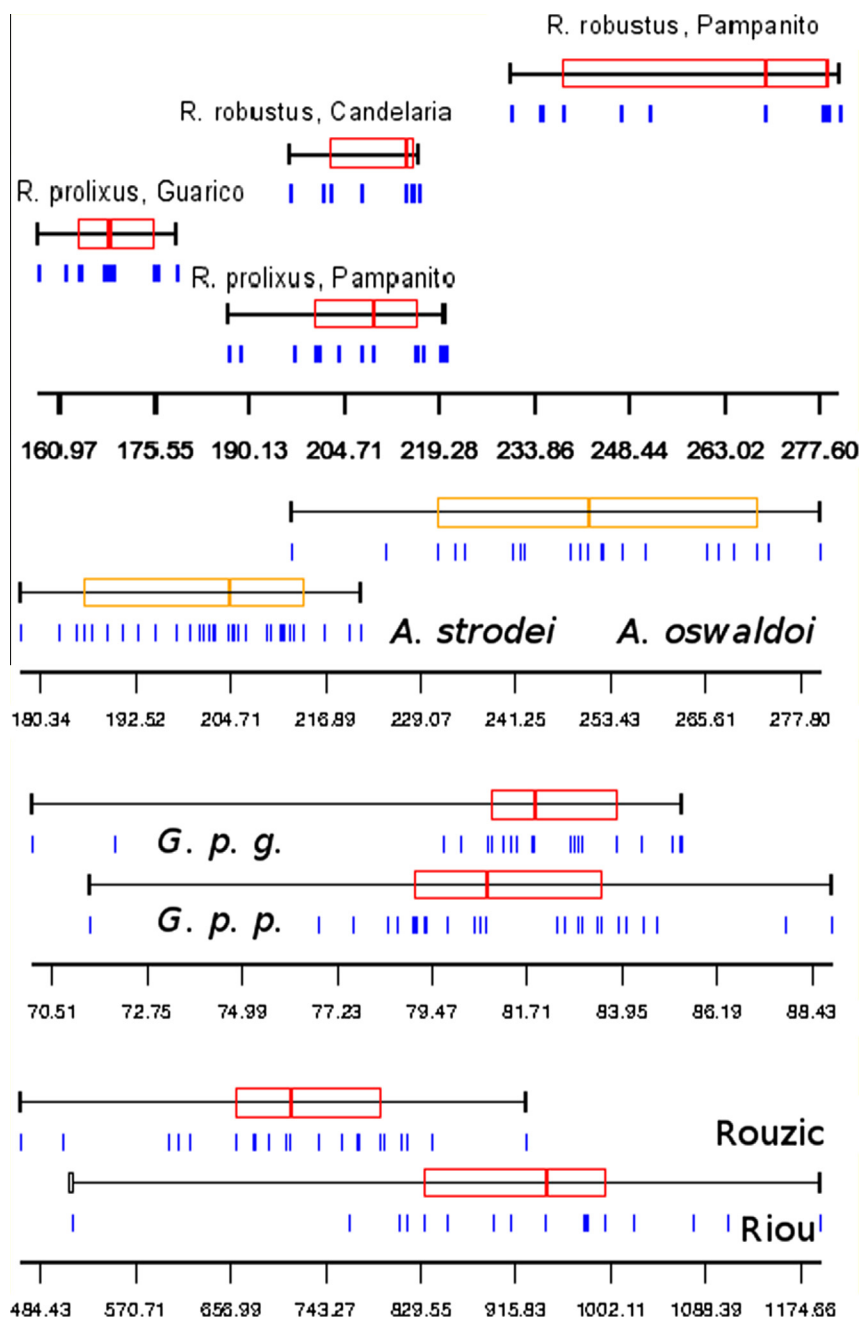


Fig. 5. Size variation as estimated by the square root of outline areas. From top to bottom: kissing bugs (*Rhodnius robustus* and *R. prolixus*), mosquitoes (*Anopheles strodei* and *A. oswaldoi*), tsetse flies (*G. p. p.* = *Glossina palpalis palpalis*, and *G. p. g.* = *Glossina p. gambiensi*s) and ticks (*Ornithodoros maritimus* from two localities, Riou and Rouzic).

arthropods (Yee et al., 2011; Palestini et al., 2012; Stephens and Juliano, 2012). The main benefit is that both landmarks and semi-landmarks can be arranged in one data set submitted to a single analysis. The discriminating power has been shown to be similar to the elliptic Fourier analysis (EFA) and some other outline methods (Sheets et al., 2006). The semi-landmark approach considers both curves and landmarks. As long as it requires true landmarks in order to describe the curves, it is a landmark-dependent method. The outline-based approach is completely different, as outlines may or may not include landmarks; it is a landmark-free method. Thus, for corresponding structures having very few landmarks or simply lacking landmarks, it is advisable to turn to the classical outline analysis based on EFA. We showed here that this option does not mean lower discriminating power. The EFA applies to closed contours only. Other statistical techniques can be applied

to curves, closed or not (Lohmann, 1983), as reviewed by Rohlf (1990a) and, more recently, by Sheets et al. (2006). The attractive feature of the EFA algorithm, which is used here, is that points need not to be equidistant nor equal in number.

4.1. Data collection

In both approaches, we used manual data collection: a manual collection of landmarks, and a manual digitization of contours. For the landmark approach, it is not advisable to mix data from different users because of the “user effect”: small but persistent differences may arise between different users in pointing to the exact location of some landmarks, with effects on the final output (Dujardin et al., 2010). Because of the high comparability of each separate landmark, it is mandatory to collect each one very care-

fully. Since the comparability of points (pseudolandmarks) is not a problem in outline digitization, it might be expected that the user effect is lower. However, it has been shown that different users, hence different ways to capture the contour, may have a more (Firmat et al., 2010) or less (Qing-Bin and Xin-Li, 2012) significant effect on the final output. We did not test for the compared magnitude of the user effect between landmark and outline techniques, but we show that, for the same images, both techniques suffered from a more or less similar amount of measurement error.

For both landmark and outline approaches, the automated capture of data could appear as the solution, but no such solution has been widely adopted so far. Automated identification of landmarks was recently proposed for Dipteran wings (Houle et al., 2003; Palaniswamy et al., 2010), but it may be a very difficult task when applied to wings covered with scales (the *Anopheles* in this study) or when crosses between veins are wide zones instead of points (kissing bugs). Automated curve tracing is already part of many imagery software and might look easier to generalize. It is a possible task when the contour is the external boundary of the body, as can be the case for rounded bodies as for instance in mussels (Ferson et al., 1985; Claude, 2008). The problem becomes more difficult when the perimeter to be digitized is a substructure of the main image, an internal part of the body. These and other obstacles in performing automatic captures of outlines have led some authors to rely on the more flexible manual collection (Sheets et al., 2006; Firmat et al., 2010).

TENTATIVE Even if adopting the same starting point is not mandatory for the EFA method we used, we digitized the contour trying to start from the same point. This strategy, when possible, allows other methods to be applied to the same set of pseudolandmarks, as for instance the Procrustes superposition (after equalizing the number of points) used for our graphical output (Figs. 1–4).

4.2. Statistical analyzes

The reclassification procedure used in the present study was based on the discriminant analysis. This analysis requires that there be more specimens than variables in the smallest group. As we were unable to increase the sample sizes, we used a principal component analysis (PCA) to reduce the dimensionality of the data: the scores of a limited number of PC was used instead of the original data. The criterion to determine the number of PC selected was simply the number of specimens in the smallest group less one. Thus, in our study, the selected number of PC can be deduced from Table 1 by looking at the sample sizes in each comparison (see the “n” column). More sophisticated procedures exist (Baylac and Frieß, 2005), some of them not based on the PCA method (Sheets et al., 2006).

Because of the PCA-based dimension reduction method, only a part of shape variation, although generally a consistent part, was used to classify individuals, which probably lowered the power of the analyzes. There are two more reasons to think that low sample sizes probably represented a heavier handicap for the outline approach than for the landmark one. First, according to Baylac and Frieß (2005), the first PCA axes of Fourier coefficients rarely account for a significant proportion of the between-group differences. Second, we were not even able to use the maximum possible number of harmonics because the total sample was too low for the PCA to include them all. Table 1 indicates the number of harmonics used for the PCA (see the column “H”; which can also be deduced by computing the total sample of each group less 1, and divided by 4). Between close or cryptic species, and *a fortiori* between conspecific populations, we expect to have discrete, localized differences, if any. Such differences are captured by the higher level harmonics, while the first few ones generally describe more global differences in shape (Lestrel, 1989).

Thus, poor sample sizes could have reduced the discriminating power of outlines relative to landmarks. In spite of that, the outline classification showed satisfactory results.

4.3. Compared scores

The morphometric methods used here to build shape representation remove the isometric change of size, but were not designed to remove the allometric effect of size on shape variation. Thus, shape variables, be they partial warps or normalized Fourier coefficients, contain an allometric residue. Here, the effect of this residue on shape discrimination was estimated by regressing the shape-derived discriminant factor on size variation; which is reported in the column “r2%” of Table 1. On average, it was slightly less present in the landmark approach, which suggests the landmark-based approach might provide a slightly greater independence between size and shape.

4.3.1. *R. prolixus* and *R. robustus*

Previous morphometric studies based on landmarks could distinguish the two species to some extent, but in the favorable situation of allopatry (samples coming from distinct geographic areas) (Matias et al., 2001; Villegas et al., 2002; Márquez et al., 2011). In our material, we compared the two species from samples collected in the same village (Pampanito, Venezuela).

Morphological differences between the two taxa were frequently thought to be related to their separate environment, domestic for *prolixus* and silvatic for *robustus*. In our material, we could compare a sample of domestic *R. prolixus* (Pampanito) with another one collected in silvatic conditions in another locality (Guárico). In spite of coming from different habitats and localities, the two *R. prolixus* samples were similar, and remained distinct from *R. robustus*.

Both landmarks and outlines did a relatively good job, with again better results obtained from outlines. Actually, the outline-based distinction was almost perfect, since only one *R. robustus* out of 18 was confounded with *R. prolixus*, and no *R. prolixus* were confounded with *R. robustus*. Moreover, these outline-scores were not affected by subdividing the *R. robustus* sample according to its geographic origin. Within both species, the stability of the outline was remarkable: it was constant whatever the size (Fig. 5), the habitat and the geographic origin (Table 1).

As for previous studies on these two close species, the shape differences could not be clearly separated from the size variation (see the column “r2%” of Table 1). Feliciangeli et al. (2007) showed that in silvatic conditions size variance of *R. prolixus* may be significantly smaller. Size is generally considered more evolutionarily labile than shape, but it seems to have played a major role in the evolutionary divergence between *R. prolixus* and *R. robustus* (Fig. 5).

4.3.2. *Tsetse flies*

Males of *G. p. palpalis* and *G. p. gambiensis* were unsatisfactorily discriminated by both landmarks and outlines. Since each individual of this study was not identified by a more accurate criterion than its geographic origin, we cannot certify that the two groups were completely homogeneous. However, in the case of these two subspecies, for which the taxonomic status is still discussed, the problem concerns particularly the females. Males indeed can be recognized by observable variations of the genitalia (terminal dilations of inferior clusters). Fortunately, it appears that, for females, modern morphometrics can contribute to their identification, especially by using the boundary of the central cell (80%–85%).

4.3.3. *Mosquitoes*

Mosquitoes represent a group of insects where very few outline-based morphometric analyses have been published (Garros

and Dujardin, 2013). Yet the first application of EFA to the shape of living organisms was the quantitative analysis of the boundary of mosquito wings (Rohlf and Archie, 1984). The material of this seminal study was a set of drawings coming from an entomological book (plates 1 to 127 in Carpenter and LaCasse (1955)). As far as we know, no other outline studies have been performed on mosquitoes, except a study of the genital leaflet of *C. neavei* (Boussès et al., 2013; Garros and Dujardin, 2013), and the use of sliding-landmarks combining the posterior edge of the wing with a set of true landmarks in *Aedes aegypti* and *Aedes albopictus* (Stephens and Juliano, 2012). The outline used in our mosquito sample was this posterior edge united to the anal vein of the wing (Fig. 3). None of the landmarks used for the landmark-based approach was included in this contour, but it could still discriminate the two close species.

4.3.4. Soft ticks

Fifteen landmarks of the ventral face were used, all of them however of type II, i.e. landmarks providing less homology (Bookstein, 1991). In spite using low quality landmarks, good scores were obtained, but the contour of the dorsal face of the tick provided even better scores. Whatever the approach, significant shape divergence was disclosed between geographic populations of the same species. These differences could reflect adaptation to different hosts and/or geographic isolation (two remote islands). From these initial results, we expect this approach may perform well in species discrimination within the *O. capensis* group.

5. Conclusion

Our four case-studies do not (and cannot) claim superiority of one technique over another, but do suggest that one should not overlook the outline approach for discriminating close forms. It produced high scores of correct group allocation, it suffered from a relatively low measurement error and could be applied to various organs of the body, including or not relocatable landmarks. Our study suggests that if the main objective is to distinguish morphologically close entities, and even if a landmark-based approach is possible, one should consider also to test the outline technique.

In some organisms other than arthropods where such comparisons have been performed, either the two methods were considered as equivalent (Jensen et al., 2002) or the outlines were said to perform better (Loy et al., 2000; Pavlinov, 2000; Baylac and Frieß, 2005; Albutra et al., 2012). A recent study on Hymenoptera wings showed that landmarks performed better than (internal wing cells) outlines, and that their combination provided the best results (Francoy et al., 2012). In our various examples involving arthropods, similar or even better discrimination scores were attained using the outline-based approach. Although our sample sizes were generally low, similar performances of outlines in four comparisons involving quite different organisms (ticks, tsetse flies, mosquitoes and kissing bugs) is a significant signal. It suggests that for discriminating close forms, the choice of an outline-based analysis can be as relevant as the choice of a landmark-based one. Ideally, the outline should be selected in areas of the body where landmarks are lacking. This is not to say that, in other applications than simple discrimination, the outline approach would be less informative. In general, there was a powerful biological signal in the variation of outlines for arthropods (Foster and Kaesler, 1988; Monti et al., 2001; Baltanas et al., 2002; Faille et al., 2007; Tatsuta et al., 2009), as well as for other organisms like fishes (Loy et al., 2000), birds (Sheets et al., 2006), mammals (Renaud et al., 1996, 1999; Pavlinov, 2000; Renaud and Michaux, 2003; Friess and Baylac, 2003) and plants (Yamanaka et al., 2001; Jensen et al., 2002; Iwata et al., 2010; Iwata, 2011).

The objective of this study was to illustrate the interest of a relatively neglected method in modern morphometrics as applied to arthropods. We show that outline analyses can be applied in complement or as an alternative to the landmark-based approach.

Acknowledgments

To Fernando Monteiro for kindly providing us the wings of the *Rhodnius* specimens he submitted to molecular analyses (Monteiro et al., 2003). Thierry Boulonier and David Gremillet are thanked for help with tick collections. This work benefited from international collaboration through the ECLAT and LITRN networks.

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