Review

Geometric morphometrics in mosquitoes: What has been measured?

Camila Lorenz [a,b,*], Fabio Almeida [a,1], Fernanda Almeida-Lopes [a,1], Caroline Louise [a,c,1], Stella N. Pereira [a,c,1], Vivian Petersen [a,b,1], Paloma O. Vidal [d,1], Flávia Virginio [a,b,1], Lincoln Suesdek [a,c]

[a] Instituto Butantan, Av. Vital Brazil 1500, Butantã, São Paulo, SP CEP 0553-900, Brazil
[b] Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes 1374, Butantã, São Paulo, SP CEP 05508-900, Brazil
[c] Instituto de Medicina Tropical, Universidade de São Paulo, Av. Dr. Enéas de Carvalho Aguiar 470, Jardim América, São Paulo, SP CEP 05403-000, Brazil
[d] Faculdade São Francisco de Barreiras, Av. São Desidério, 2440, Ribeirão, Barreiras, BA CEP 47808-180, Brazil

ABSTRACT

The field of morphometrics is developing quickly. Recent advances have enabled geometric techniques to be applied to many zoological problems, particularly those involving epidemiologically-relevant mosquitoes. Herein, we briefly introduce geometric morphometric (GM) techniques and then review selected groups of mosquitoes (Culicidae) to which those techniques have been applied. In most of the reviewed cases, GM was capable of satisfactorily discriminating among the tested groups primarily when the studies considered differences within and among species, sexual dimorphism, treatments and the separation of laboratory strains. Although GM approaches have developed quite rapidly, some caution must be taken during data processing for a reliable biometrical approach, such as allometry and asymmetry analyses, scale removal and wing clarification staining for landmark digitization. We also critically forecast directions in this field and discuss how the creation of image databases should enhance species identification in culicids.

© 2017 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Contents

---

1. Introduction

Investigations of body shape have been central to the biological sciences in understanding systematics, taxonomy and evolution. In a strict formal sense, shape is the geometry of an object after removing its localisation, orientation and size (Kendall, 1977). In a zoological context, as geometric morphometrics (GM) has emerged, there has been a dramatic increase of studies that quantitatively address shapes. GM is an effective and relatively simple technique that has become popular because of the current availability of cheap computational power, specialised software (Rohlf, 2003, 2004, 2006; Dujardin, 2008; Klingenberg, 2011; Adams et al., 2013) and digitizing applications suitable even for less experienced users.

The GM approach is a powerful tool for evaluating correlations between shape and environmental or genetic variables. That is particularly true for mosquitoes and others insects, especially the family Culicidae, which possess nearly bi-dimensional organs suitable for morphometrical descriptions: the wings. Wings do not become deformed when slide-mounted, a fact that enhances high accuracy and repeatability even when wings are mounted and digitized by distinct operators (Lorenz and Suesdek, 2013).

In this study, we review a variety of GM applications in mosquitoes, especially studies that address differences within and among species, detection of parasites, sexual dimorphism, treatments, separation of laboratory strains and technical remarks. Because the GM literature on mosquitoes is already large and is growing rapidly, we have focused this review on scientific articles published between 2002 and 2016. It is not our intention to survey the entire field of morphometrics applied to biology in the current study (for such a comprehensive review, see Rohlf, 1990; Rohlf and Marcus, 1993; Bookstein, 1996; O'Higgins, 2000; MacLeod, 2002; Richtsmeier et al., 2005). Instead, this review aims to shed light on which biological issues of mosquitoes that have been approached using GM worldwide. We also conjecture on future directions in this field and comment on the launching of a GM image databank that will facilitate species identification.

1.1. Historical

Morphological analyses changed from descriptive to quantitative over the years (Bookstein, 1998). In the late 20th century, most precisely in 1971, the book "Multivariate morphometrics" (Blackith and Reyment, 1971) was launched, which deals with morphometrics using distance variables analysed in multivariate way. Although it was not yet based on landmarks, it was a revolutionary milestone, in the morphometrics field (Adams et al., 2004). With the emergence of more sophisticated statistical methods such as the correlation coefficient (Pearson, 1895), analysis of variance (Fisher, 1935) and analysis of principal components (Pearson, 1901; Hotelling, 1933), it became possible to describe biological variation from a quantitative perspective.

Despite the availability of such powerful statistical tests, distance-based morphometrics still exhibited serious problems. For example, (a) there was no general agreement between the different methods used for size correction; (B) the linear distances measured were not always homologous, making it difficult to compare structures; (C) early on, it was not possible to graphically represent shape using linear distances; thus, several aspects of the original shape were lost (Adams et al., 2013).

These difficulties made it necessary to develop a new method for measuring phenotype variations while preserving the original traits of the form. Thus, in the end of 1980s the field of morphometrics witnessed another revolution with the invention of coordinate-based methods, the proposal of "statistical theory of shape" (Mitteroecker and Gunz, 2009; Bookstein, 1998; Rohlf and Marcus, 1993) and the thin plate spline (Duchon, 1976). This new morphometric approach was called Geometric Morphometrics (Rohlf and Marcus, 1993) because it preserved the original geometry of the shape and allowed scholars to

represent the statistical results directly in the shape of the structure (Mitteroecker and Gunz, 2009). The studies developed in the field of GM have been substantially derived from the studies of Kendall (1989), who proposed appropriate mathematical and statistical approaches to use landmarks and showed the special importance of Procrustes distance. Subsequently, Bookstein (1996) drew attention of biologists to the usefulness of these approaches.

Noticeably, the use of GM in the biological and medical sciences has grown rapidly in recent years (Fig. 1), especially to address problems such as differences within and between species and sexual dimorphism. This technique has been increasingly applied to mosquitoes, specifically, to compare their wings.

Numerous GM methods also have been developed in recent decades. The Procrustes method is one of the most widespread methods used in mosquito studies (Bookstein, 1996; Small, 1996; Dryden and Mardia, 1998); it superimposes landmark configurations using least-squares estimates for scaling, translation and rotation parameters. Other frequently used method is Fourier analysis (Lestrel, 1981; Rohlf and Archie, 1984), used to digitize points on contours and outlines and then to allow comparisons among the curves using the coefficients of the functions as shape variables in multivariate analyses (Adams et al., 2004).

1.2. Why use geometric morphometrics?

Developed as a technique for fusing geometry and biology (Bookstein, 1982), GM addresses the study of form of biological structures in two or three spatial dimensions, it permits several statistical assessments and it allows graphical depicting of shape and size. As mentioned earlier, this technique preserves the physical integrity of the shape and avoids collapsing it into linear measurements that do not represent the structure as a whole (Richtsmeier et al., 2002). The GM is based on the coordinates of identifiable landmarks (LMs) which are, usually but not necessarily, evolutionary correspondent (homologous). Another type of labelling used for morphometric analyses (not represented by LMs) are the locations of points placed on curves, which are called semilandmarks. When the location of a landmark on a smooth curve or surface cannot be clearly identified, it may be treated as a SL that is allowed to subjectively slide along its curvature. In those cases, only the position perpendicular to the curved surface bears a biological signal (Mitteroecker and Gunz, 2009).

In mosquito studies (Culicidae), the wing is widely used for morphometric comparisons because of its two-dimensional shape and because it contains veins that encompass natural anatomical landmarks that are ideal for marking the LMs (Fig. 2). In addition, most veins present conspicuous landmarks and are homologous, so that they can be found in all representatives of the Culicidae family. Using GM it is possible to observe exactly where the largest source of variation occurs; moreover,

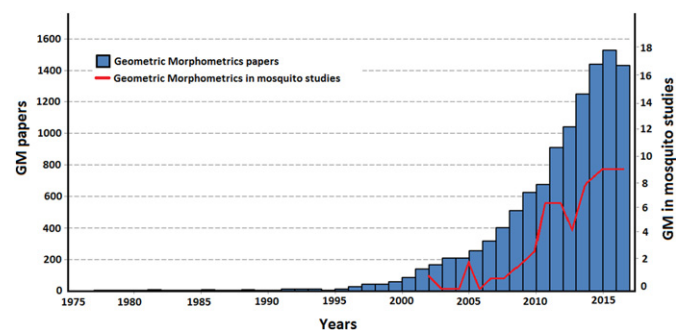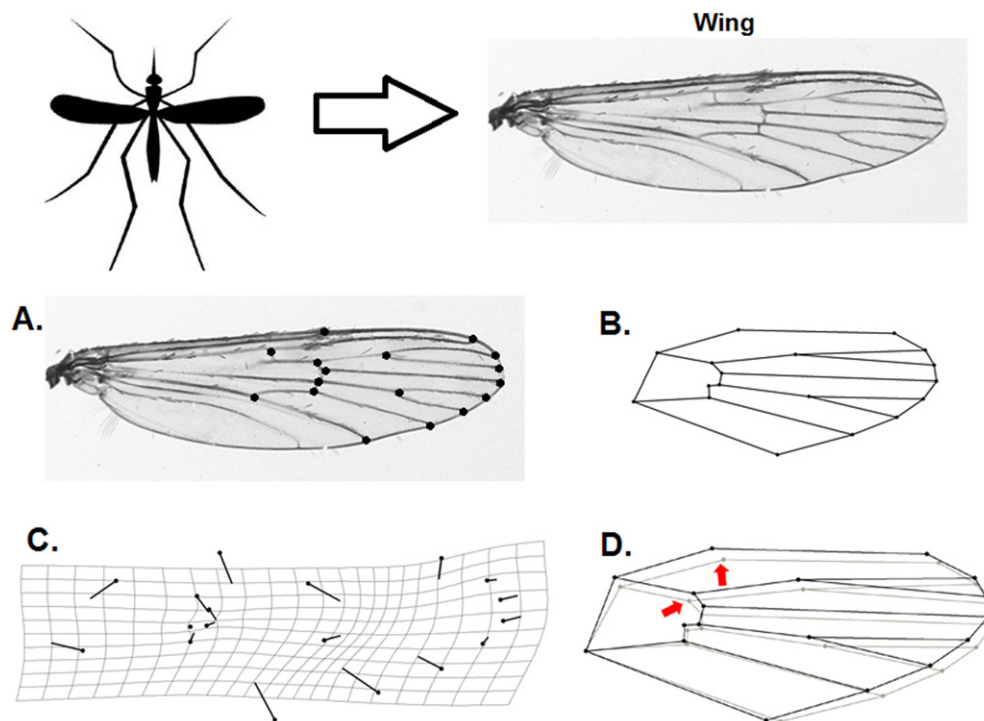
Fig. 1. Geometric morphometrics studies per year from 1980 to 2016 (blue bars) and studies that used GM techniques on mosquitoes (red line). Data from Google Scholar (2016). Total of 11.900 "hits", searching topic = "geometric morphometrics".

Fig. 2. Geometric morphometrics of mosquitoes' wings. (A) Marking the LMs in the wing veins crosses, which are natural anatomical landmarks. The number of LMs used in each analysis varies according to the study, as shown in Table 2. (B) Geometric diagram representing the wing portion considered for GM. The lines are not part of the data; they are just to help see the relationships among the LMs. (C) An example of graph which shows the shifts of landmark positions with straight lines (the strokes are proportional to the points of greatest variation). Deformation grid representing the shape variation in a wing. (D) Another way of representing the form variations: overlapping of the consensuses. The arrows indicate the most variable LMs in this comparison. *Figures A–D are derived from distinct datasets and are then uncorrelated.

this variation can be quantified for phylogenetic or biogeographic comparisons (Rohlf, 1993).

In addition to these advantages, the GM technique is fast, relatively inexpensive and simple: digitizing the coordinates of LMs indeed requires knowledge of the organism but it does not require much technical experience. Another benefit of GM is that through multivariate regression analysis, the allometric effect (residue) can easily be removed from the shape analyses, making it possible to compare shapes with minimum interference from differing sizes (Adams et al., 2004). Table 1 provides definitions of some frequently used terms in GM.

2. Geometric morphometrics applications in mosquitoes

The shapes of organisms may present either similarities or differences, depending on gender, geographical location, phylogenetic relationship, ecological relationships and types of treatments suffered. GM makes it possible to identify morphological variations and to explore their causes both within and between populations (Lawing and Polly, 2010). In this section, we briefly review some of the mosquito studies in which GM has been used to address differences within and between species, to detect parasites, sexual dimorphism and treatments, and to separate laboratory strains. Approximately half the studies available online explore intraspecific variations among mosquito populations (Fig. 3). Further details are available in Table 2.

2.1. Intraspecific variations

Intraspecific variability in mosquitoes is a phenomenon that has been studied for a long time and on that has been enhanced by the advent of molecular techniques. Many researchers are interested in exploring mosquitoes' phenotypic and genotypic adaptations to specific environments and the biogeographical differences that could lead to future speciation. The number of studies devoted to investigating

correlations between phenotype, environment, allelic frequencies and molecular markers is expanding, and such studies are currently being combined with GM data.

For instance, Gómez et al. (2014) studied the *Anopheles albimanus* Wiedmann mosquito from several different eco-regions in Colombia, comparing wing geometry, microsatellites and environmental variables such as elevation, relative humidity and precipitation. They found that wing size could be influenced by elevation and relative humidity, whereas wing shape was affected by those two variables and also by rainfall, latitude, temperature and eco-region. Considered together, wing traits and microsatellite data consistently support *An. albimanus* as a panmictic population, despite environmental heterogeneity. Motoki et al. (2012) also studied different eco-regions in Brazil and hypothesized that environmental and ecological factors may affect wing evolution in *Anopheles darlingi* Root.

Wing shape has been used as an indicator of population structure in some species (Morais et al., 2010; Sendaydiego et al., 2013; Demari-Silva et al., 2014; Petersen et al., 2015; Carvajal et al., 2016; Rodríguez-Zabala et al., 2016; Krtinić et al., 2016). In contrast, wing size is more sensitive to environmental changes and should be interpreted with caution in such studies. Morales-Vargas et al. (2013), studying populations of *Aedes albopictus* Skuse in Thailand, found that wing size is apparently under the influence of climatic factors, while wing shape can provide information on heritable intraspecific and geographic differences.

According to Dujardin (2008) centroid size can be used as a direct indicator of adult body size. Although there are some reports of weak correlation between wing size and body weight (Koella and Lyimo, 1996) from Dujardin (2008), it is theoretically possible to use GM to predict important biological mosquito characteristics such as flight capacity, gametes production (Ernsting and Isaaks, 2002) and their potential to transmit viruses. For example, the larger the mosquito is, the higher the viral load is that it can bear (Alto et al., 2008). By analysing

Table 1
Glossary. Some key terms used in GM analysis and their meanings (modified from Mitteroecker and Gunz, 2009; Slice and Bookstein, 2007).

Procrustes superimposition	Superimposition of configurations of LMs to compute shape coordinates. Through this superposition the sum of the quadratic differences is minimized.
Mahalanobis distance	Synonym for generalized distance. It takes into consideration the variance and correlation of variables within the a priori defined groups and measuring distances between points.
Procrustes distance	Distance between two configurations of Procrustes coordinates as a metric measure of shape difference.
Allometry	Any shape changes in response to size changes.
Landmarks (LMs)	Specific identifiable points on a biological form (or image of a form) located according to some rule.
Centroid size	Square root of the sum of squared distances between the centroid of a biological form (or image of a form) and each landmark.
Canonical variates analysis (CVA)	Method of discriminant analysis in which the variation among groups is expressed relative to the pooled within-group covariance matrix. CVA finds linear transformations of the data which maximize the among group variation relative to the pooled within-group variation.
Principal components analysis (PCA)	Analysis of the sample covariance or correlation matrices. The first PC is the linear combination with the largest variance of all those with coefficients summing in square to 1. It yields the best (at least squares sense) low dimensional approximation to the full multidimensional space.
Deformation grid	Deformed regular grid illustrating the shape differences between two configurations of LMs.
Thin-plate spline (TPS)	An interpolation function modelling the differences between two shapes as a smooth deformation. It is used to compute deformation grids.
Relative warp analysis	Principal component analysis of Procrustes shape coordinates.
Kendall shape space	(Nonlinear) the space defined by Procrustes distances, induced by a set of shape coordinates.
Repeatability test	Method for evaluating the accuracy of LMs repeatedly marked by a single operator.

populations of *Aedes aegypti* Linnaeus, an important vector of arboviruses, Morales-Vargas et al. (2010) showed that in natural conditions the size of this species is not only the manifestation of larval habitat quality, larval competition or temperature but also critically depends on the relative humidity during embryonic development, which can arguably affect the capacity of virus transmission in adulthood.GM is also able to detect subtle variations such as altitude gradients (Kuclu et al., 2011; Demirci et al., 2012; Lorenz et al., 2014) or temporal variations (Vidal et al., 2012; Hidalgo et al., 2015) within a single population. Louise et al. (2015) analysed populations of *Ae. aegypti* by correlating GM with microsatellite markers and observed seasonal changes in

Fig. 3. Types of studies using GM in mosquitoes (data from Google Scholar, 55 studies published between 2002 and 2016. Searching topic = "geometric morphometrics" AND "mosquito" OR "Culicidae").

wing shape. Such changes over time, detectable by GM, may indicate that microevolution is occurring in the species (Dujardin, 2008). Francuski et al. (2016) studied *Aedes vexans* Meigen in Europe and found a way to differentiate between northern and southern populations. Moreover, integrated genetic and morphometric data from a spatial analysis suggested that the populations can be grouped into three clusters, one of which consisted of Swedish and Hungarian populations.

The evolutionary rates of different markers may be different, and the variations detected by GM may not be the same as those found using molecular markers (e.g., Demirci et al., 2012; Vicente et al., 2011). Although GM is a very useful tool for identifying populations, in some cases it is unable to find clearly differentiating patterns (Vidal and Suesdek, 2012) because of canalising mechanisms or persistent gene flow among the genetic information in populations. Henry et al. (2010) analysed populations of *Ae. aegypti* and *Ae. albopictus* around the world and found that the morphology of these mosquitoes - both size and shape - appeared to be well conserved. In their interpretation, strong canalising mechanisms could account for the observed patterns of this relatively uniform morphology.

The GM method is also a particularly efficient approach for studying fluctuating asymmetry because the geometric quantification of shape captures more subtle differences in asymmetry than do traditional morphometric techniques (Klingenberg and McIntyre, 1998; Mardia et al., 2005; Zelditch et al., 2008). Measures of fluctuating asymmetry can serve as indicators of low fitness or genetic stress when they are correlated with low genetic diversity in an organism (Lawing and Polly, 2010). Galbo and Tabugo (2014) investigated the species *Culex quinquefasciatus* Say from Philippines and revealed high rates of fluctuating asymmetry in all analysed populations, which could be an indication of species fitness and its status as a pathogen vector.

2.2. Interspecific variations

Several mosquito species are difficult to identify because they lack conspicuous anatomical differences; the distinctive characteristics are mostly restricted to the male genitalia. Consequently, researchers spend much time identifying unknown species using traditional methods due to the vast number of specimens needing identification, relatively few characteristics in a dichotomous key, and a limited number of taxonomists. GM is increasingly used as a complement to the older diagnostic techniques applied in medical entomology. For example, in mosquitoes of the genus *Culex*, Börstler et al. (2014) showed that there are significant differences between *Culex pipiens* Linnaeus and *Culex torrentium* Martini, two species in which the females are morphologically almost identical, and Laurito et al. (2015) discriminated among four Neotropical species. Both studies used only wing GM. The difficulty in identifying sympatric species is even greater, but GM has nevertheless been shown to be efficient in this situation as well. For example, using only GM, Lorenz et al. (2012) were able to differentiate three species of the genus *Anopheles* that occur in sympatry and that are somewhat morphologically similar. Morphometrics has also been used to identify complex species that are indistinguishable using traditional morphology. Gómez et al. (2013), for example, used GM and DNA barcoding to evaluate members of the *Albitarsis* complex, genus *Anopheles*, in Colombia; however, the multiple marker analysis did not support the existence of more than one species of the complex.

Most females of the subgenus *Anopheles* (Nyssorhynchus) are difficult to identify in the field using the available taxonomic keys. Using GM, Jaramillo et al. (2015) were able to differentiate 11 species of the genus *Anopheles* using only the wing shape, showing that GM can discriminate between both sibling and sympatric species that are almost impossible to identify with dichotomous keys. In that work, the authors tested different combinations of LMs, seeking a better approach to recognising cryptic species within that genus. This work revealed the potential of GM for determining the taxonomy of *Anopheles* (Nyssorhynchus) similar to a prior work that used combinations of
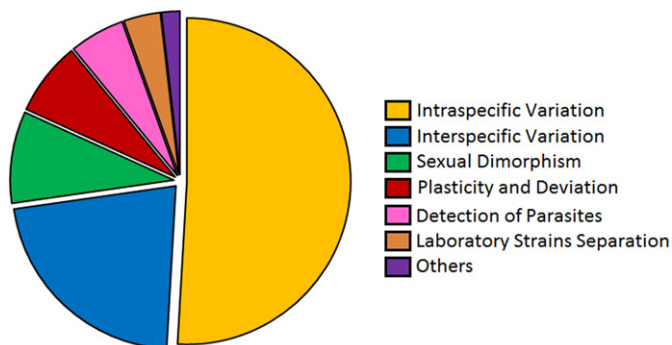
Table 2
Information about selected studies in mosquitoes which GM has been applied to answer questions of general interest.

Study	Number of LMs used	Species	References
Intraspecific variation	12, 13, 18, 19, 20, 21	*Ae. aegypti, Ae. albopictus, Ae. vexans, An. coluzzii, An. darlingi, An. atroparvus, An. funestus, An. cruzii, An. albimanus, Cx. coronator, Cx. theileri, Cx. pipiens, Cx. quinquefasciatus, Oc. scapularis*	Morais et al. (2010), Kuclu et al. (2011), Morales-Vargas et al. (2010, 2013), Henry et al. (2010), Vicente et al. (2011), Motoki et al. (2012), Vidal and Suesdek (2012), Vidal et al. (2012), Demirci et al. (2012), Sendaydiego et al. (2013), Lorenz et al. (2014), Galbo and Tabugo (2014), Gómez et al. (2014), Demari-Silva et al. (2014), Louise et al. (2015), Hidalgo et al. (2015), Petersen et al. (2015), Carvajal et al. (2016), Rodríguez-Zabala et al. (2016), Krtinić et al. (2016), Francuski et al. (2016)
Interspecific variation	12, 13, 17, 18	(*Cx. bidens, Cx. interfor, Cx. mollis, Cx. tatoi*)[a], (*Cx. quinquefasciatus, Cx. nigripalpus*)[a], (*Cx. pipiens s.s., Cx. torrentium*)[a], (*An. albitarsis I, An albitarsis F*)[a], (*An. albimanus, An. albitarsis F, An. aquasalis, An. braziliensis, An. benarrochi B, An. darlingi, An. nuneztovari, An. oswaldoi s.l., An. rangeli, An. strodei, An. triannulatus*)[a], (*An. cruzii, An. homunculus*)[a], (*An. cruzii, An. homunculus, An. bellator*)[a], (*Ma. dives, Ma. bonneae*)[a]	Vidal et al. (2011), Ruangsittichai et al. (2011), Lorenz et al. (2012), Gómez et al. (2013), Garros and Dujardin (2013), Börstler et al. (2014), Laurito et al. (2015), Jaramillo et al. (2015), Lorenz et al. (2015a), Sumruayphol et al. (2016), Wilke et al. (2016)
Parasite detection	15, 18	*An. flavirostris* (filaria detection), *Cx. quinquefasciatus* (filaria detection), *Ae. aegypti* (Wolbachia detection)	Sendaydiego et al. (2014), Yeap et al. (2014), Sendaydiego and Demayo (2015)
Sexual dimorphism	13, 18, 23	*Cx. quinquefasciatus, Cx. gelidus, Oc. scapularis, Ae. aegypti, Ae. albopictus, An. albitarsis l.s., An.cruzii, An. homunculus, An. strodei l.s., An. triannulatus l.s., Cx. nigripalpus, Ma. dives, Ma. bonneae, Ae. fluviatilis*	Manimegalai et al. (2009), Devicari et al. (2011), Ruangsittichai et al. (2011), Vidal et al. (2012), Dhivya and Manimegalai (2013), Lorenz et al. (2014), Virginio et al. (2015), Christe et al. (2016)
Plasticity and deviation	19 (with 39 SLs), 20, 22	*An. superpictus, Ae. aegypti, Ae. albopictus*	Jirakanjanakit et al. (2007), Aytekin et al. (2009), Stephens and Juliano (2012), Dogan et al. (2016)
Separation of laboratory strains	12, 16	*Ae. aegypti, An. dirus, An. cracens*	Jirakanjanakit and Dujardin (2005), Kitthawee and Rungsri (2011)
Genetic information	10, 12, 18	*D. melanogaster, An. funestus*	Palsson and Gibson (2000), Birdsall et al. (2000), Zimmerman et al. (2000), Carreira et al. (2011), Ayala et al. (2011)

[a] In studies of interspecific variation, GM was used to separate among these groups of species.

LMs to improve the discriminatory power of GM. Another suitable use of GM in species identification occurs when samples are not perfectly preserved, as suggested by Vidal et al. (2011). Using only the GM technique, these authors were able to differentiate two species of the genus Culex, *Cx. quinquefasciatus* and *Cx. nigripalpus* Theobald, which can occur sympatrically in urban and semi-urban locations.

To gain an even more accurate method for identifying species of insect vectors of pathogens, combined analyses of genetic and morphological markers have been encouraging. Ruangsittichai et al. (2011) used genetic (DNA barcoding) and phenetic (GM) techniques to identify two species of the genus *Mansonia* (*Mansonia bonneae* Edwards and *Mansonia dives* Schiner), which are evolutionarily close and difficult to identify using traditional morphological characteristics. According to these authors, the risk of misidentification during epidemiological studies leads to the need for complementary techniques to distinguish the two species. Both techniques were effective in differentiating the two species and provided information relevant to their ecology. According to these results, the authors suggest that GM can be used as a first approach in the identification of these species because it is cheap and fast, while DNA analysis can be reserved for use with dubious species.

Another study used GM and the mitochondrial cytochrome oxidase subunit I gene to determine a means of differentiating between species of *Anopheles* living in sympatry in the municipality of Cananeia, Sao Paulo, Brazil (Lorenz et al., 2015a). The authors verified that the anatomical landmarks associated with the wing shape were consistent with the molecular phylogeny of *Kerteszia* species, indicating that GM can distinguish phylogenetic relationships within the genus *Anopheles*.

Ae. aegypti, Ae. albopictus and *Ae. scutellaris* Walker are important mosquito vectors of dengue and chikungunya viruses. These species are morphologically similar and often live in sympatry. Sumruayphol et al. (2016) showed that GM can increase the reliability of morphological identification of these *Aedes* species. Another recent study, using only GM, differentiated species among three epidemiologically important genera: *Aedes*, *Anopheles* and *Culex* (Wilke et al., 2016). The authors showed differences in wing shape between genders. Furthermore, wing

GM was also efficient for discriminating between the lower hierarchical levels (subgenera and species).

GM may not be as precise as genetic sequencing is for all cases of species identification, but the results have shown that it can be highly informative. Because it is a cheap and quick technique, accessible even to minimally-trained operators, it can be used even in the field to solve ecological and taxonomic issues. According to several authors, the approach to species identification should be multidisciplinary, combining biological, ecological and molecular data to correctly determine a species, especially when assessing members of a taxonomical complex (Montoya-Lerma et al., 2011; Schlick-Steiner et al., 2010; Garros and Dujardin, 2013).

2.3. Parasite detection

Recently, GM techniques have also been used on the main mosquito vectors (i.e., *Anopheles*, *Culex*, and *Aedes*) to verify whether parasite-infected mosquitoes exhibit different wings than uninfected mosquitoes. Sendaydiego and Demayo (2015) analysed filaria-infected and non-infected female *Anopheles flavirostris* Ludlow specimens from the Philippines and showed that there is a tendency for the wings of infected mosquitoes to be slender and long, while those of non-infected mosquitoes were shorter. However, this result was not able to serve as a diagnosis of infection because there was great overlap between the groups. The authors also argued that despite the observed differences, environmental variables such as altitude and the non-removal of allometry effects could have biased the results.

Another study using GM was performed for this same goal in *Cx. quinquefasciatus* females from the Philippines (Sendaydiego et al., 2014) in which the opposite tendency was found compared to that observed in *Anopheles*, i.e., the wings of filaria-infected females were wider. However, this difference cannot be used as a diagnosis because, as the authors argue, environmental variables may be related to these differences. Another fact discussed in this study was that because filarial

infection makes individuals heavier, they would not fly to high altitudes, but that could also be related to physiological differences.

A GM study in *Ae. aegypti* was carried out to separate populations of infected and uninfected *Wolbachia* females in Australian populations (Yeap et al., 2014). Although *Ae. aegypti* was not found to be naturally infected by *Wolbachia*, in a previous work, the authors had used an artificial infection technique to infect them. Then, they released the infected population to nature with the goal of replacing the uninfected endemic population (the study showed that *Ae. aegypti* infected by *Wolbachia* had less transmission capacity). The results of this work showed that the wings of uninfected mosquitoes have different shapes and even larger differences in sizes in different environmental conditions, whereas the wings of infected insects did not vary in either shape or size in nature. In addition, it was noted that in warm temperatures infected females tended to have larger wings than the uninfected ones, but in males, this difference did not exist. In cooler temperatures uninfected insects - both males and females - also tend to be larger. Despite these observations, the conclusion was that *Wolbachia* infection could not be accurately detected by GM analyses of mosquitoes' wings.

In general, one can conclude that none of the mentioned studies were able to demonstrate the efficacy of GM to discriminate parasitically infected groups of individuals from uninfected groups. Perhaps the premise that pathogens consistently affect wing shape is not valid, or perhaps the GM technique is not the most appropriate method for detecting such differences between populations.

2.4. Sexual dimorphism

Although the Culicidae family comprises insects with high sexual dimorphism (SD) and this distinction is highly important to public health field, studies on this subject are still scarce. This family has some structures that show sexually dimorphic patterns, including wings. These differences have been described occasionally in taxonomic keys for mosquitoes, but their specific patterns and variability are insufficiently known.

In insects, morphological effects of SD can be observed in size (Fairbairn, 2007), shape (Benítez et al., 2013) and allometry (i.e., the interaction between body size and shape) (Gidaszewski et al., 2009). Most researchers do not remove the allometric effect when studying interspecific differences, because the effect of size or shape can be an important species-specific characteristic (Dujardin, 2008). Moreover, when the goal is to analyse shape and size separately, primarily between sexes, allometric effects can also be removed, as performed by Ruangsittichai et al. (2011), Devicari et al. (2011), Lorenz et al. (2014) and Virginio et al. (2015).

Ruangsittichai et al. (2011) used GM to investigate the SD in two species of filariasis vectors: *Ma. bonneae* and *Ma. dives*. These species showed both wing size and shape SD. There was a major size variation between males and females in *Ma. bonneae*, although, remarkably, they showed less sexual dimorphism in shape. Although some studies suggest that both SD types may be adaptive (Gidaszewski et al., 2009; Benítez et al., 2013; Klingenberg, 2011) there is still no clear explanation for this phenomenon. The authors concluded that the SD shape could not be satisfactorily interpreted as an allometric effect and suggested that their observations could indicate that sexual selection plays a significant role in the speciation of some members of the *Mansonia* genus.

Virginio et al. (2015) analysed SD in ten culicid species from the genera *Culex*, *Aedes*, *Anopheles* and *Ochlerotatus* collected in Brazil (Fig. 4), observing a sex-specific phenotypic expression of wing shape. They concluded that although there an allometric effect exists, it did not significantly contribute to shape SD. They also observed that females are larger than males in most species and that size is probably more variable and less canalized than shape. In insects, SD is considered to be a genetic variation. In *Drosophila* for example, studies indicate that the genetic architecture of wing shape is sexually dimorphic. Moreover, the genetic

basis of wing shape is relatively independent of wing size (Carreira et al., 2011).

GM detection of SD has also been reported in *Aedes scapularis* Rondani (Devicari et al., 2011), a vector of Rocio virus in Americas, and *Aedes fluviatilis* Wilkerson (Christe et al., 2016), a potential vector of yellow fever virus. Those authors assessed populations from different locations of Brazil and observed absolute segregation between females and males in both species. Manimegalai et al. (2009), studying *Cx. quinquefasciatus* from Coimbatore city, India, observed a clear distinction in wing shape between sexes. Based on thin-plate spline they could see that male wings vary in the middle and at the distal ends while females vary only at the distal end of the wing. A morphometric analysis based on LMs was also performed for *Culex gelidus* Theobald mosquitoes (Dhivya and Manimegalai, 2013), a vector of japanese encephalitis virus. Here, however, both females and males showed variations in both the middle and distal wing regions.

Other studies have evaluated SD in populations over time. Vidal et al. (2012) showed that over a four-year period, male and female wings of *Ae. albopictus* decreased in size. They also observed disparities in wing shape and found different rates of change between the sexes. Lorenz et al. (2014) evaluated populations of hill and plateau *Anopheles cruzii* Dyar & Knab, revealing that despite the variation over time, the species always exhibits strong SD; in this study a comparison of wing shape after Procrustes superimposition revealed that the most influential LMs in the majority of populations were localized in the wing border.

In general, the SD studies cited here demonstrate that: (A) the shape variations of LMs are mainly concentrated between the middle and distal regions of the wing and are less frequent in the proximal region; (B) most females presented larger wings than males; (C) most authors conclude that males and females have different ecological roles in the environment and use their wings differently; this could motivate sex-specific natural selection; (D) the evolutionary rates of change in size and shape seem to be different, and wing size seems to be influenced more by environmental factors than does wing shape.

2.5. Plasticity and deviation

GM is also used to detect variations that may occur in the size and shape of the wings of mosquitoes that are reared in stressful environments. Aytekin et al. (2009) observed the development of cohorts of *Anopheles superpictus* Grassi from egg stage to adult emergence at different temperatures and verified that the development time of the larvae was inversely proportional to the temperature to which they were exposed (i.e., the higher the temperature, the shorter the development time). In addition, wing deformations were found mainly at the central points of the wings for both sexes and the deformations were accentuated as the temperature increased. They also observed that rearing those mosquitoes at high temperatures resulted in smaller individuals with smaller wings.

Ae. aegypti populations were analysed for larval density and food supply during rearing (Jirakanjanakit et al., 2007) by varying the larval densities and food availability in experiments. From GM analyses, the authors observed that higher larval density resulted in smaller wing sizes (and vice versa) and that the insects grew larger wings when the amount of food was larger. The shape in the central area of the wing follows the same trends as the size. Still analysing larval density, Stephens and Juliano (2012) studied the wing geometry of *Ae. aegypti* and *Ae. albopictus* under intra- and interspecific competition at different temperatures. The wing shape of *Ae. albopictus* did not vary much during larval density experiments, but GM analyses could differentiate groups of mosquitoes raised at different temperatures. For *Ae. aegypti*, changes in the shapes at the semilandmarks were very slight on the posterior edge in the competition tests and varied with the temperature. The wing sizes varied between species: smaller sizes were observed in *Ae. albopictus*, while *Ae. aegypti* wings were larger.
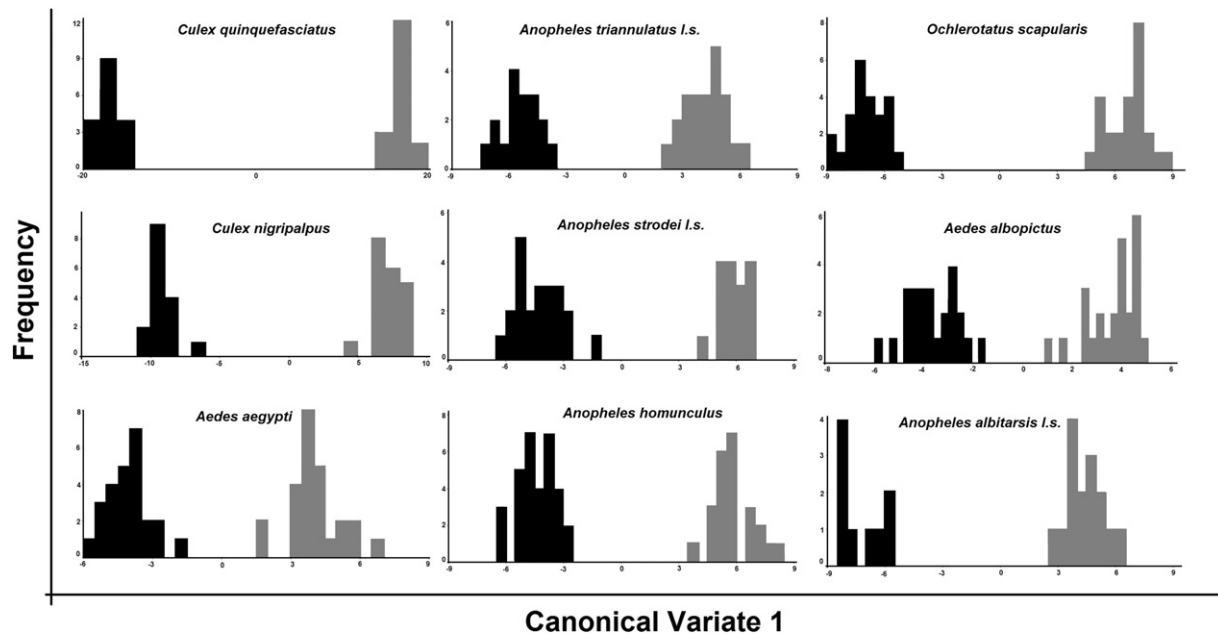
Fig. 4. Wing shape diagrams of first canonical variable from the comparison of males (black) and females (grey). In all species of Culicidae depicted here the separation between males and females was 100% (modified from Virginio et al., 2015).

In a recent study, Dogan et al. (2016) used GM analyses to define all phenotypic differences that occurred on the wing size and shape morphology of *Ae. albopictus* at four different larval densities (1, 2, 3 and 4 larvae per ml) on the preimaginal development. They found no significant difference in wing shapes among the different densities in either males or females. Nevertheless, a significant difference in the sizes of females and males was observed for populations reared at low densities versus the other densities, which has also been observed by other authors (Jirakanjanakit et al., 2007).

All these studies showed that shape changes occur easily, and the size of mosquitoes' wings can also easily change in controlled laboratory conditions. Such changes can influence mosquito dispersion, facilitate flying, mating and its vector competence, making mosquitos more able to spread mosquito-borne diseases such as malaria, dengue and zika virus. The wing alterations observed in nature may be caused by incomplete population control initiatives; that is, by reducing the number of larvae in breeding locations, larvae that survive may lead to larger mosquitoes, which can aggravate problems related to public health (Aytekin et al., 2009; Stephens and Juliano, 2012).

2.6. Separation of laboratory strains

The quantitative morphological characteristics of mosquitoes are basically products of the interactions between their genetic information and their environmental conditions. The use of lineages maintained in the laboratory is very common among researchers for studies related to species characterization. Among the advantages are the relatively simple and inexpensive maintenance of specimens and the fact that individuals are subject to the same conditions, thus, avoiding variations derived from the varying environmental conditions found in nature. In addition, the increasing use of GM has been shown to be effective in identifying of laboratory strains and species complexes (Kitthawee and Rungsri, 2011; Villegas et al., 2002).

Jirakanjanakit and Dujardin (2005) collected *Ae. aegypti* larvae at four different locations in Thailand and maintained each sample in the laboratory through several generations (17, 95, 260 and 564 generations, respectively). They observed a decrease in wing size as the number of generations increased. Using GM analyses, they could distinguish each of the four mosquito strains.

Kitthawee and Rungsri (2011) used GM associated with wing vein patterns to identify two colonies of cryptic and naturally allopatric species belonging to the *Anopheles dirus* Peyton & Harrison complex in Thailand. The specimens of *An. dirus* and *An. cracens* Sallum & Peyton came from laboratory colonies established in 1983 and 1978, respectively. The researchers found little difference in wing size between species, probably because they were raised in the same laboratory conditions as were used years ago. In contrast, the wings showed large divergences in shape that were sufficiently consistent to segregate the two species.

Both experiments cited here stress that the results of studies with laboratory strains should not be compared to those obtained with field samples. Maintenance for several generations under restricted conditions may have altered the wing shape of the population over the time elapsed since they were first collected in the wild.

3. Genetic information

3.1. Genes responsible for wing shape

Studies are currently scarce regarding the genetic origin of the patterns and variations in the wing shapes of mosquitoes. Most of the similar studies are concentrated in the model organism *Drosophila melanogaster*. However, these target gene research studies are quite valuable for understanding the factors related to polymorphism in Culicidae because they may follow similar patterns. Palsson and Gibson (2000) tested whether a group of 15 loci in *D. melanogaster* could influence variations in wing shape. The influence of the mutant genes on wings was captured through relative warps corresponding to the anatomical landmarks of the wing veins. In this study, a possible influence of the genes *dpp*, *tkv*, *EGFR*, *argos*, *elbow* and *hedgehog* was observed. Most likely, these genes act through growth factor signalling. In addition, it was observed that epistatic interactions have little contribution to wing shape variations.

Another important finding in this field is that the actions of genes influence wing shape more than do environmental factors. Birdsall et al. (2000) carried out experiments to characterize the variations in the wing shape of individuals of *D. melanogaster* using 12 isolations developed at two different temperatures (18 °C and 25 °C) and found that wing shape, specifically, the prevalence of the lineage-specific

characteristics, was little affected by the temperature. Zimmerman et al. (2000) tested 35 candidate genes and found evidence that at least 13 of these genes influence the wing shape in the female and male of *D. melanogaster* regardless of the ambient temperature at which individuals were raised. In the same study, it was observed that the shape of the wing should be considered as a characteristic composed of several development units (polygenetic action). Carreira et al. (2011) studied 191 lines of homozygotes for the insertion of p-element (transposable element) in *D. melanogaster* using GM as an analysis tool and found that more than 63% of the induced mutations affected wing shape expression in males and females but that only 33% were expressed in both sexes. The wing, therefore, is not only controlled by polygenic action but also by the expression of genes that differ by sex.

Wing shape development in mosquitoes is probably also influenced by several genes and their expression. Ayala et al. (2011) in research with *Anopheles funestus* Giles observed a correspondence between chromosomal polymorphism (chromosomal inversion) and wing shape variations in individuals from eight eco-regions in Cameroon. This ring-shaped richness probably occurs in mosquitoes due to selective pressures from the specific environments. Although it is known that wing shape is influenced more by genetic than by environmental factors, the extents of these influences are not yet known. In studies with *Triatoma infestans* Klug it was found that the host species blood food source (i.e., humans, cattle, or birds) influences the wing shape of the offspring of the same population (Dujardin et al., 2015). The same was observed in butterfly populations *Ectomyelois ceratoniae* (Mozaffarian et al., 2007). The studies reviewed here all suggest the need for additional focused studies on mosquitoes, both to identify which genes are responsible for the variation in the wing shape and to measure the genetic roles of these variations.

3.2. Genetics versus morphology

Although limited, the fixation index (F_{st}) is widely used for measuring the genetic diversity; consequently, F_{st} and related statistics are among the most widely used descriptive statistics in population and evolutionary genetics. However, a little-known index similar to F_{st} is used to measure morphological diversity: the Q_{st}. There are few studies comparing these two indexes, but when there is such a comparison, Q_{st} was usually much larger than F_{st} (Dujardin, 2008), as observed in the studies by Vidal and Suesdek (2012), Lorenz et al. (2015a) and Petersen et al. (2015). This is because, within each species, the characteristics that undergo greater selective pressure are expected to be more divergent from molecular F_{st} (Dujardin, 2008). Thus, genetic selection would act to modify the wing geometry differently in different populations and the Q_{st} index serves to measure these variations.

In addition to Q_{st}, it is possible to estimate morphological diversity within a population in another way. Suesdek [in Petersen et al. (2015)] proposed that morphological diversity of *Aedes* (*Ochlerotatus*) *scapularis* populations could be measured by using the "amount of dispersion" of individuals in the principal component morphospace. Theoretically, this amount of dispersion of individuals (of a single set) in the morphospace is proportional to the morphological variability and, therefore, the centroid size of the polygon formed by this dispersion would be an estimator of the diversity (Fig. 5). This morphological diversity value can be compared to indices of genetic diversity such as haplotypes or nucleotides.

It is practically a consensus among researchers that different markers must be combined to better understand the evolutionary history of a given group. However, for mosquito species such as *Ae. aegypti*, this is not an easy task. Genetic and morphological markers are often contradictory and, as discussed earlier, GM is often unable to show clear patterns of population differentiation. Vidal and Suesdek (2012) analysed populations of *Ae. aegypti* in São Paulo (Brazil), but found that wing geometry failed to distinguish the samples. These data suggest that microevolution in this species may differentially affect genetic

and morphological characteristics, and the wing shape did not present variations because it is stabilized by selective pressure. In addition to the different evolutionary rates for genetic markers and wings, persistent gene flow and canalising mechanisms may make wings quite homogeneous among populations, as found by Henry et al. (2010).

4. Problems in geometric morphometrics analysis

Although GM has a simple execution technique, some care must be taken to avoid errors during execution and analysis. The erroneous recognition and marking of LMs, for example, may result in an overestimated variation between the groups studied. It can be difficult to see the LMs in mosquitoes that have wings with dark spots or many scales. Lorenz and Suesdek (2013) evaluated the wing marking efficiency before and after scale-removal treatments and observed that physical or chemical treatment effectively increases the visualization of LMs in *Anopheles*. Physical and/or chemical scales removal treatments have been widely used in studies of mosquitoes of the genus *Aedes* (Morales-Vargas et al., 2010; Kuclu et al., 2011; Vidal and Suesdek, 2012; Vidal et al., 2012; Louise et al., 2015), *Anopheles* (Aytekin et al., 2009; Vicente et al., 2011; Lorenz et al., 2012; Lorenz et al., 2014; Lorenz et al., 2015a; Virginio et al., 2015), *Culex* (Demirci et al., 2012) and *Mansonia* (Ruangsittichai et al., 2011).

Allometry, the change in shape as a function of differences in size, also requires attention in GM studies. Although size and shape are not independent attributes, allometry can be assessed using linear regression techniques. Dujardin (2008) explained that, in studies of insects belonging to the same species, size variations are often environmentally induced and observed shape variations can be a consequence of variations in size. Although the allometric residue acts as a bias in shape analyses, Virginio et al. (2015) have observed that maintaining the size variation in studies of sexual dimorphism may improve gender distinction in species-specific ways (see Sexual Dimorphism session).

Another consideration involves the use of both left and right wings in the same analysis. Mosquitoes present bilateral asymmetry but may exhibit high levels of fluctuating asymmetry or even directional asymmetry when subjected to environmental stress during development (Peruzin, 2009; Galbo and Tabugo, 2014). Therefore, when symmetry is relevant to the study under development, it is best to perform a test of asymmetry before proceeding with the GM analyses to see if it is possible to work with left and right wings in the same analysis. Using the right and left wings from the same specimen in the same analysis is not recommended because that would duplicate the information from each individual and bias the results; both left and right wings are highly correlated and hence redundant.
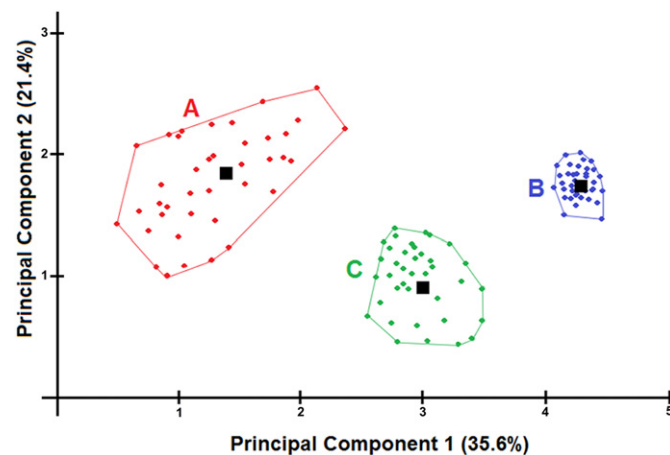

Fig. 5. Morphological diversity estimated from the morphospace of PCs, where DA > DC > DB. The dark square in each polygon represents the centroid. The centroid size, which uses the centroid as a reference, is a direct estimator of the size of morphological diversity.

5. Future research directions

5.1. Databases and automatic identification system

As stated earlier, GM's main goal is not to describe the organisms but to compare among them and to statistically analyse variation of shape as well as its covariation with other variables. Consequently, it would be both interesting and extremely useful for researchers to have access to as many wing images as possible, for example, when contrasting different species or populations. In view of this objective, databases for the storage of insect morphological information (CLIC Bank - http://mome-clic.com/) and specifically, mosquitoes (WingBank - http://www.wingbank.com.br/), are currently being developed.

Dujardin et al. (2010) consider that rather than storing coordinates, which may be affected by different operator's errors ("user effect"), databases should contain original pictures so that each user is capable of taking his/her own measurements and coordinates. Despite the fact that image-based databanks can circumvent the problem of measurement errors, they cannot prevent errors stemming from different and personalised mounting and photographic techniques.

The WingBank, another example of database, comes with the proposal that user upload their own landmark notes, to enable initial tests of an automatic insect identification system, which does not preclude the possibility of the other users ignore this notes and use their own measurements (Virginio & Suesdek, unpublished results), besides having the original coordinates allow someone to check the computations performed in a prior study. Moreover, the WingBank enables the user to add not only the species name but also a set of information such as gender, geographical location, data of image capture, related journal of publication, etc. The multivariate style of this databank permits users to enrich their studies, allowing one to investigate intraspecific variation, for instance.

Another recently improved idea is automatically marking the LMs in the wings, because manual marking of these points can be time consuming for large amounts of data. Tofilski (2004) developed a software application called DrawWing that enables automatic identification of twenty junctions. It analyses an image of an insect wing and outputs a list of the coordinates of these junctions and a wing diagram that can be used as an illustration. The coordinates of junctions extracted by DrawWing from wing images were used to discriminate between vein patterns of *Dolichovespula sylvestris* Scopoli and *Dolichovespula saxonica* Fabricius (Tofilski, 2004), two wasp species that can be considered pests to humans. Houle et al. (2003) created a software application called WINGMACHINE that can measure the positions of all the veins and the edges of the wing blade of Drosophilid flies. This software can fully complete the measurement of one wing per minute, including handling, imaging, analysis and data editing. Another application that can automatically obtain the LMs from *Drosophila* wings with high efficiency and accuracy is TINA (Palaniswamy et al., 2007). However, mosquito wing tests using these applications have not yet been performed. Mosquito wing automatic measurements may be less accurate due to the presence of scales that can impair automatic spot detection, what can consequently jeopardise the development of specific software to mosquitoes. Accordingly, Lorenz and Suesdek (2013) showed that mechanical removal of the scales as well as the chemical treatment of wings are effective for improving visualization of the landmarks.

An automatic identification system for LMs leading to the correct mosquito species identification is a daring goal and is currently a solution much-sought-after by researchers, mainly due to the medical importance of the Culicidae family. Software already exists that can use image databases to discriminate bees (Francoy and Fonseca, 2010), butterflies (Wang et al., 2012) and stoneflies (Larios et al., 2008). In the future, we expect that similar software will be created and tested for its effectiveness in identifying mosquitoes more quickly, because traditional identification keys are both very subjective and accessible only to experienced professionals.

Another approach that has been tested for separating cryptic species and characterizing sympatric populations is outline-based morphometrics, which captures the contours of forms through a sequence of close semilandmarks (Dujardin et al., 2014). Semilandmarks, which describe contours or boundary outlines, do not depend on the presence of true anatomical LMs; they can exist with no anatomical LMs at all or can include one or more LMs. In this way, the comparison of structures is not restricted to the anatomical frames of wings but is appropriate for any structure that has a contour. Dujardin et al. (2014) analysed four case-studies and found that the outline approach provided accuracies similar to those obtained by the landmark-based approach. That study suggested that if the main objective is to distinguish morphologically close entities, the outline technique should be considered even when a landmark-based approach is possible.

5.2. Artificial neural networks and mosquito identification

Generally, discriminant analysis is the statistical method most used in the evaluation of morphometric data. However, recently, studies using artificial neural networks (ANN) have been shown to have greater or equal efficiency when applied to the problem of discriminating mosquito species. The ANN technique, which was adopted from the computational intelligence field, is being used with increasing frequency to classify biological organisms (Dobigny et al., 2002; Marcondes and Borges, 2000) because of its efficiency in handling large datasets and its generalizability. Lorenz et al. (2015b) combined PCs from morphometric analysis with ANN to identify 17 species of mosquitoes of the genera *Anopheles*, *Aedes* and *Culex*. The results showed better or equal reclassification rates compared to the use of classical discriminant analysis; however, the ANN approach had advantages in both speed and efficiency when dealing with large amounts of data. For ANN, sample sizes are very important in the training phase because the algorithm needs to recognize natural patterns and "to learn" how to classify the data. For a more complete and accurate identification, using a combination of techniques would be ideal.

6. Synthesis and conclusions

Despite its mathematical sophistication, geometric morphometric techniques are easy to adopt due to the availability of easy-to-use, quality software and to the intuitive nature of the visual methods for depicting the results. In entomology, more specifically in mosquitoes, GM has been used increasingly to solve a series of biological problems by considering the positions of the LMs in the wing veins. In most cases, this technique can satisfactorily discriminate groups, especially when applied to sexual dimorphism, treatments, and separation of laboratory strains. For intraspecific variation, in some cases GM is unable to show clear patterns of differentiation, probably due to canalising mechanisms or persistent gene flow among the populations. For interspecific variation, GM is quite useful in separating the great majority of organisms, but when dealing with members of complexes or very closely related species GM must be combined with other biological data to achieve an accurate identification. While some studies have tested the effectiveness of GM in identifying mosquitoes infected with various parasites, more studies are needed to prove this association.

Although GM is a quick and easy-to-use technique, some care must be taken when capturing the data. Erroneous marking of LMs, for example, may result in an overestimated variation between the studied groups; some species have wings with dark spots or many scales that may make it difficult to see these marks. Allometry and asymmetry between right and left wings are other factors that may impair morphometric analysis. Researchers are currently seeking a method to detect LMs automatically and to automatically identify species of Culicidae based on wing vein patterns; such methods already exist for other groups of organisms. Regarding genetic information, very little is known regarding the genes responsible for the wing form in Culicidae;

most studies have focused on Drosophilidae but mosquitoes are likely to follow a similar pattern. Recently, molecular markers and allelic frequencies have been combined with GM data to increase the robustness of analyses. As seen in the examples reported here, GM could provide new tools that improve our understanding of the biology of medically important mosquitoes.

Acknowledgements

References

Adams, D.C., Rohlf, F.J., Slice, D.E., 2004. Geometric morphometrics: ten years of progress following the 'revolution'. Ital. J. Zool. 71 (1), 5–16.

Adams, D.C., Rohlf, F.J., Slice, D.E., 2013. A field comes of age: geometric morphometrics in the 21st century. Hystrix 24 (1), 7–14.

Alto, B.W., Lounibos, L.P., Mores, C.N., Reiskind, M.H., 2008. Larval competition alters susceptibility of adult Aedes mosquitoes to dengue infection. Proc. R. Soc. Lond. B Biol. Sci. 275 (1633), 463–471.

Ayala, D., Caro-Riaño, H., Dujardin, J.P., Rahola, N., Simard, F., Fontenille, D., 2011. Chromosomal and environmental determinants of morphometric variation in natural populations of the malaria vector Anopheles funestus in Cameroon. Infect. Genet. Evol. 11 (5), 940–947.

Aytekin, S., Aytekin, A.M., Alten, B., 2009. Effect of different larval rearing temperatures on the productivity (Ro) and morphology of the malaria vector Anopheles superpictus Grassi (Diptera: Culicidae) using geometric morphometrics. J. Vector Ecol. 34 (1), 32–42.

Benítez, H.A., Sanzana, M.J., Jerez, V., Parra, L.E., Hernández, C.E., Canales-Aguirre, C.B., 2013. Sexual shape and size dimorphism in carabid beetles of the genus Ceroglossus: is geometric body size similar between sexes due to sex ratio? Zool. Sci. 30 (4), 289–295.

Birdsall, K., Zimmerman, E., Teeter, K., Gibson, G., 2000. Genetic variation for the positioning of wing veins in Drosophila melanogaster. Evol. Dev. 2 (1), 16–24.

Blackith, R.E., Reyment, R.A., 1971. Multivariate Morphometrics. Academic Press, London-New York.

Bookstein, F.L., 1982. Foundations of morphometrics. Annu. Rev. Ecol. Syst. 13 (1), 451–470.

Bookstein, F.L., 1996. Biometrics, biomathematics and the morphometric synthesis. Bull. Math. Biol. 58 (2), 313–365.

Bookstein, F.L., 1998. A hundred years of morphometrics. Acta Zool. Acad. Sci. Hung. 44 (1–2), 7–59.

Börstler, J., Lühken, R., Rudolf, M., Steinke, S., Melaun, C., Becker, S., ... Krüger, A., 2014. The use of morphometric wing characters to discriminate female Culex pipiens and Culex torrentii. J. Vector Ecol. 39 (1), 204–212.

Carreira, V.P., Soto, I.M., Mensch, J., Fanara, J.J., 2011. Genetic basis of wing morphogenesis in Drosophila: sexual dimorphism and non-allometric effects of shape variation. BMC Dev. Biol. 11 (1), 32.

Carvajal, T.M., Hernandez, L.F.T., Ho, H.T., Cuenca, M.G., Orantia, B.M.C., Estrada, C.R., ... Watanabe, K., 2016. Spatial analysis of wing geometry in dengue vector mosquito, Aedes aegypti (L.) (Diptera: Culicidae), populations in Metropolitan Manila, Philippines. J. Vector Borne Dis. 53 (2), 127.

Christe, R.O., Wilke, A.B.B., Vidal, P.O., Marrelli, M.T., 2016. Wing sexual dimorphism in Aedes fluviatilis (Diptera: Culicidae). Infect. Genet. Evol. 45, 434–436.

Demari-Silva, B., Suesdek, L., Sallum, M.A.M., Marrelli, M.T., 2014. Wing geometry of Culex coronator (Diptera: Culicidae) from South and Southeast Brazil. Parasit. Vectors 7 (1), 174.

Demirci, B., Lee, Y., Lanzaro, G.C., Alten, B., 2012. Altitudinal genetic and morphometric variation among populations of Culex theileri Theobald (Diptera: Culicidae) from northeastern Turkey. J. Vector Ecol. 37 (1), 197–209.

Devicari, M., Lopes, A.R., Suesdek, L., 2011. Wing sexual dimorphism in Aedes scapularis (Diptera: Culicidae). Biota Neotrop. 11 (2), 165–169.

Dhivya, R., Manimegalai, K., 2013. Wing shape analysis of the Japanese encephalitis vector Culex gelidus (Diptera: Culicidae) at the Foot Hill of southern western Ghats, India. World J. Zool. 8 (1), 119–125.

Dobigny, G., Baylac, M., Denys, C., 2002. Geometric morphometrics, neural networks and diagnosis of sibling Taterillus species (Rodentia, Gerbillinae). Biol. J. Linn. Soc. 77 (3), 319–327.

Dogan, M., Gunay, F., Puggioli, A., Balestrino, F., Oncu, C., Alten, B., Bellini, R., 2016. Establishment of a satellite rearing facility to support the release of sterile Aedes albopictus males. I. Optimization of mass rearing parameters. Acta Trop. 159, 62–68.

Dryden, I.L., Mardia, K.V., 1998. Statistical Analysis of Shape. Wiley.

Duchon, J., 1976. Splines minimizing rotation invariant semi-norms in Sobolev spaces. Constructive Theory of Functions of Several Variables. Oberwolfach, pp. 85–100.

Dujardin, J.P., 2008. Morphometrics applied to medical entomology. Infect. Genet. Evol. 8 (6), 875–890.

Dujardin, J.P.A., Kaba, D., Henry, A.B., 2010. The exchangeability of shape. BMC Res. Notes 3 (1), 266.

Dujardin, J.P., Kaba, D., Solano, P., Dupraz, M., McCoy, K.D., Jaramillo-O, N., 2014. Outline-based morphometrics, an overlooked method in arthropod studies? Infect. Genet. Evol. 28, 704–714.

Dujardin, J.P., Lam, T.X., Khoa, P.T., Schofield, C.J., 2015. The rising importance of Triatoma rubrofasciata. Mem. Inst. Oswaldo Cruz 110 (3), 319–323.

Ernsting, G., Isaaks, J.A., 2002. Gamete production and sexual size dimorphism in an insect (Orchesella cincta) with indeterminate growth. Ecol. Entomol. 27 (2), 145–151.

Fairbairn, D.J., 2007. Sexual dimorphism in the water strider, Aquarius remigis: a case study of adaptation in response to sexually antagonistic selection. Sex, Size and Gender Roles: Evolutionary Studies of Sexual Size Dimorphism. Oxford Univ. Press, Oxford, UK, pp. 97–105.

Fisher, R.A., 1935. The fiducial argument in statistical inference. Ann. Hum. Genet. 6 (4), 391–398.

Francoy, T.M., Fonseca, V.L.I., 2010. A geometric morphometry of wings and an automatic identification of species of bees. Oecologia Australis 14 (1), 317–321.

Francuski, L., Milankov, V., Ludoški, J., Krtinić, B., Lundström, J.O., Kemenesi, G., Ferenc, J., 2016. Genetic and phenotypic variation in central and northern European populations of Aedes (Aedimorphus) vexans (Meigen, 1830) (Diptera, Culicidae). J. Vector Ecol. 41 (1), 160–171.

Galbo, K.R., Tabugo, S.R., 2014. Fluctuating asymmetry in the wings of Culex quinquefasciatus (Say) (Diptera: Culicidae) from selected barangays in Iligan City, Philippines. AACL Bioflux 7 (5), 357–364.

Garros, C., Dujardin, J.P., 2013. Genetic and phenetic approaches to Anopheles systematics. Anopheles Mosquitoes – New Insights Into Malaria Vectors, pp. 81–105.

Gidaszewski, N.A., Baylac, M., Klingenberg, C.P., 2009. Evolution of sexual dimorphism of wing shape in the Drosophila melanogaster subgroup. BMC Evol. Biol. 9 (1), 110.

Gómez, G., Jaramillo, L., Correa, M.M., 2013. Wing geometric morphometrics and molecular assessment of members in the Albitarsis Complex from Colombia. Mol. Ecol. Resour. 13 (6), 1082–1092.

Gómez, G.F., Márquez, E.J., Gutiérrez, L.A., Conn, J.E., Correa, M.M., 2014. Geometric morphometric analysis of Colombian Anopheles albimanus (Diptera: Culicidae) reveals significant effect of environmental factors on wing traits and presence of a metapopulation. Acta Trop. 135, 75–85.

Henry, A., Thongsripong, P., Fonseca-Gonzalez, I., Jaramillo-Ocampo, N., Dujardin, J.P., 2010. Wing shape of dengue vectors from around the world. Infect. Genet. Evol. 10 (2), 207–214.

Hidalgo, K., Dujardin, J.P., Mouline, K., Dabiré, R.K., Renault, D., Simard, F., 2015. Seasonal variation in wing size and shape between geographic populations of the malaria vector, Anopheles coluzzii in Burkina Faso (West Africa). Acta Trop. 143, 79–88.

Hotelling, H., 1933. Analysis of a complex of statistical variables into principal components. J. Educ. Psychol. 24 (6), 417.

Houle, D., Mezey, J., Galpern, P., Carter, A., 2003. Automated measurement of Drosophila wings. BMC Evol. Biol. 3 (1), 25.

Jaramillo, O.N., Dujardin, J.P., Calle-Londono, D., Fonseca-González, I., 2015. Geometric morphometrics for the taxonomy of 11 species of Anopheles (Nyssorhynchus) mosquitoes. Med. Vet. Entomol. 29 (1), 26–36.

Jirakanjanakit, N., Dujardin, J.P., 2005. Discrimination of Aedes aegypti (Diptera: Culicidae) laboratory lines based on wing geometry. Southeast Asian J. Trop. Med. Public Health 36 (4), 858.

Jirakanjanakit, N., Leemingsawat, S., Thongrungkiat, S., Apiwathnasorn, C., Singhaniyom, S., Bellec, C., Dujardin, J.P., 2007. Influence of larval density or food variation on the geometry of the wing of Aedes (Stegomyia) aegypti. Tropical Med. Int. Health 12 (11), 1354–1360.

Kendall, D.G., 1977. The diffusion of shape. Adv. Appl. Probab. 9 (3), 428–430.

Kendall, D.G., 1989. A survey of the statistical theory of shape. Stat. Sci. 87–99.

Kitthawee, S., Rungsri, N., 2011. Differentiation in wing shape in the Bactrocera tau (Walker) complex on a single fruit species of Thailand. ScienceAsia 37, 308–313.

Klingenberg, C.P., 2011. MorphoJ: an integrated software package for geometric morphometrics. Mol. Ecol. Resour. 11 (2), 353–357.

Klingenberg, C.P., McIntyre, G.S., 1998. Geometric morphometrics of developmental instability: analyzing patterns of fluctuating asymmetry with Procrustes methods. Evolution 1363–1375.

Koella, J.C., Lyimo, E.O., 1996. Variability in the relationship between weight and wing length of Anopheles gambiae (Diptera: Culicidae). J. Med. Entomol. 33 (2), 261–264.

Krtinić, B., Francuski, L., Ludoški, J., Milankov, V., 2016. Integrative approach revealed contrasting pattern of spatial structuring within urban and rural biotypes of Culex pipiens. J. Appl. Entomol. 140 (10), 757–774.

Kuclu, O., Aldemir, A., Demirci, B., 2011. Altitudinal variation in the morphometric characteristics of Aedes vexans Meigen from northeastern Turkey. J. Vector Ecol. 36 (1), 30–41.

Larios, N., Deng, H., Zhang, W., Sarpola, M., Yuen, J., Paasch, R., ... Shapiro, L.G., 2008. Automated insect identification through concatenated histograms of local appearance features: feature vector generation and region detection for deformable objects. Mach. Vis. Appl. 19 (2), 105–123.

Laurito, M., Almirón, W.R., Ludueña-Almeida, F.F., 2015. Discrimination of four Culex (Culex) species from the Neotropics based on geometric morphometrics. Zoomorphology 134 (3), 447–455.

Lawing, A.M., Polly, P.D., 2010. Geometric morphometrics: recent applications to the study of evolution and development. J. Zool. 280 (1), 1–7.

Lestrel, P.E., 1981. A Fourier analytic procedure to describe complex morphological shapes. Prog. Clin. Biol. Res. 101, 393–409.

Lorenz, C., Suesdek, L., 2013. Evaluation of chemical preparation on insect wing shape for geometric morphometrics. Am. J. Trop. Med. Hyg. 89 (5), 928–931.

Lorenz, C., Marques, T.C., Sallum, M.A.M., Suesdek, L., 2012. Morphometrical diagnosis of the malaria vectors *Anopheles cruzii*, *An. homunculus* and *An. bellator*. Parasit. Vectors 5 (1), 257.

Lorenz, C., Marques, T.C., Sallum, M.A.M., Suesdek, L., 2014. Altitudinal population structure and microevolution of the malaria vector *Anopheles cruzii* (Diptera: Culicidae). Parasit. Vectors 7 (1), 581.

Lorenz, C., Patané, J.S., Suesdek, L., 2015a. Morphogenetic characterisation, date of divergence, and evolutionary relationships of malaria vectors *Anopheles cruzii* and *Anopheles homunculus*. Infect. Genet. Evol. 35, 144–152.

Lorenz, C., Ferraudo, A.S., Suesdek, L., 2015b. Artificial neural network applied as a methodology of mosquito species identification. Acta Trop. 152, 165–169.

Louise, C., Vidal, P.O., Suesdek, L., 2015. Microevolution of *Aedes aegypti*. PLoS One 10 (9), e0137851.

MacLeod, N., 2002. Phylogenetic signals in morphometric data. Morphology, Shape and Phylogeny. 100, p. 138.

Manimegalai, K., Arunachalam, M., Udayakumari, R., 2009. Morphometric geometric study of wing shape in *Culex quinquefasciatus* Say (Diptera: Culicidae) from Tamil Nadu, India. J. Threat. Taxa 1 (5), 263–268.

Marcondes, C.B., Borges, P.S., 2000. Distinction of males of the *Lutzomyia intermedia* (Lutz & Neiva, 1912) species complex by ratios between dimensions and by an artificial neural network (Diptera: Psychodidae, Phlebotominae). Mem. Inst. Oswaldo Cruz 95 (5), 685–688.

Mardia, K.V., Bookstein, F.L., Moreton, I.J., 2005. Statistical assessment of bilateral symmetry of shapes (vol 87, pg 285, 2000). Biometrika 92 (1), 249–250.

Mitteroecker, P., Gunz, P., 2009. Advances in geometric morphometrics. Evol. Biol. 36 (2), 235–247.

Montoya-Lerma, J., Solarte, Y.A., Giraldo-Calderón, G.I., Quiñones, M.L., Ruiz-López, F., Wilkerson, R.C., González, R., 2011. Malaria vector species in Colombia: a review. Mem. Inst. Oswaldo Cruz 106, 223–238.

Morais, S.A.D., Moratore, C., Suesdek, L., Marrelli, M.T., 2010. Genetic-morphometric variation in *Culex quinquefasciatus* from Brazil and La Plata, Argentina. Mem. Inst. Oswaldo Cruz 105 (5), 672–676.

Morales-Vargas, R.E.M., Ya-umphan, P., Phumala-Morales, N., Komalamisra, N., Dujardin, J.P., 2010. Climate associated size and shape changes in *Aedes aegypti* (Diptera: Culicidae) populations from Thailand. Infect. Genet. Evol. 10 (4), 580–585.

Morales-Vargas, R.E.M., Phumala-Morales, N., Tsunoda, T., Apiwathnasorn, C., Dujardin, J.P., 2013. The phenetic structure of *Aedes albopictus*. Infect. Genet. Evol. 13, 242–251.

Motoki, M.T., Suesdek, L., Bergo, E.S., Sallum, M.A.M., 2012. Wing geometry of *Anopheles darlingi* Root (Diptera: Culicidae) in five major Brazilian ecoregions. Infect. Genet. Evol. 12 (6), 1246–1252.

Mozaffarian, F., Sarafrazi, A., Ganbalani, G.N., 2007. Sexual dimorphism in the wing shape and size of the carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae). J. Entomol. Soc. Iran 26 (2), 61–73.

O'Higgins, P.A.U.L., 2000. Quantitative approaches to the study of craniofacial growth and evolution: advances in morphometric techniques. Linnean Society Symposium Series vol. 20, pp. 163–186.

Palaniswamy, S., Thacker, N.A., Klingenberg, C.P., 2007. Automatic identification of morphometric landmarks in digital images. BMVC, pp. 1–10.

Palsson, A., Gibson, G., 2000. Quantitative developmental genetic analysis reveals that the ancestral dipteran wing vein prepattern is conserved in *Drosophila melanogaster*. Dev. Genes Evol. 210 (12), 617–622.

Pearson, K., 1895. Contributions to the mathematical theory of evolution. III. Regression, heredity, and panmixia. Proc. R. Soc. Lond. 59 (353–358), 69–71.

Pearson, K., 1901. Principal components analysis. The London, Edinburgh and Dublin Philosophical Magazine and Journal 6 (2), 566.

Peruzin, M.C.J., 2009. Análises comparativas populacionais de Culex quinquefasciatus de dois locais do Estado de São Paulo. (Masters thesis). Instituto de Ciências Biomédicas da Universidade de São Paulo, São Paulo, Brazil.

Petersen, V., Devicari, M., Suesdek, L., 2015. High morphological and genetic variabilities of *Ochlerotatus scapularis*, a potential vector of filarias and arboviruses. Parasit. Vectors 8 (1), 128.

Richtsmeier, J.T., Burke Deleon, V., Lele, S.R., 2002. The promise of geometric morphometrics. Am. J. Phys. Anthropol. 119 (S35), 63–91.

Richtsmeier, J.T., Lele, S.R., Cole III, T.M., 2005. Landmark morphometrics and the analysis of variation. Variation: A Central Concept in Biology, pp. 49–69.

Rodríguez-Zabala, J., González, R., Correa, M.M., Gómez, G.F., 2016. Análisis morfométrico de dos poblaciones de *Anopheles* (Anopheles) *calderoni* (Diptera: Culicidae) del suroccidente colombiano. Rev. Mex. Biodivers. 87 (3), 966–971.

Rohlf, F.J., 1990. Morphometrics. Annu. Rev. Ecol. Syst. 21 (1), 299–316.

Rohlf, F.J., 1993. Relative warp analysis and an example of its application to mosquito wings. In: Marcus, L.F., Bello, E., Garcia-Valdecasas, A. (Eds.), Contributions to Morphometrics. Museo Nacional de Ciencias Naturales, Madrid, pp. 131–159.

Rohlf, F.J., 2003. TpsRegr, Shape Regression, Version 1.28. Department of Ecology and Evolution, State University of New York at Stony Brook.

Rohlf, F.J., 2004. TpsDig. Department of Ecology and Evolution State University of New York, Stony Brook, NY.

Rohlf, F.J., 2006. Tps Series. Department of Ecology and Evolution, State University of New York at Stony Brook, New York.

Rohlf, F.J., Archie, J.W., 1984. A comparison of Fourier methods for the description of wing shape in mosquitoes (Diptera: Culicidae). Syst. Zool. 33 (3), 302–317.

Rohlf, F.J., Marcus, L.F., 1993. A revolution morphometrics. Trends Ecol. Evol. 8 (4), 129–132.

Ruangsittichai, J., Apiwathnasorn, C., Dujardin, J.P., 2011. Interspecific and sexual shape variation in the filariasis vectors *Mansonia dives* and Ma. bonneae. Infect. Genet. Evol. 11 (8), 2089–2094.

Schlick-Steiner, B.C., Steiner, F.M., Seifert, B., Stauffer, C., Christian, E., Crozier, R.H., 2010. Integrative taxonomy: a multisource approach to exploring biodiversity. Annu. Rev. Entomol. 55, 421–438.

Sendaydiego, J.P., Demayo, C.G., 2015. Describing Variations in Wing Shapes of *Anopheles flavirostris* Detected Positive and Negative of Filaria Using Relative Warp and Euclidean Distance Matrix Analysis.

Sendaydiego, J.P., Torres, M.A.J., Demayo, C.G., 2013. Describing wing geometry of *Aedes aegypti* using landmark-based geometric morphometrics. Int. J. Biosci. Biochem. Bioinforma. 3 (4), 379.

Sendaydiego, J.P., Gorospe, J.M., Torres, M.A.J., Amparado Jr., R.F., Demayo, C.G., 2014. Describing wing shape among *Culex quinquefasciatus* Say (Diptera: Culicidae) detected positive and negative for filaria using relative warp analysis. J. Med. Bioeng. 3 (1).

Slice, D.E., Bookstein, F.L., 2007. A Glossary for Geometric Morphometrics (Online).

Small, C.G., 1996. The Statistical Theory of Shape. Springer Science & Business Media.

Stephens, C.R., Juliano, S.A., 2012. Wing shape as an indicator of larval rearing conditions for *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol. 49 (4), 927–938.

Sumruayphol, S., Apiwathnasorn, C., Ruangsittichai, J., Sriwichai, P., Attrapadung, S., Samung, Y., Dujardin, J.P., 2016. DNA barcoding and wing morphometrics to distinguish three *Aedes* vectors in Thailand. Acta Trop. 159, 1–10.

Tofilski, A., 2004. DrawWing, a program for numerical description of insect wings. J. Insect Sci. 4 (1), 17.

Vicente, J.L., Sousa, C.A., Alten, B., Caglar, S.S., Falcutá, E., Latorre, J.M., ... Toma, L., 2011. Genetic and phenotypic variation of the malaria vector *Anopheles atroparvus* in southern Europe. Malar. J. 10 (1), 5.

Vidal, P.O., Suesdek, L., 2012. Comparison of wing geometry data and genetic data for assessing the population structure of *Aedes aegypti*. Infect. Genet. Evol. 12 (3), 591–596.

Vidal, P.O., Peruzin, M.C., Suesdek, L., 2011. Wing diagnostic characters for *Culex quinquefasciatus* and *Culex nigripalpus* (Diptera, Culicidae). Rev. Bras. Entomol. 55 (1), 134–137.

Vidal, P.O., Carvalho, E., Suesdek, L., 2012. Temporal variation of wing geometry in *Aedes albopictus*. Mem. Inst. Oswaldo Cruz 107 (8), 1030–1034.

Villegas, J., Feliciangeli, M.D., Dujardin, J.P., 2002. Wing shape divergence between *Rhodnius prolixus* from Cojedes (Venezuela) and *Rhodnius robustus* from Mérida (Venezuela). Infect. Genet. Evol. 2 (2), 121–128.

Virginio, F., Suesdek, L., WingBank: a wing image database (unpublished results).

Virginio, F., Vidal, P.O., Suesdek, L., 2015. Wing sexual dimorphism of pathogen-vector culicids. Parasit. Vectors 8 (1), 159.

Wang, J., Ji, L., Liang, A., Yuan, D., 2012. The identification of butterfly families using content-based image retrieval. Biosyst. Eng. 111 (1), 24–32.

Wilke, A.B.B., de Oliveira Christe, R., Multini, L.C., Vidal, P.O., Wilk-da-Silva, R., de Carvalho, G.C., Marrelli, M.T., 2016. Morphometric wing characters as a tool for mosquito identification. PLoS One 11 (8), e0161643.

Yeap, H.L., Axford, J.K., Popovici, J., Endersby, N.M., Iturbe-Ormaetxe, I., Ritchie, S.A., Hoffmann, A.A., 2014. Assessing quality of life-shortening *Wolbachia*-infected *Aedes aegypti* mosquitoes in the field based on capture rates and morphometric assessments. Parasit. Vectors 7 (1), 58.

Zelditch, M.L., Wood, A.R., Bonett, R.M., Swiderski, D.L., 2008. Modularity of the rodent mandible: integrating bones, muscles, and teeth. Evol. Dev. 10 (6), 756–768.

Zimmerman, E., Palsson, A., Gibson, G., 2000. Quantitative trait loci affecting components of wing shape in *Drosophila melanogaster*. Genetics 155 (2), 671–683.