Scientific Note

Geometric morphometrics for the differentiation of females of the Pipiens Assemblage in Argentina

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Complexes or assemblages of culicid species and subspecies from the southern region of Latin America are difficult to differentiate morphologically (Harbach 2012, Laurito et al. 2017). These complexes include taxa with differences in their vectorial capacity and therefore in their epidemiological importance (Dujardin and Schofield 2004). However, morphological variation within the species and superposition of diagnostic characters based on classical structures often do not allow for a correct identification.

In Argentina, the Pipiens Assemblage includes *Culex pipiens* with its variant *Culex molestus* and *Culex quinquefasciatus* (Harbach 2012). Both species have been incriminated as potential vectors of several arboviruses, including the West Nile virus and the St. Louis encephalitis virus, and have differences in their eco-physiological features and geographic distribution. *Cx. quinquefasciatus*, a tropical and subtropical species, is distributed from the center to the north of Argentina, whereas *Cx. pipiens*, which is a temperate species, is distributed from the center to the south of the country. Between latitudes 30° 36' S (Córdoba Province) and 36° 13' S (La Pampa Province), and between longitudes 57° 57' W (La Plata City) and 64° 48' W (Córdoba Province), both distributions overlap and these species co-exist along with viable hybrids (Almirón et al. 1995, Diez et al. 2012).

Most studies have aimed to differentiate the two above-mentioned species and the hybrids morphologically by using a morphometric index of male genitalia and/or the siphon of immature stages (Vinogradova 2003, Diez et al. 2012). Dehghan (et al. 2016) has proposed to differentiate adult females by traditional morphometry, considering the relative position of subcosta/costa intersection and R2+3 vein bifurcation. However, this method is not completely effective and the geometric shape analysis not only offers a precise and accurate description but also serves the equally important purposes of visualization and interpretation (Zelditch et al. 2004).

Geometric morphometry is a tool that has allowed identification and discrimination between species and species complexes of mosquitoes (Vidal et al. 2011, Laurito et al. 2015). The wings are most used in this tool because of their approximately flat characteristics that minimize the error when locating landmarks in the veins, facilitate the use of homologous points (Bookstein 1991), and their insights into ecological and evolutionary points of view (De Riva et al. 2001, Baylac et al. 2003). The aim of this work was to evaluate, through geometric morphometry, differences in the wing shape of female mosquitoes belonging to the Pipiens Assemblage (*Cx. pipiens* and *Cx. quinquefasciatus*) of Argentina.

Female *Cx. quinquefasciatus* were obtained from several domiciliary sites of two localities, Colonia Aurora (27° 28' S, 54° 31' W) and Eldorado (26° 24' S, 54° 38' W), Misiones Province, Argentina. *Cx. pipiens* females were collected in cemeteries from Puerto Madryn City (42° 45' S, 65° 02' W) in Chubut Province, Argentina. Both species were obtained from the exclusive region of their distribution. We avoided taking samples in areas of a hybrid zone where both species and their hybrids were present (Figure 1).

Immatures of both species were collected from artificial habitats and reared in semi-natural conditions (ambient temperature, with water from larval habitats and fed with yeast solution) until the adult stage. Dead larvae and exuviae of the 4th instars were preserved in 97% alcohol. Taxonomic keys (Rossi et al. 2002) were used for the identification to species. To identify between *Cx. quinquefasciatus* and *Cx. pipiens*, both the geographical origin and the siphonal index (SI) were considered. The SI was calculated as the ratio between the length, measured from the siphon base to the midpoint of the siphon tip, and the siphonal width, measured at the widest point (Brogdon 1981). Species classification was made according to the ranges proposed by Brogdon (1981), S.I range: 3.1-3.7 for *Cx. quinquefasciatus* and S.I range: 4.3-4.7 for *Cx. pipiens*.

A total of 71 dead 4th instar larvae and their exuviae were used to measure the SI, and 44 females were used for geometric morphometrics as follows: 35 larvae and 21 adults from Misiones Province (*Cx. quinquefasciatus* exclusive zone) and 36 larvae and 23 adults from Chubut Province (*Cx.*
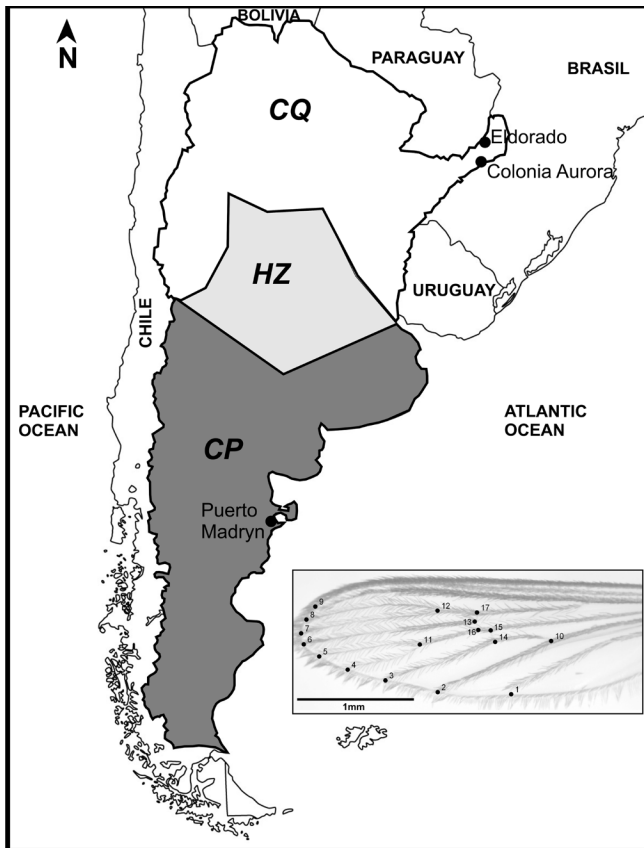
Figure 1. Distribution zones of the species of the Pipiens Assemblage in Argentina by Diez et al. (2012) and collection sites (solid circles). CQ = Distribution of *Cx. quinquefasciatus*, HZ = Hybrid zone and species overlapping and CP = Distribution of *Cx. pipiens*. The insert shows a photo of the wing of *Cx. pipiens* with 17 landmarks used for geometric morphometrics.

pipiens exclusive zone). Whenever possible, larval exuviae and adult traits were analyzed in the same individuals. For morphometric analysis we used those females that were in a better state of conservation after rearing. Digital photos of both the siphons and the left wings were taken with a camera coupled to a stereoscopic microscope. A total of 17 landmarks were selected on each digital image of the wings (Figure 1) and Cartesian coordinates were generated with the software tps-DIG® 2.16. The landmark configurations were transferred, rotated, and scaled according to the Procrustes superimposition method (Bookstein 1991) by using the software MorphoJ® 1.05 (Klingenberg 2011). This allowed generating Procrustes coordinates that were used as shape variables.

The SI means were compared with non-parametric Wilcoxon test for independent samples, using Infostat®statistical software. Only the wing shape (and not size) was analyzed because the shape was more evolutionarily informative and less affected by environmental factors (Dujardin 2008). However, allometry (shape-size relation) was tested by a multivariate linear regression and a permutation test associated with the regression analysis using the null hypothesis of complete independence between the dependent and independent variables (Zelditch et al. 2004). A principal component analysis (PCA) was applied on shape variables. To evaluate differences between shapes of categorized *a priori* individuals, discriminant analysis (DA) and canonical variate analysis (CVA) were performed (Zelditch et al. 2004). We used a permutation test with 10,000 rounds of iterations over Procrustes and Mahalanobis distances. To visualize the relative morphological changes between populations, wing wireframe schemes were used through the MorphoJ® software (Zelditch et al. 2004, Klingenberg 2011). The multivariate
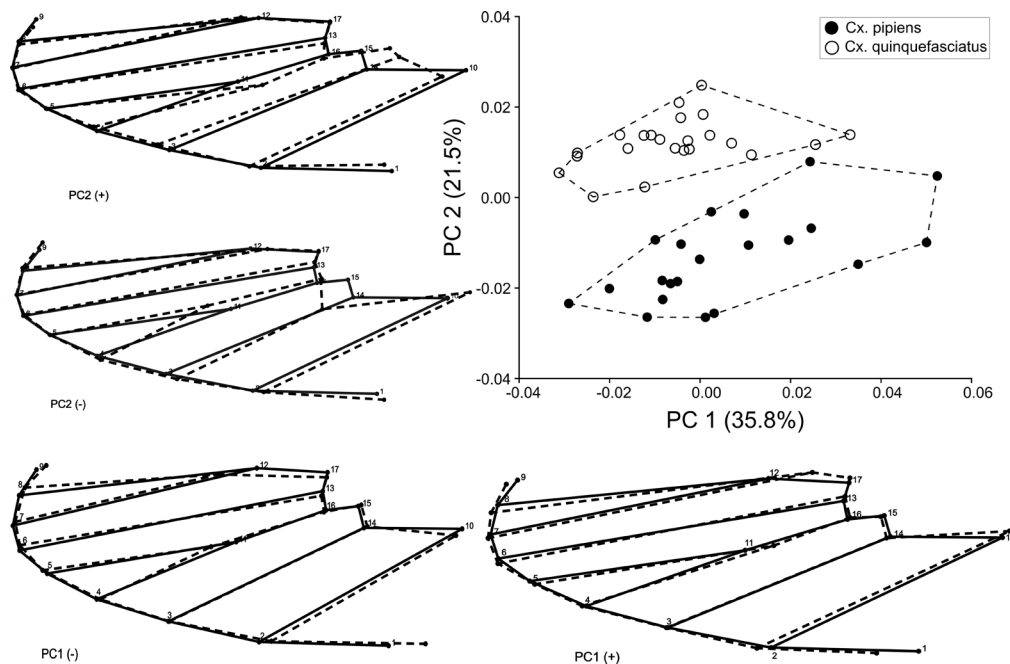

Figure 2. Scatter plot showing the wing shape distribution of *Culex* species in the morpho-space for the first two principal components. Wireframe diagrams in the laterals indicate the trend of the shape change (dotted lines) with respect to the mean shape (continuous lines), along principal components 1 and 2 for both positive (+) and negative (-) values.
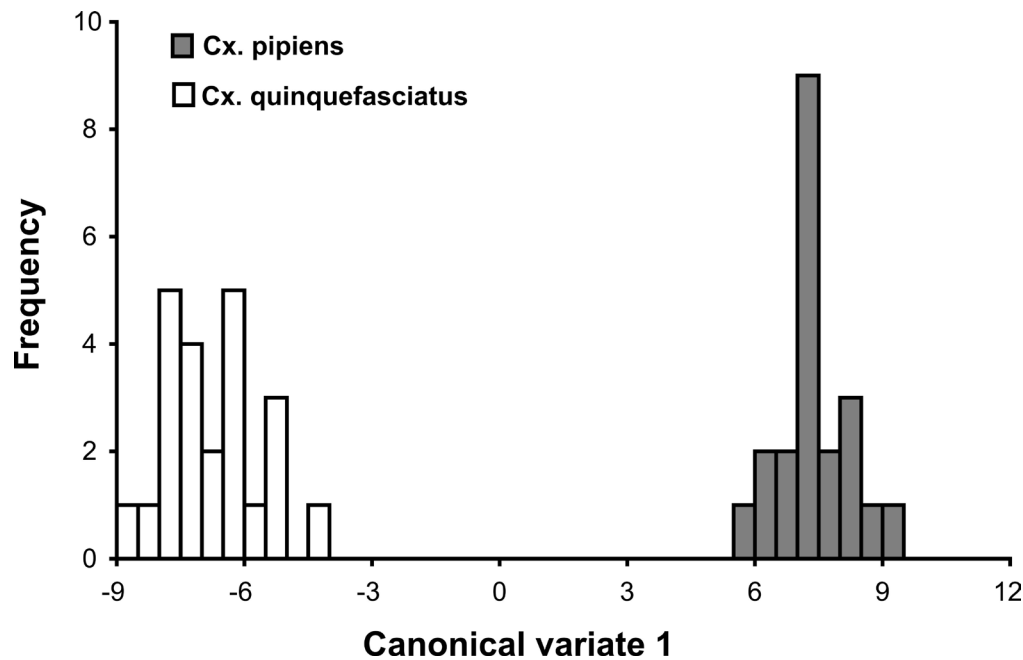
Figure 3. Canonical variate analysis (CVA) for the two samples categorized *a priori*: *Cx. pipiens* and *Cx. quinquefasciatus*. All mosquitoes were correctly classified.

analysis and permutation test were performed with the statistical package included in MorphoJ® (Klingenberg 2011). The level significance for all the statistical tests was 0.05.

The mean SI for samples from Chubut Province (mean±SD = 4.35±0.26; n = 36; min = 3.95; max = 5.16) was higher (Wilcoxon test, W = 2027; p<0.0001) than those from Misiones Province, (mean±SD 3.73±0.20; n = 35; min = 3.38; max = 3.92). The SI support that the individuals, collected from the northern and southern regions of Argentina, belonged to *Cx. quinquefasciatus* and *Cx. pipiens* respectively. Although according to Brogdon (1981), in the case of *Cx. quinquefasciatus,* the mean of siphonal index was at the end of the range.

The allometry test was significant (p = 0.003, r² = 0.09); thus, the residuals of the multivariate regression were used as shape variables not affected by size. The first two components of the PCA accumulated 57.3% of the total variability of shape (PC1: 35.8% and PC2: 21.5%). In the second axis (PC2), the shape of the individuals of *Cx. pipiens* was separated from *Cx. quinquefasciatus* (Figure 2). The wireframe scheme shows that the wings of *Cx. pipiens* along the negative values in PC2 were broader than those of *Cx. quinquefasciatus* along the positive values in PC2. The main relative change was observed in the center of the wing with LMs 11, 14, 15, and 10, and LM 1 in the posterior edge (Figure 2). The discrimination between the two species was successful when the individuals were categorized *a priori* in the CVA (Figure 3). The permutation test was significant for the discrimination, with a Mahalanobis distance of 14.08 and p-value<0.0001 and Procrustes distance of 0.028 and p-value<0.0001. This was consistent with the cross validation of DA, where the misclassified rate was 0% for both species (not shown).

The results indicate that the wing shape of *Cx. pipiens* was different from *Cx. quinquefasciatus* and the application of geometric morphometry allowed differentiating between them. This discrimination at the level of females provides new information that could be complemented with traditional morphological methods. Differences between these species could suggest particular ecological adaptations to the environment within its distribution. In *Aedes albifasciatus*, for example, the presence of morphological variations in the wing for populations that inhabit in contrasting climates seems to indicate an adaptation that favors its active dispersal (Garzón and Schweigmann 2018). Regarding the species studied in the present work, as *Cx. pipiens* remains in diapause during winter (Vinogradova 2003), wide wings could improve their dispersive capacity (Wootton 1992) of this species in its limited active season (summer). In butterflies, females with wider wings than males have a higher individual relative flight force that improves their flight performance and dispersive capacity (Berwaerts et al. 2002). It has been demonstrated that the edges of the wing have a lot of influence in the aerodynamic flight mechanisms (Bomphrey et al. 2017). In contrast, the thinner wings of *Cx. quinquefasciatus* could favor a higher beat frequency in warmer environments with higher average temperatures, as also observed in flies (Azevedo et al. 1998).

The particular wing shape of each species could act or contribute to some degree of sexual barrier in nature due to its recognized function during copulation (Gibson and Russell 2006). However, it has been shown that fertile hybrids exist between sympatric species of the *Cx. pipiens* complex both in Argentina (Almirón et al. 1995) and elsewhere in the world (Harbach 2012). Therefore, the shape of the wing may not contribute as a pre-reproductive barrier directly, although it could contribute to the eco-physiological variants between both species. For example, wider width in the wings of *Cx. pipiens* could favor flight performance in external environments (Berwaerts et al. 2002) since this species would be exophilic, resting outside, and eurygamous, mating in open spaces (Harbach et al. 1985).

Although molecular tools are available to differentiate these species, a simple, low-cost alternative method may be useful in the absence of a molecular laboratory. A rapid identification using this technique between females of these

species is convenient in a context of epidemiological risk or vector surveillance, especially since they differ in their vector competence. On the other hand, it would be interesting to apply this method to study the annual population dynamics in temperate areas where both species overlap and generate hybrids, since the temperature is a limiting environmental factor which affects the distribution of *Cx. pipiens* and *Cx. quinquefasciatus*.

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