

Article

## Relative warp analysis of wing shape variations in three selected populations of *Aedes aegypti* Linnaeus

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### Abstract

Population variations in the dengue vector mosquito, *Aedes aegypti* (Linnaeus) was examined using landmark-based Geometric Morphometric methods of the left and right wings for both sexes from among three locations in the city of Cagayan de Oro, Philippines. Relative Warp Analysis (RWA) was conducted on both wings based on shape scores for the detection of variations within and between the three populations. Canonical variate analysis (CVA) of the relative warp scores yielded Wilk's lambda that were very near zero and Pillai trace that were at or near values of 1 indicating that wing shape scores among the three populations of mosquito had means that were different from each other. Discriminant analysis have shown the three *Aedes aegypti* populations differ significantly (>70% correct classification) based on the male's left and right wings and the females' right wing. The rural population was also correctly classified based on the right wings of both sexes. What is interesting in the results is that all three populations were not correctly classified based on the female's left wing. These indicate that the wings of the rural male and all the female populations of *Aedes aegypti* were asymmetrical in shapes which may be due to genetic, developmental, or as a result of environmental processes and are "probably normally adaptive". These findings strongly demonstrate strong infraspecific variations in wing structures of *Aedes aegypti* at different areas of Cagayan de Oro City.

**Keywords** Warp; Wilk's lambda; Pillai trace; discriminant; CVA

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### 1 Introduction

*Aedes aegypti* has been described to be the most variable of all mosquito species (Mattingly, 1957; Katyal, 1996; Henry et al., 2010; Jupp et al., 1991) recognized as having three forms: a black form, known as *Aedes aegypti* subspecies *formosus*, Walker (1948), a pale form, known as *Aedes aegypti* variety *queenslandensis*, Theobald (1901) and the intermediate form known as *Aedes aegypti aegypti* or the *Sensu strict* or type-form

Mattingly (1957). Recent studies however show these types are impossible to find because in most cases the populations of either kinds are more variable or are more mixed than can be expected (based on studies of field-collected and laboratory grown *Aedes aegypti* from 33 different countries (McClelland, 1974). Analysis showed that current classification schemes of *Aedes aegypti* based on the presence/absence of scales at the 1<sup>st</sup>-2<sup>nd</sup> abdominal tergites and other scaling patterns were inadequate to describe the range of observed variations in the *Aedes aegypti* populations from different countries. The early system of classification did not discriminate infraspecific variations therefore the typological approach to the classification of subspecies as suggested by McClelland (1974) and Powell and Tabachnick (2013) will have to be abandoned. Genetic analyses of polymorphic microsatellite loci from populations across 24 different locations in 13 countries in 5 continents by Brown et al. (2010) have shown that there is high genetic distance between *Aedes aegypti aegypti* (s. str.-type form) and *Aedes aegyptiformosus*. This study however, did not use *a priori* subspecies designation of the collected *Aedes aegypti* samples resulting to the grouping of all specimens collected outside of Africa as one cluster called *pantropical cluster*. Because of the lack of morphological information gathered during the study, the results of these analyses fell short of further defining or redefining the subspecies of *Aedes aegypti*. The authors suggested there should be more detailed morphological studies on *Aedes aegypti*.

In a geometric morphometric study using relative warp analysis of wing shape of *Aedes aegypti*, two relative warps RW1 and RW2 accounted for the highest RW values of *Aedes aegypti* (95.82%) indicating variability in shapes of the wings within the species. Since wings are considered the most excellent structures for studying morphological variations because wing vein intersections provide well-defined landmarks suitable for morphometric studies, these metric properties of the wings provide very precise information for analyzing population variations. This is argued to provide precise quantitative information for the identification of species complexes as well as within-species variations as shown by several studies for other species (Calle et al., 2002; Villegas et al., 2002; Tofilski, 2004; Jirakanjanakit et al., 2008; Demayo et al., 2011; Torres et al., 2013). For *Aedes aegypti*, geometric morphometric methods such as the use of landmark-based analysis has been successfully used for distinguishing different *Aedes aegypti* lines from laboratory-reared populations over many generations (Jirakanjanakit et al., 2008) and for *Aedes* species determination (Vidal et al., 2011, 2012), for determining interspecific overlapping between the two major dengue vectors world-wide, *Aedes aegypti* and *Aedes albopictus* (Henry et al., 2010). The method also was used for showing wing shape changes in other species of insects that are suggestive of genetic drift (Jirakanjanakit et al., 2008), for discrimination of sibling species (Demayo et al., 2011), as well as for showing intraspecific variations (Torres et al., 2013). Thus, in this paper, it is argued that urbanization especially in the city of Cagayan de Oro City, causes environmental alterations that usher population changes in *Aedes aegypti*. This is further aggravated by the fact that with unplanned developments in the city creates “urban-to-rural gradients” as a result of the very little control measures of the disease vector and its transmission of the disease is included.

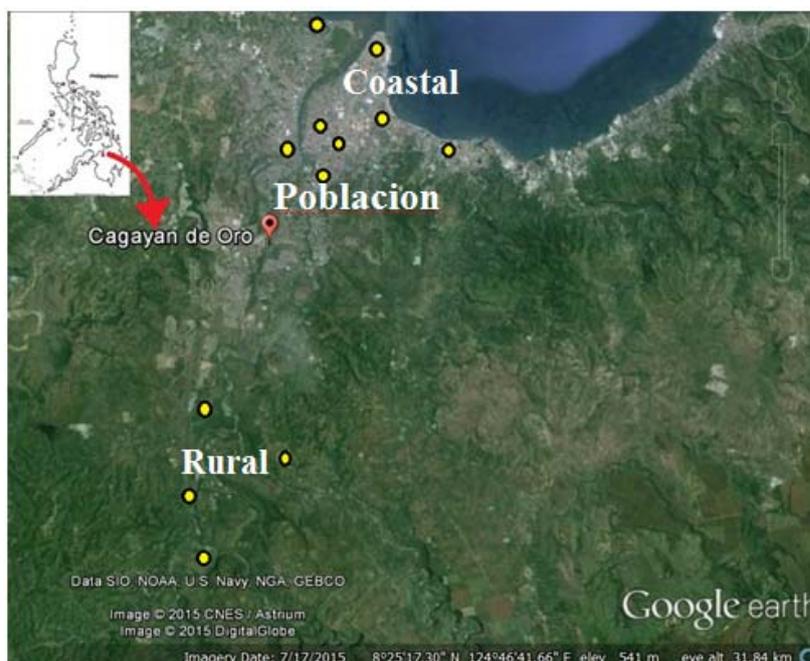
It is hypothesized that considerable variations in the geometry of the shape of the wings of *Aedes aegypti* would be manifested. It was therefore the primary objective of this study to determine population variations in *Aedes aegypti* sampled from different locations in Cagayan de Oro City using relative warp analysis. It was hypothesized that the different populations of *Aedes aegypti* may each have distinct morphometric differences in wing shapes using landmark-based geometric morphometrics.

## 2 Materials and Methods

Specimens of *Aedes aegypti* were collected from 3 ecologically and socioeconomically distinct areas in Cagayan de Oro city (Gualberto et al., 2015) in order to represent what may be distinct *Aedes* populations, namely: coastal, poblacion, and rural (Fig. 1). Although this study did not include analyses of the effects of

microclimatological and socioeconomic factors found in the selected sampling sites, the selection of the sampling sites were based on basic distinctions of these areas that are arbitrarily described as follows:

- A. Coastal areas – these comprises of a narrow 25 km stretch of 11 political territories, called barangays, fronting the Macajalar bay of Cagayan de Oro City. These areas were often subject to desiccating salt-bearing breeze that comes from the sea and temperatures that fluctuate daily due to daily rhythms of land-bound and sea-bound winds in coastal regions. The microclimates found in these areas may pose developmental effects to the *Aedes* mosquitoes. Factories, storage sites (bodegas), small stores and human habitations mostly belonging to families below the poverty line were abundant and squatter areas commonly line some segments of the unclaimed coastal areas.
- B. Poblacion – these were flat inland territories located at the lowland areas of Cagayan de oro that was no more than 10 meters above the sea-level. This area, about 4214 km<sup>2</sup>, was divided into 40 small barangays, and were heavily congested with long-established residences (in the 1960s or before) and stores, malls and markets. Temperatures and humidity were less changeable in these areas except during long periods of dry spells or heavy rains. Most of the residents belong to the middle socioeconomic class with well-maintained water pipe systems
- C. Rural areas – these areas were located in the upland areas of Cagayan de Oro City which were characterized by mountains, hills, plateaus and gorges. The areas are covered with a lot of vegetation and forests with paved and unpaved roads. A good number of high-income households were found among low-income households in the rural areas. The current developments in these areas included production plants and human housing complexes or subdivisions.



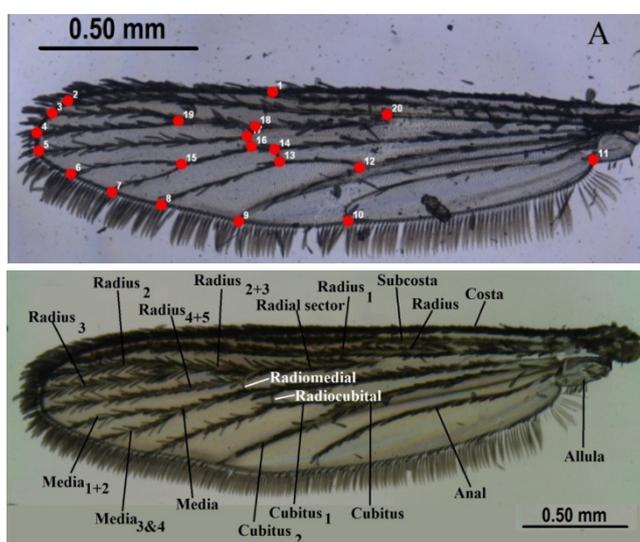
**Fig. 1** Google-earth Satellite image of Cagayan de Oro City and the locations (in yellow dots) of sampling sites in coastal, poblacion and rural areas of the city.

## 2.1 Collection of mosquito samples

Immatures of *Aedes aegypti*, mostly pupae, were collected mainly from tire habitats that were commonly found in vulcanizing shops in the three selected barangays and reared into adults where they are immediately collected and processed. The adult mosquitoes were sorted according to sex and with the use of a binocular stereomicroscope, the left and right wings were removed, mounted and secured with the use of glass slides. A

total of mounted wings from 30 female and male adults were mounted and images of individual wings were captured as jpeg images under 4x objective lens with Leica DM2700 Microscope coupled to a digitizing software LAS EZ 3.0. The jpeg images of left and right wings were then saved in separate folders for geometric morphometric analyses.

For each jpeg images of wings, 20 landmarks in the *Aedes* wings were used following Jirankanjanakit et al. (2008) using tpsDig software (Rohlf, 2004a) (see Fig. 2). The landmarking was done thrice to yield a total of 90 jpeg images of both left and right wings. The raw coordinates of the 20 landmarks were subjected to Procrustes superimposition and thin-plate spline analyses to generate “relative warp” scores (Rohlf, 1998). Affine variation (the “uniform component”), were computed separately, and added to the partial warp scores to constitute the final set of shape variables, i.e. variables describing the displacement of each landmark relative to the consensus wing.



**Fig. 2** (a) *Aedes aegypti* wing with 20 landmarks created from TpsDig2 software, and (b) labelled generalized mosquito wing, image courtesy of WRBU Mosquito Identification software, WRBU.

**Table 1** The designated number and location of landmarks used in the study.

No.	Location of landmark	No.	Location of landmark
1	Junction of costa and subcostal	11	Axillary incision (distal notch of the alula)
2	Distal end of the first branch of radius	12	Posterior point of the mediocubitalcrossvein
3	Distal end of the second branch of radius	13	Anterior point of the mediocubitalcrossvein
4	Distal end of the third branch of radius	14	Forkpoint between M (median vein) and M <sub>3+4</sub>
5	Distal end of radius <sub>4+5</sub>	15	Fork point of the M <sub>1</sub> and M <sub>2</sub>
6	Distal end of M <sub>1+2</sub> branch	16	Posterior point of the radiomedialcrossvein
7	Distal end of M <sub>3+4</sub>	17	Anterior point of the radiomedialcrossvein
8	Distal end of the first branch of the cubitus (Cu1)	18	Forkpoint between R <sub>2+3</sub> and R <sub>4+5</sub>
9	Distal end of the second branch of the cubitus (Cu2)	19	Forkpoint of R <sub>2</sub> and R <sub>3</sub>
10	Distal end of the anal vein	20	Forkpoint of R <sub>1</sub> and R <sub>s</sub> (radius sector)

## 2.2 Analysis of data

### 2.2.1 Landmark-based geometric morphometric analysis

Initially, the x and y coordinates of the 20 landmark points taken from all left and right wing of female and male specimens were used to generate thin-plate spline (tps) files and links files using TpsDig software (Rohlf, 2004a). Separate tps files of coastal, poblacion and rural wing samples were then appended and pooled using tpsUtil software (Rohlf, 2004b). These pooled tps files were then run in tpsRelw(Rohlf,1998) separately to generate the relative warp scores for each female and male *Aedes aegypti* of their left and right wings. Thin-plate spline images were saved from tpsRelw visualization plots that were also generated from pooled tps files for female and male *Aedes aegypti* specimens collected from coastal, poblacion and rural sites in Cagayan de Oro City.

### 2.2.2 Relative warp analysis

In generating the relative warp scores in tpsRelw, the landmark configurations were scaled, translated, and rotated against consensus configuration by General Least Squares – Procrustes superimposition method in 2D. The differences in thin-plate spline ordination plot were then used to describe the scaled wing shape differences between sexes of the two dengue vector species. Only relative warp scores above 5% were described and compared.

### 2.2.3 Multivariate Analysis of Variance

Pooled relative warp scores that were generated in tpsRelw from pooled tps files of coastal, poblacion and rural *Aedes aegypti* populations were run in Multivariate Analysis of Variance (MANOVA/CVA) using Paleontological Statistics, PAST version 2.17c (Hammer and Harper, 2013). But prior to MANOVA, the scores were first tested for normality with Shapiro-Wilk statistics. Shapiro-Wilk statistics that have  $p$  values that are less than the alpha level (0.05) means that the null hypothesis must be rejected and that evidence indicates that data tested are not a normally distributed population (Wikipedia). The wing shape scores were then run in MANOVA. MANOVA is often used to test for differences among groups. It tests whether there are statistically significant mean differences among groups on a combination of dependent variables based on the analysis of the relative warp scores of wing shape in left and right wings of female and male *Aedes aegypti* samples from different geographic locations in Cagayan de Oro City, namely: coastal, poblacion and rural. MANOVA is the multivariate analogue to Hotelling's  $T^2$ . The purpose of MANOVA is to test whether or not the means for two or more groups are sampled from the same sampling distribution. There are two common multivariate test criteria that were used in MANOVA: 1) Wilks' lambda and 2) Pillai's trace. The Wilk's lambda is derived from the comparison of the error variance/covariance matrix and the effect of variance/covariance matrix, determining the relationship between variables. A small Wilk's lambda (close to 0) indicates that the groups are well separated, but a large Wilk's lambda (close to 1) points to the fact the groups of variables are poorly distinguishable from one another. Pillai trace determines the independence between groups of variables. The greater the value of Pillai's trace, the more the given effect contributes to the model. A posthoc test with Hotelling's pairwise comparison were also generated in MANOVA to determine which variable sets were significantly different from each other.

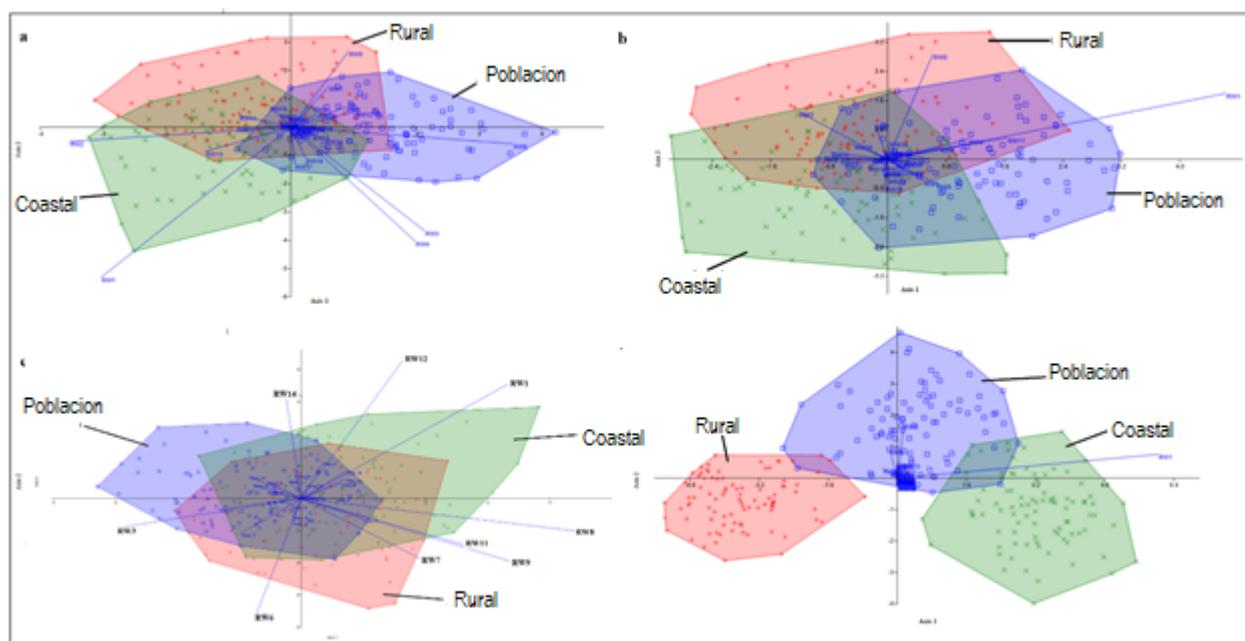
## 3 Results and Discussion

Canonical variate analysis (CVA) of the relative warp scores yielded Wilk's lambda ( $p < 0.05$ ) that were very near zero and Pillai trace that were at or near values of 1 (Table 2). These values implied that wing shape scores among the three populations of mosquito had means that were different from each other. Pillai trace values further supported that there was probable independence between the wing shape scores. As shown in the CVA scatter plot with convex hulls for both sexes showed patterns of clustering that appeared to reflect

geographic differentiation of mean in wing shapes (Fig. 3). As indicated by the results comparing the variations between the 3 populations showed that all the means of wing shape scores from the different areas of Cagayan de Oro City have significant differences (Table 3). These findings strongly demonstrate strong infraspecific variations in wing structures of *Aedes aegypti* at different areas of Cagayan de Oro City.

**Table 2** Wilk’s lambda and Pillai Trace values for the left and right wing shape scores in female and male populations of *Aedes aegypti*.

	Male left wing	Male right Wing	Female left Wing	Female Right Wing
Wilk’s lambda	0.3237	0.3751	0.5927	0.04502
df1	72	72	60	72
df1	464	464	476	464
F	4.883	4.077	2.371	23.93
P(same)	2.408E-26	1.636E-20	2.639E-07	1.23E-116
Pillai Trace	0.8417	0.7729	0.8417	1.486
df1	72	72	72	72
df1	466	466	466	466
F	4.703	4.076	4.703	18.7
P(same)	4.352E-25	1.579-20	4.352E-25	1.021E-98



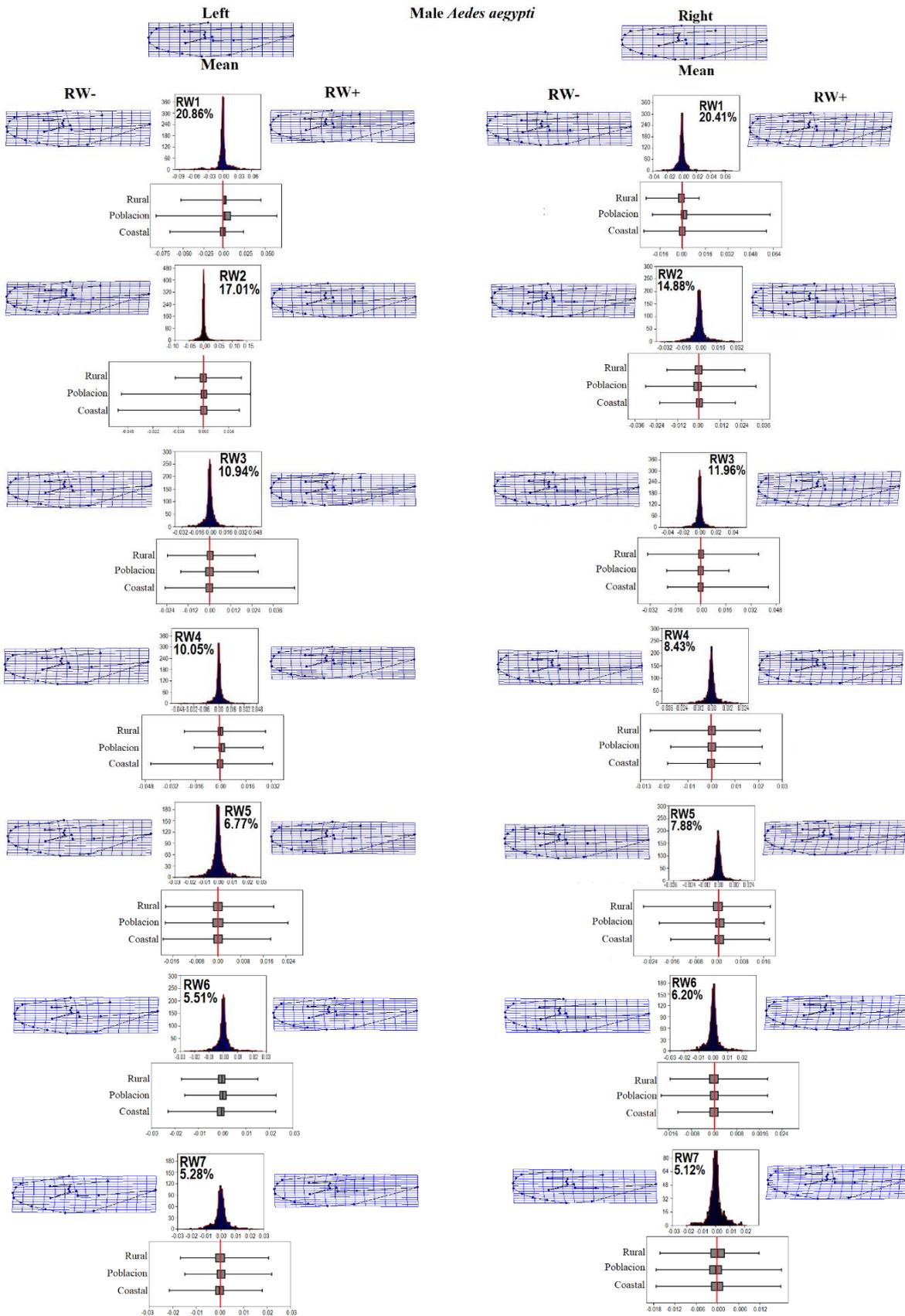
**Fig. 3** Morphological spaces of first two canonical variables (CV) 1 and 2 originated from the comparison of wing shape across all three populations of *Aedes aegypti*.

**Table 3** Pairwise comparisons of scores in the left and right wing shape between *Aedes aegypti* populations.

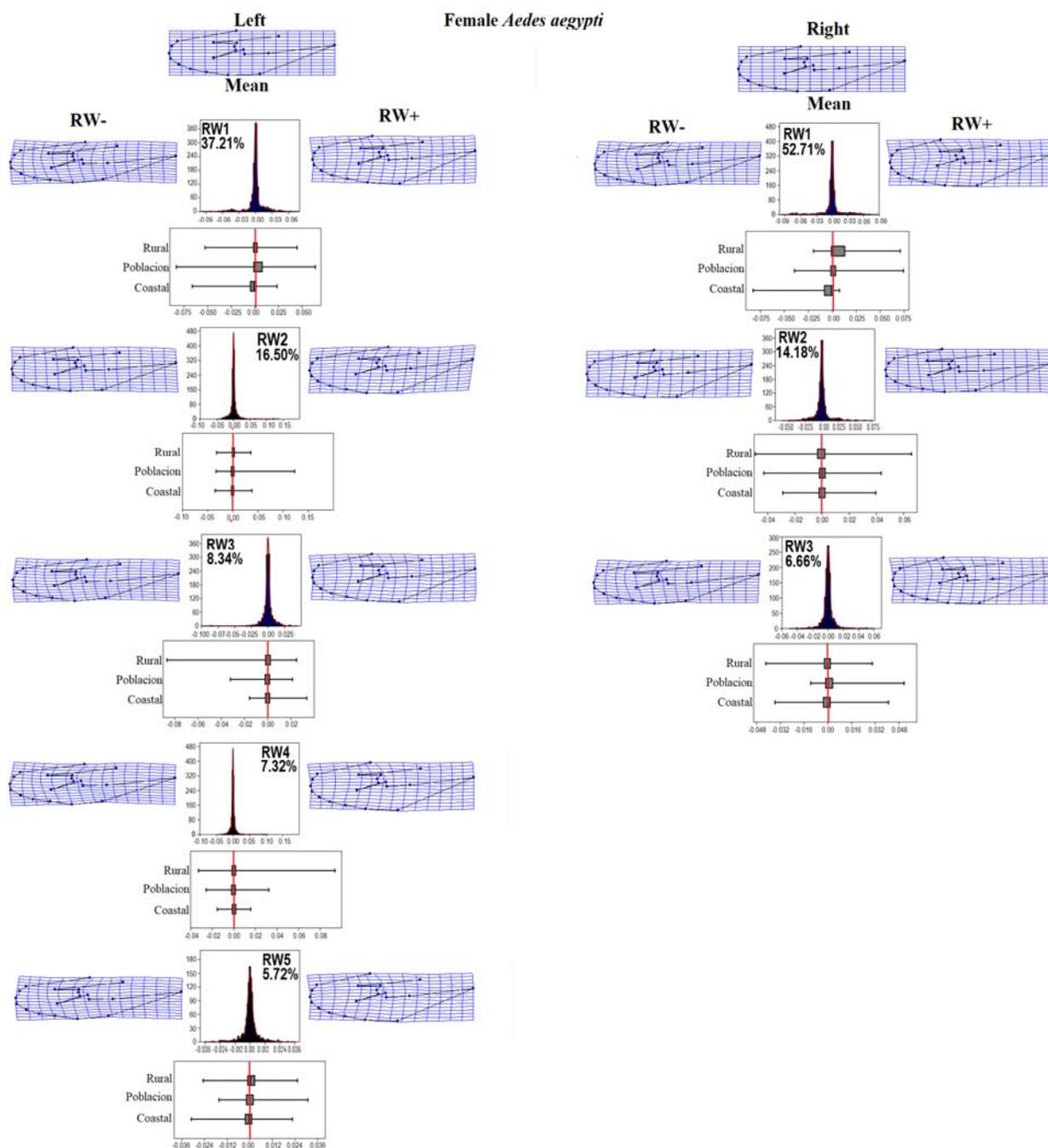
	POBLACION	COASTAL
	Male Left Wing	
Rural	4.35E-13	1.01E-05
Poblacion		2.06E-15
	Male Right Wing	
Rural	1.36E-09	4.38E-07
Poblacion		7.18E-10
	Female Left Wing	
Rural	0.001182	0.080263
Poblacion		1.37E-06
	Female Right Wing	
Rural	1.00E-42	1.29E-62
Poblacion		2.70E-33

The observed variations between the three populations using the visualization plots of vectors generated by tpsRelw for principal components of relative warps for both sexes of *Aedes aegypti* showed that the greatest source of wing shape variation in female *Aedes aegypti* were large displacements of landmarks 1 at the left and right wings (Fig. 4, Table 4). In the right wing of female *Aedes aegypti*, landmark displacements were most pronounced at the landmark 1. All other displacements in landmarks 9, 10, 11, 18 and 19 were all common to both left and right female *Aedes aegypti* wings. These wing changes appear to extend the camber (convex upper surface and concave lower surface) in the left wing more than in the right wings. Cambered wings are said to perform better in flight than flat wings (Combes, 2010). For the male *Aedes aegypti*, landmark displacements were greater in landmarks 1, 10, 12, and 20 of the left wing than the right wing (Fig. 5, Table 4). Landmarks 8, 9 and 10 at the tailing edge of the left male wing of *Aedes aegypti* have vectors that reduce the chord length (distance between the leading edge and tailing edge of the wing).

When the three *Aedes aegypti* populations were compared, the poblacion and coastal populations were significantly discriminated (>70% acceptability) based on the male's left and right wings and the females' right wing. For the rural population, these were discriminated based on the male and female right wings. What is interesting in the results is that all three populations were not correctly classified based on the female's left wing. These indicate that the wings of the rural male and all the female populations of *Aedes aegypti* were not similar in shapes and are asymmetrical which may be genetic, developmental, or as a result of environmental processes (Palmer and Strobeck, 1986; Palmer, 1986, 1992) and are "probably normally adaptive" (Van Valen, 1962; Windig and Nylin, 1999; Graham et al., 1993). The urbanization of the city may have exerted environmental pressures in the populations of the mosquitoes thus affecting their habitats and eventually their development expressed as variations observed in the shapes of their wings (Clarke, 1998; Graham et al., 2010; Leamy and Klingenberg, 2005; Graham et al., 2010). These variations may also have resulted from microevolutionary effects of genetic drift, mutation and or natural selection acting on the populations (Jirakanjanakit et al., 2008; Henry et al., 2010; Stephens and Juliano, 2012); Dujardin et al., 2007; Klingenberg and McIntyre, 1998).



**Fig. 4** The mean wing shape and wing shape variations of left and right male *Aedes aegypti* wings showing relative warps, histogram and boxplots.



**Fig. 5** The mean wing shape and wing shape variations of left and right female *Aedes aegypti* wings showing relative warps, histogram and boxplots.

**Table 4** Descriptions and variations in the landmarked-based points of left and right *Aedes aegypti* wing shape.

Relative warp	Left wing variations	Relative Warp	Right wing variations
FEMALE			
RW 1 (37.21%)	The wings were mostly altered at landmarks 1 (juncture between Costal vein and Subcostal vein) and 11. Ordination plot showed that leading edge of the left female wing is extended due to apical displacement of landmarks 1 and 11. Significant extension may have occurred at leading edge of the female wing.	RW 1 (52.71%)	Large displacements of landmark 1 at the leading edge and slight LM 9 and 10 that are oriented basally compresses the wing against the apically-oriented movements of LM 15, 18, and 19. These displacements contorts the wing at the radio- and medio-cubital veins and extends the camber at the apical edge of the wing..
RW 2 (16.50%)	Greatest displacements are occurring at the landmarks 1 and 11 wherein their vectors are toward each other. Slight apically oriented displacements also occurred at landmarks 8,9 and 10 of the <i>Aedes</i> wing, and a displacement at the landmark 11 found at the base of the wing.	RW 2 (14.18%)	Wing shape variation involves expanding displacements due to uniform directions of the large apically-oriented displacement at LM 1 and similar minor movements at landmarks 15, 16, 17 and 18. LM 6,7,8,9 and 10 at the trailing edge of the wing decreases the chord length of the wing here.
RW 3 (8.34%)	Landmark 1 displacement is opposite to RW 2, its displacement runs in the same direction now as in landmark 11. Considerable movements are also perceptible at LM 7, 8 9 and 10. The greatest expansion occurs at the landmark 7 and 8 at the lower distal region of the wing and at landmark 11 at the wing base.		
RW 4 (7.32%)	Landmark 1 displacement is opposite to RW 2, its displacement runs in the same direction now as in landmark 11. Considerable movements are also perceptible at LM 7, 8 9 and 10. The greatest expansion occurs at the landmark 7 and 8 at the lower distal region of the wing and at landmark 11 at the wing base		
MALE			
Relative warp	Left wing variations	Relative warp	Right wing variations
RW1 (20.86%)	The largest vectors or displacements are in LM1, 20 and 12. LM1 is displaced toward LM20, LM likewise moves against the direction of LM1. A shortening at the middle of wing therefore occurs at the leading edge of the wing. LMs 8, 9 and 10 at the trailing edge of the wing are also displaced anteriorly, thereby increasing camber of the wing and at the same reducing the chord-length of the wing at the midsection.	RW1 (20.41%)	Landmark 13, 14, 16, 17, 19, and 20 are displaced only slight towards the base of the wing. But a relatively displacement at LM 11 at the base of the wing, causes compression of the wing at the middle basal half.
RW2 (17.01%)	Very small displacements of LM1, 13, 14, 16, and 17 are the most obvious vectors in the wing, all of which are distally oriented. These movements slightly elongated the wing span.	RW 2 (14.88%)	The vector units that are most displaced are landmarks 13, 14, 16, and 17 at radio-cubitus and medio-cubitus regions. These displacements are causing expansion at the middle region of the wing shape.
RW 3 (10.94%)	Landmarks 1, 12 and 20 that are in compressing directions. Slight distal displacements of LM 15 and 18 slightly pulls the compression of the midwing distally. LM 9 and 10 anterior movements at the trailing edge of the wing has also increased camber and decreased chord length of the wing shape.	RW 3 (11.96%)	Mainly due to landmarks 12 displacement.
RW 4 (10.05%)	This variation is due to the same displacements of landmarks as in RW 4.	RW 4 (8.4%)	This variation is due to displacements of landmarks 2, 8, 9, 12 and 20. Their relative shearing vectors

			resulted to expansion/dilation near the medio-cubitus vein and the extension of the lower distal edge of the wing.
RW 5 (6.77%)	This variation is due to the same displacements of landmarks as in RW 4..	RW 5 (7.88%)	Landmarks 9 and 10 are the most displaced causing small expansion at the tailing edge of the wing.
RW 6 (5.51%)	This variation is due to the same displacements of landmarks as in RW 4.	RW 6 (6.2%)	The largest displacement in this variant occurs at landmark 9 and 10 at the tailing edge of the wing causing expansion.
RW 7 (5.28%)	This variation is due to the same displacements of landmarks as in RW 4.	RW 7 (5.12%)	The distal displacements of landmark 15 and 18 towards the distal end of the wing results to an expansion at the distal region of the wing shape.

**Table 5** Reclassification of individuals of *Aedes aegypti* across all three populations sampled.

	Male left wing*			
	Rural	Poblacion	Coastal	Total
Rural	62 (68.89)	13 (14.44)	15 (16.67)	90
Poblacion	4 (4.44)	79 (87.78)	7 (7.78)	90
Coastal	16 (17.78)	9 (10.0)	65 (72.22)	90
	Male Right Wing			
Rural	66 (73.33)	10 (11.11)	14 (15.56)	90
Poblacion	10 (11.11)	69 (76.67)	11 (12.22)	90
Coastal	13 (14.44)	9 (10.0)	68 (75.56)	90
	Female Left Wing			
Rural	48 (53.33)	17 (18.89)	25 (27.77)	90
Poblacion	17 (18.89)	60 (66.67)	13 (14.44)	90
Coastal	18 (20.0)	16 (17.78)	56 (62.22)	90
	Female Right Wing			
Rural	88 (97.78)	2 (2.22)	0 (0.00)	90
Poblacion	2 (2.22)	86 (95.56)	2 (2.22)	90
Coastal	0 (0.0)	5 (5.56)	85 (94.44)	90

Note: Values inside the parenthesis are in percentage.

#### 4 Conclusion

Results of this study comparing the three local populations of *Aedes aegypti* using landmark-based geometric morphometric methods particularly relative warp analysis showed all three populations vary in both left and right wings. Based on statistical analysis using CVA of the relative warp scores have yielded low Wilk's lambda and Pillai trace. These were at or near values of 1 indicating that wing shape scores among the three populations of mosquito had means that were different from each other. Discriminant analysis however have shown the three *Aedes aegypti* populations differ only significantly (>70% correct classification) based on the male's left and right wings and the females' right wing. The rural population was only correctly classified based on the right wings in both sexes. These observed asymmetry indicate that the wings of the rural male and all the female populations of *Aedes aegypti* were asymmetrical in shapes which can be argued to be possibly due to genetic, developmental, or as a result of environmental processes and are "probably normally adaptive".

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