Published in final edited form as:

Acta Trop. 2014 July ; 135: 75–85. doi:10.1016/j.actatropica.2014.03.020.

Geometric morphometric analysis of Colombian *Anopheles albimanus* (Diptera: Culicidae) reveals significant effect of environmental factors on wing traits and presence of a metapopulation

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Abstract

Anopheles albimanus is a major malaria mosquito vector in Colombia. In the present study, wing variability (size and shape) in *An. albimanus* populations from Colombian Maracaibo and Chocó bio-geographical eco-regions and the relationship of these phenotypic traits with environmental factors were evaluated. Microsatellite and morphometric data facilitated a comparison of the genetic and phenetic structure of this species. Wing size was influenced by elevation and relative humidity, whereas wing shape was affected by these two variables and also by rainfall, latitude, temperature and eco-region. Significant differences in mean shape between populations and eco-regions were detected, but they were smaller than those at the intra-population level. Correct assignment based on wing shape was low at the population level (<58%) and only slightly higher (>70%) at the eco-regional level, supporting the low population structure inferred from microsatellite data. Wing size was similar among populations with no significant differences between eco-regions. Population relationships in the genetic tree did not agree with those from the morphometric data; however, both datasets consistently reinforced a panmictic population of *An. albimanus*. Overall, site-specific population differentiation is not strongly supported by wing traits or genotypic data. We hypothesize that the metapopulation structure of *An. albimanus* throughout these Colombian eco-regions is favoring plasticity in wing traits, a relevant characteristic of species living under variable environmental conditions and colonizing new habitats.

Keywords

Anopheles albimanus; Microevolution; Geometric morphometrics; Microsatellites; Environment; Phenotype

1. Introduction

Malaria is the most important parasitic vector-borne disease and it is also the third-pathogen-specific cause of human deaths per year worldwide (Hill et al., 2005). Among Latin American malaria vector mosquitoes, *Anopheles albimanus* Wiedemann, 1820 is a dominant species (Sinka et al., 2010) and it constitutes one of the main Colombian vectors (Gutiérrez et al., 2008; Montoya-Lerma et al., 2011; Quiñones et al., 1987) typically found in low coastal lands under 400 m (Fleming, 1986), but it has also been recorded at high altitudes (995–1941 m) (González and Martínez, 2006; Martínez-Palacios and Pletsch, 1963). It is a generalist species (Fuller et al., 2012); immature stages develop in a wide variety of breeding sites and can tolerate a wide range of water conditions such as salinity, turbidity and pollution (Faran, 1980; Henderson, 1948). Adult females exhibit plasticity in blood-feeding behavior, biting humans or animals, indoors or outdoors (Olano et al., 1997), and can fly up to 32 km (Breeland, 1972). Additionally, their populations differ in *Plasmodium* susceptibility, longevity, and insecticide resistance (Cáceres et al., 2011; Collins et al., 1976; Frederickson, 1993; Grieco et al., 2005; Warren et al., 1977).

In Colombia, *An. albimanus* is mainly distributed in the Pacific and Caribbean Coasts that are part of Chocó (CBP) and Maracaibo (MBP) bio-geographic provinces, respectively (Morrone, 2006). Both regions present differences in topography, vegetation cover, forest types, land use and weather conditions (IDEAM, 2001). The Pacific humid tropical forest is one of the rainiest regions globally, characterized by high relative humidity and precipitation. In contrast, the Caribbean dry tropical forest is drier and has higher temperatures (IDEAM, 2001; IGAC, 2002). In these two regions, *An. albimanus* plays a different role in malaria transmission (Gutiérrez et al., 2008). All current evidence supports single taxon status for *An. albimanus* throughout its geographic distribution, with low population genetic structuring and little genetic differentiation among Colombian populations (De Merida et al., 1999; Gutiérrez et al., 2009; Loaiza et al., 2010a,b).

For infected anophelines, flight performance plays a major role in host seeking dispersal, maneuverability and injection of *Plasmodium* sporozoites into the vertebrate host (Dudley, 2000). Therefore, wing morphology (size and shape) is expected to be adapted for an optimum flight performance under different environmental conditions (Norberg, 1995). However, these wing traits not only depend on the genetics but also on the environment, selective pressures and random events at the molecular level (Fusco and Minelli, 2010). Therefore, the exploration of phenotypic and genetic differences in *An. albimanus* populations could shed light on malaria transmission dynamics.

Geometric morphometrics (GM) can provide valuable information on phenotypic variability and population structure with the advantage of being a low-cost and rapid tool (Dujardin, 2011); but despite its proven utility for insect wing analysis there have been very few studies

of *Anopheles* species (Dujardin and Garros, 2013). In GM, the size and shape of the wing are analyzed independently for heritability and environmental sensitivity (Klingenberg, 2010). Different from size, shape provides more reliable indirect information about genetic variation of natural populations exhibiting high and stable heritability (Bitner-Mathe and Klaczko, 1999; Dujardin, 2008; Falconer and Mackay, 1996; Moraes et al., 2004a,b). Consequently, phenotypic data are of interest, especially when analyzed in relation to genetic markers.

We hypothesized that Colombian *An. albimanus* populations vary at the bio-geographical eco-regional level and that some environmental variables (temperature, relative humidity, rainfall, longitude, latitude and elevation) affect wing size and shape. To test this hypothesis, the phenetic structure of *An. albimanus* was evaluated using morphometric traits, and the environmental effect on wing size and shape was determined. Furthermore, wing shape variation was compared to microsatellite data (Gutiérrez et al., 2009), to test for congruence between the phenetic and genetic structure.

2. Materials and methods

2.1. Source of specimens

Specimens were from the Molecular Microbiology Laboratory collection, Universidad de Antioquia; details of collection procedures, identification and bioethics have been published (Gutiérrez et al., 2008, 2009). Specimens represented seven municipalities, four in MBP and three in CBP (Fig. 1 and Table 1). Distances among localities measured applying a straight line, found that the most distant municipalities were Tumaco, Nariño Department (NAR, CBP) and Santa Marta, Magdalena Department (MAG, MBP), separated by approximately 1112 km and the nearest were Moñitos, Córdoba Department (COR) and Turbo, Antioquia Department (ANT), both in MBP, located approximately 142 km apart. Molecular species confirmation of 11% randomly selected specimens used in the morphometric analysis matched the expected restriction pattern for *An. albimanus* (Cienfuegos et al., 2008; Zapata et al., 2007), ensuring accurate taxonomic status.

2.2. Environmental data

Environmental data (temperature, relative humidity and rainfall) for sites in Nariño (NAR), Chocó (CHO), Antioquia (ANT) and Córdoba (COR) were obtained from nearest weather stations of the Instituto de Hidrología, Meteorología y Estudios Ambientales-IDEAM, Colombia; data used corresponded to the previous month to a collection (Barrera et al., 1999). Latitude, longitude and elevation were recorded using GPS or Google Earth®.

2.3. Wing geometric morphometric analysis

2.3.1. Sample preparation, landmark collection and repeatability—The wings of *An. albimanus* females were mounted on microscope slides with coverslips using commercial glue. To avoid possible intra-individual variation interference in the GM analysis (Zelditch et al., 2004), only the right wing was analyzed, photographed using a digital camera (Moticam 2500, Motic China Group Co., Ltd.), coupled to an Olympus® Trinocular Microscope SZ61. Thirteen wing type I landmarks (Bookstein, 1991) located at

vein intersections were digitized for each specimen (Fig. 2). Landmark repeatability was tested in 30 wings selected randomly, digitized twice by the same person (GFG); the measurement error was computed as $1-R$, with R as the repeatability index (Arnqvist and Mårtensson, 1998). The mounted wing slides were deposited in a collection of the Laboratorio de Microbiología Molecular, Universidad de Antioquia, Colombia, and the wing images were deposited in the CLIC image bank (<http://mome-clic.com/clic-bank/>).

2.3.2. Morphometrics data acquisition—For digitized landmarks, raw coordinates were superimposed using a generalized least-squares algorithm. This procedure separates shape information (partial warps) from variation in size, position and orientation (Zelditch et al., 2004). To reduce the dimensionality of wing shape data, partial warps were first subjected to principal component analysis (PCA), and only the principal components (relative warps, RW) accounting for 95% of the total shape variation were used for further analyses. To test whether the projection onto tangent space was a good approximation of the fitted coordinate configurations in Kendall's shape space, the correlation between Procrustes and Euclidean distances was computed.

2.3.3. Size analysis—The size variable was the isometric estimator known as centroid size (CS), defined as the square root of the sum of the squared distances of the landmarks from the center of gravity of a configuration (Bookstein, 1991). CS was used as a measure of overall wing size. Since CS data violated the assumption of normality and homoscedasticity, variation in CS was tested among populations using a nonparametric Kruskal–Wallis test based on 1000 permutations. Post hoc pairwise comparisons following a significant Kruskal–Wallis test were performed using the Bonferroni correction. In addition, we compared CS variances using a non-parametric test based on 1000 permutations (Caro-Riño et al., 2009). The relationship between CS and the environmental variables was explored using a Spearman correlation for the four populations for which data were available. The level of statistical significance was set at $p < 0.05$. Several additional linear regressions were performed to infer size variation across the longitude, latitude and elevation to explore possible clinal variation related to CS. The statistical significance of geographical distances and CS was evaluated by a Mantel test. A generalized linear mixed model (GLMM) was applied to the wing CS data. The R^2 (coefficient of partial determination) values for single-factor models were calculated, followed by the partial R^2 for each environmental variable.

2.3.4. Allometry—The allometric effect or the change in shape associated with size differences (Klingenberg, 1998) was evaluated with a multivariate regression of shape variables onto size. The statistical significance was estimated by non-parametric permutation tests using 1000-runs (Good, 2000). Since a significant allometric effect was found in the dataset, a multivariate analysis of covariance (MANCOVA) was performed to compare allometric trends among populations. The slopes did not differ significantly; therefore, the allometric effect was controlled by computing allometry-free variables.

2.3.5. Shape analysis—A multivariate analysis of variance (MANOVA; 1000 permutations) and pairwise tests were carried out on the landmark data to compare wing

shape among populations. To assess wing shape differences between populations, the allometry-free variables were used as input for a Canonical Variate Analysis (CVA), and to obtain Procrustes and Mahalanobis distances. CVA determines whether the pre-defined seven populations can be statistically distinguished based on the relative warps matrix. The success of the CVA in assigning specimens to populations was determined using a cross-validation procedure in which each specimen is omitted from the initial calculation of the CV axes and used as a test set. The omitted specimen is then treated as an unknown and assigned to the population whose mean is closest using the CV axes (Webster and Sheets, 2010). The shape means for the seven populations were plotted along the two canonical variate axes based on the Procrustes distance matrix.

The statistical significance of the Mahalanobis distances was computed by a nonparametric permutation test (1000 runs) with Bonferroni correction. Correlation among geographic and Mahalanobis distances was examined by a Mantel test (10,000 permutations) using straight line geographic distances between collection sites. The Procrustes distance matrix was used to build an unrooted Neighbor-Joining (NJ) tree. Individual MANOVAs were used to test the effect of environmental variables on *An. albimanus* wing shape. Finally, Q_{ST} values, a quantitative trait analog for F_{ST} values (Miller et al., 2008), were calculated to measure the phenotypic divergence for CS and wing shape variables.

2.4. Combining phenotypic, genotypic and spatial data

Microsatellite genotypes and allele frequencies for each population (Gutiérrez et al., 2009) were used to compute pairwise genetic distances among seven populations with Nei's genetic distances. A NJ tree was built using the Phylip package with 1000 bootstrap replicates (Felsenstein, 2005).

Georeferenced phenotypic and genetic data were integrated with an approach based on a statistical model to decipher the intraspecific structure of Colombian *An. albimanus* populations. This model assumes the existence of several clusters that display homogeneity in terms of genetic and phenotypic variation (Guillot et al., 2012). The dataset, consisting of 280 specimens genotyped at four microsatellite loci (Gutiérrez et al., 2009) and 245 with morphometric data, was analyzed using data combinations of phenotypic (P), genetic (G) and spatial (S) matrices, as follows: (i) the phenotypic data under the spatial model PS, (ii) the genetic data under the spatial model GS and (iii) phenotypic and genetic data under the spatial model PGS. For each scenario, 10 independent Markov chain Monte Carlo runs of 100,000 iterations were performed, discarding the first 10,000 iterations as burn-in (Guillot et al., 2012). The correlation between the phenotypic (Mahalanobis distances) and genetic distances (F_{ST}) was estimated as the coefficient of determination using a linear regression.

2.5. Software

The digitization of landmarks, Procrustes superimposition, and shape and size analyses were performed using the CLIC package (Dujardin, 2008). PAST software was also used for CS comparison through a nonparametric ANOVA, detection of possible outliers and Mantel test (Hammer et al., 2001). The validation of the tangent space was tested in tpsSmall by Jim Rohlf (<http://life.bio.sunysb.edu/ee/rohlf/software.html>). Pairwise geographical distances

between collection sites were calculated using the GPS coordinates in DIVA-GIS (Hijmans et al., 2001). The Phylip package was used to construct the unrooted NJ trees (Felsenstein, 2005). Collection sites were plotted according to geographic coordinates on a map using ESRI ArcGIS® software version 10. MANOVAs were performed in SAS® version 9.0.2 software (SAS Institute Inc., Cary, NC, USA). Additional regressions were computed in PAST. The integration of phenotypic and genetic data under several models was done in Geneland (Guillot et al., 2012).

3. Results

3.1. Geometric morphometrics

3.1.1. Repeatability—Comparison of two digitization sets for the same specimens showed good repeatability for x , y coordinates ($R \approx 0.90$), centroid size ($R = 0.99$) and the relative warps ($R = 0.92$).

3.1.2. Wing size—The morphometric analyses of 245 *An. albimanus* showed a significant difference in CS among populations (Kruskal–Wallis test, $p < 0.0001$), with overlap in their distribution; however, the median CS for the ANT population was slightly significantly larger (Fig. 3 and Table 2). The CHO–BOL comparison was also significant. Pairwise comparison pooling CS data for all MBP and CBP populations did not reveal any statistically significant difference (Mann–Whitney U test, $p = 0.30$). Variance analysis of CS did not detect significant differences between the populations, except for the MAG–NAR pair (Table 2). There was a low but statistically significant correlation between CS and elevation (Spearman rank correlation; $r = -0.27$, $p < 0.0001$), and CS and relative humidity (Spearman rank correlation; $r = 0.32$, $p = 0.0002$). The GLMM analysis including these two variables revealed that only approximately 11.6% of the variance in CS is accounted for by their linear combination.

A linear regression analysis showed that elevation was significant ($p = 0.001$) and negatively related to CS; *An. albimanus* had larger wing size at lower elevation. In contrast, the linear regressions did not show significant trends of CS with latitude ($p = 0.081$) or longitude ($p = 0.524$). Therefore, Bergmann's rule (Shelomi, 2012) was violated.

3.1.3. Wing shape—Regression of Procrustes distances onto the tangent space proved to be a good approximation of the fitted coordinate configurations. The slope of the regression was 0.999 with a correlation of 1. Therefore, the interpretation of results from relative warp morphospace is accurate. The analysis indicated that 22 RWs accounted for the total wing shape variation. RW1 and RW2 overlapped in morphospace and together explained 40.07% of the variation. The first 12 RWs together accounted for 95% of the variation; therefore, they were used for subsequent analysis. Variation in wing shape was depicted as a landmark movement from a consensus configuration using deformation grids (Fig. 4). RW1 axis detected the greatest movement of landmarks (4 and 8) along the medial area of the wing; albeit other landmarks on the edge of the wing and landmark 1 showed movement, and RW2 showed the greatest movement with landmarks 1, 4, 8 and 13.

MANOVA detected significant differences in shape variables among populations (Wilk's lambda: 0.3358, F : 3.827, $p < 0.001$) with all mean wing shape pairwise comparisons producing statistically significant differences ($p < 0.001$), as did all the populations pooled by eco-region (i.e., MBP or CBP) (Wilk's lambda: 0.8050, F : 2.4227, $p < 0.001$).

Discrimination among populations in the morphospace defined by the CV axes showed that the first two CV axes accounted for 67% of the total shape variation. They were projected without evident separation of populations, largely overlapping, however, the CHO locality specimens remained the most distinct (Table 3 and Fig. 5). The majority of pairwise population comparisons using the Mahalanobis distances were statistically significant different ($p < 0.05$), with some exceptions, i.e., population pairs MAG–COR, MAG–BOL, COR–BOL, COR–ANT, all in MBP. Also, NAR (CBP)–COR (MBP) populations were not significantly different. In addition, Procrustes distances comparison yielded similar but non significant results for population pairs COR–BOL and COR–ANT, but differed with respect to comparisons of MAG–BOL and MAG–NAR, also non-significant. Of the above phenetic distance analyses (i.e., Mahalanobis or Procrustes distances) (Table 3), the CHO population was the most divergent. The NJ tree based on Procrustes distances (Fig. 6A), clustered these geographical populations into two main groups: (i) ANT–COR and VDC–NAR, and (ii) BOL–MAG, with CHO separate from both clusters. There was no significant relationship between Mahalanobis and geographic distances (Mantel test, $p = 0.939$), indicating that wing shape is not influenced by distance.

In general, discriminant analysis based on Mahalanobis distances resulted in a poor assignment of *An. albimanus* populations, with the highest correct assignment being for CHO specimens (57.5%), and the lowest for BOL specimens (13.16%) (Table 4). However, pooling the specimens by eco-regions in the supervised classification resulted in correct assignment $>70\%$. Individual MANOVAs revealed a significant effect of all environmental variables on *An. albimanus* wing shape except longitude (Table 5).

3.2. Inferring *An. albimanus* population structure

Nei's distances derived from genetic data clustered the CBP population (NAR, VDC, CHO) separately from the others (Fig. 6B). The ANT population was more related to the CBP cluster than to the MBP populations (ANT, COR, BOL, MAG). Furthermore, MBP populations did not form a unique cluster in the NJ tree.

The dataset of georeferenced genetic and phenotypic markers showed good congruence in the several independent MCMC runs. The analyses, using data combinations in Geneland, produced different numbers of clusters (Fig. 7). The PS and PGS models inferred a single *An. albimanus* population in MBP (Fig. 7A and C), whereas the GS model assigned the ANT population to a different and unique cluster (Fig. 7B). The pattern for CBP populations under the GS and PGS models (Fig. 7B and C) was similar, grouping the CHO and NAR *An. albimanus* populations together and assigning the VDC population to a different cluster. In general, the F_{ST} values among the three clusters identified with the PGS model (Fig. 7C) showed little differentiation (MBP populations vs. NAR–CHO: $F_{ST} = 0.045$; MBP populations vs. VDC: $F_{ST} = 0.030$; VDC vs. NAR–CHO: $F_{ST} = 0.022$). The overall genetic distance F_{ST} was 0.033, and was not related to geographical distance (Mantel test, $p = 0.56$).

(Table 6). The quantitative phenotypic divergence or Q_{ST} value derived from CS was 0.86, and for all shape variables it was 0.52. In addition, linear regression revealed that genetic distances were not correlated with phenotypic distances ($p = 0.52$).

4. Discussion

This study found (i) an overall wing size and shape similarity among *An. albimanus* populations, (ii) a significant effect of environmental factors on wing traits, (iii) a lack of population differentiation using genetic and wing shape data, and (iv) incongruence between morphometric and genetic patterns, not related to geography.

Centroid size was used as a measure of overall wing size differences among *An. albimanus* populations. The slight variation in wing size among populations suggests that either this trait is not a target of strong differential selection or that wing size in *An. albimanus* is well suited to changing environmental conditions (Whitman and Agrawal, 2009). Particularly, the wing size variations detected could result from environmental changes experienced by immature stages during their development in breeding sites (Falconer and Mackay, 1996; Lynch and Walsh, 1998; Okech et al., 2007). For example, food availability and larval competition have been reported as influencing CS variation in adult mosquitoes (da Silva Araújo et al., 2012; Jirakanjanakit et al., 2007; Kweka et al., 2012; Paaajmans et al., 2009; Schneider et al., 2004).

The ANT *An. albimanus* had considerably larger wings than the other populations. Previous reports do not provide a clear consensus on a positive relationship between wing size and body size (Koella and Lyimo, 1996); however, it has been suggested that larger anopheline females have life history traits that in general confer fitness, and in some cases influence the ability to transmit the malaria parasite successfully. Among these traits, higher fecundity (Lyimo and Takken, 1993), longer lifespan (Takken et al., 1998), and an increase their chances of mating (Okanda et al., 2002). Larger females also have the ability to generate an effective immune response (Suwanchaichinda and Paskewitz, 1998) and to express insecticide resistance (Oliver and Brooke, 2013). In this study, the reasons for and implications of the ANT population having larger wings was not determined. Further, for *An. albimanus*, the association of wing CS variation with malaria transmission has not been studied. A complex species-specific pattern of larger mosquitoes related to higher malaria parasite infection has been observed in *Anopheles gambiae*; in contrast, in *Anopheles stephensi* this correlation was not detected (Takken et al., 2013).

Although mean wing shape comparisons revealed significant differences between populations, clear site-specific population differentiation is not evident in this species. However, an eco-regional pattern in wing shape variation was detected when populations were pooled, improving their correct assignment (>70%). At the population level, the morphospace of the first two canonical variables showed that there was no clear-cut separation between the groups, which is in agreement with a high proportion of misassignments and supports the findings of low population structure, as demonstrated by Geneland analysis using phenotypic and spatial data (one cluster). Coincidentally, wing shape changes in *An. albimanus* were in the medial area that was also the most variable area

in *Anopheles artroparvus* (Vicente et al., 2011) and *Anopheles superpictus* (Aytekin et al., 2009). Overall, most populations in MBP (ANT, BOL or MAG) did not differ significantly in mean wing shape, with the exception of COR. Previous studies of genetic variation in *An. albimanus* (Gutiérrez et al., 2009) and other Colombian *Anopheles* species have found contemporary gene flow among various MBP populations (Gutiérrez et al., 2010; Jaramillo et al., 2011; Rosero et al., 2012). Gene flow tends to homogenize the genetic background of populations, including those genes involved in phenotypic traits (Hartl and Clark, 2007), therefore, and as reported (Fusco and Minelli, 2010), the slight wing shape differences detected may be the result of phenotypic plasticity.

Given the complexity and number of environmental factors having an effect on wing traits, results show that the variables evaluated provided relevant information for the Colombian *An. albimanus* populations. Our data indicated that 11.6% of the variance in CS was explained by relative humidity (RH) and elevation. The effect of RH on size, as determined by CS, has been reported for other mosquito models (Morales Vargas et al., 2010; Kennington et al., 2003). Among the other variables, rainfall and mean air temperature did not show any significant effect on CS. This was expected since wing size seems to be more affected by the aquatic microclimate of the immature stage (Loetti et al., 2011; Stephens and Juliano, 2012; Sutcliffe and Benedict, 2012).

Environmental gradients produced by variables such as altitude or latitude are well known to impose spatially varying selective pressures on populations distributed along the gradient (Cheng et al., 2012), as observed for several Dipteran species (Hoffmann and Weeks, 2007; Huey et al., 2000). Thus, the relationship between latitude and CS (Bergmann's rule) was explored, with no findings of the effect of this rule or its inverse, i.e., the countergradient variation pattern (Blanckenhorn and Demont, 2004), acting on *An. albimanus* populations CS. Although in this study the populations spanned the distribution of *An. albimanus* in Colombia, perhaps the latitudinal effect should be reevaluated for a larger geographic area across Latin America. In contrast, a significant negative tendency between CS and elevation was detected, i.e., the *An. albimanus* population with the largest mean wing size was from the ANT collection site located 3 m above sea level.

All the environmental variables except longitude showed a significant effect on wing shape variation. Similarly, significant results were found for *An. funestus* in Africa for elevation, rainfall and temperature and other variables (Ayala et al., 2011). Although the relationship between wing shape and environment remains unclear, one possible explanation is that this phenotypic trait, controlled by numerous genetic factors (Birdsall et al., 2000; Zimmerman et al., 2000), might be sensitive to environmental stressors during development (Klingenberg, 2010). Additionally, seasonal and temporal variations can affect the wing size and shape of insects (Francuski et al., 2011; Schachter-Broide et al., 2009; Vidal et al., 2012). Because the mosquitoes analyzed in this study were caught over a three year period, covering dry (December–February and June–August) and rainy (March–May and September–November) seasons, the effect of time and seasonality cannot be discarded. However, an additional analysis of BOL population specimens collected during both seasons did not show significant differences on wing shape (data not shown), which suggest that

perhaps seasonality is not affecting this trait. On the other hand, the temporal influence on wing size and shape variation for *An. albimanus* remains to be tested.

Differential population histories could have influenced wing morphological evolution in *An. albimanus*. Mitochondrial marker *COI* detected signals of differential population histories for both MBP and CBP, i.e., MBP was more related to Central American than to CBP populations (Gutiérrez et al., 2009; Loaiza et al., 2010b). Congruently, a higher correct classification was obtained for specimens at the eco-regional level (MBP or CBP) than for geographical populations, at the collection site level, indicating that there are slight intraspecific differences in wing shape between these eco-regions. It is worth noting that the different models employed to combine phenotypic, genetic and spatial data did not agree in their clustering pattern. This could be explained because phenotypic traits commonly represent direct targets of natural selection, providing information not obtained with neutral molecular markers. Current evidence supports an *An. albimanus* metapopulation with gene flow among demes (De Merida et al., 1999; Gutiérrez et al., 2009; Loaiza et al., 2010b), i.e., the expectation is that plasticity is favored over local specialization under a broad range of conditions (Sultan and Spencer, 2002). The low F_{ST} values between the clusters suggest that slight wing morphology differences could be reflecting plastic responses more than genetic divergence in *An. albimanus*. These results may imply that *An. albimanus* exhibits plasticity to successfully colonize novel environments, as suggested for other species (Fierst, 2011; Loaiza et al., 2011).

In this study, population relationships inferred from genetic and morphometric data did not agree for several possible reasons. For example, and contrary to the neutral character of microsatellite data, the phenetic pattern could be affected by factors such as differential selection, non-additive genetic variation, differential mutation rates, environmental effects, and regulatory variation (Reed and Frankham, 2001). The finding that $Q_{ST} > F_{ST}$ implies that the degree of differentiation in wing traits exceeds what is expected by genetic drift alone, with natural selection favoring different phenotypes (i.e., wing shape) in the different populations (Pujol et al., 2008; Whitlock, 2008). This is expected because variation in wing traits could modify flight performance and related functions such as the dispersion and success in finding mates in *Anopheles* (Sanford et al., 2011). Further research using common garden experiments will be required to quantify the roles of selection and plasticity on these traits during development (Porcher et al., 2004). The lack of a significant relationship among geographic, genetic and phenetic distances suggests that geographical distance cannot explain the slight wing shape differentiation pattern among *An. albimanus* populations. It is possible that the short flight range of *An. albimanus* (Hobbs et al., 1974) and similar environmental conditions among some localities favor wing shape similarity.

5. Conclusions

In this study, Colombian *An. albimanus* populations showed slight variability in wing size and shape. This is likely a result of current gene flow that favors plasticity of wing traits. Taken together, wing traits and microsatellite data consistently support *An. albimanus* as a panmictic population, despite environmental heterogeneity. However, the effect of environment on wing traits needs further evaluation to elucidate patterns of environmental

gradients acting upon *An. albimanus* across its Neotropical distribution. Further, the geometric morphometric approach constitutes a complementary tool to genetic data that may provide relevant insights into the phenetic structure of medically important vector species such as *An. albimanus*. Integrative approaches, as the one used here, are needed to better understand the complex phenomena that underlie anopheline differentiation processes. Finally, the results of our study expand knowledge of the Colombian *An. albimanus* population structure, information useful to guide malaria control strategies.

Acknowledgments

This work was supported by Comité para el Desarrollo de la Investigación-CODI, Grant No. 8700-689 to MMC and Estrategia para la Sostenibilidad de Grupos 2013–2014, Universidad de Antioquia Grant No. E01719. GFG received financial support for his doctoral studies from Departamento Administrativo de Ciencia, Tecnología e Innovación-COLCIENCIAS, Colombia, Concocatoria 511, 2010. We thank Nelson Naranjo at Laboratorio de Microbiología Molecular-UdeA for microsatellite genotyping, Sara Bickersmith and William Lainhart at the Wadsworth Center's Griffin Laboratory for reading and critical comments on earlier versions of the manuscript. We are grateful to three anonymous reviewers that made valuable comments on the manuscript.

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Fig. 1.

Collection sites for *An. albimanus*. In the Colombian map are depicted the Departments (letter codes) and municipalities (circles) for collection sites. MAG: Magdalena (Santa Marta), BOL: Bolívar (Santa Rosa de Lima), COR: Córdoba (Moñitos), ANT: Antioquia (Turbo), CHO: Chocó (Nuquí), VDC: Valle del Cauca (Buenaventura), NAR: Nariño (Tumaco).

Fig. 2.
Right wing of *An. albimanus*. Numbers represent the position of landmarks. Scale = 1 mm.

Fig. 3.

Wing centroid size distribution for *Anopheles albimanus* females by sampling site. Vertical lines under the quantiles represent specimens. Each box denotes the median as a line across the middle and the quartiles (25th and 75th percentiles) at its ends. Scale is in mm.

Fig. 4. Deformation grids showing the wing shape variation. Visualization of landmark movement using deformation grids for the PCA positive extremes of the RW1 and RW2 axes; there is no magnification.

Fig. 5. Canonical Variate Analysis (CVA) for wing shape of *An. albimanus*. Scatter plot from canonical variate analysis of wing shape for seven *An. albimanus* populations.

Fig. 6. Neighbor-Joining trees. NJ trees obtained from: (A) morphometric [Procrustes' distances] dataset and (B) genetic [Nei's distances]. Numbers at the branches (B) refer to bootstrap values (resampling method available only for genetic data).

Fig. 7. Patterns of population structure inferred using Geneland software. Numbers depict populations assigned to a specific cluster (different colors for the color version). (A) Phenotypic data under the spatial model – PS; (B) genetic data under the spatial model – GS; (C) phenotypic and genetic data under the spatial model – PGS. K: number of clusters. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Data for Colombian *Anopheles albimanus* analyzed in this study.

Bio-geographic province ^a /coast	Department	Municipality (locality sampled)	<i>n</i>	Time of collection month/ year	Longitude	Latitude	Elevation ^b (m)	<i>T</i> (°C) ^c	RH (%) ^c	Rainfall (mm) ^c
Chocó/Pacific	Nariño (NAR)	Tumaco (Alto Guandipa)	36	12/2005	2°29' N	78°26' W	2	25.60	86	1.49
	Valle del Cauca (VDC)	Buenaventura (Puerto España)	37	08/2006	4°02' N	77°26' W	11	–	–	–
Maracaibo/Caribbean	Chocó (CHO)	Nuquí (Nuquí, Panguí)	40	6, 11/2005	5°42' N	77°16' W	5	24.77	93	33
	Antioquia (ANT)	Turbo (Camerún)	34	11/2007	8°08' N	76°43' W	3	27.55	88	3.09
	Córdoba (COR)	Moñitos (Peri-urban areas)	25	09/2005	9°15' N	76°06' W	46	27.86	84	4.64
	Bolívar (BOL)	Santa Rosa de Lima (Ciénaga, El Hatillo)	38	7, 9, 10/2005 03, 06/2006	10°26' N	75°21' W	53	–	–	3.87
	Magdalena (MAG)	Santa Marta (Los Achíotes)	35	08/2005 02/2006	11°15' N	73°36' W	119	–	–	–

^a Bio-geographic province as described by Morrone (2006).

^b Elevation data from Google Earth[®].

^c Average values of the month prior to the collection date.

Table 2

Wing size comparisons between Colombian *An. albimanus* populations.

	MAG	BOL	COR	ANT	CHO	VDC	NAR
MAG	–	0.176	0.324	0.010	0.467	0.147	0.001
BOL	0.064	–	0.879	0.141	0.499	0.882	0.060
COR	0.305	0.559	–	0.154	0.667	0.786	0.075
ANT	0.000	0.000	0.000	–	0.034	0.176	0.718
CHO	0.131	0.000	0.008	0.000	–	0.413	0.011
VDC	0.360	0.289	0.719	0.000	0.007	–	0.085
NAR	0.942	0.021	0.129	0.000	0.051	0.210	–

Comparison of *p* values of variances (above diagonal) and means (below diagonal).

Results in bold denote significance after Bonferroni correction (*p*-value < 0.0024).

Table 3

Mahalanobis and Procrustes distances among mean wing shape configurations of *An. albimanus* populations.

	MAG	BOL	COR	ANT	CHO	VDC	NAR
MAG	–	0.0055	0.0091	0.0098	0.0142	0.0113	0.0076
BOL	1.0034	–	0.0072	0.0083	0.0140	0.0101	0.0092
COR	1.2266	1.0211	–	0.0048	0.0144	0.0104	0.0097
ANT	1.5480	1.5213	0.8596	–	0.0142	0.0119	0.0098
CHO	1.8890	1.8824	2.1039	2.1825	–	0.0174	0.0147
VDC	1.9817	1.6651	1.7995	1.9795	2.1413	–	0.0090
NAR	1.3443	1.4088	1.4483	1.5727	1.8890	1.3360	–

Above diagonal: Procrustes distances; below diagonal: Mahalanobis distances derived from CVA scores computed from 12 RWs. Significant values after Bonferroni correction are in bold.

Table 4

CVA classification matrix for *An. albimanus* with a Jackknife cross-validation.

<i>A priori</i> assignments	<i>A posteriori</i> assignments										
	MAG	BOL	COR	ANT	CHO	VDC	NAR				
MAG	31.43 (11)	20 (7)	5.71 (2)	11.43 (4)	17.14 (6)	5.71 (2)	8.57 (3)				
BOL	23.68 (9)	13.16 (5)	15.79 (6)	10.53 (4)	18.42 (7)	13.16 (5)	5.26 (2)				
COR	12 (3)	16 (4)	16 (4)	28 (7)	12 (3)	8 (2)	8 (2)				
ANT	5.88 (2)	8.82 (3)	26.47 (9)	32.35 (11)	8.82 (3)	8.82 (3)	8.82 (3)				
CHO	2.5 (1)	10 (4)	7.5 (3)	2.5 (1)	57.5 (23)	2.5 (1)	17.5 (7)				
VDC	10.81 (4)	10.81 (4)	5.41 (2)	8.11 (3)	5.41 (2)	43.24 (16)	16.22 (6)				
NAR	13.89 (5)	11.11 (4)	5.56 (2)	5.56 (2)	8.33 (3)	22.22 (8)	33.33 (12)				

Percentages and number of specimens (in parentheses) assigned according to populations using the first 12 RWs derived from allometry-free variables. Percentages of correctly classified specimens are in bold.

Table 5
Results of MANOVAs testing the effect of environmental factors and eco-regions on wing shape of *An. albimanus*.

Factor	Wilks' lambda	F statistics	DF ₁	DF ₂	p value
Elevation	0.1967	3.1	132	1269.8	<.0001
Latitude	0.0217	1.46	594	3446.8	<.0001
Longitude	0.1568	1.12	330	2298.5	0.0826
Temperature	0.1539	2.98	88	433.49	<.0001
Relative humidity	0.1539	2.98	88	433.49	<.0001
Rainfall	0.1539	2.98	88	433.49	<.0001
Eco-region	0.4227	3.33	66	657.84	<.0001

DF: degrees of freedom. Statistically significant values ($p < 0.05$) are in bold.

Table 6

Pairwise F_{ST} values (upper diagonal) derived from microsatellite analysis and geographic distances in km (lower diagonal) for *An. albimanus* populations.

	MAG	BOL	COR	ANT	CHO	VDC	NAR
MAG	–	0.008	0.016	0.036	0.081	0.054	0.084
BOL	211.8	–	0.009	0.013	0.056	0.042	0.063
COR	352.9	155.3	–	0.019	0.049	0.032	0.060
ANT	487.1	296.8	141.6	–	0.010	0.022	0.024
CHO	734.7	564.1	412.0	273.9	–	0.027	0.005
VDC	907.6	748.5	599.0	463.2	190.1	–	0.025
NAR	1112.1	948.2	796.3	657.0	384.2	205.2	–

Statistically significant pairwise F_{ST} values are in bold for $p < 0.05$ after 1000 permutations.