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Wing Shape Analysis of the *Japanese encephalitis* vector *Culex gelidus* (Diptera: Culicidae) at the Foot Hill of Southern Western Ghats, India

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Abstract: A landmark-based morphometric analysis was carried out in female and male wing of *Culex gelidus* mosquitoes collected from Alwarkurichi, Tirunelveli district. In this study, shapes and sizes of wings were studied using the landmark based geometric morphometric method of thin plate spline, multivariate analysis of partial warp scores and centroid sizes. Both female and male wing showed variation in the middle region and distal region of the wing. Principal Component Analysis (PCA) and Canonical Variate Analysis (CVA) also showed variation between female and male wing of *C. gelidus*. It may be concluded from the study that in landmark-based geometric morphometrics, spatial information is contained in the data, because the data are coordinates of landmarks and have many advantages and are useful in the separation of closely related species of insects.

Key words: Mosquito · Thin Plate Spline · Multivariate Analysis · Partial Warp Score

INTRODUCTION

Vectors generally present high degree of morphological similarity at adult stage and many times difficult to identify based solely on examination of adult females using the available morphological keys [1]. In epidemiological studies it is necessary to identify the species of adult females, which are found near humans. Traditionally, morphometric studies have contributed substantially in resolving taxonomic problems in mosquito identification [1-3].

Wings of various species of insects vary in form; often they are membranous, with clear pattern of veins. Wing shape and vein pattern is species specific and is used taxonomically [4] and also in studies of fluctuating asymmetry [5]. Before the shape of a wing can be analysed it should be quantified. This often involves choosing landmarks-vein functions or vein ends. Usually a computer mouse is used to print the landmarks on a computer screen and a program provides the coordinates. Some programs, for example tpsDig is used to obtain any type of landmark [6].

Geometric morphometrics is a powerful tool for capturing the shape characteristics in several morphological aspects, particularly head, wings and genitalia [7]. Insect wings are very appropriate a structure for studies because their two dimensional flattened shape bears several useful landmarks [8, 7]. When suitable measurements and statistical methods were used, the geometric morphometrics studies will contribute in taxonomy research in determining the species and subspecies [9].

The relatively new field of geometric morphometrics represents an important new paradigm for the statistical study of shape variation and its covariation with other variables. The geometric morphometric analysis is very informative concerning biogeographical variability, morphometric discrimination of populations [10].

The field of morphometrics has undergone a rapid change and now provides a solid framework of powerful method to quantify the size and shape of organisms [11, 12]. Applying morphometrics, which traditional morphometric distances has been used in systematics and evolutionary biology, in new developmental contexts opens up a wide and unexplored range for future studies [13].

The landmark based geometric morphometrics method captures shape information more effectively, as it analyzes shapes in ways that preserve their integrity and thus avoids collapsing the shapes into a series of linear

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measurements. Combining geometric morphometric methods with multivariate statistical procedures represents a very powerful tool for testing and graphically displaying differences in shape [14-16].

Wing shape exhibits a high heritability in nature [17, 18] wing morphology is of a primary importance to entomologists interested in systematics. Landmark based geometric morphometric technique is considered to be the most rigorous morphometric method [19, 20] and a useful tool in accurate identification and delimination of numerous insect species [21-23].

MATERIALS AND METHODS

Specimen Collection: The locality selected for the present study was Alwarkurichi, Thirunelveli district, Tamil Nadu. Collection of mosquitoes was done by using sweep nets from 5.30pm to 6.30pm during the study period. The mosquitoes were anesthetized using cotton soaked in 0.5ml of chloroform, which was introduced into the sweep nets and the collected mosquitoes were kept in plastic boxes for morphometric study.Adults collected from the locality were identified morphologically to separate the *Culex gelidus* from other species. Mosquitoes were identified by using the keys of Barraud and Christophers [24, 25]. The morphometric study was carried out using 10 male and 10 female wings each of *C. gelidus* from Alwarkurichi.

Wing Preparation: Each wing was cut off pointing on the articulation with the thorax by a scalpel constituted by a vise holding a sharpened insect pin. The wings thus removed were dry mounted between two microscopic slides. Once fixed, the wings were examined under a microscope to describe venation. The wing of each specimen was photographed using a research trinocular microscope (Nikon Lab Phar-2).

Image Processing: The microscopic slide with wing samples was positioned on Nikon-Optiphot fluorescent and phase contrast stereomicroscope and the wing image was captured using FX-35D, UFX-DX-Nikon-Optiphot. Images of each wing were subsequently digitized and the Cartesians co-ordinates (homologous landmark defined by vein intersection) were recorded automatically using tpsdig software (Version 1.39) [26]. The X,Y co-ordinates were subjected to Generalized Procrustes Analysis (GPA) and subsequently to Thin Plate Spline analysis using tpsrelw software (Version 1.35) and tpsregr (Version 1.26) allowing visualization of shape differences as deformation grid.

Metric Data: Wings are relatively rigid and well preserved structures even after dissection and are suitable structures for land mark data analysis in mosquitoes. Microphotographs of the left and right wing were taken on a microscope at a magnification that allowed maintenance of a consistent plane of focus to control distortion. In Figure 1, we identified 23 possible land marks based on Rohlf and Slice [27] classification. The co-ordinates of landmarks were digitized using tpsdig (Version 1.5, [28].

Size Variation: Wing sizes among the species were compared using the isometric estimator known as "Centroid size" (CTR) and this is derived from co-ordinate data. Standardizing size using CTR allows the computation of shape variables. Because of the isometric nature of CTR, shape variables obtained may still include allometrics [29].

Shape Variation: The raw landmark co-ordinates were first superimposed using a generalized procrustes super position algorithm, where by the sum of squared distances between each object and a reference configuration were iteratively minimized by translations and rigid rotation [30]. The partial warp (Generalized procrustes super position algorithm) scores of these super imposed data were used as shape variables [31].

The Thin plate Spline (TPS) interpolation function derived from the mean of the superimposed data was applied to a squared grid overlaying the mean landmark configuration to provide a direct and quantitative implementation of the D'Arcy-Thompson transformation grids [32].

Analysis: Correlations between the procrustes and tangent shape distances were calculated using tpsSmall [28], to ensure that the amount of shape variation in the original data set was adequately represented after projection in the tangent space. The sample showed perfectly correlated distances ($R^2 = 1.000$), allowing further statistical testing using the projected dataset [28. As a measure of overall size variation of the wings, the centroid size (the square root of the sum of the squared inter landmark distances) was calculated for each population [7, 11, 32, 33]. Centroid size was calculated using tpsRelw [34] and tested for normality using the Shapiro-Wilk test.

For analyzing wing shape variation within and among species, principal component analysis (PCA) and canonical variates analysis (CVA) were conducted on the landmark coordinates data set. PCA as a tool for exploring patterns of variation within population using variancecovariance matrix and CVA for analyzing and testing differences between species. A MANOVA and two-group permutation tests (2000 permutations) were performed and calculated on the landmark coordinates data set using PAST [35] to determine whether geographically separated populations from different countries differ in wing shape.

All statistical analyses were performed in PAST (Paleontological Statistics) version 1.57 [35] and SPSS (version 15.0.1, 2006). Graphical depictions of wing-shape transformations in tpssplin [36] and IMP [37] and of the phenograms were generated in NTSYSpc (Version 2.1, [38].

RESULTS

The dataset consist of 10 wing-specimens of each female and male of *C. gelidus* from Alwarkurichi, Thirunelveli District. There was p = 23 landmarks corresponding to points at which wing veins either branch or intersect the margin of the wing (Fig. 1). The standard nomenclature for the veins and also the positions were based on Rohlf and Slice [27]. Mean wing configuration plotted in deformation grids after procrustes superimposition showed that in females, distances between landmarks 11 and between 12 proportionally lower compared to male.

In tpsrelw program the values of relative warp variations of *C. gelidus* female is 11.99%, 21.48% and 40.41% and that of *C. gelidus* male is 10.27%, 12.08% and 63.03%. The relative warp using $\alpha = 0$ explained the variance among the specimens based on the matrix of singular values, D, from the singular value decomposition of the 'W' matrix. The results obtained using procrustes matrix, $\alpha = 0$ were quite difference from the result of the matrix $\alpha = 1$.

In female, using the tpsregr program the sum of squared procrustes chord residual distance for 10 specimen is Ref d^2 = 0.02060 and Resid d^2 = 0.017990. The sum d2 of predicted fit = 0.002704 and Generalized Goodall F-test is F = 1.2024, df = 42,336; P = 0.1911. In male the sum of squared procrustes chord residual distance for 10 specimen is Ref d^2 = 0.021752 and Resid d^2 = 0.019864. The sum d2 of predicted fit = 0.001896 and Generalized Goodall F-test is F = 0.7635, df = 42,336; P = 0.8562. There was a difference between squared procrustes chord residual distance for female and male wing of *C. gelidus.*

An ordination plot of tpsrelw showed variation in the middle region and distal region of the wing. Regression visualization plot of shape of female and male wing showed variation in the middle region of the wing. The relative warps of female and male wing of *C. gelidus* mosquitoes were illustrated as thin-plate spline in Fig 2 and 3.

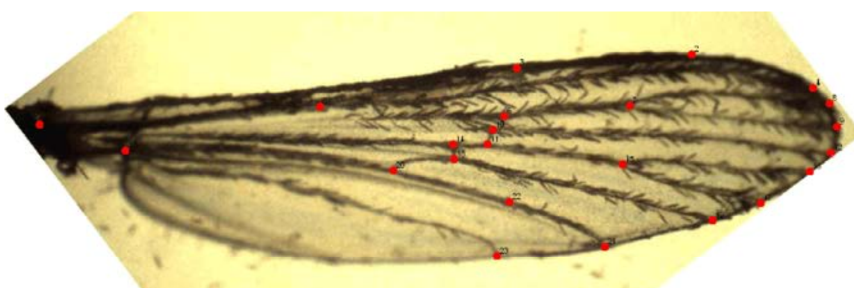

Fig. 1: Landmarks of the right wing of the female mosquito *Culex gelidus*

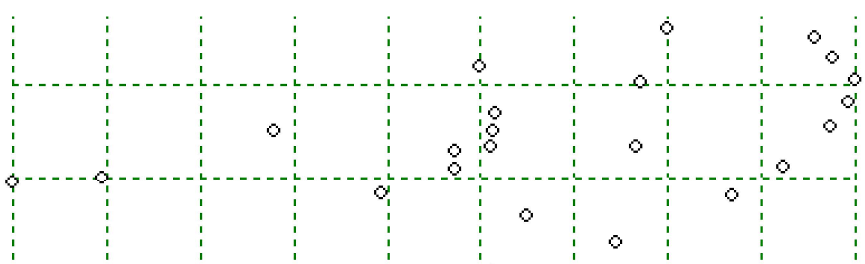

Fig. 2: Thin plate spline of female wing of *Culex gelidus*

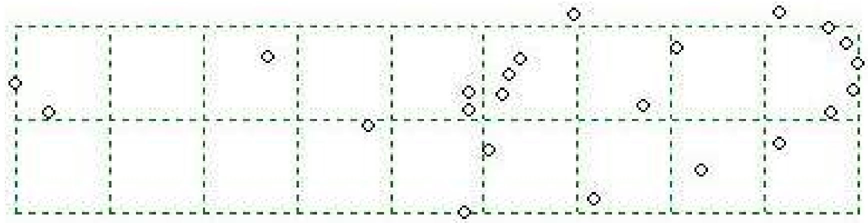

Fig. 3: Thin plate spline of male wing of *Culex gelidus*

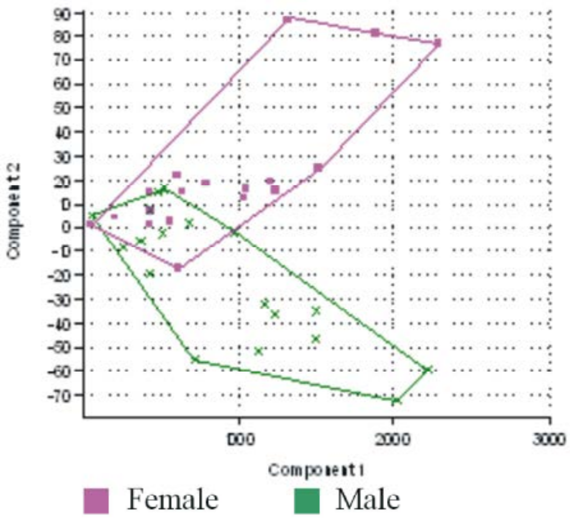

Fig. 4: Principal component analysis scatter diagram of female and male wing of *Culex gelidus*

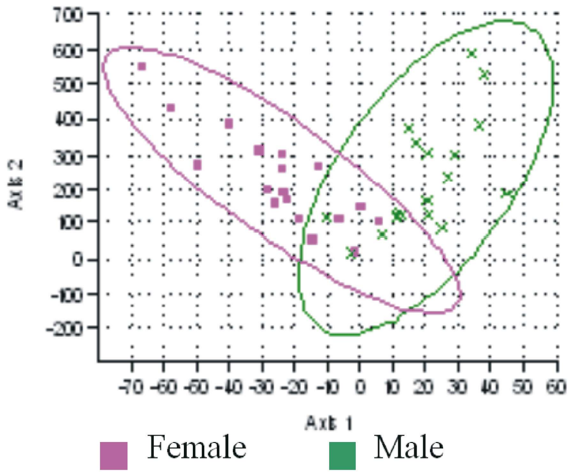

Fig. 5: Canonical variate analysis scatter plot of female and male wing of *Culex gelidus*

Procrustes distance and tangent distance were calculated using the tpssmall program. In female mosquito the mean value for procrustes distance was 0.066436 and tangent distance was 0.066420. In male mosquito the mean value for procrustes distance was 0.063652 and tangent distance was 0.063621.

PCA automatically classifies the relative warps in decreasing order of their specific values. A Multivariate Analysis of Variance (MANOVA) was carried out to see whether any morphological differences were detected between the female and male wing of *C. gelidus*. The PCA scatter diagram of female and male of *C. gelidus* are shown in figure 4 and this also showed variation in female and male wing. The CVA scatter diagram of female and male of *C. gelidus* are shown in figure 5.

DISCUSSION

Generalized Procrustes Analysis (GPA) addresses these issues by translating and rotating each specimen to minimize the squared, summed distances (squared procrustes distance) between corresponding landmarks on each configuration and an iteratively computed mean (or consensus) configuration. Specimens are individually scaled to the same, unit centroid size, the square root of the sum of squared distances of the landmarks in a configuration to their average location [32]. The present study reveals that the shape variation was high in the middle region and distal region of the female wing compared to male wing using the thin-plate spline.

Present results indicated that both female and male wings of *C. gelidus* are distinct. Regarding the wing shape, relative warps revealed a natural arrangement, which was more conspicuous in females. The relative positions of landmarks were also found to be more informative. Similar work was carried out by [1] in which they evaluated the discrimination of adult female of different species of Nyssorhynchus isolated mainly from Southern Colombia, using morphometric analysis. The morphological characteristics of the immature stages allowed the identification of four species of the subgroup Oswaldoi: *Anopheles rangeli, An. oswaldoi, An. benarrochi and An. triannulatus.* Morphometric analysis allowed differentiation of the females of all species using principle component analysis of 10 wing and leg variables, followed by canonical variate analysis.

In another study the wing shape variation with the help of geometric morphometric methods in 17 laboratory strains, was analysed by [39] representing 11 closely related species of the *Drosophila virilis* group. Overall shape estimated using Procrustes coordinates of 14 landmarks was highly variable among strains and very similar in females and males.Using wing geometry as a tool a study on the population structure of dwarf honey bees (*Apis Florea* Fabricius, 1876) in Iran was carried out by [40]. They found that the populations from different states were significantly different for both fore and hind wings. Based on the fore wing shape, all populations were significantly different.

Multivariate analysis of the female and male wing of *C. gelidus* in the present study showed a significant differentiation which was similar to the observation made by [41]. Though the shape analysis of the wing of both female and male was found to be significantly different, the centroid size data showed that female wings are relatively larger in size than the male wing. Similar study was undertaken by [42] in which they examined morphological differentiation between eight populations of *Apis cerana* with worker's wings using multivariate morphometrics. Principle component analysis and factor analysis showed that eight populations differed significantly from one another. A multivariate analysis was performed by [43] in which the morphological variation of six populations of the rice stem borer, *Chilo suppressalis* using 15 and 10 landmarks selected from the fore and hind wing respectively were analysed. The analysis showed a significant difference between the sexes and between the populations of Guilan and Mazandran provinces.

To conclude, the geometric morphometric employed in the present study has some advantages over other tedious taxonomic techniques for separation of closely related species of insects. This technique can also be employed to identify various species in one genus in taxon having problems in dichotomous keys which are currently available. These results suggest that the geometry of the wing contains useful information to identify the possible source of reinfesting specimens

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