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# Fluctuating asymmetry in the wings of *Culex quinquefasciatus* (Say) (Diptera: Culicidae) from selected barangays in Iligan City, Philippines

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**Abstract**. Fluctuating asymmetry (FA) measures deviations from the ideal state of symmetry, and is therefore thought to reflect the level of genetic and environmental stress experienced by individuals or populations during development. It measures the variations from symmetry of the right and left sides of bilateral organisms since, both sides of a symmetrical structure are said to be genetically identical, with similar history of gene activity and experiencing the same environment. It is a most common tool used for measuring developmental instability. In this study, the potential for FA as a biomarker of stress and as an indicator of developmental instability was evaluated in the wings of the common household mosquito, *Culex quinquefasciatus* from three different breeding sites. This is to understand the nature and variation of natural populations of *C. quinquefasciatus* found in Iligan City. The samples consist of 90 individuals (females), 30 from each site. FA of each sample from every site was measured for comparison. In this study, anatomical landmarks were subjected to Procrustes superimposition and Principal Component Analysis (PCA) using "Symmetry and Asymmetry in Geometric Data" (SAGE) program. Results yield highly significant FA in the wings of *C. quinquefasciatus* collected from the three barangays. This implies that species present in the three areas may exhibit inability to buffer stress in its developmental pathways and manifest increase level of FA hence, have possible implications on species fitness and its status as a vector.
**Key Words**: environmental stress, genetic stress, developmental instability, bilateral organisms, biomarker.

**Introduction**. Developmental stability is defined as the ability of an organism to moderate its development against genetic or environmental conditions and produce the genetically determined phenotype. Fluctuating asymmetry (FA) are fine and random deviations from perfect symmetry of organism's morphology. It is considered a reliable factor for measuring developmental stability because it reflects both genetic and environmental stress and this has been an important theory in evolutionary biology for decades (Palmer 1994). FA is normally distributed differences, about a mean of zero, between the left and right sides of the organism (Valen 1962). It provides an estimate of developmental "noise" (Waddington 1942) that has been used as a measure of developmental stability and to assess the influence of environmental and genetic stress on development (Palmer & Strobeck 1986). In general, increased fluctuating asymmetry is associated with increased homozygosity or environmental stress; however, exceptions to these relationships are common and few studies directly associate fluctuating asymmetry, selection, fitness and evolutionary change. Interspecific hybrids generally have significantly greater fluctuating asymmetry than members of parental populations, despite the inverse relationship between the degree of fluctuating asymmetry and percentage of protein heterozygosity and the higher heterozygosity levels in interspecific hybrids. Increased heterozygosity suggests higher fitness (Merila & Bjorklund 1995).

In addition, the quest for easily measured biomarkers has resulted in the investigation of asymmetry of morphological characters as a possible biomarker for stress and the most widely used measure of asymmetry is FA. It is perceived that FA measures the capacity of the organism to buffer its developmental pathways against any environmentally derived and genetic stressors. It is believed that the presence of either of the said stressors during ontogeny may impair the effectiveness of these buffering mechanisms. This may affect normal developmental process and could be manifested as increase levels of FA of an otherwise bilaterally symmetrical character (Mpho et al 2000). There is a direct relationship between FA and developmental instability

Herewith, FA was investigated for *Culex quinquefasciatus*, an important vector of periodic filariasis in some parts of the world (Belkin 1968). In the Philippines, there were many recorded incidence relating to mosquitoes particularly, in Misamis Oriental, Mindanao. However, there were no studies pertaining to the nature and variation of *C. quinquefasciatus,* populations found in Iligan City. These mosquitoes were said to be ubiquitous as for its selection of breeding sites. They were considered as strongly anthropophilic and can also be harmful to humans. They usually breed in organically rich and polluted surface waters or artificial containers (Weinstein et al 1997). Breeding sites include shallow ponds within streams (Derraik 2005) and artificial habitats such as drains and drain sumps, wells, oxidation ponds at sewage treatment plants (Derraik & Slaney 2005) stock drinking troughs, septic tanks, rain water containers, tires and various other small containers (Lee et al 1989). They were also found to be utilizing the same container for breeding as other species.

*C. quinquefasciatus,* is known to carry and transmit *Wuchereria bancrofti* to some degree of efficacy in many regions of the globe. *W. bancrofti* is a filarial nematode that can cause lymphatic filariasis. Currently, worldwide there are approximately 120 million cases of lymphatic filariasis. The mosquito picks up the microfilaria from an infected vertebrate. The nematode develops inside the mosquito, and is passed on to another vertebrate (Foster & Walker 2002). Besides, this species also is suspected to be a vector of dog heartworm for cats in Rio de Janeiro (Labarthe & Guerrero 2005) West Nile virus and Saint Louis encephalitis virus (Flores et al 2010). In the Philippines, *W. bancrofti* and *Brugia malayi* caused the lymphatic filariasis. It is endemic in 40 provinces in the Philippines; 76% of the municipalities in these provinces are considered poor. In 2005, of the 645,232 cases of lymphatic filariasis infection reported, 56% were in Mindanao (WHO 2014). Studies have reported that the vectorial status of *C. quinquefasciatus* is complex and changing (Hamon et al 1967; Pedersen 2008) such that, variation among natural populations of *C. quinquefasciatus*is are associated with different vectorial capacities (Morais et al 2002). Thus, it is important to study the nature and the variation among natural populations.

In this regard, this study was done to assessed the potential for FA as a biomarker of stress and determine developmental instability in the wings of *C. quinquefasciatus* collected from selected barangays (San Miguel, Palao and Del Carmen) in Iligan City. The wings were investigated because it has contributed much to the unparalleled success of insects. In the recent years, much interest has also been devoted to the determination and examination of FA as an indicator of individual quality. Here, a hypothesis assumes that fluctuating asymmetry may reflect quality of individuals. Thus, this study provides information on the nature and variation of natural populations of *C. quinquefasciatus* found in selected barangays in Iligan City. Results of FA in the wings may imply the state of adaptation and co-adaptation of *C. quinquefasciatus.* Data obtained on the nature and population status of *C. quinquefasciatus* may help in disease control initiatives of local government agencies in the Philippines.

**Material and Method**. Adult mosquitoes were collected from housing units in San Miguel, Palao and Del Carmen, Iligan City. Collection of adult mosquito samples was done using aerial nets. The adult mosquitoes that were trapped inside the net were transferred into plastic bottles for easy transport to the laboratory. Sampling was done early in the morning and late afternoon. The collected adult mosquitoes were sorted and identified in the laboratory using a stereoscope and digital microscope. Only female *C. quinquefasciatus* adults were utilized in this study. Only 30 individuals from each site were used.

The wings of identified *C. quinquefasciatus,* were gently detached from the thorax using forceps, placed on a glass slide and secured with another glass slide to cover.

Wings were photographed under a dissecting microscope with consistent magnification, and the digital images were kept on file for data analysis. Identification of *C. quinquefasciatus* was done through existing protocols on illustrated keys presented for identification of female (culex) mosquitoes.

For morphometric analysis, eighteen anatomical landmarks in the wings of female *C. quinquefasciatus* were used. Landmarks were digitized using TPSdig software. Descriptions of identified landmarks are presented in Table 1. The landmarks were placed at intersections of wing veins or intersections of veins and the wing margin (Figure 1).

Table 1

Description of assigned landmarks on *Culex quinquefasciatus* wings

| *Landmark #* | *Description of landmark* |
|---|---|
| 1 | intersection of costa (C) |
| 2 | distal end of the radius (R) |
| 3 | radial branch 2 |
| 4 | radial branch 3 |
| 5 | distal end of radial branches 4 & 5 |
| 6 | distal end of M1 & 2 |
| 7 | distal end of M3 & 4 |
| 8 | distal end of cubital vein 1 |
| 9 | distal end of cubital vein 2 |
| 10 | Anal vein |
| 11 | origin of cubital 1 |
| 12 | midpoint branch of cubital 3 |
| 13 | medio-cubital cross vein |
| 14 | midpoint branch of medial vein |
| 15 | radio-sectoral vein |
| 16 | radio-medial cross vein |
| 17 | midpoint branch of radial vein |
| 18 | origin of radius branches 2 & 3 |

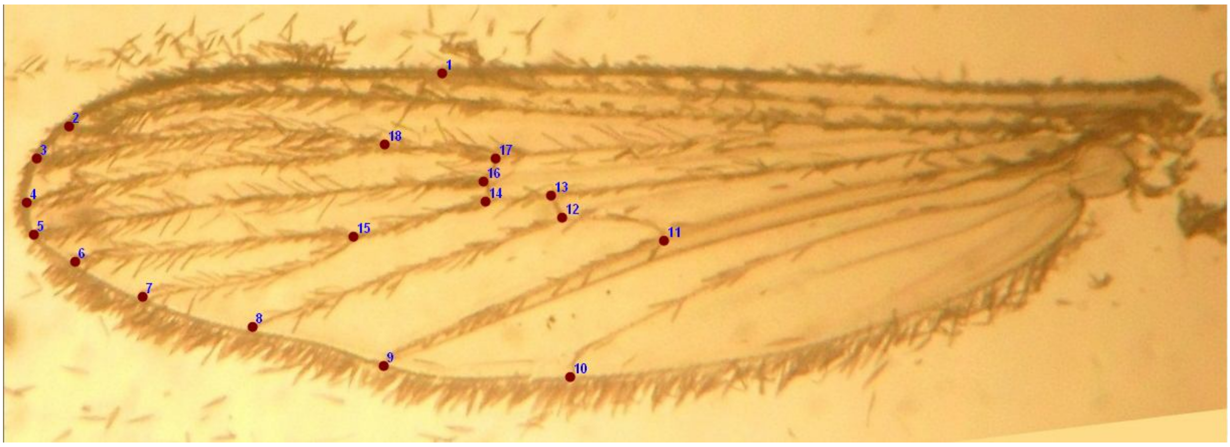

Figure 1. Location of eighteen landmarks in the wing of *Culex quinquefasciatus.*

The levels of FA were measured using the "Symmetry and Asymmetry in Geometric Data" (SAGE) program, version 1.0. This software analyzed the x- and y-coordinates of landmarks per individual. Procrustes superimposition analysis is performed with the original and mirrored configurations simultaneously. The least squares Procrustes consensus of set of landmark configurations and their relabelled mirror images is a perfectly symmetrical shape, while FA is the deviation from perfect bilateral symmetry. The squared average of Procrustes distances for all specimens is the individual contribution to the FA component of variation within a sample. To detect the components of variances and deviations, Procrustes ANOVA was used (Marquez 2006; Klingenberg et al 1998).

Principal Component Analysis (PCA) as an important step in geometric morphometrics was also performed in this study as a tool to understand overall patterns of shape variation and as a means for producing mathematically uncorrelated shape variables to use in subsequent analyses of wings. Even though PCA shape variables are mathematically uncorrelated, they may be phylogenetically correlated when shape is sampled in populations or species that are phylogenetically structured.

PCAs of the covariance matrix associated with the component of FA variation were performed for each population to carry out an interpolation based on a thin-plate spline to visualize shape changes as landmark displacement in the deformation grid (Marquez 2006).

**Results and Discussion**. The sampling sites were found to have suitable water available for breeding of the filarial vector *C. quinquefasciatus*. Often open cesspits, drains and temporary water bodies were present in the areas and were considered by these adult mosquitoes as preferred breeding sites. It was observed that they were also found to be utilizing the same container for breeding as other species. Such that, they were found along with *Aedes aegypti.* Three 3 out of 40 adult mosquitoes collected in San Miguel, 5 out 39 in Palao and 5 out of 38 in Del Carmen were *A. aegypti*, the rest were *C. quinquefasciatus.* Only female individuals were utilized for the study.

It was observed that adult *C. quinquefasciatus* vary in length from 3.9 to 4.3 mm. The color is brown with the proboscis, thorax, wings, and tarsi darker than the rest of the body. The head is light brown with the lightest portion in the center. The antennae and the proboscis are about the same length, but in some cases the antennae are slightly shorter than the proboscis. The flagellum has thirteen segments that have few to no scales (Lima et al 2003; Sirivanakarn & White 1978). The scales of the thorax are narrow and curved. The abdomen has pale, narrow, rounded bands on the basal side of each tergite. The bands barely touch the basolateral spots taking on a half-moon shape (Darsie & Ward 2005). Both males and females take sugar meals from plants. Then, following mating, the female seeks a blood meal. *C. quinquefasciatus* are considered as opportunistic feeders, feeding on mammals and/or birds throughout the night. Males survive only on sugar meals, while the female will take multiple blood meals. Thus, only female individuals were utilized for this study. After a female mosquito digests the blood meal and the eggs develop, she finds a suitable place to lay her eggs, and the cycle begins again (Gerberg et al 1994).

There are three types of biological asymmetry: fluctuating asymmetry, directional asymmetry, and antisymmetry. Fluctuating asymmetry is characterized by small random deviations from perfect bilateral symmetry. These small random deviations result in a normal or leptokurtic distribution of asymmetry around a mean of zero. Directional asymmetry is characterized by a symmetry distribution that is not centered around zero but is biased significantly, towards larger traits either on the left or the right side. Antisymmetry is characterized by being centered around a mean of zero. Directional symmetry and antisymmetry are developmentally controlled and therefore likely to have adaptive significance while fluctuating asymmetry is not likely to be adaptive as symmetry is expected to be the ideal state. An underlying assumption of fluctuating asymmetry analysis is that the development of the two sides of a bilaterally symmetrical organism is influenced by identical genes and, therefore, non-directional differences between the sides must be environmental in origin and reflect accidents occurring during development (Palmer 1994; Valen 1962; Gangestad & Thornhill 1999). Fluctuating asymmetry of the right and left wings of female mosquitoes were assessed through Procrustes method. The levels of FA were obtained using the “Symmetry and Asymmetry in Geometric Data” (SAGE) program, version 1.0. This software analyzed the x- and y-coordinates of landmarks per individual. Sides (directional asymmetry; DA), Individual x sides (fluctuating asymmetry; FA), and their respective error were included as effects. Procrustes superimposition analysis was performed with the original and mirrored configurations of the wings simultaneously using the SAGE program. This software analyzed the coordinates of the landmarks per individual, using a configuration protocol for both wings. The least squares Procrustes consensus of set of landmark configurations and their relabelled mirror images is a perfectly symmetrical shape, while FA is the deviation from perfect bilateral symmetry (Marquez 2006; Klingenberg et al 1998). The squared average of Procrustes distances for all specimens is the individual contribution to the FA component of variation within a sample. To detect the components of variances and deviations, a Procrustes ANOVA was used. The ANOVA used most frequently for fluctuating asymmetry is a two-way, mixed-model ANOVA with replication. The main fixed effect is *sides* (*S*), which has two levels (left and right). The block effect is *individuals* (*I*), which is a random sample of individuals from a population. The *sides by individuals interaction* (*S x I*) is a mixed effect. Finally, an error term (*m*) represents measurement error (replications within *sides by individuals*). The effect called *sides* is the variation between the two sides; it is a measure of directional asymmetry. The effect called *individuals* is the variation among individual genotypes; the *individuals* mean square is a measure of total phenotypic variation and it is random. Meanwhile, the *individual by sides interaction* is the failure of the effect of individuals to be the same from side to side. It is a measure of fluctuating asymmetry and antisymmetry thus, a mixed effect. The error term is the measurement, and is a random effect (Samuels et al 1991; Palmer & Strobeck 1986, 2003; Carpentero & Tabugo 2014). Procrustes ANOVA results yield significant FA in the wings of the specimens collected from the three sites (barangays). Only Individual x Sides interaction denotes fluctuating asymmetry (FA) (Table 2).

Table 2

Procrustes ANOVA results of *Culex quinquefasciatus* in three different barangays

| *Effect* | *SS* | *dF* | *MS* | *F* | *REmarks* |
|---|---|---|---|---|---|
| | | | **Del Carmen** | | |
| Sides | 0.0052954 | 32 | 0.00016548 | 1.359* | |
| Individual x Sides | 0.113 | 928 | 0.00012177 | 2.314****** | Highly significant |
| Measurement error | 0.10103 | 1920 | 5.26E-05 | - | |
| | | | **Palao** | | |
| Sides | 0.016068 | 32 | 0.00050211 | 3.6973* | |
| Individual x Sides | 0.12603 | 928 | 0.0001358 | 0****** | Highly significant |
| Measurement error | 0.1272 | 1920 | 6.63E-05 | - | |
| | | | **San Miguel** | | |
| Sides | 0.013025 | 32 | 0.00040703 | 3.1328* | |
| Individual x Sides | 0.12057 | 928 | 0.00012993 | 1.6483****** | Highly significant |
| Measurement error | 0.15134 | 1920 | 7.88E-05 | - | |

Side - directional asymmetry, individual x sides interaction - fluctuating asymmetry, * P<0.001, ns – statistically insignificant (P>0.05), significance was tested with 99 permutations.

The F value suggested highly significant FA for individuals in the three barangays as indicated by low mean square value of 'measurement error' in every sampling site compared to the 'individuals x sides' mean square value of these populations. This suggests that species present in these areas have poor developmental homeostasis at the molecular, chromosomal and epigenetic levels. Such that species present in these areas have inability to buffer its developmental pathways against any environmentally derived and genetic stressors or could have been exposed to stressful environments and thus, may be considered as developmentally unstable. Hence, may suggest not fit enough as competent vectors. Here, fluctuating asymmetry may be used as an indicator of individual quality thereby, also demonstrating the potential for FA as a biomarker of stress and as an indicator of developmental instability. The cause of developmental instability are well studied and include a range of environmental factors (e.g. deviant climatic conditions, food deficiency, parasitism, pesticides) and genetic factors (e.g. inbreeding, hybridization, novel mutants) (Mpho et al 2000).

Moreover, another way to examine the variability of landmark points in tangent space is through principal component analysis (PCA) on the tangent coordinates derived from Procrustes analysis. This method may be more reliable for visualizing variation in landmarks than superimposition methods. The red dots represent the morphological landmarks used in the study while the blue arrows indicate the direction as well as the magnitude of the fluctuation. The percentage values of PCA represent the level of variability in the data (Table 3 & Figure 2). Based on the percentage of overall variation exhibited by PC 1 and PC 2, the population from Del Carmen exhibited less variation compared to Palao and San Miguel. Thus, higher FA was also exhibited by samples coming from Palao and San Miguel compared to samples from Del Carmen. PC 1 accounts for most of the variation. Analyses of the fluctuating asymmetry by PCA suggest that there really is a variation among individuals in their left-right asymmetry and can be due to random developmental perturbations or environmentally derived and genetic stressors.

Table 3

Variance explained by first two principal components of *Culex quinquefasciatus* in three barangays

| *Barangay* | *PC 1 (%)* | *PC 2 (%)* | *Overall (%)* |
|---|---|---|---|
| Del Carmen | 49 | 19 | 68 |
| Palao | 53 | 17 | 70 |
| San Miguel | 60 | 10 | 70 |

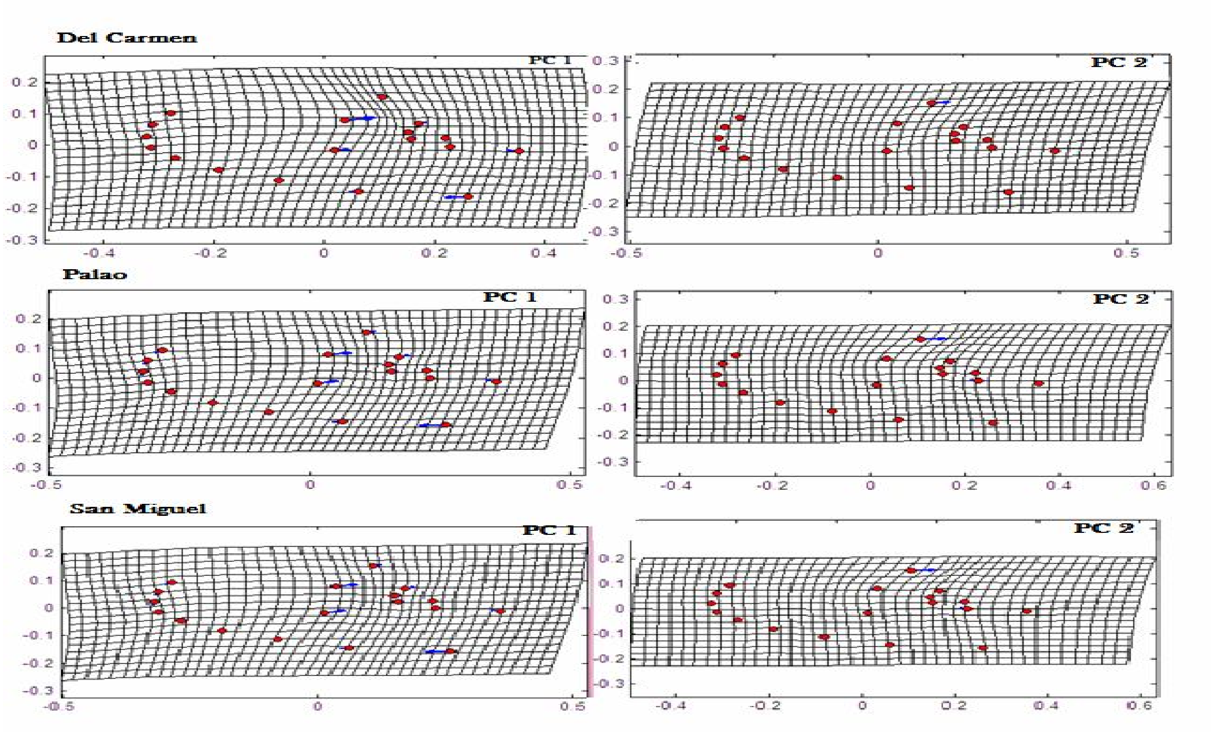


Figure 2. PCA implied deformation for *Individual x Side* interaction of fluctuating asymmetry of *Culex quinquefasciatus* in three barangays.

**Conclusions**. Developmental stability is defined as the ability of an organism to moderate its development against genetic or environmental conditions and produce the genetically determined phenotype. In this study, Fluctuating Asymmetry (FA) was obtained using Procrustes ANOVA as a measure of developmental instability and there is an inverse relationship between developmental stability and FA. Results showed that *C. quinquefasciatus* found in Barangays San Miguel, Palao and Del Carmen have high FA. It could be that *C. quinquefasciatus* found in these areas have poor developmental homeostasis and have inability to buffer its developmental pathways against any environmentally derived and genetic stressors as exposed to stressful environments thus, affecting developmental stability. Samples exhibit low developmental stability. Increased level of FA, have possible implications to reduced individual fitness and vectorial capacity. This study provides information on the nature and variation of natural populations of *C. quinquefasciatus* found in selected barangays in Iligan City.

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