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Describing Symmetry in *Brontispa longissima* Wings Utilizing Symmetry and Asymmetry in Geometric Data (SAGE) Analysis

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ABSTRACT

Background: Coconut Leaf Beetle *Brontispa longissima* Gestro, 1885 is an economically significant pest that is not yet fully understood. Studying patterned variations of the left and right side parts or asymmetry of *Brontispa longissima* could give us a picture of its mode of adaptation to different environments and its success as a pest, the ability to maintain developmental homeostasis. **Objective:** This study aimed to evaluate and quantify left and right variation patterns of *Brontispa longissima* hind wings using the advanced geometric morphometric tool – Symmetry and Asymmetry in Geometric Data (SAGE). **Results:** Procrustes Analysis of Variance (ANOVA) revealed that individual variation and directional asymmetry were present in male and female populations in all sampling locations for wing shape and size, although slight fluctuating asymmetry was noted. Principal component analysis also showed that the movements of landmarks were localized only to landmarks 3, 4 and 14. **Conclusion:** Based on the results of this study, *Brontispa longissima* hind wings have undergone lesser environmental or genetic perturbations because of the presence of directional asymmetry which by far, is genetically controlled. But based on individual variations of metasternum and wings, *B. longissima* is able to counterbalance the external pressures evidenced by shape variability of the studied traits.

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INTRODUCTION

Wings play a major contribution in the success of insects as it is used for flight. It is connected to the thorax via complex joints and the thoracic musculature provides control of the wings as well as high power production (Chapman, 2013). The detailed structure and form of the wings mainly mirror the insect's adaptation for flight although other ecological factors may provide different selective pressures resulting to wing modifications. This organ is able to counter different environmental conditions with its own adaptive variation (Cui & Liu, 2001) thus, widely used as a model in measuring distinctive patterns of variations (Klingenberg, 2003) aside from its taxonomic use (Francoy *et al.*, 2009).

Wing asymmetry analysis has been applied in several insect species as a descriptor of developmental instability, variability and fitness. This approach was applied pest species *Diabrotica virgifera virgifera* species (Benitez *et al.*, 2014), Rice bug *Leptocorisa oratorius* (Torres *et al.*, 2010), cotton aphid *Aphis gossypii* (Babbitt, 2008), gall thrips *Oncothrips tepperi* (Crespi & Vanderkist, 1997), *Onthophagus taurus* and *Bubas bison* horns (Hunt & Simmons, 1998) and several other insects. Among the studied pest species however, hardly any research have tackled on its agricultural applications as they are subject to the principle of asymmetry alone and only serve as models. Moreover, connections on its successful infestation and stabilized development are yet to be tackled. This study aimed to determine and measure patterns of wings variations of Coconut leaf beetle *Brontispa longissima* (Gestro, 1885) and attempts to relate the results to its success as a pest.

Coconut leaf beetle, one of the most serious pest to the coconut palms (*Cocos Nucifera L*) (Nakamura *et al.*, 2006) have invaded the Philippines, causing downfall in the coconut industry of the country. The larvae and adults thrive in the closed or partly opened leaflets where they feed on the soft tissues of the youngest palm.

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This causes the affected leaves to dry up, curl, turn brown and form necrotic patches causing leaf growth retardation and subsequent fruit productivity losses. High infestation levels may eventually result to plant death (Vanhan, 2007; APFISN 2008). Coconut leaf beetle is believed to be understudied since in the past years there have been few scientific reports rerecorded. Ayri and Ramamamurthy (2012) studied basically on the diagnostic morphological features on CLB at high resolutions wherein sexual dimorphism was observed. Murata *et al.* (2011) have studied about the survival advantage of CLB while Kawazu (2012) and Thu Giang & Nakamura (2009) studied its mating sequence and developmental stages, respectively. Others have focused on its host preference (Yamashita *et al.*, 2010, Takasu *et al.*, 2009). In terms of this pest's invasiveness, only Tabugo *et al.*'s study (2012) was noted.

Recent advances in Geometric Morphometrics (GM) have brought much development in the field of biological shape analysis, retaining the geometric information while providing a more powerful and efficient statistical analysis of a morphological structure. This approach utilizes landmark configurations (anatomical points) which correspond to 'x and y' coordinates, for direct visualization of the shape or morphology of the structure, in contrast to traditional morphometrics which is limited to distances, ratios and angles (Slice, 2007; Adams, *et al.*, 2004). GM has found to be crucial in describing and quantifying phenotypic diversity. Through this, identifying the relationship between morphology and ecology is made easier, allowing additional implications on the evolution of the specimens.

In this study, Symmetry and Asymmetry in Geometric Data (SAGE) v.1.04 (Marquez, 2008) tool was used to quantify the asymmetry and shape variability of Coconut leaf beetle *Brontispa longgisima* hind/inner wings. The coconut leaf beetle samples were collected in five (5) areas in Mindanao Island, Philippines; Aloran, Misamis Occidental, Pagadian City Zamboanga del Sur, Parang Maguindanao, Sibutad Zamboanga del Norte and Wao Lanao del Sur where the infestation is heavily observed. Comparison of the samples as well as testing for correlation to sex and location was also done. Studying the developmental homeostasis of *Brontispa longgisima* hind wings could give us a picture of its adaptive ability. Fundamental studies about the biological background of Coconut leaf beetle such as this could contribute to pest management.

MATERIALS AND METHOD

Study Areas:

Male and female samples of coconut leaf beetle were collected from 5 areas in Mindanao Island; Aloran, Misamis Occidental, Pagadian City Zamboanga del Sur, Parang Maguindanao, Sibutad Zamboanga del Norte and Wao Lanao del Sur (Figure 1).



Fig. 1: Map of the Philippines and Mindanao (boxed) with red circles signifying the sampling locations.

Sample Processing:

The collected individuals were placed in eppendorf tubes with 70% ethyl alcohol for preservation. The wings were dissected thoroughly. Digital imaging of the wings was done using a built in Digital SLR Camera (E-410, Zuiko Digital 14-42mm) on the Olympus SZ stereomicroscope. It was noted that all wing images were conformed to the same position/direction. In this case, all wing images were flipped with the proximal side facing left. The captured images were digitized using TPS Utility Program (tpsUtil) (Rohlf, 2009) and saved as TPS file. After which, TPS version of the images was opened with TPS Dig v.2.12 (tpsDig2) (Rohlf, 2009) where landmark points and pseudolandmarks were plotted to obtain the 2D ("x" and "y") coordinates of the corresponding morphological landmark points. The images were copied in triplicates to lessen inconsistencies or error in plotting landmark points.

Landmark and Protocol Selection:

Establishment of landmarks in this study were designated to the homologous wing vein ends and intersections. Pseudolandmarks were assigned to the points at extremes of curvature of the veins and structure outline. A total of 22 landmark points were used to represent the whole shape and vein structure of *B. longissima* wings as shown in Figure 2. The landmark points with its corresponding descriptions were summarized in Table 1. The analysis done was specifically for matching symmetry – a structure with two separate copies as mirror image of one another. Symmetry axis does not pass through in matching symmetries (Graham *et al.*, 2010). Two protocols were established; specimen identification protocol and side identification protocol.

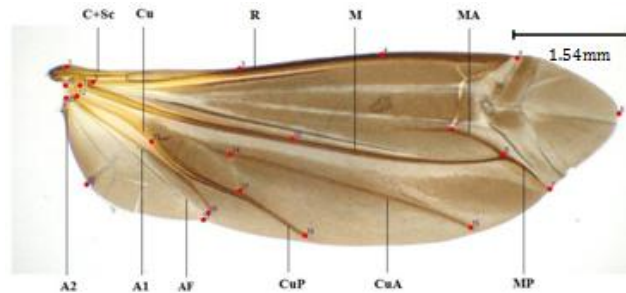


Fig. 2: Assigned landmarks and pseudolandmarks on *Brontispa longissima* hindwing. Legend: C+Sc = Costa+Subcosta, R = Radius, M = Media, MA = Media Anterior, MP = Media Posterior, Cu = Cubitus, CuA = Cubitus Anterior, CuP = Cubitus Posterior, AF = Anal fold, A1 = Anal Vein1, A2 = Anal Vein2.

In specimen identification protocol, the numbers of specimens in the dataset were labelled with the same ID for the replicates in data file (notepad), saved as indivprot1.txt. On the other hand, side identification protocol consists of the same number of entries as column as in the individual identification protocol (.txt) but with entries equal to zero or one corresponding to different sides. The side identified with zero will be labeled as the right side, and the one identified with one will be labeled as the left side or vice versa. The landmark configurations on one side were first adjusted in scale, rotation and orientation of *B. longissima* wings employed through Procrustes Superimposition (Figure 3-Left) followed with the reflection of the left and right parts (Figure 3-Right). The asymmetry of the entire object is computed as the difference between the original and the mirror configurations.

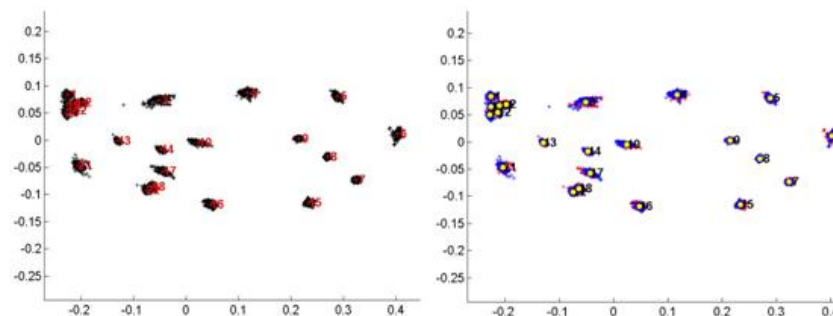


Fig. 3: (Left) Procrustes fit of original data on *B. longissima* wings. (Right) Procrustes fit of original (red) and reflected (blue) data. Light marks at the center of each point suggest bilaterally symmetrical points

Landmark-based Analysis:

The TPS version of the landmarked images was subjected to Symmetry and Asymmetry in Geometric Data (SAGE) v.1.04 software to analyze the wing shape and size with the SAGE manual as a reference (Marquez, 2008). Each of the aligned configurations which correspond to x- and y-coordinates was used for further analysis. Procrustes two-way mixed model ANOVA with three replicates with 99 permutations was used to quantify the shape asymmetry of metasternum. The “individuals” effect is the individual variation of size/shape. The main effect is “Sides” which indicates the variation between sides and is the measure of directional asymmetry. The “individual by sides interaction” is the mixed effect, the failure of the individuals to be the same between sides (left and right). This measures the fluctuating asymmetry. Lastly, measurement error was included. Table 1 summarizes the landmark points of *B. longissima* inner wings with its corresponding descriptions.

Table 1: Description of the anatomical landmark points of *B. longissima* wings.

LANDMARK NO.	DESCRIPTION
1	Proximal end of Costa+Subcosta
2	Proximal end of Radius; intersection point between Radius and Media
3	Distal end of Costa+Subcosta
4	Midpoint of LM 2 and 4
5	Distal end of Radius
6	Distal point of wing
7	Distal end Media Posterior
8	Branching point of Media Anterior and Posterior from the major vein
9	Sharp curve on Media Anterior
10	Midpoint of LM 8 & 11 on the Media
11	Proximal end of Media
12	Sharp curve near the proximal end of Cubitus
13	Branching point of Cubitus Anterior from the main vein
14	Vein protrusion forming Crossvein- like intersection in Cubitus Anterior
15	Distal end of Cubitus Anterior
16	Distal end of Cubitus Posterior
17	Vein protrusion forming Crossvein- like intersection in Cubitus Posterior
18	Distal end of Anal vein 1
19	Proximal end of Anal vein 1
20	Promixal end of the Anal vein 2 (proximal lateral margin)
21	Sharp curve on the proximal lateral margin
22	Depression on the wing margin at the distal end of Anal fold

RESULTS AND DISCUSSION

Procrustes ANOVA shows the mean squares for the mixed-model ANOVA and its corresponding effects. Fisher's exact test (F-test) was used to determine the significance of null hypothesis of no difference between variance products (XY) in both left and right side. The "individuals" effect is the variation among individual samples and can be interpreted as shape/symmetry variation. The results measuring the directional asymmetry - DA ($p < 0.05$) is designated by the significant factor 'side' box in the SAGE main panel while fluctuating asymmetry (FA) is denoted by a significant p value ($P < 0.05$) of the interaction of 'side' and 'individual' (Individuals x sides).

Table 2. Procrustes ANOVA for shape of *B. longissima* wing shape, characterized by matching symmetry

EFFECT	SS	dF	MS	F	P
Female					
Individuals	0.3416	5560	0.00006	1.0417	0.063823
Sides	0.0739	40	0.00185	31.324	<0.0001***
Individuals x sides	0.3280	5560	0.00006	3.5938	<0.0001***
Measurement error	0.3677	22400	0.00002	-	-
Male					
Individuals	0.3028	5440	0.00006	1.0503	0.035293*
Sides	0.0422	40	0.00106	19.905	<0.0001***
Individuals x sides	0.2883	5440	0.00005	3.9246	<0.0001***
Measurement error	0.2960	21920	0.00001	-	-

Note: side = directional asymmetry; individual x sides interaction = fluctuating asymmetry; * $P < 0.05$, statistically significant, *** $P < 0.05$, highly significant; significance was tested with 99 permutations

Table 3. Procrustes ANOVA results in *Brontispa longissima* wing size in terms of sex.

EFFECT	SS	dF	MS	F	P
Female					
Individuals	313662580	139	2256565	0.89246	0.74836
Sides	315281757	1	315281758	124.69	<0.0001***
Individuals x sides	351460120	139	2528490	7.2821	<0.0001***
Measurement error	194443775	560	347221	-	-
Male					
Individuals	401595625	136	2952909	1.287	0.071266
Sides	106838302	1	106838302	46.5632	<0.0001***
Individuals x sides	312049012	136	2294478	19.6893	<0.0001***
Measurement error	63860745	548	116534	-	-

Note: side = directional asymmetry; individual x sides interaction = fluctuating asymmetry; * $P < 0.05$, statistically significant, *** $P < 0.05$, highly significant; significance was tested with 99 permutations

In this study, Procrustes ANOVA (Table 2) shows that significant L-R within-individual size and shape variation is present (Individuals $p < 0.05$) in male samples. The asymmetry variations in male and female samples were due to directional asymmetry and fluctuating asymmetry, indicated by the significant factor 'side'

($P \lll 0.05$) and significant factor 'Individuals x sides' ($P \lll 0.05$), respectively. The observed deviations are not sex specific. Since there cannot be both fluctuating asymmetry and directional asymmetry present in a single character, the next basis for evaluating asymmetry is the mean square value. In that case, directional asymmetry explains L-R variations of male and female samples (*Side MS* \gggg *Individuals x sides MS*). The same is true in terms of wing size (Table 3) where DA and FA is significantly present ($p \lll 0.05$). Again, directional asymmetry was qualified to be governing the patterns of asymmetry as explained by greater mean square value. In general, *B. longissima* hind wings shape and size exhibit directional asymmetry.

Table 4: Procrustes ANOVA results in *B. longissima* wing shape by location.

EFFECT	SS	dF	MS	F	P
Aloran, Misamis Occidental					
<i>Individuals</i>	0.1202	2120	0.0001	0.9113	0.98371
<i>Sides</i>	0.0677	40	0.0017	27.2005	<0.0001***
<i>Individuals x sides</i>	0.1319	2120	0.0001	3.0576	<0.0001***
<i>Measurement error</i>	0.1759	8640	0.0001	-	-
Pagadian City Zamboanga del Sur					
<i>Individuals</i>	0.1129	2080	0.0001	1.3042	<0.0001***
<i>Sides</i>	0.0935	40	0.0023	56.1547	<0.0001***
<i>Individuals x sides</i>	0.0866	2080	0.0001	3.0147	<0.0001***
<i>Measurement error</i>	0.1171	8480	0.00001	-	-
Parang, Maguindanao					
<i>Individuals</i>	0.1140	2400	0.00005	1.2504	<0.0001***
<i>Sides</i>	0.0306	40	0.00076	20.13	<0.0001***
<i>Individuals x sides</i>	0.0912	2400	0.00004	2.7545	<0.0001***
<i>Measurement error</i>	0.1346	9760	0.00001	-	-
Sibutad, Zamboanga del Norte					
<i>Individuals</i>	0.0837	1960	0.00004	0.9281	0.95077
<i>Sides</i>	0.0143	40	0.00036	7.7484	<0.0001***
<i>Individuals x sides</i>	0.0902	1960	0.00005	3.5045	<0.0001***
<i>Measurement error</i>	0.1050	8000	0.00001	-	-
Wao, Lanao del Sur					
<i>Individuals</i>	0.0961	2320	0.00004	0.9079	0.99002
<i>Sides</i>	0.0166	40	0.00041	9.071	<0.0001***
<i>Individuals x sides</i>	0.1059	2320	0.00005	3.2849	<0.0001***
<i>Measurement error</i>	0.1312	9440	0.00001	-	-

Note: side = directional asymmetry; individual x sides interaction = fluctuating asymmetry; * $P < 0.05$, statistically significant, *** $P \lll 0.05$, highly significant; significance was tested with 99 permutations

Table 5: Procrustes ANOVA results in *B. longissima* wing size by location.

EFFECT	SS	dF	MS	F	P
Aloran, Misamis Occidental					
<i>Individuals</i>	2095873	53	39545	0.47335	0.99631
<i>Sides</i>	166.4751	1	166.475	0.00199	0.96456
<i>Individuals x sides</i>	4427785	53	83543	9.3458	<0.0001***
<i>Measurement error</i>	1930845	216	8939	-	-
Pagadian City Zamboanga del Sur					
<i>Individuals</i>	6656678	52	128013	1.2951	0.17704
<i>Sides</i>	10309122	1	1030912	104.30	<0.0001***
<i>Individuals x sides</i>	5139743	52	98841	5.818	<0.0001***
<i>Measurement error</i>	3601630	212	16989	-	-
Parang, Maguindanao					
<i>Individuals</i>	10822865	60	180381	1.1146	0.33785
<i>Sides</i>	10894183	1	108941831	673.16	<0.0001***
<i>Individuals x sides</i>	9710159	60	161836	10.452	<0.0001***
<i>Measurement error</i>	3777979	244	15484	-	-
Sibutad, Zamboanga del Norte					
<i>Individuals</i>	34894748	49	7121377	1.3461	0.1508
<i>Sides</i>	42418422	1	4241842	8.0179	0.0067016
<i>Individuals x sides</i>	25923391	49	5290488	4.3707	<0.0001***
<i>Measurement error</i>	24209106	200	1210455	-	-
EFFECT	SS	dF	MS	F	P
Wao, Lanao del Sur					
<i>Individuals</i>	6898511	58	118940	0.66316	0.93966
<i>Sides</i>	10836048	1	1083605	60.417	<0.0001***
<i>Individuals x sides</i>	10402485	58	179353	6.1317	<0.0001***
<i>Measurement error</i>	6903006	236	29250	-	-

Note: side = directional asymmetry; individual x sides interaction = fluctuating asymmetry; * $P < 0.05$, statistically significant, *** $P \lll 0.05$, highly significant; significance was tested with 99 permutations

In terms of location, all of the samples (Table 4 & 5) have shown a significant directional asymmetry based variation of left and right sides of metasternum ($P \lll 0.05$). Although fluctuating asymmetry is also present across populations ($P \lll 0.05$), it was not proven to not explain all the variations in both wing shape and size. The variations in asymmetry for both wing shape and size are instead both attributed to Directional asymmetry, $Side MS \ggg Individuals \times sides MS$. In all of population groups, wing shape variation among individuals significantly differs, except for Aloran, Sibutad and Wao group. Different observation was noted for wing size in that Aloran and Sibutad group exhibit significant fluctuating asymmetries. The result for Procrustes ANOVA is similar to the study of Klingenberg and McIntyre (1998).

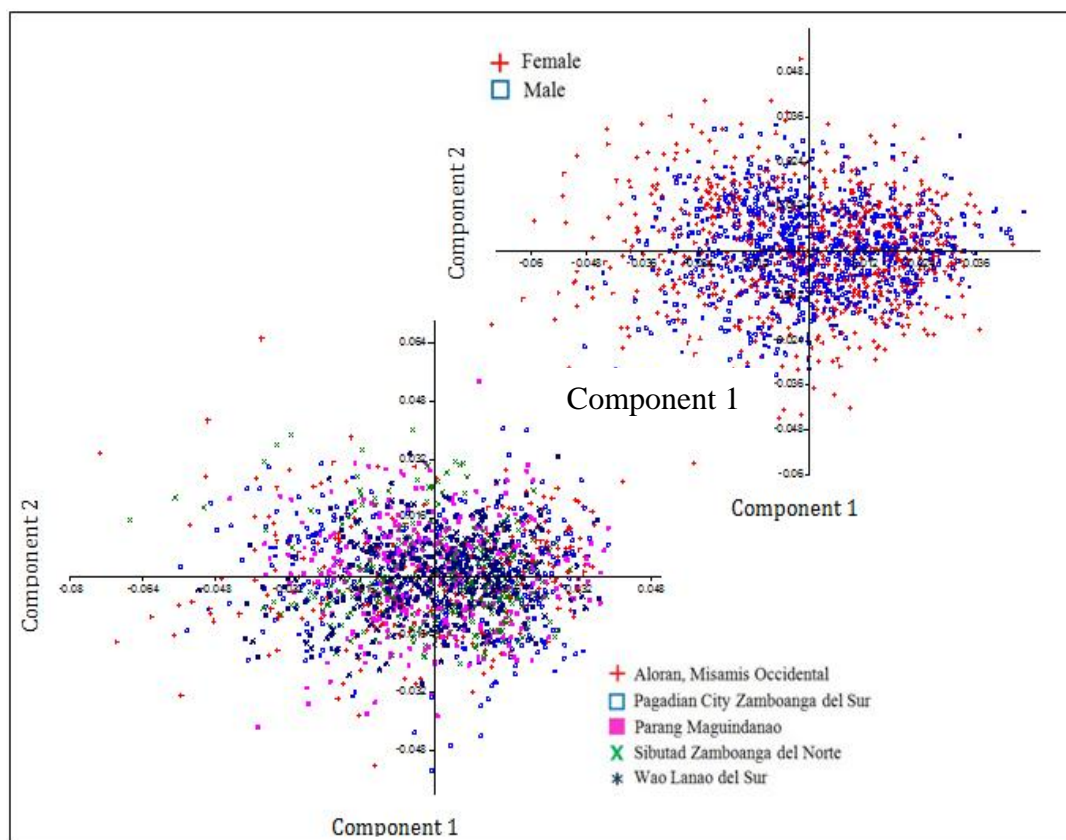


Fig. 4: Principal Component 1 and 2 scatter diagram for individual variation of *B. longissima* wings in terms of sex (above) and location (below).

Directional asymmetry was noted in *B. longissima* wing size across locations ($p \lll 0.05$) while for Aloran and Sibutad samples in terms of wing size, significant fluctuating asymmetry is present ($p \lll 0.05$). Principal component analyses were performed to the sex and location dataset to further examine the visual variation of the left-right sides of the landmark configurations. Shown in Figures 4 and is the Principal component analyses scatter plot in Individual (symmetric) variation. Only two principal components (PC1 and PC2) were presented. Individual covariance matrix revealed that female variation is more dispersed compared to male samples (Figure 4). In terms of location, the PC values were almost equally distributed around the central axis. The same holds true for fluctuating asymmetry covariance matrix (Figure 5).

Table 6: Individual variation of male and female *Brontispa longissima* hind wings.

Sex	Individual Variation (%)		
	PC1	PC2	Overall
Female	30.95	18.38	49.33
Male	30.66	16.47	47.13

Table 4 lists the Principal components for the individual variation for male and female samples. While Figure 4 displays its visual representation. Around 50% (female) and 46% (male) of the variations are represented by the movements of landmarks 3, 4 and 14.

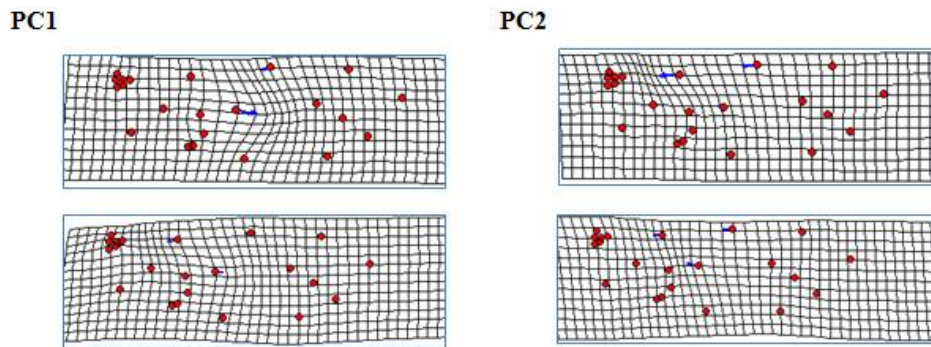


Fig. 6: PCA-implied deformation grid for individual variation (symmetric) in female (above) and male (below) *B. longissima* hind wings.

Table 7: Individual variation of *B. longissima* wings by location.

Location	Individual Variation (%)		
	PC1	PC2	Overall
Aloran	31.81	16.35	48.16
Pagadian	29.46	25.97	55.43
Parang	30.98	21.38	52.36
Sibutad	34.38	16.38	50.76
Wao	24.76	16.13	40.89

Table 5 lists the Principal components for the individual variation of populations across locations. Around 60% of the variations are represented by the movements of landmarks 3, 4 and 14 and 17 for both individual and fluctuating asymmetry. The same movements were also observed in for directional asymmetry (Fig 10).

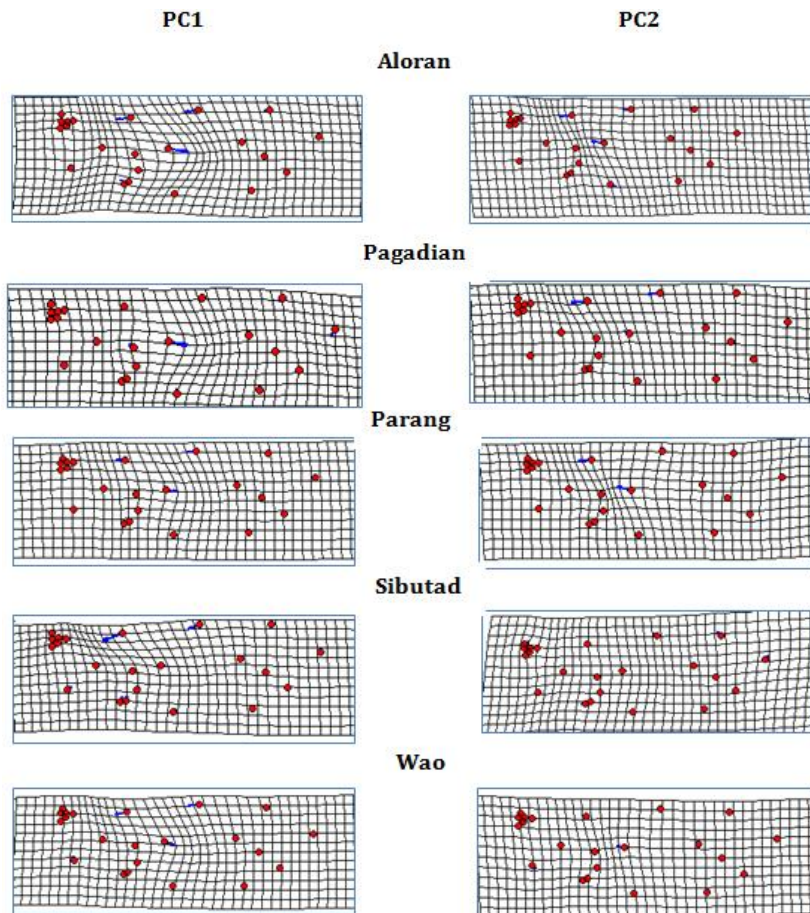


Fig 7: PCA-implied deformation grid for individual variation (symmetric) of *Brontispa longissima* wings across Locations.

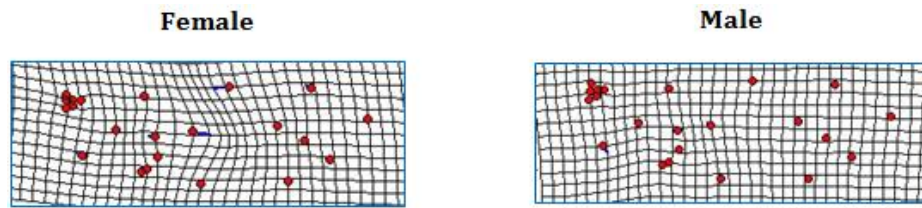


Fig. 8: PCA-implied deformation grid for directional asymmetry of *Brontispa longissima* wings between sexes (PC=100).

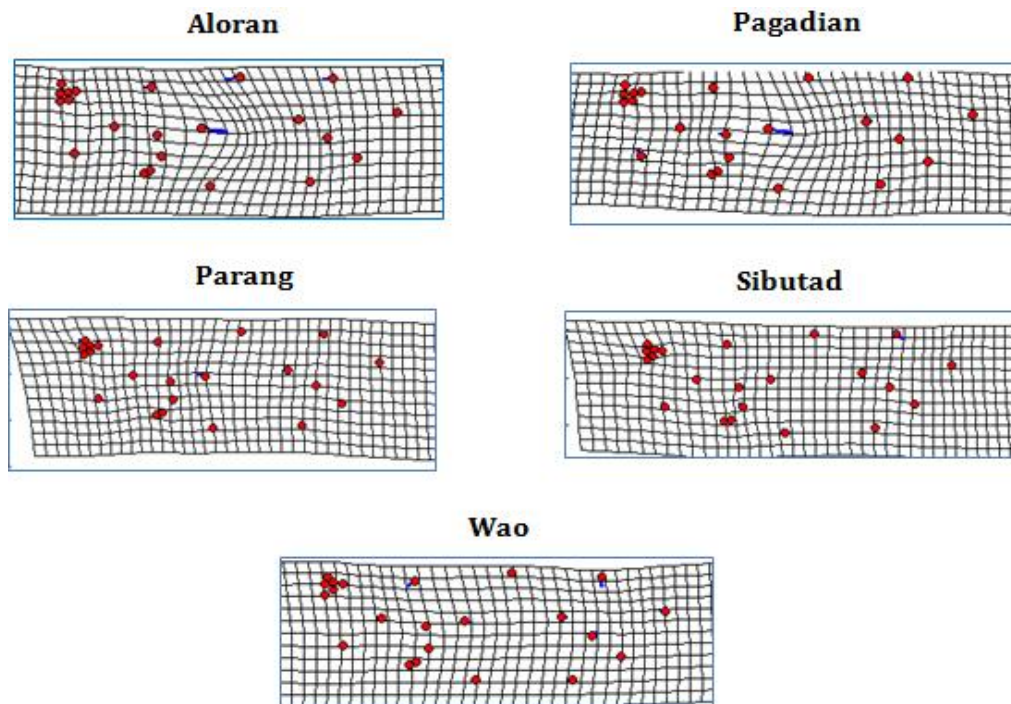


Fig. 9: PCA-implied deformation grid for directional asymmetry of *Brontispa longissima* wings across locations (PC=100).

This study revealed that *B longissima* wing shape individual variability is sex specific. Male samples are more prone to wing shape variability. It was also found out that individual shape variability is relative to environment (condition dependent) as explain Aloran, Wao and Sibutad populations ($P>0.05$). The reasons for this non-association are still to be explained. Directional Asymmetry was also observed in *B longissima* wings shape and size in almost all samples with specific subtle movements in landmarks 3, 4 and 14. This means that stabilized development is demonstrated in these wing regions might have experience lesser developmental perturbations/noise early in life caused by stressor either endogenous or exogenous (Palmer, 2003).

Conclusion and Recommendations:

The results of this study have shown patterns of variation in coconut leaf beetle *B. longissima* (Gestro, 1885) hind wing in male and female populations in Mindanao Island. It has been presented that directional asymmetry is present in all male and female populations ($P<0.05$) which might imply that coconut leaf beetle have undergone a normal development in this structure. Moreover, all of the samples coming from different locations exhibit a significant directional asymmetry. Individual variation is also common in all samples across locations. Future studies can be done to other parts of coconut leaf beetle to fully estimate developmental stability.

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