INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 11, No. 4, p. 73-84, 2017

RESEARCH PAPER OPEN ACCESS

# Determining developmental instability via fluctuating asymmetry in the shell shape of *Venerupis philippinarum* (Manila Clam)

**Kurt Marche L. Cabiluna, Sharon Rose M. Tabugo***

*Department of Biological Sciences, College of Science and Mathematics, Mindanao State University-Iligan Institute of Technology, Iligan City, Philippines*

**Key words:** *Venerupis philippinarum*, Developmental instability, Fluctuating asymmetry, Procrustes ANOVA, SAGE

http://dx.doi.org/10.12692/ijb/11.4.73-84 Article published on October 8, 2017

**Abstract**

This exploratory study look into the nature and variation of Manila Clams (*Venerupis philippinarum*), an edible species of saltwater clam native to Japan but has been cultivated, commercially harvested and considered as the second most important bivalve grown in aquaculture in the Philippines also worldwide. Fluctuating Asymmetry (FA) is apopular tool to estimate the quality, health of individuals and populations and used to measure developmental stability or the organism's ability to buffer environmental and genetic perturbations. This study demonstrated the use of FA for monitoring developmental stability of bivalve species *V. philippinarum*. It investigated the differences of FA of three different morphotypes from one population. Fifteen anatomical landmarks were used and were subjected to Procrustes superimposition and Principal Component Analysis (PCA) using "Symmetry and Asymmetry in Geometric Data" (SAGE) program. Results yield significant evidence of FA for all the morphotypes. Possible explanation for high levels of FA detected may rise from the differences in genetic composition of the populations resulting in different tolerance to stress. Results revealed that all morphotypes exhibited high FA value thus, relatively considered unstable morphotypes with poor developmental homeostasis. Hence, indicate genetic and environmental stress and has the inability to buffer such stress. In this context, it is perceived that there is a direct relationship between FA and developmental instability. Along this line, morphotype B have relatively the lowest FA compared to other morphotypes, providing that stabilizing selection is at work and thus, relatively fit for cultivation to maximize yield for food production.

* **Corresponding Author:** Sharon Rose M. Tabugo ✉ sharonrose0297@gmail.com

**Introduction**

Manila Clams (*Venerupis philippinarum*) is an edible species of saltwater clam native to Japan but has been cultivated, commercially harvested in the Philippines and even worldwide. It is considered as the second most important bivalve grown in aquaculture. It is recognized by a number of different common names which includes, "Asari" (after its Japanese name), "Japanese littleneck", "Manila clam", "steamer clam", "Filipino Venus", "Japanese cockle", and "Japanese carpet shell". These clams are extremely variable in color and pattern, white, yellow or light brown, sometimes with rays, steaks, blotches or zig-zags of a darker brown. It is a bivalve mollusc with a solid equivalve broad oval shell and anterior beaks, shells are large and solid. The height of the shell is 19-31 mm; length is commonly 28-46. Maximum length is 80 mm and width is 13-22 mm. Harvesting of Manila clam shells can be done through manual harvesting consists of raking them out of the substrate and bringing them to the surface. Mechanical harvesting is carried out by suction or elevator dredges; a tractor equipped with a lateral conveyor belt can dig and grade clams from sandy bottom (Qi, 1998; Antunes *et al.,* 2003; Goulletquer, 2005). Noteworthy, successful cultivation has led to considerably expanded distribution range although wild populations are still found in the Philippines. Successful intertidal mollusk farming requires a working knowledge on the nature and biology of the organism. Herewith, this study looked into developmental instability of populations. Developmental instability is most often estimated as random deviations from perfect bilateral symmetry, which is presumed to be the idealized phenotype in the absence of perturbation. Inability to maintain precise development creates variations in morphological characters between the right and left sides of an individual manifesting directionally random, subtle deviation from perfect symmetry, referred to as fluctuating asymmetry. Fluctuating asymmetry (FA) are fine and random deviations from perfect symmetry of organism's morphology. It is considered a reliable factor for measuring developmental instability because it reflects both genetic and environmental stresses. FA increases as the environmental stress increases and thus referred to as a tool for measuring developmental stability and environment quality (Palmer and Strobeck 1986; Zakharov, 1989; Palmer, 1994). Hence, as the level of environmental stress or instability increases, so is the level of fluctuating asymmetry (Antuaco and Leyesa, 2004).

Moreover, FA is measured via traditional measures of dispersion (variances and mean absolute deviations), landmark methods for shape asymmetry, and continuous symmetry measures. It has numerous applications in evolutionary biology, quantitative genetics, environmental biology, ecotoxicology, conservation biology, anthropology, agriculture and aquaculture, evolutionary psychology, and medicine and public health (Graham *et. al.*, 2010). FA is considered as a dependable factor for measuring developmental instability since it reflects both genetic and environmental stresses and this has been considered as an important theory in evolutionary biology for decades (Parsons, 1990). FA increases in both environmental and genetic stress even if responses may be inconsistent. Furthermore, it is significant because it reflects a population's situation of adaptation and co-adaptation (Graham *et al.*, 1993). Linear dimensions, shape variation involving landmarks are the variances that could measure deviations from perfect symmetry.

Palmer (2003) stated that the measurement of FA is complicated knowing that the magnitude and distribution are the same as the magnitude and distribution of measurement error. For such reason, it is of essence to establish that the measures of FA clarify a statistically important proportion of the observed total variance between the sides, in order to establish that real differences in symmetry rather than just measurement error being reported. In doing so, there is a need to create repeated measures of the left and right sides of the trait. With the same equipment and under the same laboratory or field conditions as those of the main data, the repeated procedures are ought to be made on the same subjects in ignorance of the initial measure.

Moreover, to eliminate bias, ideally all measurements should be made in ignorance of the measurement recorded for the side's pair. A certain criteria must be met for analysis of a possible fluctuating asymmetry data set: the measurements must represent actual deviations from symmetry and not measurement error, and the distribution of fluctuating asymmetry must conform to that expected for it, rather than for antisymmetry or directional asymmetry.

Hence, this study determined developmental instability via fluctuating asymmetry in the shell shape of the bivalve species *Venerupis philippinarum* (Manila Clam). It investigated the differences of FA on the right and left valves of three different morphotypes from one population.

In this regard, there is an assumption that fluctuating asymmetry has costs and reflects the quality of individuals. Therefore, this study present knowledge and information on the nature of the organism also on which morphotype is likely dominant and relatively developmentally stable, thus, can be cultured to maximize the production of food and augment the yield.

## Materials and methods

*Study Area and Specimen Collection, Identification and Image Processing*

Venerid bivalves were randomly collected from the municipality of Baliangao, located in Misamis Occidental, Philippines (Fig. 1).


**Fig.1.** Sampling area: Map of Baliangao, Misamis Occidental.

A total of 90 specimens were used comprising 30 individuals for morphotype A, 30 for morphotype B and 30 for morphotype C. The three (3) morphotypes were identified from one population shown in Table 1. Before the shells were sun-dried, soft tissues were removed leaving the shells totally clean. The two valves of each sample were slowly separated by carefully tearing their ligament. The left and right valves were documented dorso-ventrally. Using a standard procedure, the ventral aspect was documented where all internal shell structures are clear and distinct with the umbo oriented vertically and upward. Digital images of the left and right valves of clams were taken using standard procedure and landmarked assignment was done using tpsDig2 software. Landmarking per specimen was done in triplicates in order to quantify and minimize measurement error. Fifteen anatomical landmarks were assigned in the inner valves of *Venerupis philippinarum*. Anatomical landmarks, the most biologically informative, are points that are biologically homologous between organisms. Descriptions of identified landmarks are presented in Table 2 and Fig. 2.

*Fluctuating Asymmetry Analysis (FA) and Principal Component Analysis (PCA)*

FA levels were assessed using the "Symmetry and Asymmetry in Geometric Data" (SAGE) program, version 1.0 (Marquez, 2006). The software analyzed the x and y coordinates of the landmarks per individual, using a configuration protocol for both left and right valves of the Manila clam (*Venerupis philippinarum*). Procrustes superimposition analysis for both valves was performed (Fig. 3) with the original and mirrored configurations of the right and left inner valves simultaneously.

The least squares Procrustes consensus set of landmark configurations and their relabelled mirror images is a perfectly symmetrical shape, while FA is the deviation from perfect bilateral symmetry (Klingenberg *et al.,* 1998; Marquez, 2006). Herewith, the squared average of Procrustes distances for all specimens is the individual contribution to the FA component of variation within a sample. In order to detect the components of variances and deviations, a Procrustes Analysis of Variance (ANOVA) was used. *Sides* (DA), *individual x sides* (FA), and their respective error were included as effects. The ANOVA used most frequently for fluctuating asymmetry is a two-way, mixed-model ANOVA with replication. The main fixed effect is sides (S) has two levels (left and right).

The block effect is *individuals* (*I*) 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 byindividuals*).

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 x 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 and Strobeck, 2003; Graham *et al.*, 2010). In addition, to detect the components of variances and deviations, Principal Component Analysis (PCA) of the covariance matrix associated with the component of FA variation were also performed for the samples to carry out an interpolation based on a thin-plate spline and then visualize shape changes as landmark displacement in the deformation grid (Marquez, 2006; Albarran-Lara *et al.,* 2010).

## Results and discussion

*Measurement of FA levels*

The presence of symmetry is a major advantage of FA over other measures of developmental instability. Through Procrustes method using SAGE software, FA of the right and left valves of *Venerupis philippinarum*, collected from Baliangao, Misamis Occidental, were evaluated.

Moreover, FA is directly related to developmental instability (DI), thus, a tool in investigating the DI. Together with the product of the coordinates of the left and right homologous provided the final results of the Procrustes ANOVA the index of FA using the coordinates was determined (Table 3).

The *individual by sides* interaction is a measure of fluctuating asymmetry and antisymmetry, hence, a mixed effect. The failure of the effect of individuals that is the same from side to side is the interaction of individual by sides.

The measurement is the error term and is ought to be a random effect, furthermore, only *Individual x Sides* interaction signifies fluctuating asymmetry (FA) (Palmer and Strobeck, 1986; Galbo and Tabugo, 2014). In this connection, the interaction 'Individual x sides' presented a high value of mean square and a low value of mean square measurement error. The results of the Procrustes ANOVA showed a random variation (FA) between the left and the right sides of the landmark parts of the bivalve shells, rather than non-random differences among sides.

**Table 1.**Different morphotypes in a population.

| Morphotype | Description |
|---|---|
|  | Morphotype A- white and have thick layers of dark brown patterns. Size range of 4mm- 5.5mm. |
|  | Morphotype B- light yellow to brown and have thin layers of light to dark brown patterns.<br>Size range of 4mm- 5mm. |
|  | Morphotype C- has the darkest colors; thin brown to black and also dark patterns.<br>Size range of 4.5mm- 5.5mm. |

**Table 2.**Position of the selected landmarks in the interior valve of *Venerupis philippinarum*.

| Landmark # | Position of Anatomical Landmark |
|---|---|
| 1 | Umbo |
| 2 | dorsal tip of anterior cardinal tooth |
| 3 | dorsal tip of posterior cardinal tooth |
| 4 | antero-ventral tip of anterior cardinal tooth |
| 5 | postero-ventral tip of anterior cardinal tooth |
| 6 | antero-ventral tip of middle cardinal tooth |
| 7 | postero-ventral tip of middle cardinal tooth |
| 8 | antero-ventral tip of posterior cardinal tooth |
| 9 | postero-ventral tip of posterior cardinal tooth |
| 10 | dorsal tip of posterior adductor muscle |
| 11 | junction of ventral tip of posterior adductor |
| 12 | deepest point of pallial sinus |
| 13 | postero-ventral tip of pallial sinus |
| 14 | junction of ventral tip of anterior adductor and pallial line |
| 15 | dorsal tip of anterior adductor |

Therefore, the F value show highly significant FA for all populations of three different morphotypes of *V. philippinarum* from Baliangao, Misamis Occidental, where *P <0.001.

In the light of the results, all three morphotypes show significant FA levels which indicated that variations among the individual genotypes and asymmetry in phenotypes are mostly interaction of genes under stressful environment.

**Table 3.**Procrustes ANOVA results of *Venerupis philippinarum* from Baliangao, Misamis Occidental.

| Effects | SS | dF | MS | F | Remarks |
|---|---|---|---|---|---|
| Morphotype A | | | | | |
| Sides | 0.085531 | 26 | 0.00332897 | 19.1057 | ****** |
| Individual x sides | 0.12983 | 754 | 0.00017218 | 3.0451 | ****** |
| Measurement error | 0.17642 | 3120 | 5.65E-05 | -- | -- |
| Morphotype B | | | | | |
| Sides | 0.092254 | 26 | 0.0035482 | 17.6443 | ****** |
| Individual x sides | 0.1516 | 754 | 0.0002011 | 2.0249 | ****** |
| Measurement error | 0.3098 | 3120 | 9.93E-05 | -- | -- |
| Morphotype C | | | | | |
| Sides | 0.0374 | 26 | 0.0014 | 7.9099 | ****** |
| Individual x sides | 0.1371 | 754 | 0.000182 | 2.3087 | ****** |
| Measurement error | 0.2457 | 3120 | 7.87E-05 | | -- |

Note: side = directional asymmetry; individual x sides interaction = fluctuating asymmetry; * P < 0.001, ns – statistically insignificant (P> 0.05); significance was tested with 99 permutations.

**Table 4.** Variance explained by first two principal components among the population of three morphotypes of *Venerupis philippinarum* (manila clam).

| Morphotype | PC 1 (%) | PC 2 (%) | Overall (%) |
|---|---|---|---|
| A | 28.3106 | 16.6038 | 44.9144 |
| B | 24.5264 | 16.3425 | 40.8716 |
| C | 35.4932 | 18.7836 | 54.2768 |

The F values of "individual x sides" suggested a highly significant FA for all populations as indicated by low mean square of measurement error compared to the individual by sides interaction values. *Venerupis philippinarum* population in Baliangao, Misamis Occidental also revealed significant scores for the "individual" and "side" effects. For all the morphotypes, the effect called "sides" were all significant and refers to the variation between two sides, a measure of DA occurring in two levels (left and right valves). With this, high FA and significant DA is an indication that generations of phenotypes are interacting in a perturbed environment, hence, convey the interplay of both genotype and environment in a more stressful condition. It is important to take note that, Baliangao, Misamis Occidental showed some level of disturbance based on site inspection, considering that Baliangao is the seafood capital of Misamis Occidental, where excessive farming of marine resources is inevitable resulting to perturbations and stressors in the area. The sampling site is evidently affected by urbanization and other anthropogenic activities. Thus, exogenous factors affect the growth of the bivalve's population such as exposure to pollutants. The higher the F value the greater the stress is (Ducos and Tabugo, 2015). The stress in this field can be clearly expressed as high levels of asymmetry.

**Table 5.**Localized trait fluctuating asymmetry in the shell shape of *Venerupis philippinarum*.

| Landmark number | Character | A | B | C |
|---|---|---|---|---|
| 1 | Umbo | ns | ns | ns |
| 2 | dorsal tip of anterior cardinal tooth | ns | ns | ns |
| 3 | dorsal tip of posterior cardinal tooth | ns | *** | ns |
| 4 | antero-ventral tip of anterior cardinal tooth | *** | ns | ns |
| 5 | postero-ventral tip of anterior cardinal tooth | ns | ns | ns |
| 6 | antero-ventral tip of middle cardinal tooth | ns | ns | ns |
| 7 | postero-ventral tip of middle cardinal tooth | ns | ns | ns |
| 8 | antero-ventral tip of posterior | ns | ns | ns |
| 9 | postero-ventral tip of posterior cardinal tooth | ns | ns | ns |
| 10 | dorsal tip of posterior | *** | ns | *** |
| 11 | junction of ventral tip of posterior adductor | ns | ns | ns |
| 12 | deepest point of pallial sinus | *** | ns | ns |
| 13 | Postero ventral lip of pallial sinus | ns | ns | ns |
| 14 | junction of ventral tip of anterior adductor and pallial line | ns | ns | ns |
| 15 | dorsal tip of anterior adductor | ns | ns | ns |

***highly significant, *ns*-not significant; $p<0.05$ is significant.

A possible explanation for high levels of FA ascends from the differences in genetic composition of the populations resulting in different tolerance to stress. Based on the trait examined, observed deviations from bilateral symmetry resulted since individuals in their particular locations might have experienced developmental distresses or developmental noise in life.

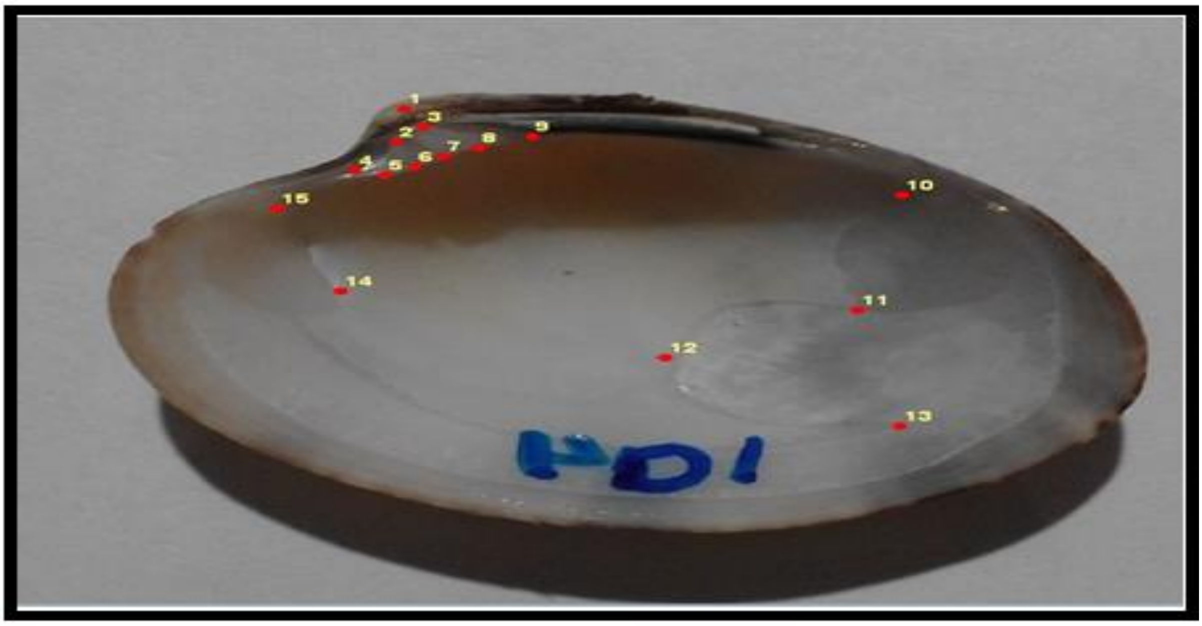


**Fig. 2.**Location of the 15 landmarks on the bivalve interior of *Venerupis philippinarum*.

According to Utayopas, 2001 exogenous and endogenous stresses such as low habitat quality to low genetic heterozygosity among others are the probable sources of developmental noise. In addition, a wide range of environmental factors and genetic factors may become the possible causes of developmental instability, in such factors stress to populations has a great tendency to increase (Mpho *et al.,* 2000). In this way, we can imply that FA could also demonstrate its potential as a bioindicator of stress and developmental instability of populations and could be a medium as a pointer of individual's quality and adaptation (Kotiaho and Tomkins, 2001).

Results further revealed that all morphotypes exhibited high FA value and thus, considered as relatively unstable morphotypes indicating that it experienced more environmental and genetic stressors and has the inability in buffering such stress. In this context, it is perceived that there is a direct relationship between FA and developmental instability (Graham *et al.*, 2010).

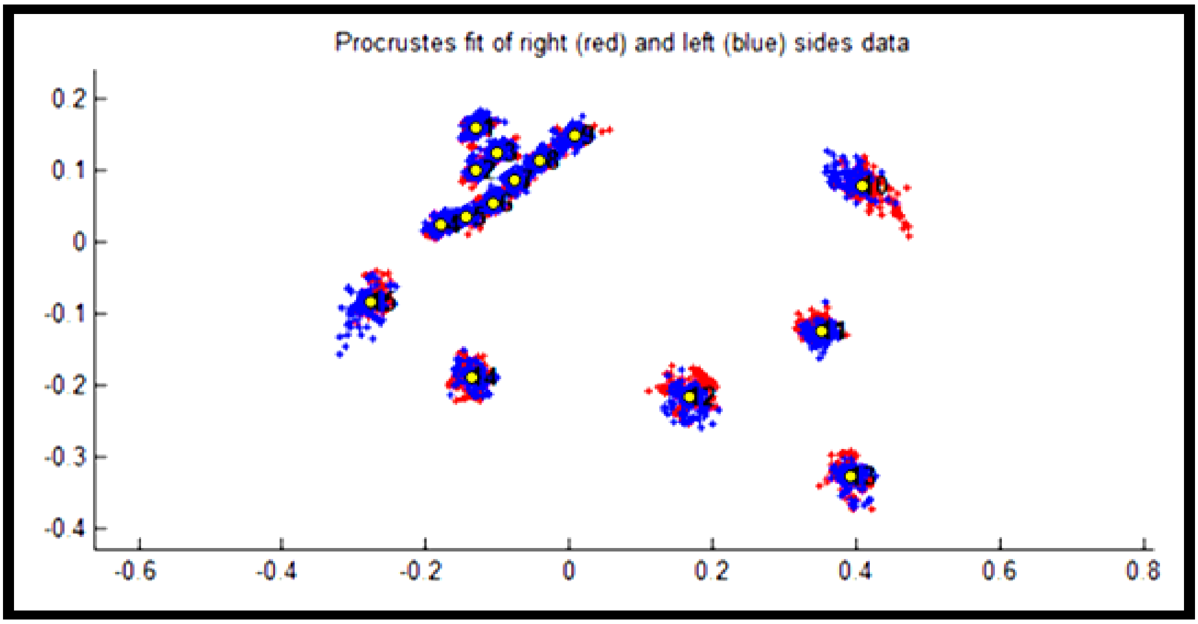


**Fig. 3.**Procrustes fitted image of *Venerupis philippinarum* done by SAGE software.

Apparently, morphotype B likely has the lowest FA compared to other morphotypes, thus, considered relatively fit for cultivation to maximize the products and yield for food production. In addition, it is perceived that stabilizing selection is at work such that it acts against extreme phenotypes, reduces variation, maintains the normality for a trait and intermediate variants are favoured (Campbell and Reece, 2005). Morphotype B has the lowest FA value, thus, an intermediate variant. With this, morphotype B is apparently favorable in a way that stressors from the environment may least disturbed its development. Herewith, particularly, for individuals with somewhat high level of heterozygosity are considered to be more fit because they show a relatively high level of stability/homeostasis. Nevertheless, suggestions are made from the literature on the association of heterozygosity and fitness (Britten, 1996) that if natural selection favours alleles in numerous loci in which interact in order to produce developmentally stable phenotypes then coadaptive gene complexes may evolve in a population (Graham, 1992). The expectation of such positive relationship among heterozygosity and fitness, is because heterozygotes has been said to exhibit the ability to buffer environmental variation and hence considered to be phenotypically plastic. In such way, they can maintain optimal phenotype in the face of environmental fluctuation (Utayopas, 2001).

In this respect, FA exhibits its potential use in conservation biology, wherein measurements of asymmetry can indicate that a population is under some kind of stress. It has also been argued that the degree of FA of morphological traits may indicate both environmental and genetic stress plus the degree of such stress (Leary and Allendorf, 1989). This study proved that the measurement of FA is an effective indicator of ecological stress and developmental instability (DI).

*Principal Component Analysis*

Moreover, principal component analysis (PCA) on the tangent coordinates that is derived from Procrustes analysis is another way to study the variability of landmark points in tangent space. It was also performed in order to see the general direction and magnitude of the fluctuation for each landmark and to visualize the covariance shape change in every principal component.

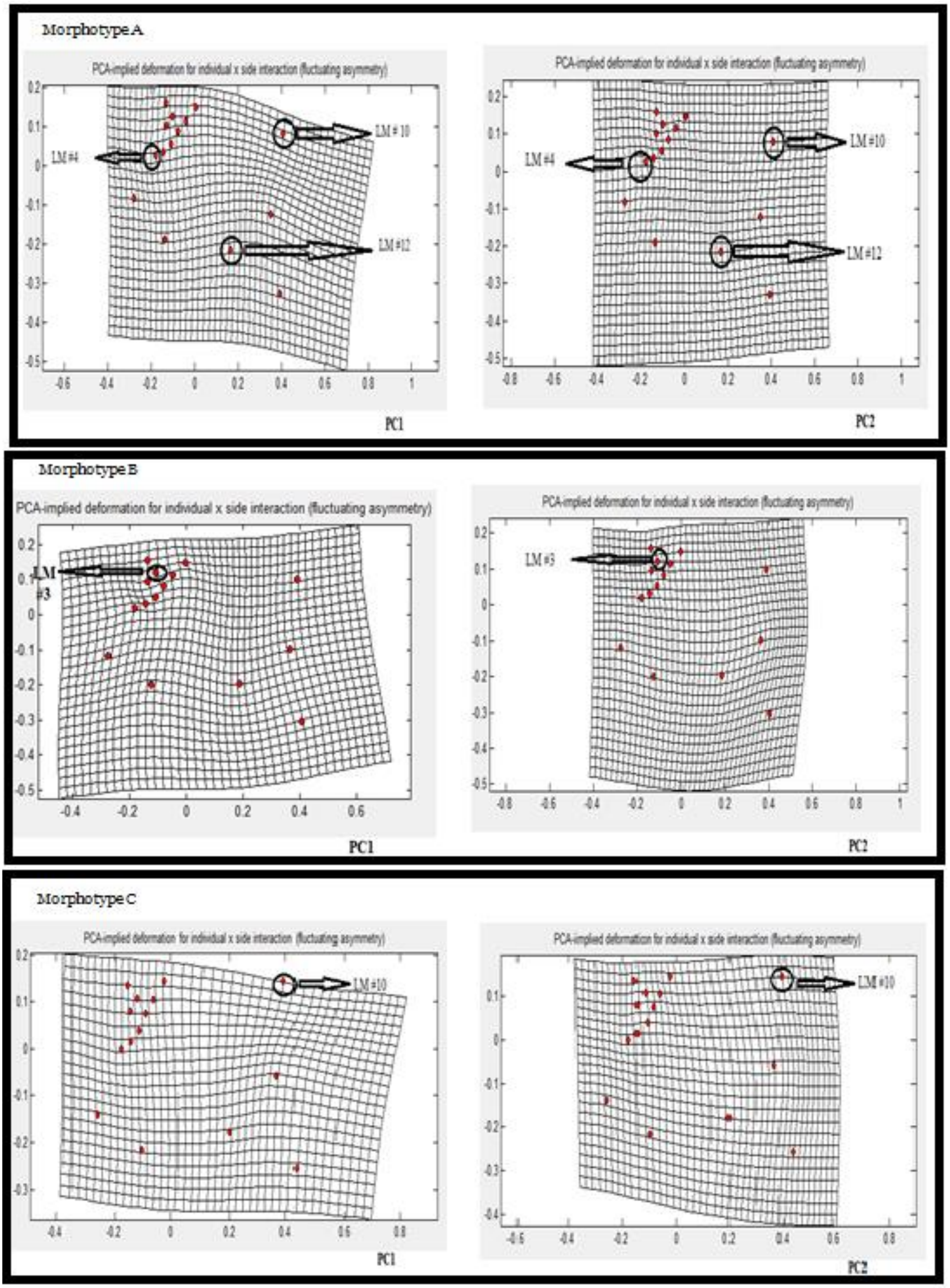


**Fig. 4.** PCA implied deformation for *individual x side* interaction of fluctuating asymmetry of *Venerupis philippinarum* showing localized landmarks with significant variations.

The level of variability in the data is represented by the percentage values of PCA. Principal component depicts vectors at landmarks that show the magnitude and direction in which that landmark is displaced relative to the others and also it depicts the difference via the thin plate splines, an interpolation function that models change between landmarks from the data of changes in coordinates of landmarks (Marquez, 2006).

Herewith, Table 4 show the amount of overall variation exhibited by PC1 and PC2. Morphotype B has the lowest variation and small FA value compared to Morphotype A and C. Moreover, morphotype C show the highest percentage of variation (Fig. 4).

*Localized Trait Fluctuating Asymmetry*

Based on FA values obtained per morphotype localized trait fluctuating asymmetries were determined (Table 5). The results revealed that morphotype Alocalized trait fluctuating asymmetry was evident on the antero-ventral tip of anterior cardinal tooth, dorsal tip of posterior and dorsal tip of posterior. These are landmarks 4, 10 and 12 respectively thus, it has also the highest fluctuating asymmetry value of 3.0451 among the other morphotypes. This implies that morphotype A is subject to more stress and has less resistance from perturbations. Moreover, morphotype B acquired variation was in the dorsal tip of posterior cardinal tooth for landmark number 3 only, while morphotype C was in the dorsal tip of posterior for landmark number 10 only. In this respect, stress in this field can be clearly manifested as high levels of FA. A plausible explanation for observed high levels of FA arises from the differences in genetic composition of the population resulting in different tolerance to stress (Ducos and Tabugo, 2014).

**Conclusion**

It is highly believed that the symmetry of an organism is directly proportional to its developmental stability, thus, the more perfectly symmetrical an organism is, it has the capacity to cope with developmental stress and is more stable.

Significant values for Fluctuating asymmetry (FA) were observed in all of the morphotypes examined. Hence, indicates developmental instability. Thus, *V. philippinarum* found in Baliangao, Misamis Occidental is relatively developmentally unstable and its inability to buffer the environmental and genetic stressors above tolerance limit have led to deviation of its relative symmetry.

Moreover, albeit Morphotype B has resulted to significant FA, it has the least significant FA value among the samples examined thus, implies being least disturbed. It is perceived that there is a direct relationship between FA and developmental instability. In this respect, Morphotype B is seemingly a favorable phenotype such that stressors from the environment pose least disturbance on developmental homeostasis.

Also it was observed that more number of individuals in the population has morphotype B, suggesting its relatively higher rate for survival and adaptation to its environment. Based on the observations, stabilizing selection is at work favoring Morphotype B and thus, relatively fit for cultivation to maximize yield for food production.

**Acknowledgment**

The researchers would like to express their heartfelt gratitude to their families and friends who become a considerable source of inspiration and determination.

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