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Landmark-based relative warp analysis of Golden Apple Snail *(Pomacea caniculata)* from Agusan del Norte and Sur rice fields, Philippines

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Abstract

The Golden Apple Snail, *Pomacea caniculata* is considered one of the serious agricultural pests of rice in Asia's countries. Its rapid invasion in many habitats suggests genetic variability and differentiation that expressed in the level of the phenotype. As this pest exists, the researchers endeavor to describe the shell shape variations of *Pomacea caniculata* sample in from different rice fields of Agusan del Norte and Agusan del Sur. A total of 200 golden apple snails (*P.caniculata*) were randomly collected in the rice fields of Bayugan City, Antongalon Butuan City, RTR and Buenavista, Agusan del Norte. To determine shell shape variations among the four (4) snail populations and coordinates were administered to relative warp analysis. Data were subjected to Multivariate Analysis of Variance (MANOVA) using the PAST Software. Results show statistically significant (*p*<0.05) from the appended ventral and dorsal portions of *P.caniculata*. These phenotypic distinctions were associated in geographic isolation, predation, and nutrient components of the gastropods. Thus, the importance of using geometric morphometric is important in describing shell shape variations of *Pomacea caniculata*.

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Introduction

The golden apple snail *(Pomacea caniculata)* is a freshwater snail listed among the top 100 most invasive species worldwide and noted agricultural and quarantine pest that causes great economic losses. It is characterized by fast growth, strong stress tolerance, a high reproduction rate, and adaptation to a broad range of environments (Liu *et al*, 2018). Interest in the golden apple snail *Pomacea caniculata* as a food resource and gourmet export item was noted as the basis of its introduction into the Philippines. But such initiative is as expected. Facts of the invasive character of the species became evident after it was seen to cause serious damage to the ricefields following its probable escape or release into the wild. It was also found out that apple snails had high adaptability to the new environment and were easier to form a new population (Dong *et al.*, 2011). Snails plant-consuming and reproductive abilities, this species is currently causing serious problems for many farmers, particularly in rice production. The types of snails in the Philippines, for example, differ from those in other parts of South America; in fact, research shows that they behaved differently than those in Argentina and Japan, taking longer to mature and hatch eggs. Studies of shell shape variation also found out that snails found in Japan and the Philippines differ in size and structure (Moneva *et al.*, 2012). Each golden apple snail's distribution pattern is an important factor in its morphological variations. Small-scale geographic patterns have been linked to shape differences within populations. As a result, the rapid growth and invasion of this species in various types of environments increases its genetic variability, resulting in speciation (e.g. genotype or phenotype).

However, the organism's inconsistent environments establish to have phenotypic advances in both biological and morphological forms that are attributed to being more receptive and adaptive to changing ecological variations (Torres *et al.*, 2013). Several studies have found that differences in shell shape are also associated with sexual dimorphism, the result of sexual selection. Sexual dimorphism is evident in *P. caniculata* due to shape differentiation, as several studies have shown (Torres *et al.*, 2013). Shape variations and covariations are important factors in determining morphological distinctions between species within the same taxon (Cabuga *et al.*, 2016).

In describing morphological variations in the shell shape of *P. Caniculata*, geometric morphometric was employed. Geometric morphometric (GM) is a tool for describing shape and shape variances among biotic elements. The method proposed for distinguishing and analyzing shape differences from the obtained coordinates assigned in the body shape of organisms while removing the effects of orientation, position, and size.

The X and Y Cartesian coordinates will be used to analyze shape variations among, between, and within organism populations. GM has established the importance of determining quantitative shape analysis of different biological forms (Moneva *et al.*, 2012). It is also an effective tool for determining sexual dimorphism among the snail shell shape of *P. caniculata* (Cabuga *et al.*, 2017). Several studies have utilized GM to quantify variances in the body shapes of aquatic organisms (Mahilum and Demayo, 2014).

Indeed, GM contributes advances over traditional measurements of examining the effects dissimilarities in orientation, location, and position of the organisms. This application is a breakthrough in science particularly in biology since biological forms are essential for evolutionary history (Cabuga *et al.*, 2017). Geometric morphometrics plays an important instrument for showing shell shape variances (Wang, 2011). The landmark-based method allows illustrating phenotypic variances and transforming dissimilarities into an image shape thus proposing three-dimensional shape variations (Webster and Sheets, 2010). Hence, this study aimed to identify the shell shape variations of *P. caniculata* from four different rice fields in Agusan del Norte and Sur through geometric morphometric using landmark-based analysis.

Materials and Methods

Study site

The golden apple snails were obtained from four different rice fields in Agusan del Norte and Agusan del Sur in Caraga region. The samples of *P. caniculata* were collected from the rice fields of Bayugan City - Agusan del Sur, Municipalities of Remedios T. Romualdez (RTR) - Agusan del Norte, Buenavista - Agusan del Norte, and Antongalon - Butuan City, Philippines. About 50 snail samples went through for relative warps (RWs), which are the main components of the covariance matrix of the partial warp scores, were calculated using the centroid size as the alignment-scaling method. PAST software (Hammer *et al.*, 2001) was used to generate histograms and boxplots from the relative warps of the shell morphologies. Histograms and boxplots are effective tools for comparing distributions. They give a concise perspective of where the data are centered and how they are dispersed over the variable's range.

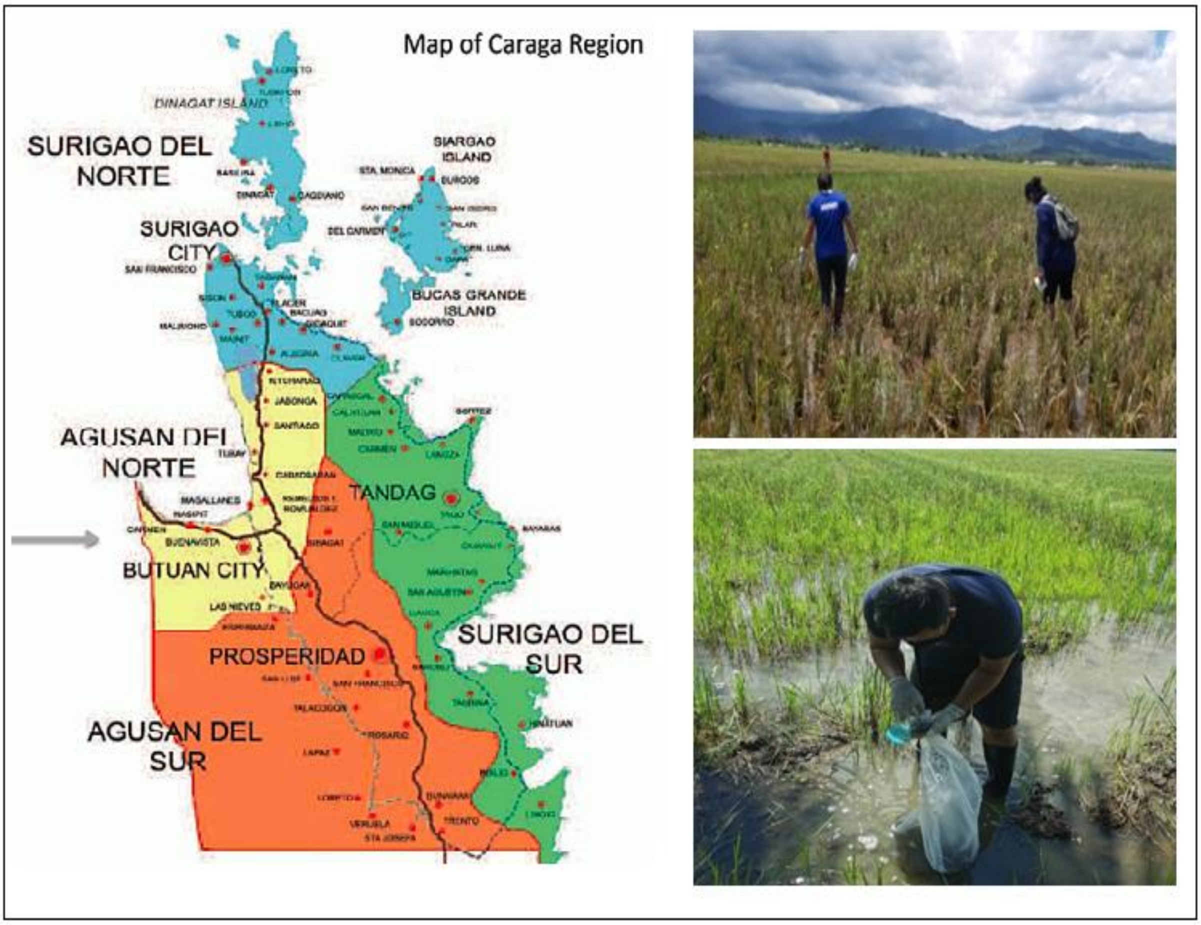

Fig. 1. Map of the study area where *P. Caniculata were* collected.

Research design

This research focuses on landmark-based geometric morphometric methods (GMMs) to analyze shell shape variation of *Pomacea caniculata* . Landmarks are cartesian coordinates of points in 2D or 3D that can be localized precisely and without ambiguity on a structure and from one form specimen to another.

The diagram above shows the flow of the study. Collections of samples were taken first at four different rice fields in Agusan del Sur and Agusan del Norte, namely; Antongalon in Butuan City, Bayugan City in Agusan del Sur, municipalities of RTR in Agusan del Norte, and Buenavista in Agusan del Norte. After samples were taken, digital imaging was done which used a 48 mp android camera. Images obtained were then translated into tps files using tps software. Translated files were appended and linked using tps Util subject for relative warp analysis.

A shape analysis was conducted using tpsRelW and computation of relevant figures was done using the paleontological tool (PAST).

Data gathering procedure

Image preparation

Snail samples were collected by handpicking. An arbitrary lower limit of size for samples collected was about 2cm to exclude juveniles. Before the digitization stage of the snail's shell, samples taken were first boiled with water and cleaned with running water while extracting the meat using forceps. After which, shells with eroded and cracks spire were excluded (Mahilum and Demayo, 2014). The images of the shell will be captured by a digital camera (Huawei P30, 48 megapixels). All pictures were taken with a scale next to the specimen and at the same distance as the camera. To make sure that scaling can be applied during tps landmarking, the ruler was used as a reference for each sample image and the use of a tripod positioned at a fixed distance from the samples was also observed for uniformity. The position of the shell was e in the columella at 900 to emphasize the aperture view or the apex is visible (Moneva *et al.*, 2012). Images of the shell were made sure to be always in the same position with the columella at 90° of the x-axis in an aperture view or in the orientation in which the apex is visible. To eliminate measurement error, the samples were tri-replicated, this means that a population of 50 was replicated thrice making the total number of images per sampling area 150. A total of four (4) populations has a total of 600 images which will be ready for the landmarking.

Landmark-based Analysis

A total of fourteen (14) and seventeen (17) anatomical landmarks points in dorsal and ventral aperture were used in this study (Fig 3). The x and y coordinates were acquired by outlining the scanned pictures of the snail's shell with sample points around its contour. Tps Dig freeware 2.12 image analysis and processing software was used to do this. Tps Dig makes it easy to acquire and store landmark data from digitized pictures, therefore facilitating statistical analysis of landmark data in morphometrics. Once landmark configurations have been acquired on sets of digital images or 3D objects, a Canonical Variance Analysis (CVA) is performed on landmark configurations and consists in minimizing the sum of squared distances between corresponding landmarks to extract shape data by removing the extraneous information of size, location and orientation. A mean shape configuration (consensus) is calculated and variation around this mean can be decomposed into components of morphological variation.

The relative warps (RWs), which are the main components of the covariance matrix of the partial warp scores, were calculated using the centroid size as the alignment-scaling method. PAST software was used to generate histograms and boxplots from the relative warps of the shell morphologies. Histograms and boxplots are effective tools for comparing distributions. They give a concise perspective of where the data are centered and how they are dispersed over the variable's range.

Seventeen (17) anatomical landmarks were selected located along the outline of the ventral (Fig. 3a) portion of the shell and fourteen (14) anatomical landmarks located along the outline of the ventral / apertural (Fig. 3b) portion of the shell was used.

Statistical treatment

The study utilized the multivariate analysis of variance or manova in determining significant differences among the sample of *P. caniculata* taken from different ricefields in Agusan del Norte and Sur. The MANOVA was also used to test differences in shape between groups of samples.

Results and discussion

Canonical Variance Analysis (CVA) scatter plots of the pooled individuals from different populations of the golden apple snails showed patterns of geographical variation. Results of the Multivariate Analysis of Variance (MANOVA) are presented in Table 1 below for the ventral portion of the shell obtained *p*-values of 1.047E-60 while the dorsal portion, MANOVA showed a *p*-value of 3.649E-31 (Table 2) for the population of *Pomacea caniculata* from varying places.

The results of multivariate analysis of variance as shown in Table 1 shows significant shell shape variations for the ventral portion of *Pomacea caniculata* taken from four different rice fields in Agusan del Norte and Sur.

All of the *p*-values are greater than the α that rejects the assumption that the population of *P. caniculata* from four different rice fields in the Caraga region is not significantly different from one another.

Table 1. Results of multivariate analysis of variance (MANOVA) *p*-values for the ventral portion of freshwater snail *P.caniculata* from four different rice fields.

	Antongalon	Bayugan	Buenavista	RTR
Antongalon		9.04E-06	3.79E-25	1.14E-13
Bayugan	9.04E-06		4.74E-28	7.54E-19
Buenavista	3.79E-25	4.74E-28		1.41E-30
RTR	1.14E-13	7.54E-19	1.41E-30	
p	1.047E-7			

Results of multivariate analysis of variance as shown in Table 2 expresses significant shell shape variations for the dorsal portion of *Pomacea caniculata* taken from four different rice fields in Agusan del Norte and Sur. All of the *p*-values are greater than the α that rejects the assumptions that the population of *P. Caniculata* from four different rice fields in the Caraga Region is not significantly different from one another. The results of MANOVA for dorsal and ventral *p*-values of the population of *P. caniculata* showed that the traits tend to be consistently different or similar between pairs of species, they are congruent, and it showed in this plot as general tendency to cluster together that would show the slightest difference between the samples (Moneva *et al.*, 2015).

Table 2. Results of multivariate analysis of variance (MANOVA) *p*-values for the dorsal portion of freshwater snail *P.caniculata*.

	Antongalon	Bayugan	Buenavista	RTR
Antongalon		5.24E-10	2.65E-13	2.09E-09
Bayugan	5.24E-10		2.15E-09	6.40E-12
Buenavista	2.65E-13	2.15E-09		1.65E-08
RTR	2.09E-09	6.40E-12	1.65E-08	
p	3.649E-31			

The variations of the shell shape are can be visualized through the CVA and relative warp yet the variations are not significantly seen in the results of MANOVA. The same study also supports that there is a correlation between the variations among the shell shapes of Golde Apple Snails that indicates their phenotypic plasticity that took advantage of their sustainability to varying environments (Guitierrez, 2017). Several studies have found that differences in shell shape are also associated with sexual dimorphism. Sexual dimorphism is evident in *P. caniculata* due to shape differentiation as several studies have shown (Torres *et al.*, 2013). The summary of the geometric morphometric analysis showing the consensus morphology and variation in the ventral shell shape pattern of different *P.caniculata* populations as produced by the relative warps (RW) is shown in Figure 3 and explained in Tables 3 and 4. On the other hand, Figure 4 showed the clusters of the samples depending on their shapes to observe underlying differences and sources of variability (Moneva, 2015) among groups in terms of congruence among characters and would be further seen in the relative warps score in Figure 5 and 6. It is important to study these characters since the conchological characters in molluscs are primarily useful in identifying taxonomic groups as well as clarifying issues on populations within a given taxon However, some findings could show that these characters could provide visual links between environmental factors and development thus increasing understanding of a species (Gala *et al*., 2015). For instance, a study on the Roman snail (*Helix pomatia*) shows that heavy metal accumulation in the hepatopancreas could affect shell height, relative shell height and whorl number. Other indications of environmental influence on conchological characters are shown in shell shape spectrum in *Margaritifera margaritifera* (L.) with river water pH and with the current for *Semisulcospirareinniana* (Yusa, 2016). The variations of each population may also be seen and further be visualized using the histogram and PCA which are used by other studies.

Table 3. Percentage variance and overall shape variation in the ventral/apertural shell of golden apple snail as explained by significant relative warps.

RW	% Variation	Ventral/apertural shell
1	28.09%	- RW1: Smaller body, elongated and wider aperture. +RW1: Globulus body, small yet circular aperture.
2	17.11%	- RW2: Smaller body, elongated and wider aperture. +RW2: Globulus body, small yet circular aperture.
3	11.51%	- RW3: Smaller body, elongated and wider aperture. +RW3: Globulus body, small yet circular aperture.
4	9.59%	- RW4: Smaller body, elongated and wider aperture. +RW4: Globulus body, small yet circular aperture.
5	6.83%	- RW5: Smaller body, elongated and wider aperture. +RW5: Globulus body, small yet circular aperture.
6	5.50%	- RW6: Smaller body, elongated and wider aperture. +RW6: Globulus body, small yet circular aperture.

The projections on the left side of the histogram are considered to be variations in shell shape foreseen as negative deviations of the mean of the axis of the relative warps on the ventral side of the snail shell. While on the right side are variations in shell shape foreseen as positive deviations of the mean in the axis of the relative warps. The topmost figure is the mean shape of the samples obtained. For the ventral part of the shell, Relative warp analysis obtained six significant relative warp axes.

Table 4. Percentage variance and overall shape variation in the dorsal shell of golden apple snail as explained by significant relative warps.

RW	% Variation	Dorsal shell
1	36.18%	-RW1: Spine whorls are parallel and wide. Has a larger aperture and a smaller body. +RW1: Spine whorls are triangular. Has a small aperture and a larger body.
2	17.94%	-RW2: Spine whorls are parallel and wide. Has a larger aperture and smaller body. +RW2: Spine whorls are triangular. Has a small aperture and a larger body.
3	9.99%	-RW3: Spine whorls are parallel and wide. Has a larger aperture and a smaller body. +RW3: Spine whorls are triangular. Has a small aperture and a larger body.
4	8.69%	-RW4: Spine whorls are parallel and wide. Has a larger aperture and a smaller body. +RW4: Spine whorls are triangular. Has a small aperture and a larger body.
5	6.69%	-RW5: Spine whorls are parallel and wide. Has a larger aperture and a smaller body. +RW5: Spine whorls are triangular. Has a small aperture and a larger body.

The first relative warp obtained 28.09%, warp #2 17.11%, relative warp #3 is 11.51%, relative warp #4 is 9.59%, relative warp #5 is 6.83%, and relative warp #6 obtained 5.50%. This means that the relative warp analysis on the ventral portion of the *P. caniculata* shows wider variation among the snails with a smaller body, elongated, and wider aperture. Further, Figure 6 shows the relative warp box plot and histogram showing variations in the shape of the dorsal portion shell of *P. caniculata* found in varying rice fields in Agusan: Bayugan, Buenavista, RTR, and Butuan City. Projections on the left side of the histogram are shows the variations in shell shape foreseen as negative deviations of the mean of the axis of the relative warps on the dorsal portion of the snail shell. Then on the right side are variations in shell shape foreseen as positive deviations of the mean in the axis of the relative warps.

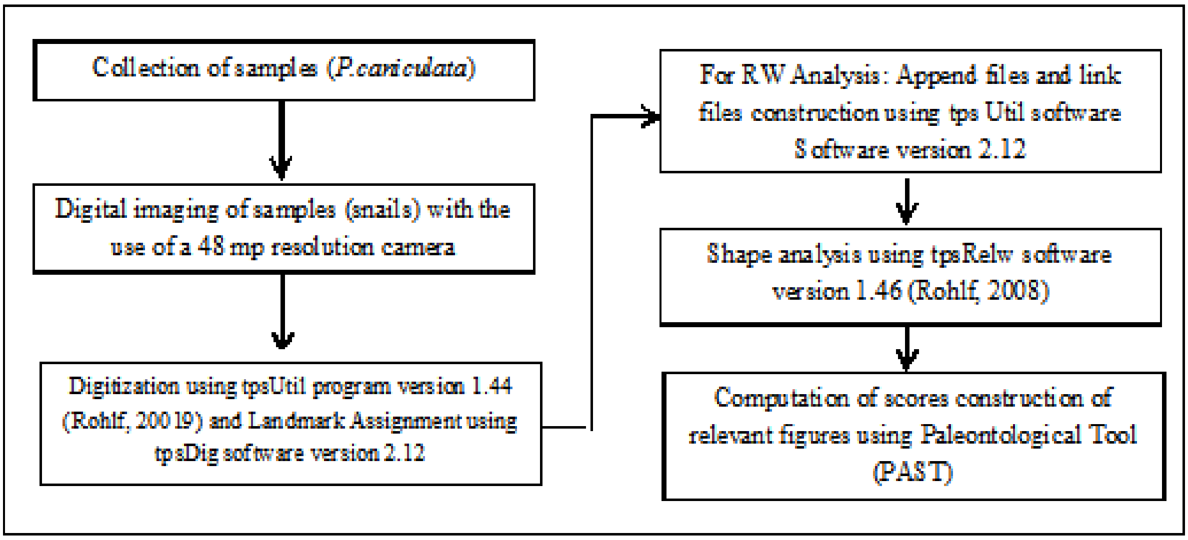

Fig. 2. Research workflow of the study.

The topmost figure is the mean shape of the samples obtained. For the dorsal part of the shell, relative warp analysis obtained five (5) significant relative warp axes as seen in Table 4.

The first relative warp obtained 36.18 %, second warp 17.94%, relative warp #3 is 9.99%, relative warp #4 is 8.69%, and relative warp #5 obtained 6.69% variation. This means that the relative warp analysis on the dorsal portion of the *P. caniculata* in negative axis shows that spine whorls are parallel and wide with a larger aperture and smaller body. While relative warp analysis on the dorsal portion of the *P. caniculata* on the positive axis shows that spine whorls are triangular and have smaller apertures and larger bodies.

The summary of the geometric morphometric analysis showing the consensus morphology and variation in the ventral shell shape pattern of different *P.caniculata* populations as produced by the relative warps (RW) is shown in Figure 6 and explained in Table 3.

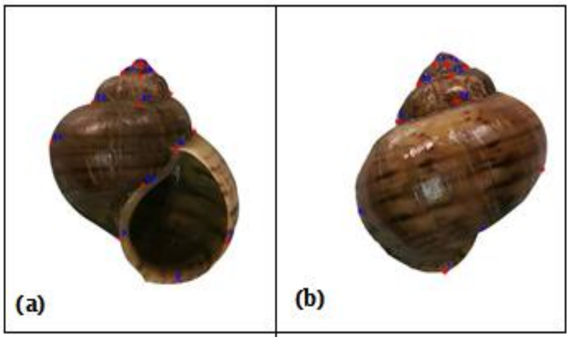

Fig. 3. Landmarks used to describe the shape of (a) ventral, (b) dorsal view of the shell of *Pomacea caniculata*.

This shell shape variation implies Golden Apple Snails having a globulus body and wider aperture is an adaptation that allows the snails to minimize the damages in its shell during tumbling and may reduce dislodging (Cagauan, 2017).

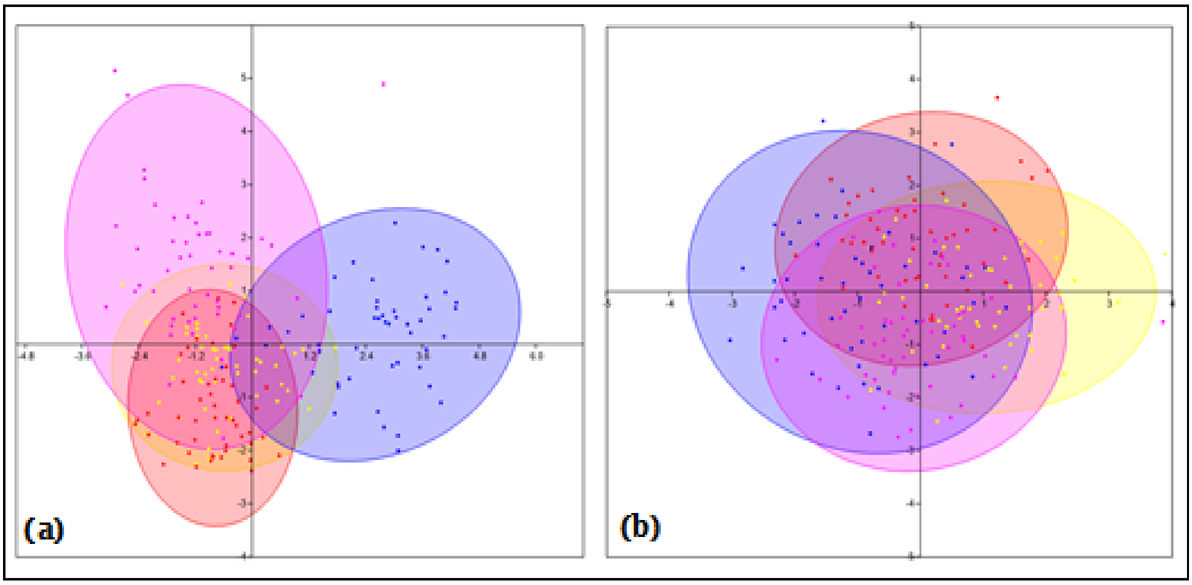

Fig. 4. CVA scatter plot of the (a) ventral and (b) dorsal portions of the freshwater snail *P.caniculata*.

Snails having the characteristic of phenotypic plasticity is a great strategy for organisms that are unable to modify their genes to change their phenotype to meet the demands of the environment (Hollander and Butlin, 2016).

As a primitive circumtropical species, *P.caniculata* possesses strong ecological plasticity with many advantages, including low-temperature resistance and drought tolerance, Which has contributed to its competitive success in resource acquisition. *Pomacea caniculata* has been reported to establish populations at temperatures ranging from 10˚C to 35˚C (Liu *et al.*, 2018). The summary of the geometric morphometric analysis showing the consensus morphology and variation in the dorsal shell shape pattern of different *P.caniculata* populations as produced by the relative warps (RW) analysis. Results showed a general tendency for each population to cluster out together implying distinction or similarities of *P. caniculata* populations in varying locations, which suggests there is no geographical variation of the species. However, these populations are highly variable in the aperture and top / whorl shell which can be seen in Figures 5 and 6 and discussed in Tables 3 and 4. The result shows how the population differs from the rest by interpreting such differences in terms of individual characters and the warping of the different populations may depend on other factors excluding their geographical location (Moneva, 2015).

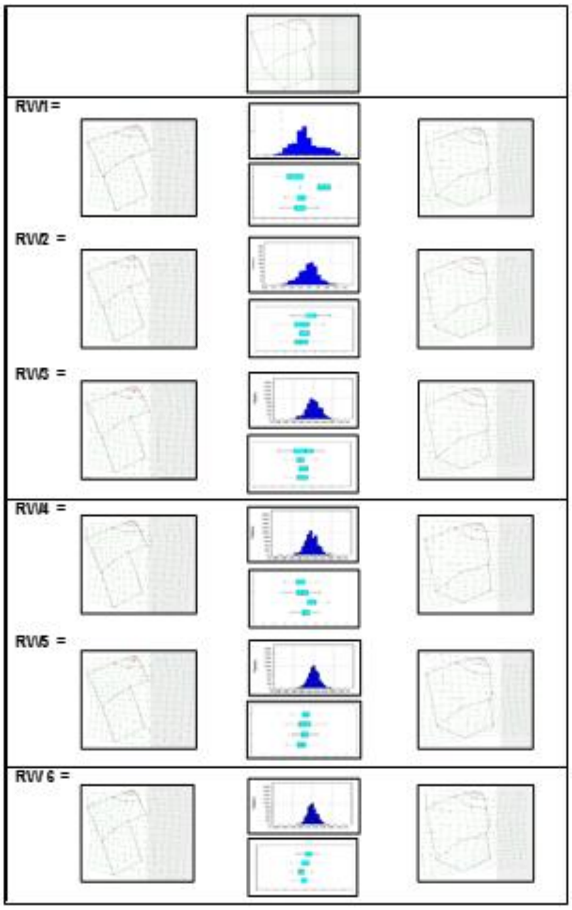

Fig. 5. Relative warp box plot and histogram showing variations in the shape of ventral / pertural portion shell of *Pomacea caniculata* found in varying rice fields in Agusan del Sur, Bayugan, Buenavista, RTR, and Butuan City.

The phenotypic plasticity of Golden Apple Snails is environmental effects such as Physico-chemical factors and predation (Minton *et al.*, 2016). The changes are often accompanied by a change in spire height and aperture size; high, narrow spires and small apertures may reduce predations in headwaters (Lagesson, 2015) which are evident in the samples collected in this study while low spires and large apertures may reduce dislodging and damages during tumbling (Haase, 2017).

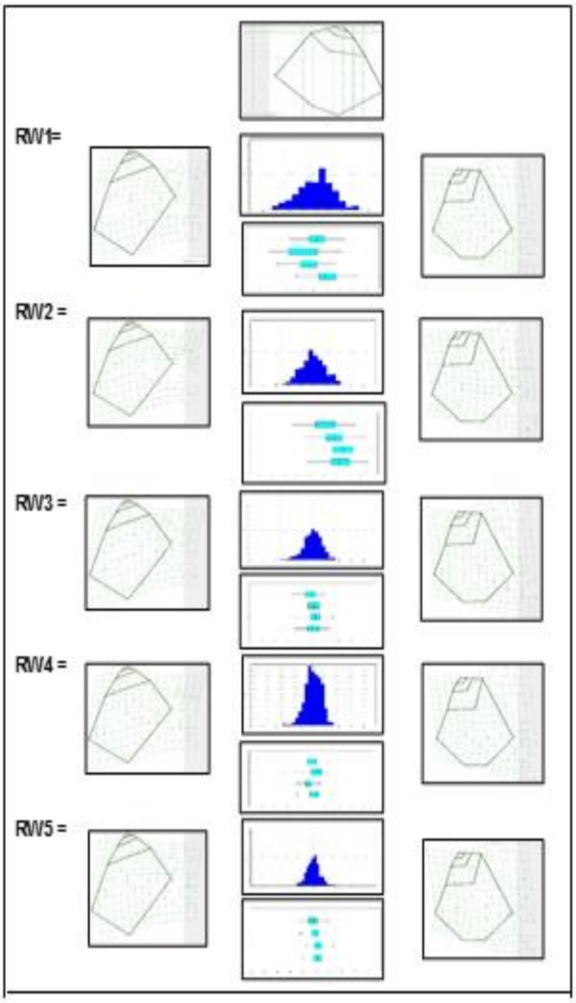

Fig. 6. Relative warp box plot and histogram showing variations in the shape of dorsal portion shell of *Pomacea caniculata* found in varying rice fields in Agusan del Sur, Bayugan, Buenavista, RTR, and Butuan City.

Thus phenotypic plasticity in Golden Apple Snails evolved to allow organisms a greater chance of survival in changing environments and leads allow to the variations and warping of the shell shape of the snails.

Conclusion

Snail shell morphology is traditionally quantified through straight-line shell measurement and ratios. However, traditional measurements can be strengthened by applying quantitative tools to provide a more reliable descriptive analysis of shell shape. Thus, the use of geometric morphometric analysis shows that *P. caniculata* shell varies its shape, the geography does not contribute to shaping the structure of the Golden Apple Snail population. Result of the MANOVA, revealed that the *p*-value in each corresponding location is greater than α (0.05). This concludes that there is no significant differences or shell shape variation among the observed *P. caniculata.* In this regard, further study on soil physicochemical analysis of snails' tissue is significant to trace the presence of heavy metals that may affect shell shape variations. It is also encouraged to future researchers to conduct water analysis along with physicochemical tests and sampling shall be done either early in the morning or late afternoon or when the sun is out for them to easily obtain the sample.

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