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Outline-based geometric morphometric analysis of shell shapes in geographically isolated populations of *Achatina fulica* from the Philippines

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ABSTRACT
The invasive giant African land snail, *Achatina fulica* collected from different geographical locations in the Philippines exhibit qualitative differences in shell shape and banding pattern configurations. To be able to describe quantitatively the differences in shapes in relation to banding patterns and geographical locations, this study used outline-based geometric morphometrics analysis, the elliptic Fourier analysis. Snails were collected from 15 different geographical locations in the Philippines and were categorized under 14 different banding patterns. The shells were photographed, digitized using tpsDig software and subjected to elliptic Fourier analysis. Results showed the variations were mainly observed on the lateral and longitudinal length aspect of the shell. A total of 16 major shape configurations were observed through variation in shell shape, aperture size, and aperture margin shape. One important finding of this study suggested some degree of intrapopulational variations as shown by the scatterplots which may suggest phenotypic plasticity in the species. Although the scatter plots revealed some degree of overlaps among populations, Kruskal-Wallis test, phenetic and cluster analysis showed significant differences among populations. The differences can be due can be due to many possible factors including genetic, biotic and abiotic factors since geographic distance cannot be attributed to the differences observed among populations.

Keywords: *Achatina fulica*, banding pattern, EFS, invasive species, land snail, outline.

1. Introduction

From the origin of *Achatina fulica* in East Africa to the Philippines, variations within and among populations may indicate that the organism may have diverged from its native population [1]. This ecologically damaging land snail [2-3] is argued to have a wide range of ecological tolerance [4] thus is considered very invasive. Qualitative observations among populations show phenotypic variations in shell shapes and banding patterns suggesting genetic and environmental influences [5-9]. Quantitative conchological variations that made use of landmark-based geometric morphometric (GM) tools [1-2] have found significant variations within populations of the species. Study on the polymorphic banding patterns observed on the shell of *A. fulica* were correlated to shell shape variation [10] and geographical locations [1]. Since GM tools were found to be useful in gastropods shells because they are accreted rather than grown [11], the outline-based shape analysis of GM was explored to study the mollusk shell. The tool is useful for structures that are generally homologous (in biological sense) or comparable in the geometric sense, but where individual of homologous points analysis of curve may be absent which could not be detected through landmark based analysis [12]. In the current study, shape variations within and between populations with varying geographical locations were quantitatively analyzed.

2. Material and Methods

2.1 Sampling area

This study was conducted from April 2013 to March 2014. Snail samples were collected from 15 different provinces across the Philippine islands: Cagayan (Tuguegarao City), Pangasinan (Calasiao), Quezon (Lucena City), and Rizal (Antipolo City) in Luzon; Bohol (Agapi, Ondol, and Kinogitan) and Southern Leyte (Sogod) in Visayas; and Compostela Valley (Las Arenas), Davao del Norte (New Corella and Panabo City), Davao del Sur (Emily Holmes, Riverside, Nova Tierra Village, Mandug in Davao City and Padada), Lanao del Norte (MSU-IIT campus and Mimbalot Falls, Iligan City), Lanao del Sur (Marawi City), Misamis Occidental (Cagayan de Oro City), South Cotabato (General Santos City), and Zamboanga Sibugay (Ipil). Sampling site locations were plotted in Figure 1.

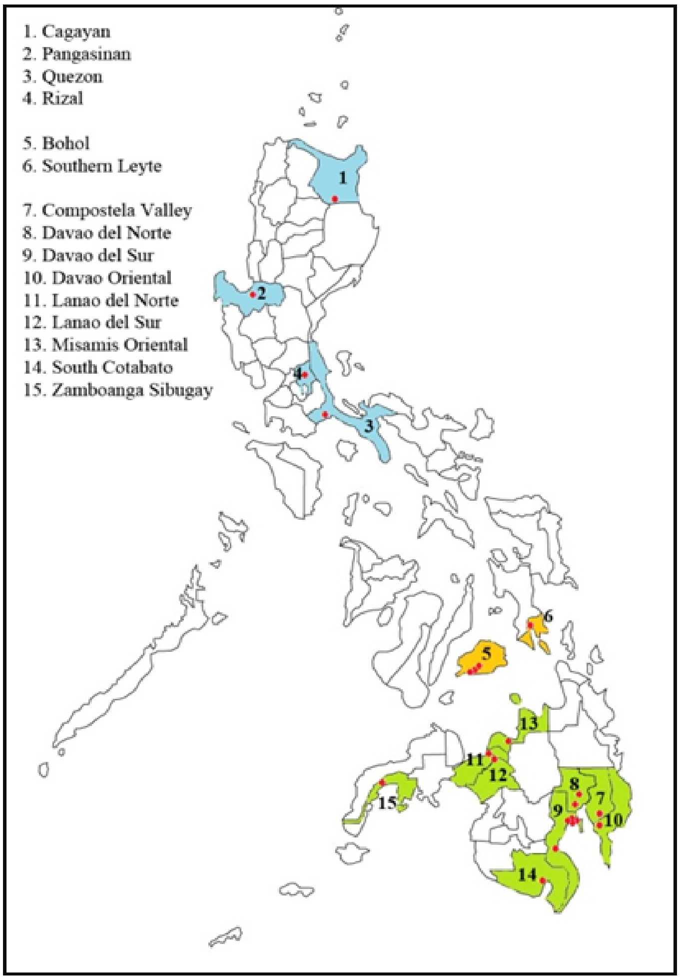

Fig 1: Map of the Philippines. Red dots correspond to locations of the different sampling sites.

2.2 Imaging, Organizing and Digitalization

A total of 1309 mature shells were collected from the different sampling sites. For imaging, shells were positioned in such a way that the columellar is at 90° of the x-axis in the aperture view and in the orientation in which the apex is visible. The digital images of the shells were then captured using a high resolution digital single-lens reflex (DSLR) camera with manual focus setting mounted on a tripod to maintain the distance for all samples, and to ascertain uniformity and minimize errors. Specimens were digitized and the images were triplicated in order to reduce the measurement error [13]. The images of the grouped samples were digitized using tpsUtil [14] and saved as thin-plate splines (TPS) files. TPS files were further pooled by the corresponding banding pattern.

2.3 Outline data acquisition

A total of 199 points were established for outlining ventral marginal shape of *A. fulica* (Figure 2) using tpsDig version 2.12 [15]. After outlining the tps curve was converted into landmark points or XY coordinates using tpsUtil [14] which served as the raw data for the succeeding analysis.

2.4 Data analysis

Thin-plate splines (TPS) technique was used to analyze and display the shell shape variations. The TPS consisted of fitting an interpolating function to the landmark coordinates of each specimen against the reference configuration so that all homologous landmarks coincide [16]. Generalized Procrustes Analysis (GPA) was then performed to superimpose the landmark configurations using least-square estimates for translation and rotation parameters [17], eliminating any variation due to differences in translation, orientation, and size [18]. Elliptic Fourier Shapes (EFS) using the algorithm in Paleontological Statistics (PAST) software version 2.13 [19] was then used as a standard method of outline analysis [20]. Outlines were smoothed 30 times (30 modes) to reproduce morphological details significantly, first eight (8) harmonics (8 shapes) per population were used.

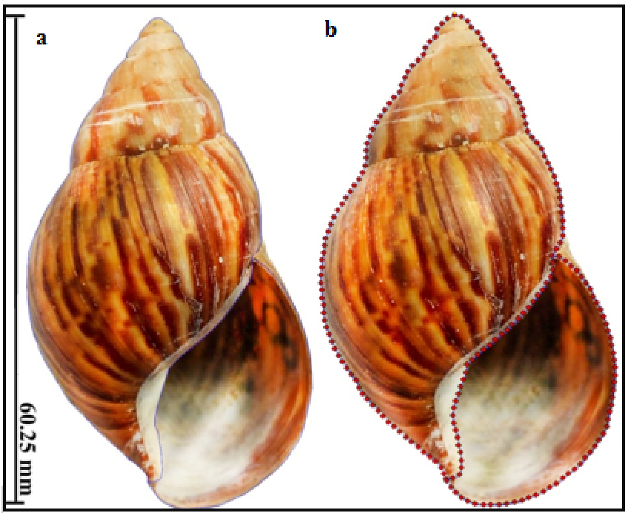

Fig 2: Outline (**A**, blue line) and coordinates (**B**, red dots) on the ventral view of the shell of the *A. fulica*.

Data after GPA was used for Principal Component (PC) Analysis to explore patterns of variation in the shells shapes of *A. fulica.* PCA from a variance-covariance matrix was computed in PAST 2.13 [19]. Scatter plots generated using the significant PC scores were used to visualize the distribution of data over the range of variables. Kruskal-Wallis Test, a nonparametric test used to compare independent groups of sample data, was used to determine significant differences in shape variations of shells. The analysis made use of the first and second PC scores of the pooled data to compare the population mean ranks [21]. The consensus data of the pooled file was used for Cluster Analysis where multivariate data were separated into a series of hierarchically related sets [22]. To further determine the relationship among populations of banding patterns and geographical locations, a phenetic analysis based on the general or major shell shape configuration was conducted. All statistical analyses were performed in PAST software version 2.13 [19].

3. Results and Discussion

Sixteen (16) general shell morphologies for the ventral view of the *A. fulica* shell (Figure 3) resulted after outline-based analysis. Variations were observed mainly on the spire-whorl length, body-aperture size, and aperture shape. Figure 4 represents Elliptic Fourier shapes produced by smoothing outlines 30 times to produce significant morphological detail for shells from different locations. The first eight (8) harmonics were used. Modes of change in shell shapes by banding patterns of the *A. fulica* were represented by elliptic Fourier shapes (EFS). Variation of color saturation from dark to light specifies representative shapes of EFS 1 to 8 of 30 modes. It can be seen from these figures that distinct observable variations were detected in lateral and longitudinal length aspect of the shell.

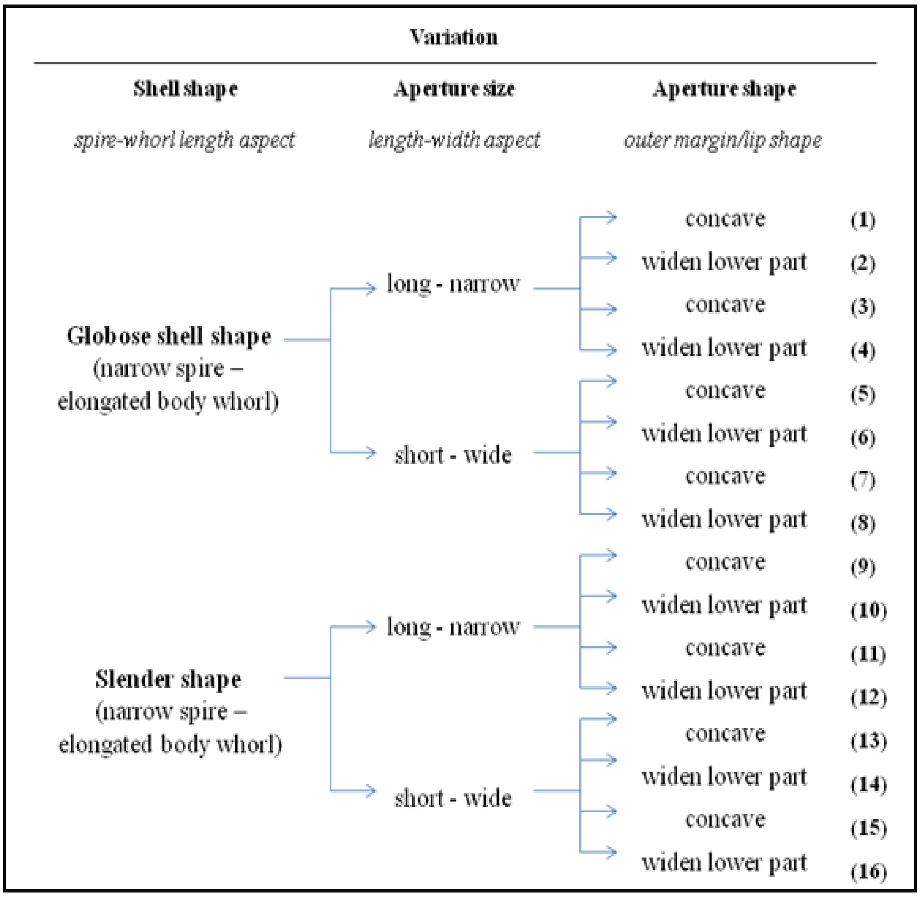

Fig 3: Summary of the major shell shape morphologies of A. *fulica*.

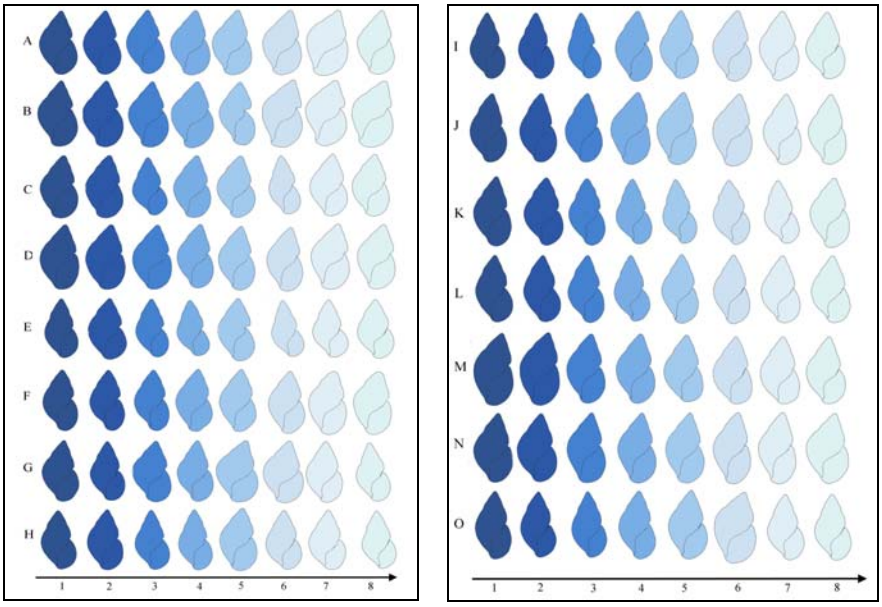

Fig 4: Modes of change in shell shapes in geographically isolated populations of the *A. fulica* represented by Elliptic Fourier Shapes (EFS); variation of color saturation from dark to light specifies representative shapes of EFS 1 to 8 of 30 modes. (*Legend : Cagayan (A), Pangasinan (B), Quezon (C), Rizal (D), Bohol (E), Southern Leyte (F), Compostela Valley (G), Davao del Norte (H), Davao del Sur (I), Davao Oriental (J), Lanao del Norte (K), Lanao del Sur (L), Misamis Oriental (M), South Cotabato (N), and Zamboanga Sibugay (O)*).

Scatter plot of the significant principal components (PC) for the different populations is presented in Figure 5. The differences cannot be ascertained from the plot thus multivariate analysis of variance (MANOVA) was done. The results show significant relationship for the two variables – differences in shape with geographical location (Wilk's Lambda = 0.6615, df1 = 28, df2 = 2264, F = 18.56, and p(same) = 7.797^{-82}). The first PC which accounts for the highest variability in the shape of the shell contributing 90.886 % of overall shape variation. Table 1 shows the percent (%) variance contributed by each principal components to the conchological variation.

Analysis of the overall variation in the shell shape was also done by analyzing the PC scores with nonparametric version of one way ANOVA, the Kruskal-Wallis test. The results shown in Table 2 revealed significant relationship between geographical location and shell shape (p=4.011^{-29}). Shell population from Lanao del Sur and Bohol were shown to be most significantly different as compared to other populations.

Table 1: Percentage variance value of the significant principal component scores in the ventral view of the outline of the shells of *A. fulica* by geographically isolated population.

Principal Components	Eigenvalue	Variance (%)	Eigenvalue	Variance (%)	Eigenvalue	Variance (%)
	A		F		K	
PC 1	0.003059	75.276	0.004291	90.886	0.005611	84.162
PC 2	0.000496	12.202	0.000265	5.6149	0.000354	5.3052
PC 3	0.000141	3.4691			0.000216	3.2434
PC 4	0.00012	2.9427			0.00014	2.1065
PC 5	6.30E-05	1.5504			0.000113	1.6877
Total		95.4402		96.5009		96.5048
	B		**G**		**L**	
PC 1	0.008222	88.634	0.002001	74.808	0.003002	80.323
PC 2	0.000365	3.9329	0.000212	7.9086	0.000245	6.5451
PC 3	0.000254	2.7401	0.000142	5.2968	0.00013	3.4703
PC 4	0.000113	1.2188	6.66E-05	2.4893	0.000122	3.2723
PC 5			5.78E-05	2.1596	6.72E-05	1.7983
PC 6			3.78E-05	1.4122		
PC 7			3.28E-05	1.2279		
PC 8			2.41E-05	0.90232		
Total		96.5258		96.2047		94.409
	C		**H**		**M**	
PC 1	0.003288	80.07	0.003183	79.112	0.00381	83.546
PC 2	0.000427	10.41	0.000326	8.0953	0.000327	7.1736
PC 3	0.000135	3.299	0.00016	3.9652	0.000133	2.9172
PC 4	6.15E-05	1.4979	9.02E-05	2.2418		
PC 5	4.81E-05	1.1713	6.99E-05	1.7374		
PC 6			2.89E-05	0.71751		
Total		96.4482		95.8692		93.6368
	D		**I**		**N**	
PC 1	0.001672	74.987	0.002608	75.932	0.001068	56.233
PC 2	0.000238	10.661	0.000306	8.9016	0.000489	25.727
PC 3	0.000194	8.6764	0.000153	4.4678	0.000144	7.587
PC 4			8.30E-05	2.4171	9.52E-05	5.0115
PC 5			6.73E-05	1.96		
PC 6			4.82E-05	1.4034		
PC 7			3.16E-05	0.92078		
PC 8			2.58E-05	0.75113		
PC 9			1.48E-05	0.42953		
PC 10			1.37E-05	0.40031		
Total		94.3244		97.58365		94.5585
	E		**J**		**O**	
PC 1	0.003773	81.091	0.001532	76.599	0.003082	78.599
PC 2	0.000323	6.9492	0.000193	9.629	0.000263	6.7154
PC 3	0.000152	3.277	9.64E-05	4.8177	0.000167	4.2606
PC 4	0.000112	2.4058			0.000141	3.5859
PC 5	8.18E-05	1.758			7.16E-05	1.8266
PC 6	4.00E-05	0.85926			3.97E-05	1.0132
PC 7	2.77E-05	0.59594				
PC 8	2.33E-05	0.50137				
TOTAL		97.4376		91.0457		96.0007

Table 2: Result of Kruskal-Wallis test on the shell outline shapes of different geographically isolated population of A. fulica based on the first and second principal component scores. Mann-Whitney pairwise comparison (Bonferroni uncorrected) of the shell shapes. *Colored cells indicate significant difference (0.05 level of significance).*

Location						Location									
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
A	-														
B	0.258	-													
C	**5.09^{-06}**	**0.0259**	-												
D	0.4504	0.9584	**0.0167**	-											
E	**3.06^{-12}**	**1.61^{-05}**	**0.0294**	**5.63^{-04}**	-										
F	0.0707	0.493	0.31	0.3199	**0.0365**	-									
G	0.0717	0.2817	**6.46^{-05}**	0.9086	**8.74^{-15}**	0.2513	-								
H	**1.80^{-08}**	**1.78^{-03}**	0.2974	**5.79^{-03}**	0.2359	0.1339	**1.72^{-09}**	-							
I	**2.95^{-06}**	0.0868	0.2939	0.0737	**2.34^{-06}**	0.6467	**3.67^{-06}**	**6.51^{-03}**	-						
J	0.2187	0.8433	**0.0428**	0.7275	**6.77^{-04}**	0.2756	0.436	**0.0107**	0.0867	-					
K	**9.067^{-04}**	0.4905	0.0949	0.1946	4.14^{-06}	**0.909**	0.0597	**3.33^{-04}**	0.1147	0.4836	-				
L	**2.94^{-10}**	**4.33^{-05}**	**0.0170**	**6.86^{-04}**	0.3993	**0.0232**	**8.94^{-13}**	0.0821	**1.65^{-05}**	**1.97^{-04}**	**3.18^{-06}**	-			
M	0.1697	0.6525	**7.61^{-03}**	0.8509	**1.49^{-06}**	0.3467	0.7472	**1.02^{-04}**	**8.25^{-03}**	0.7283	0.2122	**6.98^{-07}**	-		
N	0.5134	0.0632	**6.07^{-06}**	0.2001	**2.18^{-09}**	**0.0262**	**0.0141**	**8.59^{-08}**	**5.58^{-06}**	**0.0365**	**1.11^{-03}**	**2.68^{-09}**	**0.03817**	-	
O	**0.0102**	0.7862	**3.09^{-03}**	0.5102	**1.13^{-10}**	0.5485	0.2226	**7.25^{-07}**	**1.51^{-03}**	0.9693	0.4348	**8.19^{-10}**	0.5663	**3.19^{-03}**	-

Legend: Cagayan (A), Pangasinan (B), Quezon (C), Rizal (D), Bohol (E), Southern Leyte (F), Compostela Valley (G), Davao del Norte (H), Davao del Sur (I), Davao Oriental (J), Lanao del Norte (K), Lanao del Sur (L), Misamis Oriental (M), South Cotabato (N), and Zamboanga Sibugay *(O).*

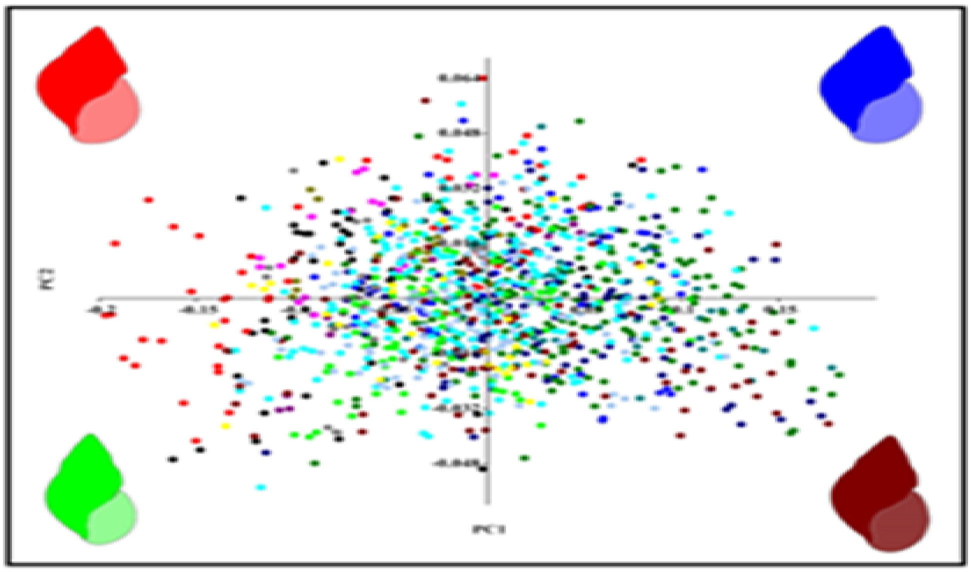

Fig 5: Principal component (PC) scatter plot showing the distribution of shell shapes of different banding patterns (a) and of different geographical locations (b) based on outline geometric morphometric analysis with corresponding shapes of each quadrant. Legend: Cagayan (black), Pangasinan (red), Quezon (blue), Rizal (pink), Bohol (green), Southern Leyte (violet), Compostela Valley (yellow green), Davao del Norte (navy blue), Davao del Sur (neon blue), Davao Oriental (brown), Lanao del Norte (maroon), Lanao del Sur (blue green), Misamis Oriental (yellow), South Cotabato (gray), and Zamboanga Sibugay (sky blue).

Phenetic analysis of *A. fulica* from different geographical locations revealed the clustering of populations by major islands and neighboring regions except that of the shell population from Quezon (Luzon) which was observed to have a globose shell with concave long-narrow aperture, globose shape shell with widen lower margin of the lone-narrow aperture, and a globose shape shell with concave long-wide aperture. Cluster analysis however of the consensus shape of the shell populations showed differences with that generated from phonetic analysis. This result however is consistent with the result of the Kruskal-Wallis test. Shells from Bohol were observed to have a slender shape with concave short-narrow aperture or a widened lower margin of the short-narrow aperture.

It can be seen from the above results from EFA that the conchological variations observed on the shells of the *A. fulica* from different geographical locations can be due to many possible factors including genetic, biotic and abiotic factors. Cluster analysis of shape data or consensus shapes showed that the differences between populations cannot be attributed to geographical distance. Unraveling the way in which populations adapt to their environments, and the processes maintaining the intraspecific variation in the species was not done and is out of scope in this study. Since *A. fulica* exhibited morphological differences both within and among populations, it is argued that the differences or variations in size, shape and colour can be attributed to environmental conditions [23]. Some researchers however claimed that the variability in shape of *A. fulica* was due to genetic anomalies [24]. Since clear mate-choice behavior was reported in *A. fulica*, it can be asserted that the mate-choice criteria could be based on the reproductive stage or a size-assortative [25] thus contribute to the observed variability. Others also argue that variation in shells of *A. fulica* is not only genetic but also affected by the growth rate and population density of the snails [1]. It may also be possible that the diversity within populations observed could be due to the numerous introduction and reintroduction of several gene pools of snails to different regions of the Philippines [1]. The variations in the snail's shell morphology could also be indicating phenotypic plasticity or genetic differentiation. Plasticity influences the evolution and adaptive responses of organisms because it can alter the relationship between the phenotype, which is the target of selection, and the genotype [9]. The response of a trait to selection depends on its heritability and its genetic correlation to other traits also under selection [26]. Plasticity can affect both of these factors and thus produce quite surprising effects on the direction and rate of evolution [9].

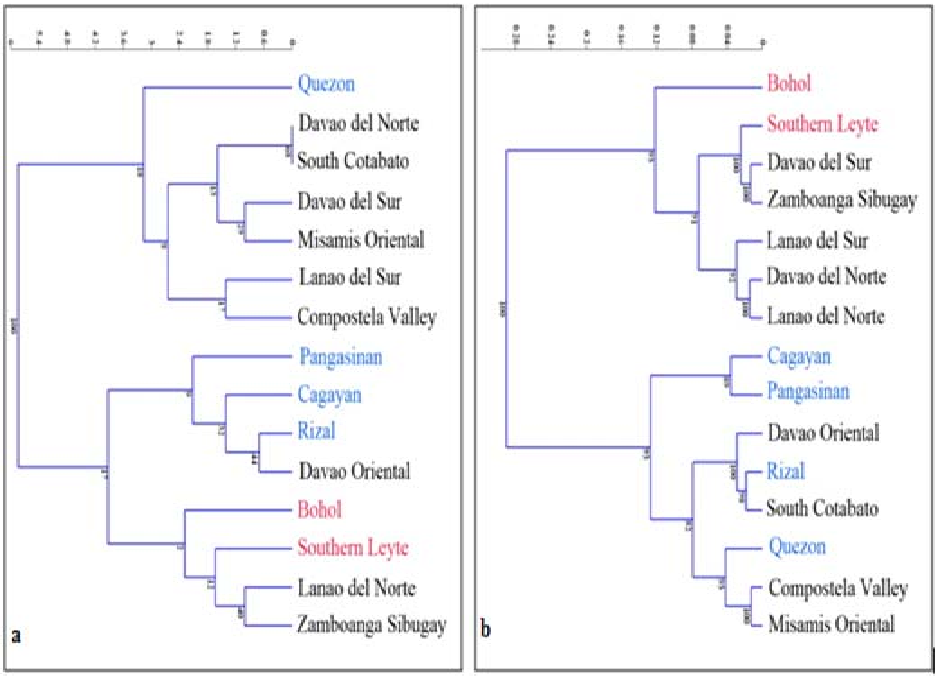

Fig 6: Result of the Cluster analysis on shell shape of the different geographically isolated populations of A. fulica based on the (a) major shell shape variations and (b) consensus data of each population. *Legend: Blue (*Luzon regions), Red (Visayas regions), and Black (Mindanao regions).

It is also possible that since the snails were collected from different geographical locations across the Philippine islands which have varied climatic and environmental factors, these may have direct effects on the snail shell shapes. Aestivation stage of the organism [27], humidity and temperature range in a habitat of the land snail [28] are factors that were argued to have influenced variations in the snail. Differences in aperture size and shape could be due to humidity and temperature since snails adapted to areas with low to almost no rainfall have smaller aperture to minimize the exposed surface area to further reduce the loss of water or humidity under the stress condition [5]. Variation in spire-body whorl length aspect could be attributed to temperature range. It was reported that *A. fulica* is active at 50% increase in humidity, a severe change in temperature and extended dry periods could promote aestivation stage to the land snail [28-30]. This is argued to be causing physiological changes which could affect the snail's development [27], especially the shell length-width relationship [28].

4. Conclusion

Using the outline-based method for specimens with the absence of homologous points on curves, the shape of the shell can be described quantitatively. Conchological investigation in *Achatina fulica* from different geographical locations in the Philippines revealed intra- and inter populational variations suggesting phenotypic plasticity in the species. The differences can be due to many possible factors including genetic, biotic and abiotic factors since geographic distance cannot be attributed to the differences observed between populations.

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