

POST NATAL CRANIAL AREAS OF EVOLUTION IN THE SMALL AFRICAN PANGOLIN (*PHATAGINUS TRICUSPIS*) ONTOGENY FROM THREE GEO-ECOLOGIES – A FIRST REPORT: ASYMMETRY, ELLIPTICAL FOURIER ANALYSES AND MODULARITY

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Abstract

Background; We here present a first intraspecific eco-variant post natal attempt at evaluation of skull signaling in the small African pangolin (Boreoeutheria) family, sub-family phatagininae emphasizing evolutionary trend in ontogeny. Forty; digitally processed skulls and foramen magnum outlines of this species from different geo-locations were assessed for asymmetry, foramen magnum shape variations and modularity hypotheses on skull areas using geometric and Elliptical Fourier analyses methods. Results; Regression of log transformed centroid size and asymmetric components suggested significant expression of asymmetry through ontogeny ($p < 0.03$) with size accounting for 81.34% of asymmetric shape changes. Multivariate analysis of regression confirmed directional but low ($p < 0.5$) fluctuating asymmetry ($F_{1539} = 3.4045$, $F_{882} = 3.2665$, for dorsal and ventral views respectively). Intraspecific ontogenetic allometric trajectories followed rostro-caudal and caudo-lateral directions; intercepts for shape/size predictions were parallel. Mahalanobis distances between centroids (2.42) of specimens were significant ($p < 0.01$). Variance-covariance matrix in ontogeny lies between 0.0017 and 0.56 suggestive of shape overlaps and variations. Foramen magnum outline descriptors by incremental harmonics explained morphologic details; the first 4 effective principal components defined 96.98% of shape properties, while (3.02%) constituted finer details. 74.1% accuracy decline after size factor elimination. Modulation PCA: of Covariance Matrix and Asymmetry component was 88.38% and 7.48% (PC1 and 2) variance % predicted 10.08%. Conclusions; The study confirmed directional 'handedness' and fluctuating asymmetries among skull samples studied, an attenuated ability to maintain paired symmetric bilaterality irrespective of geo-location. Foramen magnum shape assumed priority over size in ontogeny with profound asymmetry (from the 5th harmonic); an indication of precocity and early presumptive form for arboreal adaptation this observation in conjunction with modularity inference suggested instability and global weakness of cranial integration processes and modules. These findings will be of value for species conservation and may be vital in pangolin evolutionary systematics.

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Abstract

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Keywords- African Small Pangolins, Allometry, Foramen magnum, Asymmetry, Skull modularity, Geometric morphometrics

Background

The small African pangolin (SAP) is a poorly described scaly anteater formerly classified as a single family Manidae from the Order Pholidota (Murphy et al., 2001a). Recent research has reclassified those (*P. tricuspis*) to the Clades of Boreoeutheria, Sub-order Ferungulata, sub-family Phatagininae (Gaubert et al., 2017; IUCN, 2018) and are not related to the giant anteater (*Myrmecophaga tridactyla vermillina*) of the Order Pilosa from Central South American nativity whose familiarity to sloths is well documented. Pangolins are better related to carnivorans in evolutionary trends (Gaudin and Wible, 1999; Murphy et al., 2001b). All eight extant species of SAP are currently classified as threatened, three of these are critically endangered (IUCN, 2018). SAP are arboreal in nature with Sub-Saharan African geographic distribution (ranging from West, Central to Southern Africa), they are deeply diverged from the Asian and Indian species of the genus *Manis* (Gaubert et al., 2017; Gaudin et al., 2019). Several morphological variations of its head and cranium have brought this species to fore in current researches and further tilted her already threatened

status to critical level (Ferreira-Cardoso et al., 2020); this new status justified this study more so that morphological distinction of species is a requisite vulnerability evaluation in the instance of ecology and evolutionary status classification. The species has been declared the most trafficked in the world (Gaubert et al., 2017); this fact serves a major impetus to study its peculiar cranial morphology as well as certain developmental/ biological processes as an adjunct to molecular tracing of trafficked mammals for preservation and conservation. Tracking and tracing smuggled species skeletons and remains through illegal trade routes often require adequate information on geographic distribution, shape and sizes of preserved antiquated or archival specimen as well as season of interception to offer effective control and discourage poaching.

Attempts at preservation and conservation of critically threatened species such as the small African Pangolins (*Phataginus tricupis*) have been made through extensive captive breeding; this is partly due to shrinking ecology and urbanization and have been documented to interfere negatively with natural selection (Hewitt, 2001); a phenomenon associated with founder's events as well as increased occurrence of developmental aberrations in species (Hewitt, 2001), hybridization, is another common occurrence when wild-taken variants are introduced in breeding programs. Preservation effort may only be successful when baseline information on proper characterization and developmental peculiarities of species structural parts is possible (Richtsmeier and Deleon, 2009).

The zygomatic, maxillary and temporal bones of the skull developed from the first pharyngeal arch (Elliot, 2010) whereas the neural crest and *mesenchymal* cells forms the cranium (Le Lievre, 1980; Le Douarin and Kalcheim, 1999). Modularity hypotheses of development will be tested using landmark partitions to verify if co-variations among embryological related landmarks are lower in comparison to partitions based on anatomical proximities. A combination of three separate embryological compartments will be analyzed for dependency for all partitions in both planes.

Asymmetry as a concept may be defined by the distribution of left-to-right differences in a population (Palmer and Stroebeck, 1986). Fluctuating asymmetry is relevant in the assessment of environmental and developmental stress in organisms. Subtle and unapparent directional asymmetry (DA) has been described fairly regularly among species population using the methods of geometric morphometrics and this seems to be fairly widespread (Klingenberg et al., 1998). The present investigation is to the better of our knowledge a first post natal developmental investigation on the skull of the small African Pangolin. Calvarias development aims at achieving a symmetric paired structure (Elliot, 2010), deviations from this 'target' morphology could result from individual or population developmental stress/noise (ecologic input) (Urbanova et al., 2014) and can be useful in making inferences about such biological processes since morphological asymmetry may provide information on a variety of biological mechanisms (Polak, 2003). There is lack of documented evidence on the development of the skull in this species, thus making accurate taxonomic classification and discrimination among similar extant species conjectural especially for trafficked Pangolin. The challenge of skeletal remains recovery and failure of formulated conservation policies resulting from wrong identification created a knowledge gap to be bridged by this study. Visual observation of sample remains of various ages revealed minute subtle skull asymmetries despite the absence of masticatory mechanisms prompting such queries to warrant enquiry into the extent and certainty of developmental errors to signify the existence of developmental instability, poor integration and a suspicion of reduced ability to maintain bilaterality in paired symmetric skull structures taken from an environment with ecologic constraints, an observation relatively scarce in arboreal species.

Significant overlap of body structural similarities and divergence between *P. tricuspis* and all other extant species and between *P. tetradactyla* and *P. javanicus* respectively have been reported by Ferreira-Cardoso et al. (2020), a situation which provokes a closer investigation on its (SAP) skull characterization.

Latest available literary evidence on extant Pangolins; Manidae (Ferreira-Cardoso et al., 2020) focused on comparative skull shape variations but was not contextual with phenotype as a product of environmental, ecologic and evolutionary trends in the order Pholidota.

The following are the aims and objectives of this study:

1. This study aims to investigate the association or otherwise between environment/ecology and skull development in SAP to justify observed skull asymmetries.
2. That the possibility of ‘handedness’ in skull side’s use and development despite absence of teeth for mastication does not exist in the sample population evaluated (null hypothesis), particularly due to paucity of documented evidence of directional asymmetric investigations in the species from different ecological background under study.
3. Establish and compare variations among sample populations in foramen magnum outline allometry and possible occurrences of malformations such as dorsal and ventral notches among population for an assessment of stability in this structure.
4. Assess overall evaluated skull developmental pattern among contiguous landmarks (modularity hypothesis) to confirm or otherwise an association in embryonic skull modules compared to related anatomic structures in its integration process using two blocks Partial least squares (PLS), Overall strength of association between blocks: yielded lowest RV coefficients; Permutation test against the null hypothesis of independence with 10,000 rounds of randomization.

. In evaluation of symmetry/asymmetry in skull construction and architecture, all contributory forms of asymmetry (directional asymmetry, fluctuating asymmetry and antisymmetry) were explored (Palmer and Strocker, 1986). We also aimed at studying the foramen magnum construction in ontogeny from the three ecologies as a corroborative attempt in characterization of observed asymmetry of this structure for confirmation of in-born errors. Further, we endeavor an evaluation of ontogenetic morphological features using a range of harmonics in Elliptical Fourier Analysis in resolution of foramen magnum outlines architecture.

0. Materials and Methods

ETHICAL CONSIDERATIONS

Ethical approval for the use of these animals was granted by the Animal Care and Use, Research Ethics Committee (ACUREC), University of Ibadan, Nigeria, ethical code number UIACUREC/17/0023.

ANIMAL ACQUISITION, SKULL PROCESSING AND CATEGORIZATION

To enhance validity, samples for this study were taken from separate locations in Nigeria. All skulls were obtained after due permissions from museums curators of Universities in Nigeria; Eighteen (18), twelve (12) and six (6) from the Department of Anatomy, University of Ilorin, Kwara State, Department of Veterinary Anatomy, Federal University of Agriculture Makurdi, Benue State, and Department of Veterinary Anatomy, University of Ibadan, Oyo State, respectively. Four (4) unsexed were wild taken in Ibadan, Oyo State, categorization was based on skull size and geographical location (Fig. 1) while animal handling was in conformity with ACUREC and NIH guidelines for Use and Care of Laboratory Species. Sacrifice and maceration process were according to Catania et al., (2000) and Igado (2017).

Figure 1 Distribution areas (starred) of the small African Pangolin (*Phataginus tricuspis*) along the coast line in the tropical rain forest zone with special concentration in the Southwestern parts of Nigeria which serves as our assessment areas

A series of eleven (11) landmarks comprising of four (4) single midline (axis of symmetry) and seven (7) paired points on either side of the skulls were digitally placed on direct dorsal and ventral views (nine landmarks) of skull images (Fig.2a) using TPS Dig2 Vers. 1.40 (Rohlf, 2015) CANON EFS-1200D Camera with EF-S 18-55 IS 11 kit and HAMA tripod plumb line stand stabilizers at a focal distance of 2.8 cm and 15 cm DIN, shutter speed ISO 1/100. The study utilized Generalized Procrustes Analysis (GPA), which superimposed landmark configurations, apart from providing information on size (Centroid size, CS, calculated as the square root of the sum of squared distances of each landmark from the centroid of the landmark configuration) and shape (Procrustes distance estimates) eliminates spatial variation that does not correspond to form. Allometric data could therefore be assessed for evaluation as analysis of size and shape differentiation across the geo-ecologies.

Sage: software was used in the evaluation of asymmetric and symmetric components for this study, Sym-

metry and Asymmetry in Geometric Data Version 1.21 software (Marquez, 2014) freely available online at <http://www-personal.umich.edu/~emarquez/morph/>

Distribution, assessment, landmarks, digitization, measurement errors methods and statistical analysis

The species are freely distributed within this geography and rarely found beyond 7.430 50°N, 8.320 1°E. Images were produced in replicates and processed in Thin Plate Spline (tps) methods for dorsal and ventral skull suture digitized landmarks. Measurement errors may arise as a result of individuals in the population being evaluated and as well as from replicate residuals in the evaluation; and was accounted for by taking the landmarks in repeated procedures (replica), measurement error value contributions were also used in the determination of fluctuating asymmetry; whereas the former must be insignificant in quantity contribution when compared to the latter since both are similar in magnitude and distribution during analysis. Paleontological statistical software (PAST) version 3.0 (<http://folk.uio.no/ohammer/past/>) (Hammer et al., 2001) was employed in all statistical analyses including homoscedasticity and normality tests in this investigation.

2.4. Symmetric and asymmetric quantities

The components of asymmetry are often quantified with a two-factor analysis of variance (ANOVA) of individual measurements with the sides (left and right) and the individuals as the main effects (Leamy 1984; Palmer and Strobeck 1986), with measurement error when both sides of each individual are measured repeatedly. Directional asymmetry (DA) is quantified by the difference between the means of all the configurations from either body side (left and right) (means of all individual asymmetries) (Urbanova et al., 2014) and expressed statistically as the difference in means between the left and right sides in the two-way ANOVA.

2.5. Foramen magnum investigations

Foramen magnum rim images for the studies (without mandibles) screened out using polysiloxane putty and processed in a C++ 2018 personal computer. Images were taken at 20 cm focal distance and processed with 24-bit depth BitMap extension (bmp_work_dll) at 4014 x 2944 pixels dimensions using Microsoft paint Application, as configured for SHAPE chain coder program (<http://lbm.ab.a.u-tokyo.ac.jp/~iwata/shape/>) (Iwata and Ukai, 2002); a coding system for describing biological shapes and closed contours. A total of 2064 chain coded landmark points were evaluated on the foramen magnum outlines (Kuhl and Giardina, 1982; Chen et al., 2000), a chain code assigned numerical values between 0 to 7 around the contour outlines before binarizing a full colored image to a two-colored image (Black and white). Scanned outlines were stored on a scale of 30 mm as a pair of (y, x) clockwise in Cartesian coordinates.

2.5.1. Elliptic Fourier (EF) sequences. Reconstructions, repeatability and measurement error

According to Dixon et al. (1997) the expanded EF sequence (Elliptical Fourier) by series of harmonics y, x coordinates descriptors; the geometry of each harmonic corresponds to an ellipse thus:

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image1.emf available at <https://authorea.com/users/557515/articles/607130-post-natal-cranial-areas-of-evolution-in-the-small-african-pangolin-phataginus-tricuspid-ontogeny-from-three-geo-ecologies-a-first-report-asymmetry-elliptical-fourier-analyses-and-modularity>

Each harmonic was described by four Fourier coefficients, two each for the x - and y -axes, generating a total of $4n$ coefficients labeled a_n , b_n , c_n and d_n , where n is the number of harmonics. They combine to describe the repeated elements in a sinusoidal waveform, (a_j, b_j) and (c_j, d_j) were the four Fourier coefficients defining each harmonic (j th order); k corresponds to the maximum number of harmonics used for the Fourier decomposition and T was equal to the perimeter of the outline. An incremental number of harmonics in Fourier series converges on detailed information on the morphology of analyzed form, and numerically describes distortions and complexity of forms (from the original circle) described by the Fourier series when no harmonics are present. In this manner, Elliptical Fourier Analysis (EFA) is able to resolve and describe complex 2-Dimensional bounded outlines and also spatial orientation relation to reference planes (Ferrario

et al., 1996), allometric changes and shape (Procrustes coordinates) can be assessed when plotted against CS. The size normalization procedure consisted of a recalculation of the outlines using the same value of the enclosed area for all specimens.

Measurement error was estimated from a Procrustes ANOVA by considering individual as the main source of variation and residuals representing variation in digitized replicates as a second source of error (Cocilovo et al. 2012). To remove differences due to specimen orientation and position during data collection, and to separate size and shape components; landmark configurations are first scaled to the same size, centered at their origin and rotated to minimize the distances among the corresponding landmarks (Generalized Procrustes Analysis or GPA) (Rohlf, 2010). GPA superimposes specimen landmark configurations by translating them to a common origin, scaling them to unit centroid size (the square root of the sum of squared distances of all landmarks to the centroid of the object), and rotating them according to a best-fit criterion

Figure 2a and b Showing dorsal, ventral and direct caudal views of the small African Pangolin skull (*P. tricuspis*) and landmarks assessed 1. Posthion in the midline of the asal bone, 2. Suture of the nasal bone in the midline rostral limit of the frontal bone, 3 Parietal suture in the midline at the rostral limit of the parietal bone, 4 the Inter-parietal bone suture at the caudal limit of the parietal in the midline, 5 Dorsal limit of the foramen magnum in the midline of the occipital bone, 6 and 7 Lateral boundaries of the nasal bone sutures in the maxilla, 8 and 9 Lateral limits of the parietal bone sutures at the temporal bones, 10 and 11 Lateral limits of the Inter-parietal bone sutures with the occipital bone. The ventral view landmarks includes; 1 the Posthion in the ventral view, 2 the ventral midline boundary of the sphenoid, 3 Ventral rim of the foramen magnum in the ventral midline, 4 and 5 Ventral tips of the zygoma on both sides, 6 and 7 Vento-lateral limits of the temporal bone on both sides, 8 and 9 The lateral boundaries of the occipital condyles in ventral view (b) the caudal view of foramen magnum outline

Size normalization of descriptor coefficients of foramen outlines was achieved by the ellipse of the first harmonic described for closed shapes (Kuhl and Giardina, 1982) to be invariant of size employing CHc 2-NEF SHAPE Version 1.3. Major axis length/2, Minor axis length/2, orientation of the major axis and phase angle ($\theta = 1/2 \arctan((a_1b_1 + c_1d_1)/(a_1^2 + c_1^2 - b_1^2 - d_1^2))$) corresponding to the position elliptical first point are the four descriptors of each harmonic. A stepwise Elliptical Fourier reconstruction of foramen magnum outlines was done employing incremental number of harmonics. Size and peculiar anisotropic characteristics were determined for each harmonic (Major axis length/2 x Minor axis length/2 and Major axis/Minor axis respectively) (Buck, 1962).

Descriptor coefficients were analyzed with ‘PrinComp’ based on Variance-Covariance matrix of normalized coefficients. It was also noted that such coefficients with small variance and covariation values do not significantly explain morphological variations and are now calculated and used in principal components derivation bearing all edge/contour shapes information in the first 14 harmonics as explained by Rohlf and Archie, (1984). Summary statistics showing mean $\pm$ SD employing PAST (Hammer et al., 2013) for foramen magnum shape outline descriptors was performed. An allometric (shape vs size) based discriminant function analysis was evaluated with incremental Fourier descriptor harmonics followed by use of inverse Fourier transform to obtain graphical representation at 0.05 Bonferroni post-test level of significance.

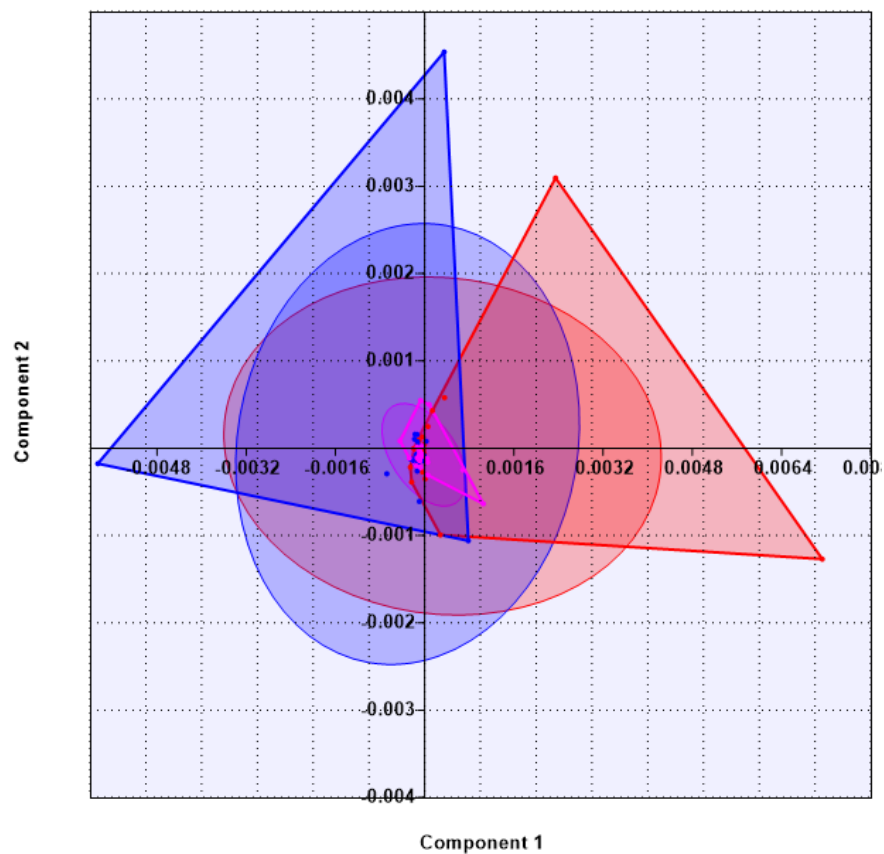
2.6 Modularity assessment methods

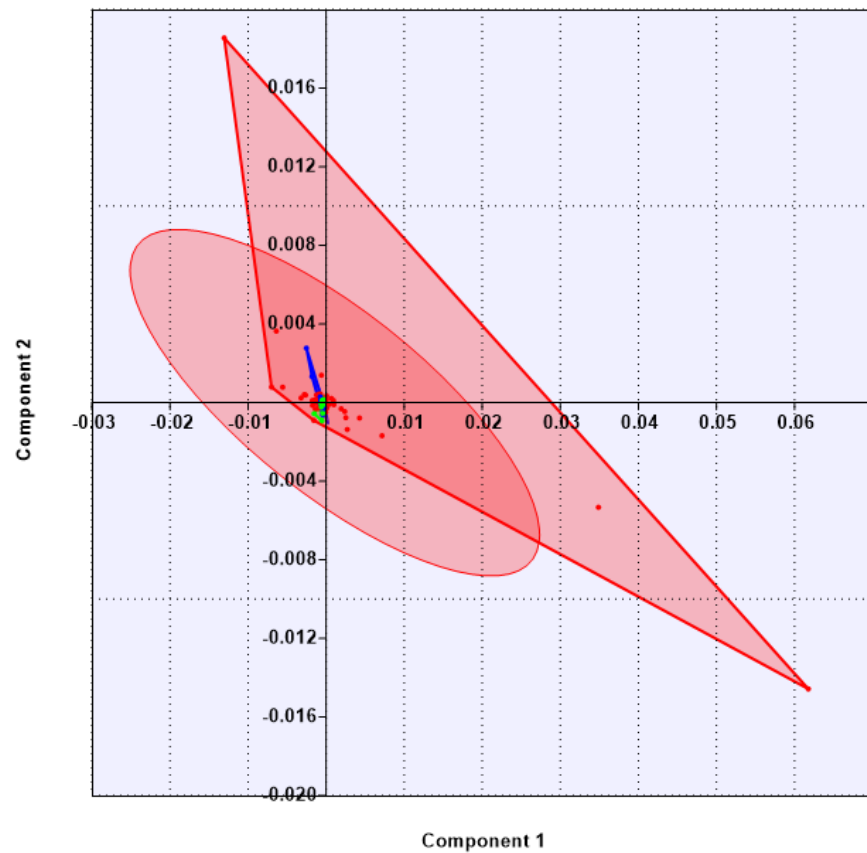
In the evaluation of modularity of contiguous skull landmarks based on the hypothesis that covariance between true modules (embryologic relations) should be lower than for other developmentally unrelated parts, the extent of such covariation is expressed in terms of the RV coefficients; RV coefficient (Relative variance); as explained by Escoufier (1973); is a similarity coefficient between positive semi-definite matrices or simply a multivariate generalization of the squared Pearson correlation coefficient (values between 0 and 1), it measures two sets of points that may be represented in a matrix; the closer to 1 the RV becomes, the more similar the two matrices. The eleven dorsal view landmarks were divided into two subsets of equal number of landmarks; only landmarks forming contiguous configurations were allowed to run with full enumerations of partitions using MorphoJ software vers. 2.0 (Rohlf, 2015) (MorphoJ is available freely under the Apache

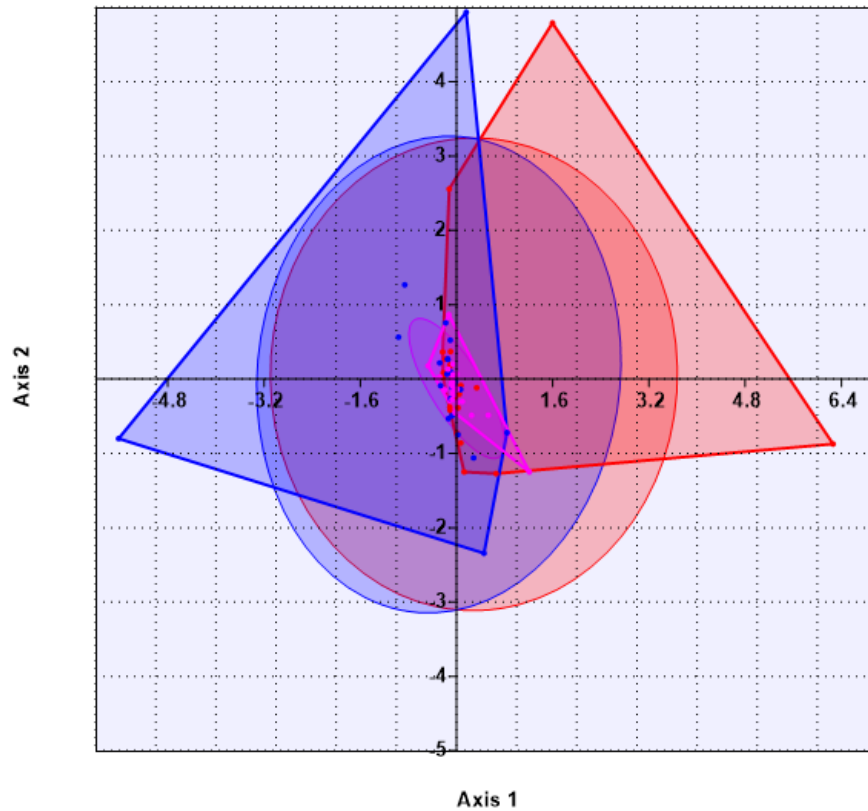
Licence, Version 2.0, from the web site http://www.flywings.org.uk/MorphoJ_page.htm)

3.0. Results

Measurement errors – Errors arising from placement of landmarks (digitization procedures) represented 3.4% of the variations in the evaluations







Figures 3 a, b and c A within (PC1=68.17%, PC2=28.04%), (b) between (PC1 99.61%, PC2= 0.31%) and (c) Multivariate analysis of variation (discriminant analysis) sampling locations Principal component analysis with convex hull at 95% ellipses showing the contributions to variations observed from the three locations (Red= Kwara), Blue and Green, Ibadan and Makurdi sample populations respectively

Size and shape variations in samples from Ibadan in Fig. 3a were more disposed on the first axis with size contributing a higher proportion of variations observed in comparison to those from Kwara and Makurdi where shape variation was an overriding factor as they were better disposed on the second axis. Between the geographical location groups (Fig. 3b), Makurdi samples demonstrated the highest population variation in both size and shape factors compared to samples from other geo-locations. Large area of overlaps existed between samples evaluated.

Analysis of similarities (ANOSIM) at 9999 permutations demonstrated significant ($P < 0.1$) Mahalanobis distance of 0.045 in Kwara samples and 0.910 in Ibadan sample population. Multivariate analysis of variance (MANOVA) (Fig. 3c) failed to reveal any significant ($P < 0.7$) discriminant values based on geography. Wilks lambda (0.936), $df=8$, $F=0.5994$

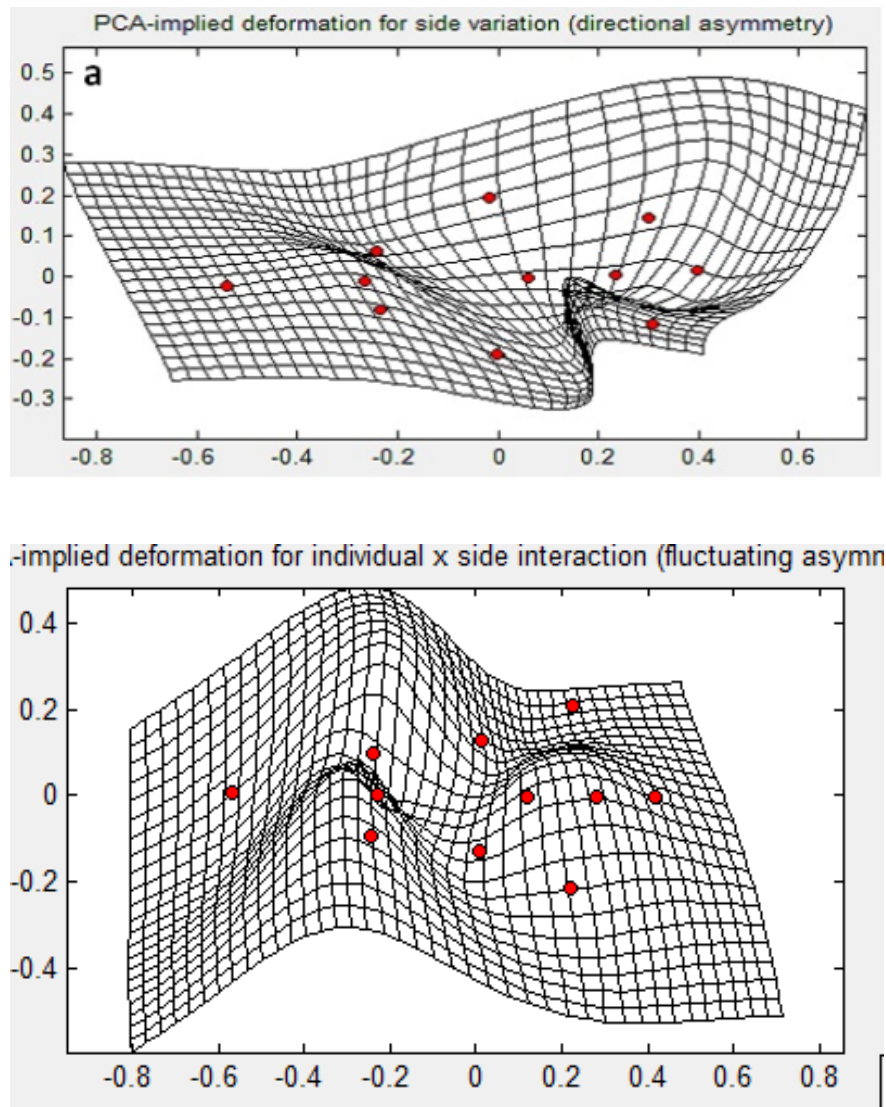
3.1. *Asymmetry in skull shape in dorsal and ventral view analyses respectively*

Procrustes ANOVA demonstrated (Table 1) that in both dorsal and ventral view analyses the results are highly repeatable ($F=10.5501$ and 4.1967 , $P=0.0001$) for dorsal and ventral views respectively as between sides variation values are larger than within sides variation (measurement error) contributions ($F=0.00034755$ and 0.0024199 , $P= -$) respectively in the same order. FA accounted for 61.77% and 72.01% of variations in dorsal and ventral cranium, respectively and was not found significant ($p > 0$), signal noise for dorsal view was observed significantly high ($F=0.00034755$) and comparable to values for FA (ns) and could not be employed for further analysis.

A regression model of log transformed centroid size and asymmetric components suggested the significant expression of asymmetry throughout ontogeny ($p < 0.03$) and that size accounted for 81.34% of asymmetric shape changes of this component. Further, a multivariate analysis of regression confirmed DA and a low significance ($p < 0.5$) of fluctuating asymmetry (FA) ($F_{1539} = 3.4045$, $F_{882} = 3.2665$) for dorsal and ventral views (Table 2). More shape changes concentrated collectively more on the rostral-dorsal and caudal areas and were right side biased (Fig. 4a and b) suggestive of a low association between environment and skull development in Small Pangolins to justify observed skull asymmetries (hypothesis 1).

Intraspecific allometric ontogenetic trajectories among samples under assessment suggested a rostral-caudal followed by a caudo-lateral growth trajectory considering the intercepts for shape versus size predictions (Fig. 5) was found parallel. Intercept (-4.43), slope (-11.168) $p < 0.0004$ while overall statistics $R^2 = 0.013$, Wilk's Lambda = $1.07E-11$, $df = 21, 7$, $F = 3.091E 10$, $p < 0.0001$. in a pooled sample population across geographical locations.

Figure 4a, b and c Grid representation of PCA implied deformation for directional asymmetry, (b) Implied deformation for individual side interaction (FA) for dorsal skull views (Mag X 10) (c) Regression model of log transformed centroid size (CS) versus asymmetric components of data in *P. tricuspis*



3.2.1. Evaluations of EF descriptors

For data decomposition, 40 covariance descriptors were analyzed with 14 harmonics. Constant descriptor coefficients for size-normalized foramen magnum outlines were a_1 , b_1 , c_1 and d_1 for forty (40) Small African Pangolins (*P. tricuspis*) of various sizes, were evaluated for size-normalized outlines, magnitude and the peculiar anisotropy of their ellipses as depicted in tables 3-5. Elliptical descriptors demonstrated the highest major and minor axes/2 magnitudes as well as peculiar anisotropy in the 1st harmonics in all samples irrespective of shapes, these values decreased generally with higher number of harmonics. The gradient decline in values from the 1st to 7th harmonics was characteristic to the more developed samples whereas the lesser developed were from 8th-13th, shapes and sizes were found more unpredictable among the latter. Descriptor values rapidly became inferior to less than or equal to 2% of the values of the 1st harmonics from the 5th harmonic finer details were shown by the minute descriptor values (after the 10th) retained in the higher harmonic orders. The third descriptor (angle of orientation of the first EF position/ellipse) between 0.26-41° could not be associated with any particular harmonic order but common in each (Tables 3 and 4). Elliptical anisotropy of 0.018-53.1 was constant with each harmonic but without any harmonic order relationship (Table 5). Size normalization results presented amplitude related profound descriptor value reduction according to harmonics (approximate factor 1/90 to 1/67000) demonstrated wide ontogenetic allometry. Angular orientation of major axis was also attenuated by 1/1.08 to 1/1.009 factors.

3.2.2. Morphological analysis of foramen magnum in African small Pangolin (*P. tricuspis*) in stepwise reconstruction

Stepwise foramen magnum outline reconstruction in *P. tricuspis* revealed better accuracy with increasing harmonic numbers from the 1st to 14th as presented visually in Fig. 5. The accuracy of this as shown by the fit index of original vs reconstructed values is about 97% (Fig. 6). Fifty-two (52) principal components coefficients were analyzed with 14 harmonics with a total variance of 1.276294E-001, the first 4 principal components yielded effective description of the foramen outlines (Table 6).

Figure 5 Contour reconstructions of the foramen magnum outlines in Small African Pangolins (*P. tricuspis*) for the first 14 harmonics (Overlapped reconstructions -2std= +std Red, mean is green, -std yellow)

Figure 6 Allometric analysis of the foramen magnum outline (*P. tricuspis*) in caudal view showing fit diagram of incremental EF harmonics evaluated from discriminant analysis

A full morphologic description of the outlines was achieved in evaluated samples by the 5th harmonic followed by finer details from the 6th to 14th harmonic. The first portion to be morphologically characterized from a graphic central point was the direct dorsal and ventral rims in a clockwise direction by the 1st harmonic, the left dorsolateral aspect in caudal perspective in relation to the central point was perfectly elucidated by PC2 whereas PC3 explained the right dorso-lateral and left ventro-lateral aspects at the condylar rims, the 4th harmonic was more diffuse and at variance focusing on the right and left lateral rims (Figs. 2b and 5). The last parts to be reconstructed were the left dorso-lateral left lateral and left ventro-lateral rims in a dorso-ventral direction (5th - 14th harmonics). Components of its asymmetry were observed by the 5th harmonic accurately. Variance-covariance matrix along ontogenetic lines was between 0.0017 and 0.56, discriminant analysis in ontogeny of the sample population in stepwise harmonic increments and Mahalanobis distance (Fig. 6) demonstrated gradient decline in distance with elliptical Fourier harmonics.

Sample proportion demonstrating significant size and shape differences before was (82.3%) and (8.2%) after size normalization thus yielding 74.1% accuracy decline with size factor elimination employing size normalization (Table 7) protocol revealed shape an overriding factor. This finding satisfies the third (3rd) hypothesis of association stated for this investigation

3.3. Modularity

Landmarks in the subsets of the a-priori modularity hypothesis Subset 2: 8, 9, 10, 11. Using two blocks Partial least squares (PLS) Overall strength of association between blocks: yielded lowest RV coefficients; Permutation test against the null hypothesis of independence with 10000 rounds of randomization were also

extremely significant ($P < .0001$) in dorsal skull landmark analyses but P-value: 0.0687 (in ventral view) with anatomical proximity and developmental origin employed in module partitioning, while correlation of PLS scores between blocks (0.99). Comparison with alternative partitions: using only contiguous partitions. All appropriate partitions were evaluated considering partitions possessing RV less than or equal to the a-priori hypothesis, (with minimal RV) this outcome confirms hypothesis 4 of the study. Principal Component Analysis: PCA: Covariance Matrix and Asymmetry component PC1= 88.38% PC2=7.48% variance, % predicted: 10.0770% Asymmetrical components demonstrated lower RV coefficients than the symmetrical components.

Table 8 Modularity hypotheses tested for dorsal and ventral skull views in the African Pangolin (*P. tricuspis*) Modularity hypothesis among contiguous landmarks evaluated by full enumerations of partitions with the eleven (11) and nine (9) landmarks divided into 2 subsets. Partition with minimal RV minimum co-variation

4.0 Discussion

4.1. Symmetry/Asymmetry, Elliptical Fourier analysis (EFA) and Modularity perspectives in skull development

Cranial asymmetry sheds light on developmental process disruptions in species (Elliot, 2010) and pathological conditions involving the skull (Elliot, 2006), it emphasizes relationship between structurally or functionally interacting elements (Benitez et al., 2020). DA and asymmetry studies are relatively scarce or non-existent in literature for small African pangolins, FA is the bilateral asymmetry that represents minute random developmental differences between a right and its left side (Benitez et al., 2020). Klingenberg (2008) showed that within the concept of geometric morphometrics studies of asymmetry can be combined with other morphological issues – modularity and integration. While modularity refers to the covariance among morphological structures that originates in independent developmental processes (so-called modules), integration; a counterpart to modularity, is a measure of the interconnection among parts in order to function as a whole unit (Klingenberg, 2008). Modules may be defined with respect to genetic, developmental, functional or evolutionary context as being currently interrogated. A low covariance (representing magnitude of interaction among modules) is expected if two modulating units possess a weak boundary suggestive of relative independence (Urbanova et al., 2014); the converse is also true. Elliptical Fourier analysis is fundamentally a mathematical application in the derivation of biological closed shape outlines evaluations and valued for its automatization of image analysis irrespective of morphological complexity for less experienced users (Schmittbul et al., 2000). The method demonstrates precise individual variations introducing scientific perspectives in characterization of closed shapes such as the foramen magnum (Samuel et al., 2017; Daegling and Jungers, 2000) in making useful inferences on developmental instability in a population of samples.

4.2. Post natal skull development in small African Pangolins

Samples obtained from Ibadan geo-location in (Fig. 3a) demonstrated more robust size and shape variations; being more disposed on the first axis of the PCA plot with size contributing a higher proportion of variations observed in comparison to similar samples from Kwara and Makurdi locations where shape variations factor overrode as they are better disposed on the second axis of the plot. Between the groups (Fig. 3b), the observed differences perhaps explain the input of environment on skull phenotypes development (Elliot, 2010; Benitez et al., 2020). Skull samples from Makurdi demonstrated the highest population variation in both size and shape factors compared to those from other geo-ecologies. Environmental prevailing temperatures, vegetation and diet variables as well as predator influences seem to favor samples from Ibadan eco-environments when compared to those from Guinea and Sudan savannah (Kwara and Makurdi) respectively. Large areas of overlap existed between the samples evaluated as shown in the MANOVA and ANOSIM analyses which failed to discriminate the species on geographical bias and was confirmed similar respectively; this is indicative of topographical area landmark similarity among population evaluated, such areas did not give any untoward developmental signal but follow phylogenetic trajectory compared to areas of non-overlap and is consistent with the report on similar extant species (Ferreira-Cardoso et al., 2020).

Skull structural flexibility potentials may have been exhibited by certain trait-variability patterns in these studied samples ontogeny which may be consequential in evolutionary trend. (Fig. 3b, Tables 1 and 2)

confirmed the suggestion of Hendrikse et al. (2007) where subtle rostrum and dorsal skull deviations from midline (Fig. 4) were detectable and attributable to genetic input and lateralization in muscle load use or other functional demands (Urbanova et al., 2014). Maximum size and shape overlap observed (Fig. 3b) among sample populations from the geo-locations suggests high similarity this was further confirmed by an analysis of similarity (ANOSIM) and a multivariate analysis of variation (MANOVA) (Fig. 3c) on both skull views understudied. Rostrum shape overlaps between the rainforest (Ibadan) and Guinea savannah (Kwara) samples seem to progress in diversity as the species geographical space goes northwards to the Sudan savannah belt, they are however not been reported in the Sahel.

During craniofacial development, cranial neural crest cells' migration to (frontal part of head) generate the facial skeleton (Le Douarin and Kalcheim, 1999) to become the mesenchyme of future face while the back of the skull is derived from a combination of neural crest-derived and mesodermal bones (Murphy et al., 2001a). Skull bones are derived from both the neural crest and the head mesoderm (Le Lievre 1980; Noden 1978; Klingenberg, 2010). Results from our study exposed some unexplained cranial asymmetries among the population investigated likely to be consequential to inconsistencies in cellular migrations; this is the most probable explanation to the observed unapparent skull asymmetries despite the absence of masticatory function in this species. Furthermore, the ecologic definition of arboreal living in species' habitat substrates to their cranio-facial morphotypes with compensatory mechanisms in skull morphologic equilibrium as adaptations to their peculiar environment (Richtsmeier & DeLeon, 2009); though insignificant ($F_{1539}=3.4045$, $F_{882}= 3.2665$); for the skull views evaluated respectively demonstrated surreptitious fluctuating asymmetry (FA) (Tables 1 and 2); a measure of developmental instability (Klingenberg and McIntyre, 1998) in minute randomly distributed anomalies associated with environmental signals (Urbanova, 2014) on either (right and left) sides of skulls in the population evaluated in an otherwise bilateral development This is further substantiated by foramen magnum asymmetry assessments where size was an overriding factor (table 6) over shape.

Occurrences of FA in paired body structures have been attributed to ecologic, habitat, on-going metabolic disease conditions among population groups (Singh and Rosen, 2001). It is also known that a sustained unilateral body-side muscular load demand preference would directly modulate external observable morphology (Urbanova, 2014). Results from this study only confirmed a weak occurrence of FA ($F=0.00034755$). Foramen magnum development in the current study further predicated its importance in accurate forensic analyses, types of abnormalities encountered, proportions and interpretations in embryologic and evolutionary contexts among species as well as allometric trajectory pattern discriminations. Such information has no prior literature evidence in Pangolins. Salient neurological, developmentally consequential information hitherto not described could be inferred from the results obtained from EFA; the dorsal-most rim, right dorso-lateral, left dorso-lateral portions presented the discriminating areas of individual shape variations (Fig. 6, Table 6). Manoel et al. (2009) documented the possibility of cerebellar protrusion resultant upon volume reduction of the posterior fossa (pre and post natal); syringomelia and other neurological disorders sequel to foramen magnum dysmorphology manifested by the occurrence of dorsal notches as a result of developmental errors. Such structural malformations have been associated with domestication attempts and captive breeding of species (Dixon et al., 1997; Hewitt, 2011); this finding satisfies the 4th objective of this study. The results of the present investigation notwithstanding; is limited by an absence of fetal skulls thus prenatal studies will further confirm the onset and form of asymmetries in these pangolins.

4.3. Allometry and Ontogeny

Unapparent skull asymmetries seem to be noticeable early in ontogeny in this species, although further studies may be necessary to confirm precisely at what point this begins. The presence of both shape and size asymmetry in dorsal and ventral skull views is undeniable in studied samples and more profoundly so in dorsal views (Table 1) despite similar level of significance after multivariate regression (Table 2). Results from the currently investigated samples (confirmed hypothesis number 2) demonstrated a persistence of directional asymmetry throughout ontogeny, we thus fail to accept the null hypothesis Further, skull shape changes concentrated collectively more on rostro-dorsal and caudal areas as growth advances, and are right

side biased, (Fig. 4a and b) is suggestive of “handedness” (a tendency to use a side of the head more than the other naturally) in *P. tricuspis*. Confirmation for this ontogenetic trajectory is consistent with Esquerre et al (2017) and does not differ in other clades of pangolin species (Ferreira-Cardoso et al., 2020). Literature evidence, though scarce or non-existent in this species, suggested sex does not significantly influence shape, size notwithstanding as a covariate in the regression model of the skull (Ferreira-Cardoso et al., 2020). Allometric regressions of shape on size in existing works revealed a significant overlap of intercepts in *P. tricuspis* with all other species and between *P. tetradactyla* and *P. javanicus* only (Ferreira-Cardoso et al., 2020) this position postulates both a similarity and divergence in skull morphology of the species to provoke a cursory look at its characterization process (Table 2; Fig. 5).

Along the first axis (Figs. 4a and b), changes involved the posthion, nasal and maxillary regions whereas the second plane portray more shape changes synonymous with fronto-temporal sutures and the parieto-occipital areas, this observation was corroborated by Murphy et al. (2001b) in placental mammals. The correlation between morphology and modularity in the present investigation suggests that adult integration patterns are the result of selection pressures rather than historical constraints, and that developmental modularity played little constraining role in diversification (Fig. 6), similar to observations reported by Alföldi et al. (2011) and Sanger et al. (2011) in studies involving other arboreal species; Anolis lizards (*Anolis carolinensis*). The right and left ventro-lateral rims of the foramen magnum presented highest portions of individual shape diversities in ontogeny of this species as currently observed (Fig. 6), asymmetry in architecture of this foramen easily appeared by the 5th harmonic (Tables 3 and 4) despite the uninformative order of the angle of orientation of the EF position. Our inferences in this regard postulate dynamic developmental outcomes yielding foramen magnum phenotypes.

4.4. Structural peculiarities and environmental input as arboreal adaptations in *P. tricuspis* skull evolution

Sanger et al (2011) suggested that similar skull modules in morphologically related arboreal species lineages have been found to evolve traits that converge on similar integration patterns as seen in the long-faced anoles (*A. carolinensis* and *A. bahorucoensis*) in the evolution of rostrum specific modules and the short-faced trunk-ground anoles (*A. sagrei* and *A. cybotes*) with no modularity at all. Average asymmetry in the current sample population seemed to be concentrated in rostral areas, the rostral limit of the nasal bone as well as the caudal limit of the parietal bone in the dorsal aspect while the posthion, ventro-lateral sutures of temporal bones and the zygoma represented major asymmetric areas on the ventral side. Considering the heterogeneity of the sampling geographical ecologies; across the rain forest belt and guinea savannah zone this may disallow putative inference on similar skull developmental errors and skull asymmetric components observed (Fig. 5).

Our results on modularity of subsisting and contiguous cranial parts partitioned based on anatomical co relations, embryologic origins suggested a profoundly stronger covariance coefficients for symmetric components for all hypothesis tested but a minimal RV coefficients for asymmetric components (Table 8) as an attestation of a global weakness of skull integration processes in asymmetry of component parts, an observation which has been documented for rodents, reptiles, insects and dogs (Drake and Klingenberg, 2010; Esquerre et al., 2017; Benitez et al., 2020). Landmark partitions based on the fore going resulted in lower co-variation in partitions based on developmental relations comparison to partitions based on anatomical proximities. A combination of three separate embryological compartments were analyzed for dependency but was found to be comparatively weaker to all other tested partitions for dorsal skull landmarks. The reverse was demonstrated in the ventral view for such hypothetical tests. A caveat could be the inadequacy of structural landmarks or limitation of assessable modules to clearly elucidate the boundaries between these partitions.

Conclusions

This investigation concludes a geo-ecological post natal cranial signal in small African Pangolin ontogeny from three geographical locations in Nigeria. Based on sampled population results we confirm directional asymmetry (DA) in SAP skulls with right side biased ‘handedness, a mild to moderate fluctuating asymmetry

(FA) exist in the species population evaluated irrespective of location. It was observed that there is strong foramen magnum outline shape consistency and developmental stability devoid of congenital malformations such as dorsal and ventral notches with less likelihood of neurological disorders. The findings of this study revealed the peculiar skull morphology and intraspecific morphotypes present in Nigeria, it further demonstrated developmental fragility in the species with a view to formulate conservatory and preservation policies for its protection. Finally, evolutionary pattern in SAP as shown in the study showed its relation to dynamic developmental phenotypes in different environments and worthy of note in systematic skull characterization.

Declarations;

? Ethics approval and consent to participate : Ethical approval for the use of these animals was granted by the Animal Care and Use, Research Ethics Committee (ACUREC), University of Ibadan, Nigeria, ethical code number UIACUREC/17/0023.

? Consent for publication : Not Applicable

Data Accessibility statement : The datasets used and/or analyzed during the current study will be deposited in Dryad and Figshare upon acceptance for publication.

? Competing interests : There are no competing interests

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? Authors' contributions : SOM conceptualized, wrote and analyzed result of this manuscript, IOO donated the specimen used for the study, AA proofread and contributed some of the skull samples from another location.

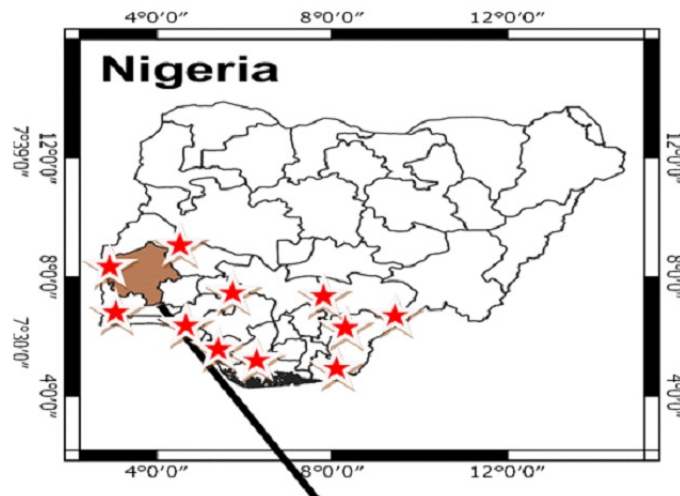
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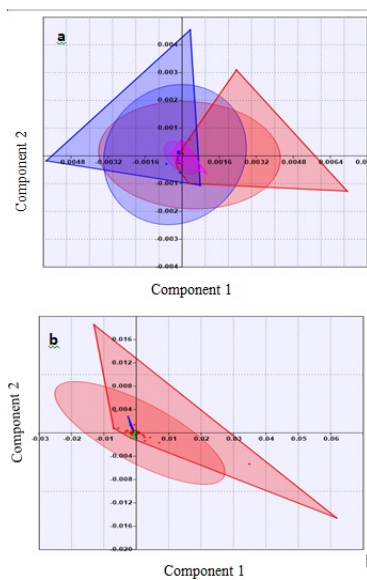
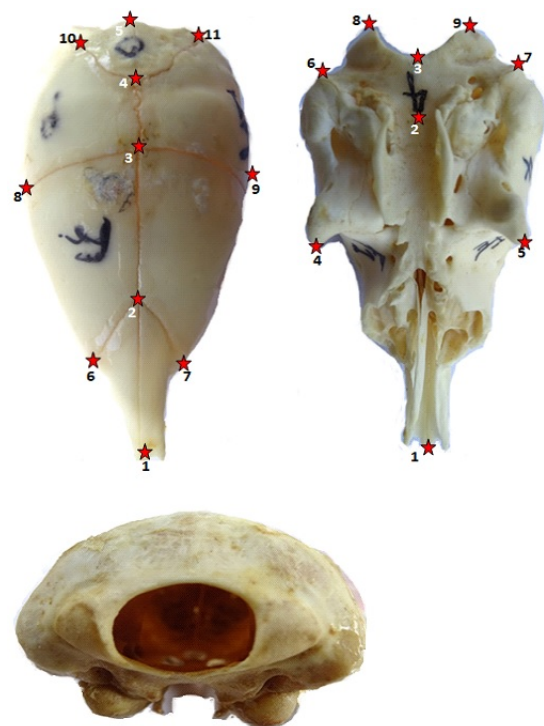
5.0. References

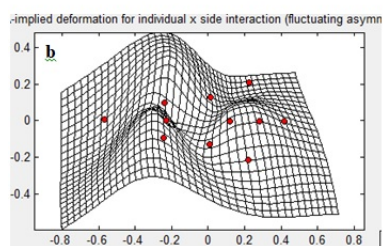
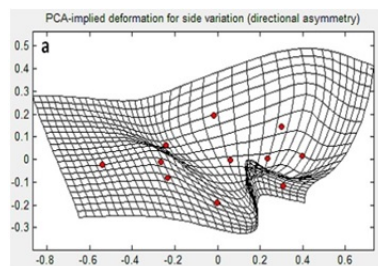
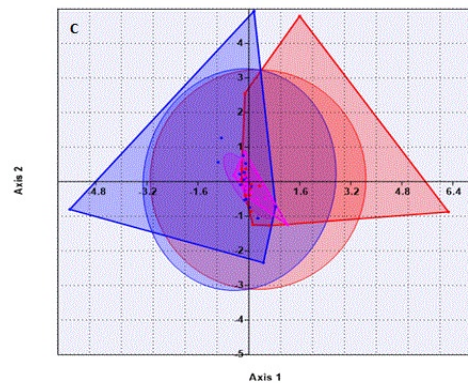
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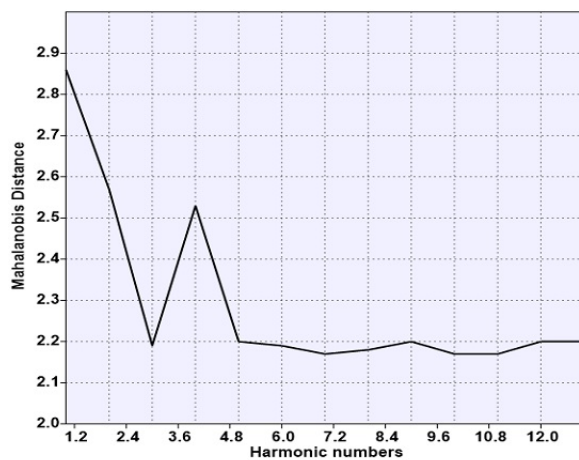
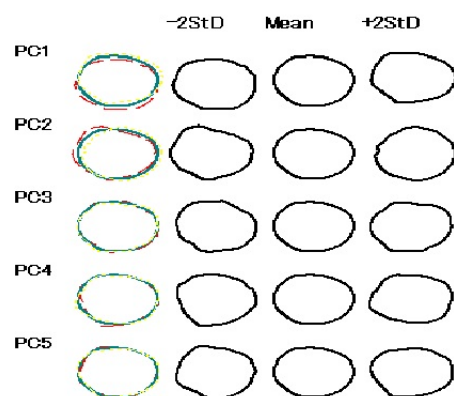
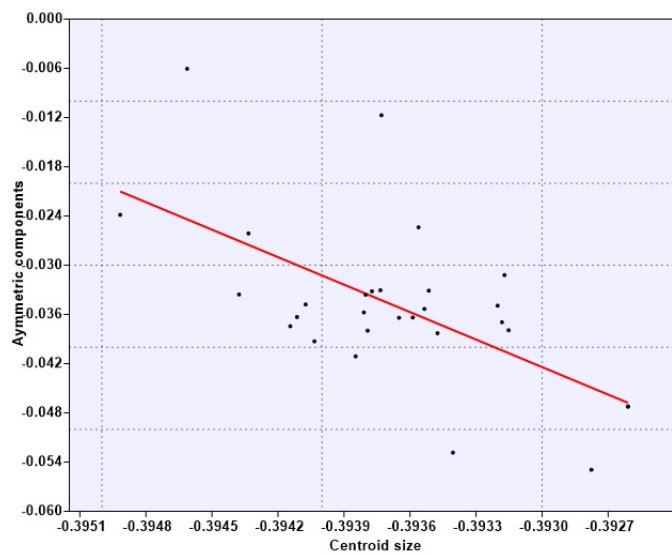
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