GEOMETRIC MORPHOMETRIC ANALYSES AND CRANIAL SHAPE EVOLUTION IN MONITOR LIZARDS

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DECLARATION

I declare the thesis entitled “Geometric morphometric analyses and cranial shape evolution in monitor lizards” is my original work and no part has been previously submitted for a degree. This work has been done in collaboration with Prof. J. Scott Keogh, Dr. Domenic D’Amore, and Dr. Marta Vidal-Garcia, and the published article is co-authored with all three people. I am, however, the main contributor to this thesis.

[Signature]

Gabrielle Heidi Openshaw
10 February 2017
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ABSTRACT

Understanding morphological change is a central theme in evolutionary, developmental, and comparative biology. Lizard heads are well suited to analyses of morphological evolution because of the complexity and diversity in the feeding system that integrates the cranium, jaw, hyolingual and cervical systems. Aside from feeding, the lizard skull is functionally associated with ecomorphology and sexual selection, and is additionally responsible for housing the major sensory systems of the body. Despite being a structurally complex and integrated system with numerous functions and roles, evolution in the lizard skull is characterized by the appearance of extreme morphologies.

Geometric morphometrics is a powerful tool for the quantification, visualization and analysis of morphological variation and change. This approach is being applied more frequently in a phylogenetic comparative context to assess the relative influence of size, ecology, function, and developmental constraints on morphological evolution. Geometric morphometric methods rely on homologous landmarks as the source of shape data, and the level of detail and accuracy increases with the amount of information contained in a landmark configuration. However, it may be possible to capture particular elements of shape variation by concentrating on different observation angles of a complex structure.

This research article examines how observation view (dorsal, ventral and lateral) influences 2D geometric morphometric analysis of interspecific cranial shape variation in monitor lizards (Varanus, Varanidae). I recover strong phylogenetic signal in all three views and general concordance in patterns of size-corrected shape diversification within the genus. However, I also find subtle but important differences among views in analyses of evolutionary allometry and shape variation, which may reflect both landmark configuration design and adaptive functional trends of the study system. This study shows that studies restricted to a 2D geometric morphometric analysis of a complex 3D biological structure can combine carefully designed 2D landmark configurations describing alternative planes to maximize shape coverage.
The original research article presented in my thesis provides the basis for addressing a wide range of questions associated with the analysis of skull morphology, dentition and biomechanical performance as it relates to diet in monitor lizards using 3D geometric morphometrics, bite force measurements and phylogenetic comparative methods. The future directions I propose examine alternative ways in which predators may optimize feeding success: through morphological adaptations (in the skull and teeth) that enhance prey capture, or through biomechanical adaptations that enable feeding on a wider variety of prey.
STUDY SYSTEM

Monitor lizards (*Varanus: Varanidae*) belong to the Order Anguimorpha (~200 extant species). This diverse group includes limbed and limbless lizards, the largest known terrestrial and aquatic lizards, as well as some of the smallest. Additionally, the clade has a fossil record dating back over 130 million years and spanning every continent (Conrad *et al.* 2011).

A Laurasian hypothesis for the evolutionary origins of *Varanus* is now generally agreed upon based on molecular and fossil evidence (Conrad *et al.* 2011; Conrad *et al.* 2012; Portik & Papenfuss, 2012; Vidal *et al.* 2012). Molecular evidence suggests a dispersal of *Varanus* to Africa approximately 41 million years ago (Mya) (standard error range 49-33 Mya) (Vidal *et al.* 2012). The oldest fossil within the extant *Varanus* radiation is *Varanus rusingensis*, from the early Miocene (17.8 Mya) of Rusinga Island, Kenya (Clos, 1995; Conrad, 2008; Conrad *et al.* 2011). *Varanus* is believed to have dispersed to Australia approximately 32 Mya (Late Eocene – Oligocene) based on molecular evidence (Vidal *et al.* 2012), but fossils do not appear in Southeast Asia and Australia until the Pliocene (Owen, 1859; Hocknull *et al.* 2009; Conrad *et al.* 2012). The relationships between the fifty or so extant species have historically been based on geography, separating the African, Asian and Australian species, and these three major groups have held up to scrutiny as phylogenetic techniques have advanced (Ast, 2001; Vidal *et al.* 2012).

Monitors are widely regarded as well suited to studies of morphological evolution, having diversified into an exceptional range of body sizes that spans four orders of magnitude in body mass between the smallest (*Varanus brevicauda*) and largest (*Varanus komodoensis*) extant species (Pianka, 1995). Even more impressive is the scale when considering the extinct *Varanus* ‘Megalania’ *priscus*, a giant Australian monitor that attained lengths of 6 – 9 m (Owen, 1859; Erickson *et al.* 2003). Body size diversification in monitors is tightly linked with ecology (habitat type and retreat choice), with terrestrial species evolving large body size, rock-dwelling species evolving small body size, and arboreal species evolving intermediate body size (Collar *et al.* 2011). In terms of body shape, monitors have positive allometric scaling between body size and limbs, suggesting
shape changes evolve in response to changing biomechanical function (Christian & Garland, 1996; Thompson & Withers, 1997; Collar et al. 2011). Larger monitors also mitigate stress on limb bones by reducing femur rotation, and by increasing the percentage of time the hind feet are grounded during movement (Clemente et al. 2011).

Openshaw & Keogh (2014) used two-dimensional geometric morphometric techniques to characterize patterns of dorsal head shape variation in 36 species (375 specimens) of monitor lizard, and test how this variation relates to size, phylogenetic history, and ecology as represented by habitat (Fig. 1). Interspecific head shape disparity is strongly allometric, and once size-effects are removed, principal component analysis (PCA) shows most shape variation relates to changes in the snout and head width. Size-corrected head shape variation has strong phylogenetic signal at a broad level, but habitat use is predictive of shape disparity within phylogenetic lineages. Size often explains shape disparity among organisms; however, the ability to separate size and shape variation using 2D geometric morphometrics has enabled the identification of phylogenetic history and habitat as additional key factors contributing to the evolution of head shape disparity among monitor lizards.

Snout shape variation accounted for a substantial amount of total head shape variation among monitor species (Openshaw & Keogh, 2014). The nares position varies both longitudinally and dorsolaterally, with each external nostril (nare) opening into a nasal capsule. Located at the base of the nasal cavity and just above the palate are the vomeronasal organs (VNOs) (Jacobson’s organs). These are paired chemosensory organs stimulated by “tongue-flicking” behaviour whereby the tongue is used to sample environmental chemicals (Filoramo & Schwenk, 2009). The derived nature of the chemosensory apparatus in monitors suggests it plays an important role in interspecific (prey detection) and intraspecific (mating, aggression) interactions.
**Figure 1.** Phylogenetic tree of the 36 *Varanus* species included in the 2D external head morphology study (based on the full phylogeny in Vidal et al. 2012). Representative species heads for each phylogenetic group are shown with a scale bar equal to 1 cm and correspond with bold names on the phylogeny. Figure reproduced from Openshaw & Keogh 2014.
INTRODUCTION

The field of morphometrics is a central component of biology that quantifies biological shape, shape variation, and its correlation with other variables (Bookstein, 1991; Adams, Rohlf & Slice, 2004; Webster & Sheets, 2010; Adams, Rohlf & Slice, 2013). Geometric morphometrics are a popular set of methods that restrict data to locations of discrete anatomical points (landmarks), and rely on a constellation of points (the landmark configuration) to sample morphology (Bookstein, 1986; Bookstein, 1993; Corti, 1993; O'Higgins, 2000). Designing an appropriate landmark configuration is a critical early step in geometric morphometric analysis and ideally, three broad goals are achieved through appropriate selection of landmarks - a configuration should cover the variation clearly observable among objects, provide a sufficiently comprehensive and biologically significant sampling of morphology, and deliberately test the hypotheses proposed (Roth, 1993; O’Higgins, 2000; Oxnard & O’Higgins, 2009; Zelditch, Swiderski & Sheets, 2013).

Geometric morphometric studies frequently use a two-dimensional landmark configuration to quantify form, even though the structure of interest is typically three-dimensional (Roth, 1993; Cardini, 2014; Klingenberg, 2015). While the 3D approach is becoming more accessible, for many it is still a more expensive and time consuming processes that requires large data storage facilities. The 2D approach has several advantages over 3D, in particular the ease of capturing 2D coordinates by placing points on a photograph using freely available, user-friendly software such as tpsDig (Rohlf, 2010a), and the intuitively understood visualizations of shape variation produced using
the thin-plate spline (Klingenberg, 2013a). It is also much faster and so it is easier to obtain much larger sample sizes to quantify variation. The obvious disadvantage of a 2D approach is the loss of information regarding depth, and both correlative and comparative studies of 2D versus 3D datasets suggest that including the third coordinate may lead to different results for some biological structures (Cardini & Thorington, 2006; Álvarez & Perez, 2013; Cardini, 2014). Despite the fact that 2D morphometric analyses of 3D structures mean the loss of tridimensional information, the relative increased costs, time and difficulty associated with 3D data collection, storage and analysis still make 2D geometric morphometrics a realistic, inexpensive, and time-effective choice for many researchers (Cardini, 2014; and references therein).

The vertebrate head is a complex structure that has received much attention in morphometric studies, perhaps due to the enormous disparity in form that has evolved among groups. At the broadest scale, the skull is comprised of three parts: the cranial vault, the cranial base, and face (de Beer, 1937). Although these different parts originate in embryologically distinct regions, they apparently grow in a morphologically integrated manner through numerous developmental and functional interactions (Lieberman, Ross & Ravosa, 2000; Bookstein et al. 2003; Sanger et al. 2012). This has implications for 2D geometric morphometric analyses of head shape diversification regarding which observation view (or combination of views) is most informative for any vertebrate group. An observation view is the orientation of the specimen in relationship to observer, and the view(s) chosen by a researcher may influence the nature of the morphometric data yielded from a given specimen. This is problematic as the numerous structural subunits of the head observable from different views may be semi-independent in ontogeny or function, and are thus expected to vary in conservative properties and respond differently to ecological and evolutionary factors (Klingenberg, 1996; Lu et al. 2014). It is not understood how profoundly 2D shape analyses of the vertebrate head are influenced by observation view(s) and the landmark configuration used to characterize them.

Lizards are suitable for such examination, as they have a remarkable diversity in cranial form that is tightly linked to the functional and constructional demands of the skull, and show a unique evolutionary trend toward increased fenestration and reduced cranial
ossification (Evans, 2003; Stayton, 2005; Herrel et al. 2007; Daza et al. 2008). The ventral morphology of lizard crania, formed by parts of the dermatocranium and neurocranium, is directly involved in the mechanics of feeding, affecting bite force through connections to the jaw musculature (Herrel et al. 2007). Jaw musculature is also associated with structures most clearly observed from a lateral view (for example the postorbital-postfrontal, quadrate, coronoid and jugal). Consequently, much shape variation observed in lateral view is strongly linked with diet, for example herbivorous lizards tend to have short snouts and high skulls, which is consistent with a slow but powerful bite relative to carnivorous lizards (Evans, 2003; Stayton, 2005). Palate morphology and the septomaxilla also reflect the evolution of the chemosensory system in general, and the vomeronasal system in particular (Rieppel, Gauthier & Maisano, 2008), and the skull roofing bones are greatly influenced by the development of the brain and other sensory organs (Haas, 1973). The most highly fenestrated cranial morphology belongs to the monitor lizards (family: Varanidae), and is known as a ‘space frame’ type of construction in engineering terms (McCurry et al. 2015). The monitor cranium has a noteworthy mixture of extremely reduced bones (such as those forming the skull roof, particularly the snout region), derived bones (such as the palpebral and dermal palate elements), and composite bones (such as the fused postorbitofrontal) (Bellairs, 1949; Frazzetta, 1962; Auffenberg, 1988; Rieppel et al. 2008; Werneburg et al. 2015). Further, 2D geometric morphometric analysis of lateral cranial shape among lizard families reveals monitors have conspicuous patterns of interspecific cranial shape disparity (Stayton, 2005).

My study examines how observation view and landmark configuration design influence 2D geometric morphometrics analysis of interspecific cranial shape variation in monitor lizards. Specifically, I quantify the covariation of species cranial shapes with phylogeny and allometry, and characterize major patterns of interspecific cranial shape variation and size-corrected diversification in morphospace. I then identify and evaluate differences among 2D views to determine the utility of combining multiple views, and to assess whether choosing different views for landmark-based morphometric analyses could influence omy quantification of shape differences among specimens or species, providing complementary morphological information to each other. I expect congruence among views for quantification of shape covariation with phylogeny to reflect developmental
constraints within the cranium. A pervasive influence of allometry on shape is predicted, and should be exemplified in monitor lizard morphology, as species differ in body mass over four orders of magnitude (Pianka, 1995). However, I predict the anatomical points sampled to reflect functionally different characters among views, and therefore to recover discrepant patterns of interspecific cranial shape variation and size-corrected diversification.

**MATERIALS AND METHODS**

**Morphometric data**

My morphometric dataset comprises images of the cranium for 29 *Varanus* species (n = 152 specimens) in lateral, dorsal and ventral views (Supp. Table S1). I only used cranial material of adult specimens (skeletal maturity judged by specimen size) and I did not know the sex of the specimens. Within a species, male and female varanids have the same shaped heads but subtle sexual dimorphism in head size has been detected in some of the larger monitor species (Frýdlová, Velenský, Šimková *et al.* 2011). Not knowing the sex in my study is unlikely to influence my results because I correct for head size and my primary interest is in interspecific shape variation rather than intraspecific size variation.

A majority of the dataset is photographs of preserved crania, obtained from collections in the American Museum of Natural History (AMNH), the Florida Museum of Natural History (FLMNH), the Field Museum of Natural History (FMNH), the National Museum of Victoria (NMV), the University of Michigan Museum of Zoology (UMMZ) and the Smithsonian Institution National Museum of Natural History (USNM). Photographs were taken with a mounted and leveled Olympus ® C-765 Ultra Zoom. Skulls were situated against a dark background with a scale bar in the same plane. The lateral view involved positioning the crania so the camera was perpendicular to the mid-sagittal plane. The dorsal view involved placing the cranium ventral side down with the teeth and palate against the surface. The ventral view involved positioning the skull so the palate was parallel to the camera lens. The dataset was supplemented with 2D images obtained from micro X-ray computed tomography (CT) scans of crania from whole, alcohol-preserved specimens from the Western Australian Museum (WAM) (11 species). All scans were made with the Xradia microCT x400 system at the Australian Microscopy and Microanalysis Research Facility (University of Sydney). CT scan data were rendered using Drishti v2.0 (Limaye,
Non-cranial volumes were digitally removed, and images of the lateral, dorsal and ventral views of the cranium were then saved (including a scale bar).

**Phylogeny**

The phylogenetic hypothesis used in this study is based on the published molecular phylogeny presented in Vidal *et al.* (2012). I assembled the published data for 26 species included in Vidal *et al.* (2012) and added three additional species from the published work of Welton *et al.* (2014) (total: 29 species) (Fig. 2; Supp. Table S1). In order to include the three additional species I generated a maximum-likelihood phylogenetic tree based on mitochondrial protein-coding genes (ND3 and ND4) and nuclear (brain-derived neurotrophic factor, bone morphogenetic protein 2, and neurotrophin 3) sequence data and constrained the major clades so that the resultant phylogeny would be consistent with the phylogeny presented in Vidal *et al.* (2012). The resulting phylogeny was used for visualizing shape diversification across *Varanus*, identifying shape differences among different groups in a phylogenetic context, and to test for phylogenetic signal.
Figure 2. Phylogenetic relationships of the 26 Varanus species included in this study. The tree is primarily based on the phylogeny of Vidal et al. (2012), with additional information from Welton et al. (2014) (see text for details).
2D Geometric morphometrics

I used a 2D geometric morphometric approach for all views, also using a 2D sliding semilandmark procedure for the ventral view (Bookstein, 1997). Landmarks and semilandmarks were collected using tpsDig v. 2.16 (Rohlf, 2010a (Fig. 3; Supp. Table S2). The semilandmarks were collected as a curve outlining the fused pre-maxillae and paired maxillae in ventral view (Fig. 3). These data were subsequently reduced to equidistant landmarks (four on the pre-maxillae and ten on each maxilla), and subsequently defined as semilandmarks using the software tpsUtil v. 1.46 (Rohlf, 2010c). I then slid the landmarks using the bending energy method (Bookstein, 1997; Gunz & Mitteroecker, 2013) implemented in the R statistics package geomorph (Adams & Otárola-Castillo, 2013). The original landmark coordinate data were aligned using a generalized Procrustes superimposition analysis (GPA), providing the centroid size and shape coordinates (Adams et al. 2013). The monitor cranium has matching symmetry in lateral view and object symmetry in dorsal and ventral views. Although shape analysis procedures for both types of symmetry can separate the symmetric (left-right averages) component of variation from left-right asymmetries within individuals (Klingenberg, Barluenga & Meyer, 2002), damage to specimens in lateral view meant I could only digitize landmarks on one side of the cranium. Shape data therefore refers to Procrustes coordinates in lateral view, and the symmetric component of shape variation in dorsal and ventral views. I then calculated the mean of the Procrustes coordinates and centroid sizes of each species, in order to generate phylomorphospace plots and allow phylogenetic comparative analyses on the shape data.
Figure 3. The three 2D landmark configurations used in this study to characterize cranial shape for (a) dorsal, (b) ventral, and (c) lateral views. A scale bar equal to 10 mm is shown, and numbers refer to the anatomical definitions of landmarks in Supp. Table S2. The example skull is Varanus glauerti specimen [WAM_R77266].
Cranium size and shape variation

Ignoring the phylogeny in preliminary transformations of species data results in substantially elevated variance and type I error (false positive) in statistical estimators (Revell, 2009). To quantify the cranial shape variation evolutionarily associated with allometry in the monitor lizard cranium, I performed a Procrustes regression of shape on size, based on the Procrustes coordinates averaged by species, using the software geomorph (Adams & Otárola-Castillo, 2013). I also used the residuals from the averaged Procrustes coordinates and centroid sizes in order to perform a phylogenetic regression of shape on size in a phylogenetic context, in order to get ‘evolutionary allometry’-corrected shape data, also using geomorph (Adams & Otárola-Castillo, 2013). Analyzing the residuals from a regression of the size-dependent trait against size is a common method of correcting for allometry in evolutionary studies (Gould, 1966; Monteiro, 1999; Revell, 2009).

Phylogenetic signal

To quantify phylogenetic signal in Varanus crania for each of the views (dorsal, ventral, and lateral), I used the K_{mult} method, which is Blomberg’s K statistic generalization appropriate for high-dimensional and/or multivariate data (Adams, 2014), using the R package geomorph (Adams & Otárola-Castillo 2013). I determined the statistical significance of K_{mult} using phylogenetic permutation with 10,000 iterations, which is calculated by permuting the shape data of the Procrustes-aligned specimens among all tips of the phylogenetic tree. I also simulated the confidence intervals of K under a Brownian Motion (BM) model of evolution using phytools (Revell, 2012), with 10,000 iterations. Under BM, K_{mult} has an approximate expected value of 1.0, and the higher the K_{mult} value is, the stronger the phylogenetic signal, indicating higher morphological variance among clades instead of within clades (Adams, 2014).

Patterns of cranial shape variation and diversification

To examine patterns of cranial shape variation among Varanus, I subjected the averaged shape coordinates to a Principal Component Analysis (PCA), both before and after size correction, for all three views. To characterize the evolutionary patterns of cranial shape diversity, I projected the phylogeny into the shape tangent space and projected it on plots
species mean size-corrected cranial shapes. This approach maps PC scores of the species cranial shapes to the phylogeny, computes the PC scores at internal nodes, and subsequently projects the branches of the phylogenetic tree onto the morphospace. Evaluating this ‘phylomorphospace’ provides insights into the history of morphological diversification for complex traits and allows a multivariate visualization of shape differences between species (Sidlauskas, 2008; Sherratt et al. 2014). To quantify the amount of shape variation and dispersion between original and size-corrected shape data, I measured the dispersion of all the individuals at three levels: (a) for the whole data set – by calculating the dispersion around the mean shape for all the specimens, (b) by species, and (c) by the phylogenetic groups identified in Vidal et al. (2012). Finally, I tested the integration between the three views, in order to assess the strength of dependency among each landmark configuration, with geomorph (Adams & Otárola-Castillo 2013).

RESULTS
Shape variation among Varanus crania
In dorsal view, original shape variation captured by the two first Principal Components (PC 1-2) relates to cranium width, relative length of the snout and parietal units, and angularity of the fronto-parietal suture and nasal-frontal boundary (Fig. 4; Fig. 5). PC 1 (accounting for 54.9% of the overall morphological variance) (Table 1) contrasts crania with a shortened snout complex and widened parietal unit in the negative direction, and crania with a lengthened snout complex and narrowed parietal unit in the positive direction. PC 2 (19.0% variance) contrasts narrow crania with a relatively short snout in the negative direction, and broad crania with a relatively long snout in the positive direction.
Table 1. Cranial shape variation in the three observation views captured in PC 1-5, both for the original and size-corrected shape data. The tabled values are the percentages of total variance (%) for which each principal component accounts.

<table>
<thead>
<tr>
<th>Axis</th>
<th>Original Shape Data</th>
<th>Size-corrected Shape Data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dorsal</td>
<td>Ventral</td>
</tr>
<tr>
<td>PC 1</td>
<td>54.939</td>
<td>47.742</td>
</tr>
<tr>
<td>PC 4</td>
<td>5.094</td>
<td>5.803</td>
</tr>
<tr>
<td>Cumulative (PC1-5)</td>
<td>89.133</td>
<td>88.811</td>
</tr>
</tbody>
</table>
Figure 4. The two major principal axes of cranial shape variation in *Varanus*, visualized as deformation grids, in dorsal, ventral and lateral views. PC axes are from a PCA of species means, and shape changes associated with the PCs are shown as extreme cranial shapes representative of the positive and negative ends of each axis. In each case the magnitude of the shape change from the mean is indicated by the extent of deformation.
Figure 5. Cranial shape diversification among *Varanus*, in (a) dorsal, (b) ventral, and (c) lateral views, for original shape data. The phylogenetic tree is superimposed onto a plot of the first two PCs of the covariance matrix based on residuals computed using the regression vector of independent contrasts. The tips of terminal branches are at the locations of species means, and are coloured according to phylogenetic group.
In ventral view, over 70% of the cranial shape variation among monitors relates to cranium width, curvature of the snout, robusticity of the palatine and ectopterygoid, and length of the pterygoid (Fig. 4; Fig. 5). For PC 1 (47.7% variance), a negative change from the mean describes crania with a short and broad snout, distally shifted and broadened suborbital bones, and a broad and long pterygoid. A positive change from the mean describes crania with an elongate and narrow snout, mesially shifted and narrowed suborbital region, and a shortened pterygoid. PC 2 (24.7%) captures relative shifts in landmarks altering pointedness of the snout, width of the posterior maxilla region, mesial-distal shifting in the palatine and length of the pterygoid.

In lateral view, shape variation captured by PC 1-2 relates to overall cranium height, length and angularity of the snout complex, convex-concave bending and length of the basal units, and robustness of the parietal unit (Fig. 4; Fig. 5). For PC 1 (31.1%), a negative change from the mean shape describes relative shifts in landmarks that flatten the cranium, lengthen the snout complex, and both shorten and project the parietal and basal units mesially. A positive change from the mean shape corresponds to a heightened cranium with a short but steeply rising snout complex, and enlarged parietal and basal units. PC 2 (22.5%) describes more subtle shape changes, contrasting crania with a lengthened snout complex, ventrally projected pterygoid, and steeply rising cranial roof in the negative direction, and crania with a shortened snout complex, mesially projected elongate pterygoid, and flattened cranial roof in the positive direction.

**Phylogenetic signal**

The results of the multivariate K-statistic calculated on the shape data are significant for each view of the *Varanus* cranium (dorsal: $K_{\text{mult}} = 0.99$, $P < 0.001$; ventral: $K_{\text{mult}} = 0.85$; $P < 0.001$; lateral: $K_{\text{mult}} = 0.83$, $P < 0.001$). K 95% confidence interval for values expected under a Brownian Motion model of trait evolution = [0.658, 1.612].

**Cranial size and shape variation**

The multivariate Procrustes regressions of shape data on centroid size were statistically significant ($P < 0.0001$) in dorsal, ventral and lateral view, indicating the presence of allometry in the *Varanus* cranium. In the quantification of evolutionary allometry (when
phylogenetic relatedness is incorporated in the regression), the \( r^2 \) values decrease substantially but remain significant for all three views (Table 2). The dorsal view recovers the largest \( r^2 \) values, followed by lateral view then ventral view, suggesting that shape variation in the monitor lizard cranium is most heavily constrained by size in the skull roof. The amount of shape variation accounted for by PC 1-5 is substantially less after evolutionary allometry is removed from average species cranial shapes (Table 1). The amount of dispersion and shape variation was very similar between original and size-corrected shape data for each of the views, for all three levels (all individuals, by species, and by phylogenetic groups; Table 3). The two-block PLS between different views pointed out very high integration between dorsal and lateral (\( r_{\text{PLS}} = 0.943, P < 0.001 \)) and between ventral and lateral landmark configurations (\( r_{\text{PLS}} = 0.921, P < 0.001 \)), and moderately high between dorsal and ventral views (\( r_{\text{PLS}} = 0.878, P < 0.001 \)).

**Size-corrected shape diversification in Varanus crania**

Superimposing the phylogeny onto a plot defined by PC 1-2 of evolutionary allometry-corrected shape variation shows cranial shape diversification across species, depicting their great morphological differences (Fig. S1). There is a moderate clustering of size-corrected species cranial shapes according to phylogenetic group in ‘phylomorphospace’ for all three-observation views. These clusters are most clearly separated from each other in dorsal view. The major difference in diversification patterns among views is attributed to the African species, which occupy a positive position on PC 2 in dorsal view, but a negative PC 2 position in ventral and lateral views. Cranial shapes of species in the Indo-Australian group show the greatest divergence from all other species cranial shapes, occupying a distinct region of each phylomorphospace. Cranial shapes of individual species from all other phylogenetic groups have diversified varying amounts in each view, as shown by branches that traverse a large proportion of the region occupied by the whole sample. These include the African species *Varanus exanthematicus* and *Varanus albigularis*, the Indo-Asian species *Varanus olivaceus* and *Varanus rudicollis*, and the Indo-Australian species *Varanus komodoensis* and *Varanus giganteus*. 
Table 2. Summary table of multivariate Procrustes regressions and phylogenetic regressions of shape data on centroid size, for each of the three views, using *geomorph* (Adams & Otárola-Castillo 2013).

<table>
<thead>
<tr>
<th></th>
<th>Dorsal</th>
<th></th>
<th>Ventral</th>
<th></th>
<th>Lateral</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>R²</td>
<td>p</td>
<td>R²</td>
<td>p</td>
<td>R²</td>
<td>p</td>
</tr>
<tr>
<td>Regression</td>
<td>0.3052</td>
<td>&lt;0.0001</td>
<td>0.1666</td>
<td>&lt;0.0001</td>
<td>0.2294</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Phylogenetic regression</td>
<td>0.2072</td>
<td>&lt;0.0001</td>
<td>0.1121</td>
<td>&lt;0.0001</td>
<td>0.1367</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
DISCUSSION
The highly fenestrated space frame construction of monitor lizard crania creates clear differences in abundance and spread of candidate landmarks among alternative views of the structure. It follows that the design of each 2D landmark configuration inevitably describes shape in a slightly different way. Phylogeny underpins patterns of gross morphological variation among monitor lizard crania, even when diluted by size-related constraints and presumably adaptive trends, which might be also phylogenetically constrained. The strength of association between cranial size and shape differs among observation angles; however, it is unclear whether these results reflect genuine evolutionary trends or slight differences on the morphological information captured by each landmark configuration design. The patterns of cranial shape variation are broadly concordant among views, suggesting dietary variation has contributed to shape diversification across the entire cranium at a higher phylogenetic level. Below, I evaluate 2D characterization of the monitor cranium, present an overview of cranial shape evolution in monitors, and discuss more broadly how 2D geometric morphometric analyses of vertebrate head shape can be improved through analysis of multiple observation views and careful landmark configuration design.

The evolution of cranial morphology in monitor lizards
A careful consideration of landmark configuration design is required when interpreting geometric morphometric results. My landmark configurations capture a dorsal-ventral ‘silhouette’ in lateral view, the medial paired and fused bones in dorsal view, and palatopterygoid morphology in ventral view (Fig. 3). A 2D characterization of monitor lizard cranial morphology is most comprehensive in ventral view, as abundant type I and type II landmarks (Bookstein, 1991) are reliably digitized, and four curves are traced to outline the fused pre-maxillae and paired maxillae. I find the lateral view to be the least reliable, because of the difficulty in accurately orienting specimens when taking photographs (Klingenberg, 2015). The requirement of 2D landmarks to be coplanar (Zelditch et al. 2013) is most limiting for landmark configuration design in lateral and dorsal view, and is the key reason why semilandmarks outlining the pre-maxillae and maxillae were only added in ventral view. There are also morphological differences clearly observable among species for numerous bones (such as the palpebral); however, such
variation should not be characterized using geometric morphometric methods (Zelditch et al. 2004).

The major monitor lizard clades match biogeographic distribution well, dividing the African, Indo-Asian, and Indo-Australian species (Fig. 2). An Asian origin for crown *Varanus* is likely, with major dispersal events occurring to Africa approximately 41 million years ago (mya), and to Australia approximately 32 mya (Vidal et al. 2012). Significant phylogenetic signal is found across observation angles, and interspecific cranial shape variation still retains substantial phylogenetic structure after correcting for evolutionary allometry; Fig. 5 shows that cranial shapes of closely related species form moderate clusters in phylomorphospace. The importance of phylogenetic affinity for explaining interspecific variation is recovered in geometric morphometric analyses of lateral, dorsal and/or ventral cranial shape in other lizard studies (Stayton, 2005; Daza et al. 2009; Openshaw & Keogh, 2014). Such clear phylogenetic structure in the morphometric data across observation angles may indicate a role for developmental constraints in diversification of monitor lizard cranial shape.

Monitors occupy a wide range of environments across their distribution, and the selective demands imposed by habitat have driven the evolution of extreme body size disparity in the genus (with extant species ranging in total body length as adults from 23 cm to 3 m) (Collar, Schulte & Losos, 2011). Strong size-shape relationships are known for monitor lizard body and head morphology (Christian & Garland, 1996; Thompson & Withers, 1997; Collar et al. 2011; Openshaw & Keogh, 2014). Initially, I found strong and significant allometry in each view of the cranium; however, controlling for non-independence of shape data due to shared ancestry reduced the estimation of evolutionary allometry in all views. This also reflects the strong phylogenetic signal in body size evolution for the group (Collar et al. 2011; Openshaw & Keogh, 2014). The inconsistency in strength of evolutionary allometry among views could reflect discrepancy in the way each observation view quantifies size: dorsal view recovers the strongest influence of evolutionary allometry ($r^2 = 0.2072$), and is described by a landmark configuration that effectively captures cranium length, but only covers cranium width at the posterior-most edges of the cranial table (Fig. 3).
The three observation views share notable similarities in patterns of interspecific cranial shape variation (Fig. 4) readily linked with diversity in diet through particular influences on cranial performance and strength. First, the shape changes captured by the primary axis of shape variation (PC 1) reveals two cranial phenotypes: (1) relatively tall and broad crania with a short and steeply rising snout; and (2) relatively flat and gracile crania with an elongate, slender snout. Monitor lizard crania with greater widths and heights perform better at biting, pulling and shaking (as exemplified by *V. komodoensis*) (Moreno *et al.* 2008; D’Amore *et al.* 2011). The most extreme examples of this morphotype belong to durophagous species like *V. exanthematicus*, which are characteristically short and wide with a tall snout (McCurry *et al.* 2015). These traits are often coupled with deep mandibles and robust dentition (Rieppel and Lambhardt 1979; D’Amore 2015; McCurry *et al.* 2015). The gracile morphotype is most clearly observed in 2D in *V. giganteus*. The elongate cranial shape likely reflects a diet consisting of large prey and carrion (McCurry *et al.* 2015). Second, there are finer scale shape differences that are expected to be important in determining the strength of a space frame construction. For example, a greater ventral projection of the palatopterygoid articulation to form an obtuse angle, and a more vertical positioning of the epipterygoid increases structural support, and is observed in semi-frugivorous species feeding like *V. olivaceous* (Auffenberg, 1988). I also recover variation in angularity of the frontoparietal suture or mesokinetic joint. Cranial sutures are forms of articulation in which the bones are rigidly joined by fibrous tissue (synarthroses) (Di leva *et al.* 2013) and are expected to have important roles in cranial kinesis and reducing stresses during biting feeding (Moazen *et al.* 2009). The concordance among observation angles and links with diet suggests feeding performance imposes selective pressure across each view, resulting in high integration between the different views, and to certain degree among the cranial subunits described by each landmark configuration (Harmon *et al.* 2005; Perez, Bernal & Gonzalez, 2006; Klingenberg, 2008; Meloro *et al.* 2011).

My analysis of dorsal, ventral and lateral cranium shape in monitors leads to subtle differences in interpretation of morphological diversification of the cranium as a whole. Given the dorsal landmark configuration describes fused and paired bones, I expect to
recover a more conserved pattern of morphological diversification. Increased conservatism is evident through the strong phylogenetic signal, the greatest size-shape constraint among views, and a more clear separation of phylogenetic groups in phylomorphospace. The interplay of lateral and ventral views is apparent, further supporting a role for some developmental constraints in morphological diversification of the monitor cranium, but also highlighting the role of diet.

**Implications for 2D landmark configuration design**

Geometric morphometric methods have been widely applied in studies of head shape diversification for numerous vertebrate groups (Slice, 2007). External head morphology is primarily landmarked using the facial features in primates, and the scales of lizards, snakes and fish (Kaliontzopoulou 2011; Kerschbaumer & Sturmbauer, 2011; Baab, McNulty & Rohlf, 2012). The skull is frequently broken down into its constituent parts (including dentition), depending on the question of interest, and described by landmarks at the contacts between bones, tips of processes, origins and attachments of muscles, locations of joints and tips of lever systems (Slice, 2007). Although landmarks are frequently collected as surface marks on one tissue (i.e. scales or bone), they may relate to different tissues whose relationships vary with evolution, development, and growth (Slice, 2007; Oxnard & O'Higgins, 2009). For example, 2D landmark configurations on the surface of bone have been utilized in marmot skulls for all three cranial observation views, and the mandible. The ventral cranium seems to be the best morphological feature for recovering phylogenetic relationships in varanids, and it seems that the largest shape modifications occur in regions directly involved in the mechanics of mastication in several other groups (Cardini & O'Higgins, 2004; Caumul & Polly, 2004; Cardini & Thorington, 2006). The lateral cranium and mandible are least strongly correlated with phylogeny, and dorsal and lateral cranium morphology together reflects brain size and dermal bones (Caumul & Polly, 2004; Cardini, Hoffman & Thorington, 2005).

The efficiency of geometric morphometrics methods to provide a detailed and accurate quantitative description of form ultimately increases with the amount of information contained within a landmark configuration (Cardini & Thorington, 2006). Outline-based geometric morphometric techniques are therefore a powerful addition to a 2D landmark
configuration, as they can capture structural information on large areas of morphology only represented by surfaces, curves or outlines (Oxnard, 1978; Roth, 1993; Adams et al. 2004; Perez et al. 2006; Webster & Sheets, 2010). The outline-based approach is less widely applied than the landmark-based approach, having mainly been utilized in anthropological studies (Slice, 2007; Baab et al. 2012). However, three limitations of outline-based methods should be considered: (1) protrusions on one individual that do not appear on another can be poorly captured by the resulting semilandmark alignment; (2) it is often not clear how many semilandmarks should be used for any given outline; and (3) outline methods only define relative features (MacLeod, 1999; McCane, 2013; Finlay & Cooper, 2015). Regardless, their utility is highlighted in studies like mine, where the clarity of results is considerably improved in the landmark configuration(s) that utilize both landmarks and outlines (Baab et al. 2012; Finlay & Cooper, 2015). By increasing the number of coordinates along a homologous contour or surface, it is ultimately possible to obtain more information on the shape of a structure. The optimal 2D observation view for analyzing cranial shape variation may thus be the view in which both landmarks and semilandmarks may be utilized.

In conclusion, my study adds to a growing body of evidence suggesting multiple carefully designed 2D landmark configurations can provide fairly accurate descriptions of interspecific variation in complex structures like the vertebrate head (Cardini & Thorington, 2006; Perez et al. 2009; Álvarez & Perez, 2013; Cardini, 2014; Finlay & Cooper, 2015; Klingenberg, 2015). The key benefits of 3D techniques may instead be in the extensions to assessing morphological patterns in internal structures (e.g. brain case), functional morphology (finite element modeling and mechanical strain) and reconstruction of ancestral shapes (evolutionary warping or morphing) that they offer (Wiley et al. 2007; Parr et al. 2012).
FUTURE DIRECTIONS
Cranio-dental morphology and maximal bite force in gape-limited predators.

BACKGROUND
In examining the evolution of a particular phenotype, biologists link morphology to biomechanical function, and biomechanical function to performance (the ability to carry out ecological tasks that influence fitness). There is therefore an implicit assumption of disruptive or directional selection on alternative morphologies that represent divergent performance optima (Dumont et al. 2014). Lizards are a suitable group for studying functional morphology, and specifically in the skull, because of the complexity and diversity in the feeding system that integrates the cranium, jaw, hyolingual and cervical systems (Schaerlaeken et al. 2011). Aside from feeding, the lizard skull is functionally associated with ecomorphology and sexual selection, and is additionally responsible for housing the major sensory systems (Stayton, 2005; Herrel et al. 2007). Despite being a structurally complex and integrated system with numerous functions and roles, evolution in the lizard skull is characterized by the appearance of extreme morphologies. Among extant lizards, a reduction in the size and/or robusticity of individual skull elements (i.e. temporal fenestration) is typically associated with cranial kinesis. Specific movements in skull elements characterize the various ‘types’ of cranial kinesis. For example, streptostyly refers to movement of the quadrate in relation to the dermocranium, often in the form of an anteroposterior rotation at its dorsal end. Although there is a lack of clear evidence on the biomechanical relevance of cranial kinesis, it is suggested to impact function in three key ways: (1) increasing bite force via increasing mechanical advantage to one or more of the adductor muscles during jaw closing; (2) increasing gape and therefore enabling consumption of larger prey through rotation of the quadrate around a transverse, sagittal or dorsoventral axis; and (3) facilitating variety in food processing and intraoral transport abilities (Smith, 1986; Metzger, 2002).

Bite force is a basic performance measure for lizards, since variation among closely related taxa should strongly reflect variation in trophic demands (Nogueira et al. 2009; Marshall et al. 2014). High bite force is suggested to be biologically optimal, as it allows
animals to eat a wider range of foods (Nogueira et al. 2009; Herrel et al. 2014). Empirical evidence of bite force in lizards suggests species that include considerable amounts of plant matter in their diet have, on average, higher bite forces and more strongly developed jaw adductor muscles than insectivores or carnivores (Herrel et al. 2014). Increasing bite force may be achieved via increasing overall body size, head size relative to body size, increasing muscle mass or mechanics of the lever system (Schaerlaeken et al. 2012). Unless animals shorten the jaw outlever and bite closer to the fulcrum, an increase in muscle mass will impose greater loads on the cranial elements that need to withstand these larger bite forces. Species with increased bite forces are therefore expected to have more robust skulls and diets consisting of tough, hard prey. However, constraints on lever mechanics mean the cranium cannot be simultaneously optimized for force generation and speed. Therefore, I still expect species preying on fast and agile prey to have long snouts (in addition to long parallel fibered muscles and long jaw-opening inlevers), despite its mechanical association with a weaker bite force (Herrel et al. 2007; Goyens et al. 2014).

Movements of the jaw, tongue and hyobranchium are strongly connected with tongue morphology and dentition in intraoral prey transport (Schaerlaeken et al. 2012). Tongue morphology makes intimate contact with the prey item during feeding, and as such, differing lizard clades having different degrees of specialization relating to diet and habitat. For example, heavy dependence on the chemosensory system for detecting prey is correlated with greater lingual elongation, deepened lingual forking, a decrease of the total surface area of the tongue and a decoupling of the tongue and hyobranchium (Cooper, 1997; Schaerlaeken et al. 2011). Lizard clades that rely heavily on the chemosensory system generally use inertial prey transport, whereby the prey is thrown into the oesophagus by rapid movements of the head, neck, and entire body. The jaws must open rapidly as the head/neck system is drawn back, and then at maximal gape, the entire head and body move forward and the jaws are closed (Metzger, 2009; Schaerlaeken et al. 2011). Dentition is critical in seizing and handling prey, and so not surprisingly, lizards exhibit a large variety of tooth shapes closely related to diet (Rieppel, 1979; Zahradnicek et al. 2014). For example, different Tupinambis lizard species have distinct dentition specialized for capturing and puncturing the chitonous exoskeletons of
insects, grasping and holding large prey, or crushing crustaceans and snail shells (Presch, 1974). Although once considered to have largely homodont dentition, numerous examples of heterodont, mammal-like dentitions are now known in lizards, with conical anterior teeth and more complex posterior teeth (Zahradnicek et al. 2014).

Monitor lizards (Varanus: Varanidae) are a genus of over fifty species that uniquely vary in body mass across four orders of magnitude, and subsequently occupy an enormous variety of habitats (Pianka, 1995). Monitors are active foraging, ‘opportunistic’ predators that feed on a variety of prey items; for example, diets may include carrion, invertebrates, aquatic prey, reptiles, birds, eggs and mammals (Losos & Greene, 1988; Arbuckle, 2008). Behavioural data supports monitors as opportunists, with animals showing an ability to adjust predatory behaviour and feeding kinematics in response to prey characteristics (mobility, mass, texture) (Schaerlaeken et al. 2011). Because monitors are adapted to feeding on relatively large prey items, the degree of tooth recurvature, as opposed to absolute tooth size, is suggested to have vital importance (Rieppel, 1979; Frazzetta, 1986). Recurved teeth enable a more secure hold on prey, however they make it more difficult for the tip of the tooth to make contact with the surface of the prey. Cranial kinesis may play a role in altering the line of action of the teeth during contact with prey items, and thus facilitate the evolution of recurved teeth (Rieppel, 1979). Indeed, monitors are often suggested as a model group for describing cranial kinesis, despite a lack of empirical consensus regarding the types, timing and magnitude of intracranial movements (Frazzetta, 1962; Rieppel, 1979; Smith, 1986; Metzger, 2002). Feeding on relatively large prey is also linked with the formation of plicidentine, which increases the surface area of the root for tooth attachment, and may help mitigate stress on cranial elements during prey capture and processing (Maxwell et al. 2011). Plicidentine is closely associated with mode of tooth replacement. In monitors, the replacement tooth grows in an interdental position posterior to the functional tooth, a condition that separates them from the majority of other lizards, whose replacement teeth move into the pulp cavity of the functional tooth through a large resorption pit (McDowell & Bogert, 1954; Rieppel, 1978).
I propose several areas for future research that specifically involve examining interspecific patterns of: (1) scaling between body size, head size and bite force; (2) skull robusticity and mandible length; and (3) dentition and cranial sutures. Considering greater bite forces enable feeding on a wide variety of prey, I might anticipate monitors to have higher than expected maximal bite forces relative to body size. However, the clear interspecific variation in skull morphology, robusticity and dentition (Fig. 6) suggests a more important role of morphology in mitigating stresses placed on cranial elements associated with feeding on a wide variety of prey. It may also suggest some species are more specialized in their diets than previously understood.

MATERIALS AND METHODS

Sampling
I have already selected, and X-ray CT scanned, the heads of fifteen Varanus species (Table S2). My sampling strongly focuses on Indo-Australian monitors because these interspecific relationships are best understood, and both the largest and smallest species belong to this lineage. However, I will ideally find and CT scan additional species from the African and Indo-Asian lineages to increase the range of diets and morphologies included.

Interspecific skull shape variation and diet
The skull morphology of each species will be described, and structural changes such as the loss of bony elements in miniaturized lineages will be assessed qualitatively. The interspecific skull shape analyses will closely follow those detailed in the research article presented here using the R package geomorph (Adams & Otárola-Castillo, 2013). I will also use the ‘evolutionary morphing’ technique implemented in the freely available software Landmark (Wiley et al. 2007), to visualize morphological changes in particular structures of interest, such as the mandible, postorbitofrontal and palpebral.

I will utilize the diet data compiled by Arbuckle (2009) for fifty monitor species and the 3D visualization software Drishti (Limaye, 2012) to highlight four variable aspects of monitor skull morphology closely related to diet: skull robusticity (quantified by bone density), mandible length (linear measurement), and dentition (overall shape category and degree of tooth recurvature).
Figure 6. Cranial morphology of seven closely related pygmy Australian monitors (subgenus Odatria) in lateral, dorsal and ventral view, highlighting substantial interspecific shape variation.
Biomechanical Performance

First, alternative ‘proxies’ for bite force will be explored, including head volume, examination of head musculature and attachment, and more sophisticated finite element modeling techniques. If no alternative is suitable, then captive bred animals for the Australian pet trade will be used for in vivo bite force measurements. While bite force studies should be set-up specific for the animal under study, I will broadly follow the bite force measurement protocols outlined for Uromastix, Dracaena, and Tupinambis lizards (Schaerlaeken et al. 2012; Herrel et al. 2014). Typically, these studies measure bite force at the tip of the jaw using an isometric Kistler force transducer mounted on a purpose-built holder and connected to a Kistler charge amplifier. To ensure I am obtaining maximal bite forces, five trial measurements will be recorded for each individual, with an inter-trial interval of at least 30 minutes, during which animals will be allowed to thermoregulate. Other lizard species are typically eager to bite when held, but I will tap gently on the sides of the jaws if individuals are hesitant. Notably, comparisons of bite-force experiments in vertebrates with padded and unpadded or non-compliant bite surfaces have shown significant differences in the forces elicited by the same-sized individuals. Such differences suggest animals generate under-motivated, and hence low-force values when biting non-padded surfaces that may damage their teeth (Erickson et al. 2014). To protect teeth and encourage aggressive biting, I will cover the plates on the bite force transducer with leather pads.

SIGNIFICANCE OF STUDY

The evolution of cranial kinesis (and temporal fenestration) has received considerable attention in the literature across vertebrates. Despite this, the patterns and biomechanical relevance of cranial kinesis is still largely unknown. Monitors are considered a crucial group for understanding cranial kinesis, given their lightweight skulls and possible affinity with snakes. Anatomical notes and descriptions exist for some monitors (for example Varanus niloticus and Varanus indicus); however, my 3D dataset provides an excellent opportunity for exploratory analysis of sutures in monitors from across the phylogeny.
Monitor lizard dentition, feeding behaviour and kinematics, specialized morphology and function of the tongue and hyoid apparatus are central discussion points in form-function studies of lizards (Rieppel, 1979; Smith & Hylander, 1985; Smith, 1986; Cooper, 1997; Elias et al. 2000; D’Amore et al. 2011; Maxwell et al. 2011; Schaeferaeken et al. 2011; Montuelle et al. 2012). This proposed study is providing key quantitative measures of how a range of species skulls performs biomechanically during feeding (either directly through in vivo bite force measurements or using proxies through the morphological data). If an allometric scaling relationship between the body, head and bite force is established, then it would allow prediction of bite forces based on body size measures in related extant and extinct specimens. This has exciting implications for understanding skull biomechanics in several extinct crown-Varanus species, which are the largest terrestrial lizards known to have ever existed.
Figure S1. Cranial shape diversification among Varanus, after correcting for allometry, in (a) dorsal, (b) ventral, and (c) lateral views. The tips of terminal branches are at the locations of species means, and are coloured according to bioregion groups.
**Table S1.** A list of the cranial material used in morphometric analysis. Institutional abbreviations: AMNH = American Museum of Natural History (AMNH), FLMNH = the Florida Museum of Natural History, FMNH = the Field Museum of Natural History, NMV = the National Museum of Victoria, UMMZ = the University of Michigan Museum of Zoology, USNM = the Smithsonian Institution National Museum of Natural History, and WAM = the Western Australian Museum.

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Table S2. Definitions of two-dimensional landmarks and semilandmarks used in the dorsal, ventral and lateral view landmark configurations.

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<tr>
<td>Dorsal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Premaxilla: anterior extremity on the midline.</td>
<td></td>
</tr>
<tr>
<td>2-3</td>
<td>Premaxilla, maxilla: meeting point on the distal edge.</td>
<td></td>
</tr>
<tr>
<td>4-5</td>
<td>Maxilla, prefrontal: anterior meeting point at the fenestra exocochoanalis opening.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Nasion: midline point on fronto-nasal suture.</td>
<td></td>
</tr>
<tr>
<td>7-8</td>
<td>Prefrontal, frontal: anterior meeting point at the fenestra exocochoanalis opening.</td>
<td></td>
</tr>
<tr>
<td>9-10</td>
<td>Prefrontal: posterior extremity on the lateral edge.</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Frontal, parietal: midline point on the frontoparietal suture.</td>
<td></td>
</tr>
<tr>
<td>12-13</td>
<td>Frontal, parietal: lateral extremities on the frontoparietal suture.</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Parietal: posterior edge on the midline.</td>
<td></td>
</tr>
<tr>
<td>15-16</td>
<td>Squamosal: lateral extremities on the posterior margin.</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Supraoccipital: posterior edge on the midline.</td>
<td></td>
</tr>
<tr>
<td>18-19</td>
<td>Otoccipital: lateral extremities on the posterior margin.</td>
<td></td>
</tr>
<tr>
<td>Ventral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Premaxilla: anterior extremity on the midline.</td>
<td></td>
</tr>
<tr>
<td>2-3</td>
<td>Premaxilla, maxilla: meeting point on the distal edge.</td>
<td></td>
</tr>
<tr>
<td>4-5</td>
<td>Premaxilla, septomaxilla: meeting point (anterior extremity of fenestra vomeronalis).</td>
<td></td>
</tr>
<tr>
<td>6-7</td>
<td>Maxilla, palpebral: proximal point along margin.</td>
<td></td>
</tr>
<tr>
<td>8-9</td>
<td>Vomer, palpebral: posterior meeting point (at the fenestra exocochoanalis opening).</td>
<td></td>
</tr>
<tr>
<td>10-11</td>
<td>Maxilla, ectopterygoid: meeting point on the distal edge.</td>
<td></td>
</tr>
<tr>
<td>12-13</td>
<td>Palpebral, pterygoid: distal meeting point (at the suborbital fenestra).</td>
<td></td>
</tr>
<tr>
<td>14-15</td>
<td>Ectopterygoid, pterygoid: proximal point along margin (at the suborbital fenestra).</td>
<td></td>
</tr>
<tr>
<td>16-17</td>
<td>Pterygoid: proximal extremity.</td>
<td></td>
</tr>
<tr>
<td>18-19</td>
<td>Pterygoid: posterior extremity.</td>
<td></td>
</tr>
<tr>
<td>c1-2</td>
<td>Premaxilla: curve along distal margin (2 landmarks, 2 semilandmarks).</td>
<td></td>
</tr>
<tr>
<td>c3-4</td>
<td>Maxilla: curve along distal margin (2 landmarks, 10 semilandmarks).</td>
<td></td>
</tr>
<tr>
<td>Lateral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Premaxilla: anterior extremity on margin of palate.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Premaxilla and maxilla: meeting point on margin of palate.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Nasal and frontal: meeting point on dorsal margin.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Maxilla, prefrontal, lacrimal: meeting point.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Maxilla, lacrimal, jugal: meeting point.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Maxilla, jugal, ectopterygoid: meeting point.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>---</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>Prefrontal: posterior extremity on dorsal process.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Postorbitofrontal: anterior extremity on dorsal margin.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Parietal: dorsal extremity (dorsal-most point of the cranium).</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Squamosal: ventral extremity on the posterior margin.</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Ectopterygoid, pterygoid: ventral extremity along the lateral contact margin.</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Epipterygoid, pterygoid: median point on the epipterygoid at the ventral contact with the pterygoid.</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Pterygoid: posterior extremity.</td>
<td></td>
</tr>
</tbody>
</table>
REFERENCES


APPENDIX 1

See attached article pdf.
Combining geometric morphometric analyses of multiple 2D observation views improves interpretation of evolutionary allometry and shape diversification in monitor lizard (Varanus) crania

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Geometric morphometrics is a powerful tool for the quantification, visualization and analysis of morphological variation and change. This approach is being applied more frequently in a phylogenetic comparative context to assess the relative influence of size, ecology, function, and developmental constraints on morphological evolution. Geometric morphometric methods rely on homologous landmarks as the source of shape data, and the level of detail and accuracy increases with the amount of information contained in a landmark configuration. It may be possible, however, to capture particular elements of shape variation by concentrating on different observation angles of a complex structure like the vertebrate cranium. Our study examines how observation view (dorsal, ventral and lateral) influences 2D geometric morphometric analysis of interspecific cranial shape variation in monitor lizards. We recover a strong phylogenetic signal in all three views and general concordance in patterns of size-corrected shape diversification within the genus. We also find subtle, but important differences, however, among views in analyses of evolutionary allometry and shape variation, which may reflect both landmark configuration design and adaptive functional trends of the study system. Our study shows that studies restricted to a 2D geometric morphometric analysis of a complex 3D biological structure can combine carefully designed 2D landmark configurations describing alternative planes to maximize shape coverage. © 2016 The Linnean Society of London, Biological Journal of the Linnean Society, 2016, 00, 000–000.


INTRODUCTION

The field of morphometrics is a central component of biology that quantifies biological shape, shape variation, and its correlation with other variables (Bookstein, 1991; Adams, Rohlf & Slice, 2004, 2013; Webster & Sheets, 2010). Geometric morphometrics is a popular set of methods that restrict data to locations of discrete anatomical points (landmarks), and rely on a constellation of points (the landmark configuration) to sample morphology (Bookstein, 1986, 1993; Corti, 1993; O’Higgins, 2000). Designing an appropriate landmark configuration is a critical early step in geometric morphometric analysis and ideally, three broad goals are achieved through appropriate selection of landmarks – a configuration should cover the variation clearly observable among objects, provide a sufficiently comprehensive and biologically significant sampling of morphology, and deliberately test the hypotheses proposed (Roth, 1993; O’Higgins, 2000; Oxnard & O’Higgins, 2009; Zelditch, Swiderski & Sheets, 2012).

Geometric morphometric studies frequently use a two-dimensional (2D) landmark configuration to quantify form, even though the structure of interest is typically three-dimensional (3D) (Roth, 1993; Cardini, 2014; Klingenberg, 2015). While the 3D
approach is becoming more accessible, for many it is still a more expensive and time-consuming process that requires large data storage facilities. The 2D approach has several advantages over 3D, in particular the ease of capturing 2D coordinates by placing points on a photograph using freely available, user-friendly software such as tpsDig (Rohlf, 2010a), and the intuitively understood visualizations of shape variation produced using the thin-plate spline (Klingenberg, 2013). It is also much faster and so it is easier to obtain much larger sample sizes to quantify variation. The obvious disadvantage of a 2D approach is the loss of information regarding depth, and both correlational and comparative studies of 2D vs. 3D datasets suggest that including the third coordinate may lead to different results for some biological structures (Cardini & Thorington, 2006; Álvarez & Perez, 2013; Cardini, 2014). Despite the fact that 2D morphometric analyses of 3D structures mean the loss of tridimensional information, the relative increased costs, time and difficulty associated with 3D data collection, storage and analysis still make 2D geometric morphometrics a realistic, inexpensive, and time-effective choice for many researchers (Cardini, 2014; and references therein).

The vertebrate head is a complex structure that has received much attention in morphometric studies, perhaps due to the enormous disparity in form that has evolved among groups. At the broadest scale, the skull is comprised of three parts: the cranial vault, the cranial base, and face (de Beer, 1937). Although these different parts originate in embryologically distinct regions, they apparently grow in a morphologically integrated manner through numerous developmental and functional interactions (Lieberman, Ross & Ravosa, 2000; Bookstein et al., 2003; Sanger et al., 2012). This has implications for 2D geometric morphometric analyses of head shape diversification regarding which observation view (or combination of views) is most informative for any vertebrate group. An observation view is the orientation of the specimen in relationship to observer, and the view(s) chosen by a researcher may influence the nature of the morphometric data yielded from a given specimen. This situation is problematic as the numerous structural subunits of the head observable from different views may be semi-independent in ontogeny or function, and are thus expected to vary in conservative properties and respond differently to ecological and evolutionary factors (Klingenberg, 1996; Lu et al., 2014). It is not understood how profoundly 2D shape analyses of the vertebrate head are influenced by observation view(s) and the landmark configuration used to characterize them.

Lizards are ideal for such examination, as they have a remarkable diversity in cranial form that is tightly linked to the functional and constructional demands of the skull, and show a unique evolutionary trend toward increased fenestration and reduced cranial ossification (Evans, 2003; Stayton, 2005; Herrel et al., 2007; Daza et al., 2009). The ventral morphology of lizard crania, formed by parts of the dermatocranium and neurocranium, is directly involved in the mechanics of feeding, affecting bite force through connections to the jaw musculature (Herrel et al., 2007). Jaw musculature is also associated with structures most clearly observed from a lateral view (e.g. the postorbital-postfrontal, quadrate, coronoid and jugal). Consequently, much shape variation observed in lateral view is strongly linked with diet, for example herbivorous lizards tend to have short snouts and high skulls, which is consistent with a slow but powerful bite relative to carnivorous lizards (Evans, 2003; Stayton, 2005). Palate morphology and the septomaxilla also reflect the evolution of the chemosensory system in general, and the vomeronasal system in particular (Rieppel, Gauthier & Maisano, 2008), and the skull roofing bones are greatly influenced by the development of the brain and other sensory organs. The most highly fenestrated cranial morphology belongs to the monitor lizards (family: Varanidae), and is known as a ‘space frame’ type of construction in engineering terms (McCurry et al., 2015). The monitor cranium has a noteworthy mixture of extremely reduced bones (such as those forming the skull roof, particularly the snout region), derived bones (such as the palpebral and dermal palate elements), and composite bones (such as the fused postorbitofrontal) (Belairs, 1949; Frazzetta, 1962; Auffenberg, 1988; Rieppel et al., 2008; Werneburg, Polachowski & Hutchinson, 2015). Further, 2D geometric morphometric analysis of lateral cranial shape among lizard families reveals monitors have conspicuous patterns of interspecific cranial shape disparity (Stayton, 2005).

Our study examines how observation view and landmark configuration design influence 2D geometric morphometrics analysis of interspecific cranial shape variation in monitor lizards. Specifically, we quantify the covariation of species cranial shapes with phylogeny and allometry, and characterize major patterns of interspecific cranial shape variation and size-corrected diversification in morphospace. We then identify and evaluate differences among 2D views to determine the utility of combining multiple views, and to assess whether choosing different views for landmark-based morphometric analyses could influence our quantification of shape differences among specimens or species, providing complementary morphological information to each other. We expect congruence among views for
quantification of shape covariation with phylogeny to reflect developmental constraints within the cranium. A pervasive influence of allometry on shape is predicted, and should be exemplified in monitor lizard morphology, as species differ in body mass over four orders of magnitude (Pianka, 1995). However, we predict the anatomical points sampled to reflect functionally different characters among views, and therefore to recover discrepant patterns of interspecific cranial shape variation and size-corrected diversification.

MATERIAL AND METHODS
MORPHOMETRIC DATA

Our morphometric dataset comprises images of the cranium for 29 Varanus species (N = 152 specimens) in lateral, dorsal and ventral views (Supporting Information, Table S1). We only used cranial material of adult specimens (skeletal maturity judged by specimen size) and we did not know the sex of the specimens. Within a species, male and female varanids have the same shaped heads but subtle sexual dimorphism in head size has been detected in some of the larger monitor species (Frýdlová et al., 2011). Not knowing the sex in our study is unlikely to influence our results because we correct for head size and our primary interest is in interspecific shape variation rather than intraspecific size variation. A majority of the dataset is photographs of preserved crania, obtained from collections in the American Museum of Natural History (AMNH), the Florida Museum of Natural History (FLMNH), the Field Museum of Natural History (FMNH), the National Museum of Victoria (NMV), the University of Michigan Museum of Zoology (UMMZ) and the Smithsonian Institution National Museum of Natural History (USNM). Photographs were taken with a mounted and levelled Olympus C-765 Ultra Zoom. Skulls were situated against a dark background with a scale bar in the same plane. The lateral view involved positioning the crania so the camera was perpendicular to the midsagittal plane. The dorsal view involved placing the cranium ventral side down with the teeth and palate against the surface. The ventral view involved positioning the skull so the palate was parallel to the camera lens. The dataset was supplemented with 2D images obtained from micro X-ray computed tomography (CT) scans of crania from whole, alcohol-preserved specimens from the Western Australian Museum (WAM) (11 species). All scans were made with the Xradia microCT ×400 system at the Australian Microscopy and Microanalysis Research Facility (University of Sydney). CT scan data were rendered using Drishti v2.0 (http://anuf.anu.edu.au/)

Figure 1. Phylogenetic relationships of the 29 Varanus species included in this study. The tree is primarily based on the phylogeny of Vidal et al. (2012), with additional information from Welton et al. (2014) (see text for details).

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ND4) and nuclear (brain-derived neurotrophic factor, bone morphogenetic protein two, and neurotrophin three) sequence data and constrained the major clades so that the resultant phylogeny would be consistent with the phylogeny presented in Vidal et al. (2012). The resulting phylogeny was used for visualizing shape diversification across Varanus, identifying shape differences among different groups in a phylogenetic context, and to test for phylogenetic signal.

2D GEOMETRIC MORPHOMETRICS

We used a 2D geometric morphometric approach for all views, also using a 2D sliding semilandmark procedure for the ventral view (Bookstein, 1997). Landmarks and semilandmarks were collected using tpsDig v. 2.16 (Rohlf, 2010a) (Fig. 2; Supporting Information, Table S2). The semilandmarks were collected as a curve outlining the fused pre-maxillae and paired maxillae in ventral view (Fig. 2). These data were subsequently reduced to equidistant landmarks (four on the pre-maxillae and ten on each maxilla), and subsequently defined as semilandmarks using the software tpsUtil v. 1.46 (Rohlf, 2010b). We then slid the landmarks using the bending energy method (Bookstein, 1997; Gunz & Mitteroecker, 2013) implemented in the R statistics package geomorph (Adams & Otárola-Castillo, 2013). The original landmark coordinate data were aligned using a generalized Procrustes superimposition analysis (GPA), providing the centroid size and shape coordinates (Adams et al., 2013). The monitor cranium has matching symmetry in lateral view and object symmetry in dorsal and ventral views. Although shape analysis procedures for both types of symmetry can separate the symmetric (left-right averages) component of variation from left-right asymmetries within individuals (Klingenberg, Barluenga & Meyer, 2002), damage to specimens in lateral view meant we could only digitize landmarks on one side of the cranium. Shape data therefore refer to Procrustes coordinates in lateral view, and the symmetric component of shape variation in dorsal and ventral views. We then calculated the mean of the Procrustes coordinates and centroid sizes of each species, in order to generate phylomorphospace plots and allow phylogenetic comparative analyses on the shape data.

Cranium size and shape variation

Ignoring the phylogeny in preliminary transformations of species data results in substantially elevated variance and type I error (false positive) in statistical estimators (Revell, 2009). To quantify the cranial shape variation evolutionarily associated with allometry in the monitor lizard cranium, we performed a Procrustes regression of shape on size, based on the Procrustes coordinates averaged by species, using the software geomorph (Adams & Otárola-Castillo, 2013). We also used the residuals from the averaged Procrustes coordinates and centroid sizes in order to perform a phylogenetic regression of shape on size in a phylogenetic context, in order to get ‘evolutionary allometry’-corrected shape data, also using geomorph (Adams & Otárola-Castillo, 2013). Analyzing the residuals from a regression of the size-dependent trait against size is a common method of correcting for allometry in evolutionary studies (Gould, 1966; Monteiro, 1999; Revell, 2009).

Phylogenetic signal

To quantify phylogenetic signal in Varanus crania for each of the views (dorsal, ventral, and lateral), we used the $K_{mult}$ method, which is Blomberg’s $K$ statistic generalization appropriate for high-dimensional and/or multivariate data (Adams, 2014), using the R...
package \textit{geomorph} (Adams & Otárola-Castillo, 2013). We determined the statistical significance of $K_{\text{mult}}$ using phylogenetic permutation with 10,000 iterations, which is calculated by permuting the shape data of the Procrustes-aligned specimens among all tips of the phylogenetic tree. We also simulated the confidence intervals of $K_{\text{mult}}$ under a Brownian Motion (BM) model of evolution using \textit{phytools} (Revell, 2012), with 10,000 iterations. Under BM, $K_{\text{mult}}$ has an approximate expected value of 1.0, and the higher the $K_{\text{mult}}$ value is, the stronger the phylogenetic signal, indicating higher morphological variance among clades instead of within clades (Adams, 2014).

\section*{Patterns of Cranial Shape Variation and Diversification}

To examine patterns of cranial shape variation among \textit{Varanus}, we subjected the averaged shape coordinates to a Principal Component Analysis (PCA), both before and after size correction, for all three views. To characterize the evolutionary patterns of cranial shape diversity, we projected the phylogeny into the shape tangent space and projected it on plots species mean size-corrected cranial shapes. This approach maps principal component (PC) scores of the species cranial shapes to the phylogeny, computes the PC scores at internal nodes, and subsequently projects the branches of the phylogenetic tree onto the morphospace. Evaluating this 'phylomorphospace' provides insights into the history of morphological diversification for complex traits and allows a multivariate visualization of shape differences between species (Sidlauskas, 2008; Sherratt \textit{et al.}, 2014). To quantify the amount of shape variation and dispersion between original and size-corrected shape data, we measured the dispersion of all the individuals at three levels: (1) for the whole data set – by calculating the dispersion around the mean shape for all the specimens, (2) by species, and (3) by the phylogenetic groups identified in Vidal \textit{et al.} (2012). Finally, we tested the integration between the three views, in order to assess the strength of dependency among each landmark configuration, with \textit{geomorph} (Adams & Otárola-Castillo, 2013).

\section*{Results}

\textbf{Shape Variation Among \textit{Varanus} Crania}

In dorsal view, original shape variation captured by the two-first PCs relate to cranium width, relative length of the snout and parietal units, and angularity of the frontoparietal suture and nasal-frontal boundary (Figs 3, 4). PC 1 (accounting for 54.9\% of the overall morphological variance) (Table 1) contrasts crania with a shortened snout complex and widened parietal unit in the negative direction, and crania with a lengthened snout complex and narrowed parietal unit in the positive direction. PC 2 (19.0\% variance) contrasts narrow crania with a relatively short snout in the negative direction, and...
Figure 4. Cranial shape diversification among Varanus, in (A) dorsal, (B) ventral, and (C) lateral views, for original shape data. Phylogenetic tree is superimposed onto a plot of the first two PCs of the covariance matrix based on residuals computed using the regression vector of independent contrasts. Tips of terminal branches are at the locations of species means, and are coloured according to phylogenetic group.
Table 1. Cranial shape variation in the three observation views captured in PC 1–5, both for the original and size-corrected shape data

<table>
<thead>
<tr>
<th>Axis</th>
<th>Dorsal</th>
<th>Ventral</th>
<th>Lateral</th>
<th>Dorsal</th>
<th>Ventral</th>
<th>Lateral</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC 1</td>
<td>54.939</td>
<td>47.742</td>
<td>31.077</td>
<td>15.242</td>
<td>21.246</td>
<td>14.443</td>
</tr>
<tr>
<td>PC 4</td>
<td>5.094</td>
<td>5.803</td>
<td>6.781</td>
<td>7.330</td>
<td>6.924</td>
<td>6.810</td>
</tr>
<tr>
<td>Cumulative (PC1–5)</td>
<td>89.133</td>
<td>88.811</td>
<td>79.248</td>
<td>50.252</td>
<td>54.152</td>
<td>47.320</td>
</tr>
</tbody>
</table>

The tabled values are the percentages of total variance (%) for which each principal component accounts.

broad crania with a relatively long snout in the positive direction.

In ventral view, over 70% of the cranial shape variation among monitors relates to cranium width, curvature of the snout, robusticity of the palatine and ectopterygoid, and length of the pterygoid (Figs 3, 4). For PC 1 (47.7% variance), a negative change from the mean describes crania with a short and broad snout, distally shifted and broadened suborbital bones, and a broad and long pterygoid. A positive change from the mean describes crania with an elongate and narrow snout, mesially shifted and narrow suborbital region, and a shortened pterygoid. PC 2 (24.7%) captures relative shifts in landmarks altering pointedness of the snout, width of the post-ital roof in the negative direction, and crania with a ventrally projected pterygoid, and steeply rising cranial roof in the positive direction.

In lateral view, shape variation captured by PC 1–2 relates to overall cranium height, length and angularity of the snout complex, convex–concave bending and length of the basal units, and robustness of the parietal unit (Figs 3, 4). For PC 1 (31.1%), a negative change from the mean shape describes relative shifts in landmarks that flatten the cranium, lengthen the snout complex, and both shorten and project the parietal and basal units mesially. A positive change from the mean shape corresponds to a heightened cranium with a short but steeply rising snout complex, and enlarged parietal and basal units. PC 2 (22.5%) describes more subtle shape changes, contrasting crania with a lengthened snout complex, ventrally projected pterygoid, and steeply rising cranial roof in the negative direction, and crania with a shortened snout complex, mesially projected elongate pterygoid, and flattened cranial roof in the positive direction.

**Phylogenetic signal**

The results of the multivariate $K$ statistic calculated on the shape data are significant for each view of the Varanus cranium (dorsal: $K_{\text{mult}} = 0.99$, $P < 0.001$; ventral: $K_{\text{mult}} = 0.85$; $P < 0.001$; lateral: $K_{\text{mult}} = 0.83$, $P < 0.001$). $K$ 95% confidence interval for values expected under a BM model of trait evolution $= [0.658, 1.612]$.

**Cranial size and shape variation**

The multivariate Procrustes regressions of shape data on centroid size were statistically significant ($P < 0.0001$) in dorsal, ventral and lateral view, indicating the presence of allometry in the Varanus cranium. In the quantification of evolutionary allometry (when phylogenetic relatedness is incorporated in the regression), the $r^2$ values decrease substantially but remain significant for all three views (Table 2). The dorsal view recovers the largest $r^2$ values, followed by lateral view then ventral view, suggesting that shape variation in the monitor lizard cranium is most heavily constrained by size in the skull roof. The amount of shape variation accounted for by PC 1–5 is substantially less after evolutionary allometry is removed from average species cranial shapes (Table 1). The amount of dispersion and shape variation was very similar between original and size-corrected shape data for each of the views, for all three levels (all individuals, by species, and by phylogenetic groups; Table 3). The two-block partial least squares (PLS) between different views pointed out very high integration between dorsal and lateral ($r_{\text{PLS}} = 0.943$, $P < 0.001$) and between ventral and lateral landmark configurations ($r_{\text{PLS}} = 0.921$, $P < 0.001$), and moderately high between dorsal and ventral views ($r_{\text{PLS}} = 0.878$, $P < 0.001$).

**Size-corrected shape diversification in Varanus crania**

Superimposing the phylogeny onto a plot defined by PC 1–2 of evolutionary allometry-corrected shape variation shows cranial shape diversification across
species, depicting their great morphological differences (Supporting Information, Fig. S1). There is a moderate clustering of size-corrected species cranial shapes according to phylogenetic group in ‘phylomorphospace’ for all three observation views. These clusters are most clearly separated from each other in dorsal view. The major difference in diversification patterns among views is attributed to the African species, which occupy a positive position on PC 2 in dorsal view, but a negative PC 2 position in ventral and lateral views. Cranial shapes of species in the Indo-Australian C phylogenetic group show the greatest divergence from all other species cranial shapes, occupying a distinct region of each phylomorphospace. Cranial shapes of individual species from all other phylogenetic groups have diversified varying amounts in each view, as shown by branches that traverse a large proportion of the region occupied by the whole sample. These include the African species Varanus exanthematicus and Varanus albigularis, the Indo-Asian species Varanus olivaceus and Varanus rudicollis, and the Indo-Australian species Varanus komodoensis and Varanus giganteus.

### DISCUSSION

The highly fenestrated space frame construction of monitor lizard crania creates clear differences in abundance and spread of candidate landmarks among alternative views of the structure. It follows that the design of each 2D landmark configuration inevitably describes shape in a slightly different way. Phylogeny underpins patterns of gross morphological variation among monitor lizard crania, even when diluted by size-related constraints and presumably adaptive trends, which might be also phylogenetically constrained. The strength of association between cranial size and shape differs among observation angles; however, it is unclear whether these results reflect genuine evolutionary trends or slight differences on the morphological information captured by each landmark configuration design. The patterns of cranial shape variation are broadly concordant among views, suggesting dietary variation has contributed to shape diversification across the entire cranium at a higher phylogenetic level. Below, we evaluate 2D characterization of the monitor cranium, present an overview of cranial shape evolution in monitors, and discuss more broadly how 2D geometric morphometric analyses of vertebrate head shape can be improved through analysis of multiple observation views and careful landmark configuration design.

### THE EVOLUTION OF CRANIAL MORPHOLOGY IN MONITOR LIZARDS

A careful consideration of landmark configuration design is required when interpreting geometric morphometric results. Our landmark configurations capture a dorsal-ventral ‘silhouette’ in lateral view, the medial paired and fused bones in dorsal view, and palatoptygoid morphology in ventral view (Fig. 2). A 2D characterization of monitor lizard cranial morphology is most comprehensive in ventral view, as abundant type I and type II landmarks (Bookstein, 1991) are reliably digitized, and four curves are traced to outline the fused pre-maxillae and paired maxillae. We find the lateral view to be the least reliable, because of the difficulty in accurately orienting specimens when taking photographs (Klingenberg, 2015). The requirement of 2D landmarks to be coplanar (Zelditch et al., 2012) is most limiting for landmark configuration design in lateral and dorsal view, and is the key reason why semilandmarks outlining the pre-maxillae and maxillae were only added in ventral view. There are also morphological differences clearly observable among species for numerous bones (such as the palpebral); however, such variation should not be characterized using geometric morphometric methods (Zelditch et al., 2004).

The major monitor lizard clades match biogeographic distribution well, dividing the African, Indo-Asian, and Indo-Australian species (Fig. 1). An Asian origin for crown Varanus is likely, with major dispersal events occurring to Africa ~41 Mya, and to Australia ~32 Mya (Vidal et al., 2012). Significant phylogenetic signal is found across observation angles, and interspecific cranial shape variation still retains substantial phylogenetic structure after correcting for evolutionary allometry; Figure 4 shows that cranial

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**Table 2.** Summary table of multivariate Procrustes regressions and phylogenetic regressions of shape data on centroid size, for each of the three views, using `geomorph` (Adams & Otárola-Castillo, 2013)

<table>
<thead>
<tr>
<th></th>
<th>Dorsal</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>$p$</td>
<td>$r^2$</td>
<td>$p$</td>
<td>$r^2$</td>
</tr>
<tr>
<td>Regression</td>
<td>0.3052</td>
<td>&lt; 0.0001</td>
<td>0.1666</td>
<td>&lt; 0.0001</td>
<td>0.2294</td>
</tr>
<tr>
<td>Phylogenetic regression</td>
<td>0.2072</td>
<td>&lt; 0.0001</td>
<td>0.1121</td>
<td>&lt; 0.0001</td>
<td>0.1367</td>
</tr>
</tbody>
</table>
shapes of closely related species form moderate clusters in phylomorphospace. The importance of phylogenetic affinity for explaining interspecific variation is recovered in geometric morphometric analyses of lateral, dorsal and/or ventral cranial shape in other lizard studies (Stayton, 2005; Daza et al., 2009; Openshaw & Keogh, 2014). Such clear phylogenetic structure in the morphometric data across observation angles may indicate a role for developmental constraints in diversification of monitor lizard cranial shape.

Table 3. Summary of Procrustes variances, accounting for the morphological disparity of all the samples for the whole data set, grouped by species (dispersion calculated within species), and grouped by regions (dispersion within each geographical region)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Original shape data</th>
<th>Size-corrected shape data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dorsal</td>
<td>Ventral</td>
</tr>
<tr>
<td>All specimens</td>
<td>0.0054</td>
<td>0.0057</td>
</tr>
<tr>
<td>Species</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Varanus acanthurus</td>
<td>0.0088</td>
<td>0.0062</td>
</tr>
<tr>
<td>Varanus albigularis</td>
<td>0.0080</td>
<td>0.0066</td>
</tr>
<tr>
<td>Varanus bengalensis</td>
<td>0.0053</td>
<td>0.0041</td>
</tr>
<tr>
<td>Varanus brevicauda</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Varanus bushi</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Varanus caudolineatus</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Varanus dumerilii</td>
<td>0.0066</td>
<td>0.0032</td>
</tr>
<tr>
<td>Varanus emeiensis</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Varanus exanthematicus</td>
<td>0.0085</td>
<td>0.0125</td>
</tr>
<tr>
<td>Varanus giganteus</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Varanus gilieni</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Varanus glauerti</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Varanus gouldii</td>
<td>0.0028</td>
<td>0.0039</td>
</tr>
<tr>
<td>Varanus griseus</td>
<td>0.0028</td>
<td>0.0034</td>
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<tr>
<td>Varanus indicus</td>
<td>0.0021</td>
<td>0.0020</td>
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<tr>
<td>Varanus homodoensis</td>
<td>0.0094</td>
<td>0.0098</td>
</tr>
<tr>
<td>Varanus mertensi</td>
<td>0.0014</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>VaranusMitchelli</td>
<td>0.0024</td>
<td>0.0021</td>
</tr>
<tr>
<td>Varanus niloticus</td>
<td>0.0022</td>
<td>0.0035</td>
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<tr>
<td>Varanus olivaceus</td>
<td>0.0042</td>
<td>0.0030</td>
</tr>
<tr>
<td>Varanus prasinus</td>
<td>0.0041</td>
<td>0.0035</td>
</tr>
<tr>
<td>Varanus rosenbergi</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Varanus rudicollis</td>
<td>0.0183</td>
<td>0.0146</td>
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<tr>
<td>Varanus salvadorii</td>
<td>0.0055</td>
<td>0.0068</td>
</tr>
<tr>
<td>Varanus salvator</td>
<td>0.0048</td>
<td>0.0042</td>
</tr>
<tr>
<td>Varanus scalaris</td>
<td>0.0056</td>
<td>0.0036</td>
</tr>
<tr>
<td>Varanusstorri</td>
<td>0.0127</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Varanus tristis</td>
<td>0.0041</td>
<td>0.0050</td>
</tr>
<tr>
<td>Varanus varius</td>
<td>0.0034</td>
<td>0.0027</td>
</tr>
<tr>
<td>Region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>African</td>
<td>0.0043</td>
<td>0.0056</td>
</tr>
<tr>
<td>Indo-Asian A</td>
<td>0.0062</td>
<td>0.0055</td>
</tr>
<tr>
<td>Indo-Asian B</td>
<td>0.0038</td>
<td>0.0030</td>
</tr>
<tr>
<td>Indo-Australian A</td>
<td>0.0070</td>
<td>0.0076</td>
</tr>
<tr>
<td>Indo-Australian B</td>
<td>0.0029</td>
<td>0.0045</td>
</tr>
<tr>
<td>Indo-Australian C</td>
<td>0.0071</td>
<td>0.0078</td>
</tr>
</tbody>
</table>

Procrustes variances were calculated for both the original morphological generalised superimposition Procrustes analysis (GPA)-aligned morphological dataset, and the size-corrected one (accounting for allometry on the GPA-aligned dataset). For each analysis, we performed 1000 permutations, using the R package geomorph (Adams & Otárola-Castillo, 2013).
Monitors occupy a wide range of environments across their distribution, and the selective demands imposed by habitat have driven the evolution of extreme body size disparity in the genus (with extant species ranging in total body length as adults from 23 cm to 3 m) (Collar, Schulte & Losos, 2011). Strong size–shape relationships are known for monitor lizard body and head morphology (Christian & Garland, 1996; Thompson & Withers, 1997; Collar et al., 2011; Openshaw & Keogh, 2014). Initially, we found strong and significant allometry in each view of the cranium, however controlling for non-independence of shape data due to shared ancestry reduced the estimation of evolutionary allometry in all views. This situation also reflects the strong phylogenetic signal in body size evolution for the group (Collar et al., 2011; Openshaw & Keogh, 2014). The inconsistency in strength of evolutionary allometry among views could reflect discrepancy in the way each observation view quantifies size: dorsal view recovers the strongest influence of evolutionary allometry ($r^2 = 0.2072$), and is described by a landmark configuration that effectively captures cranium length, but only covers the cranium width at the posterior-most edges of the cranial table (Fig. 2).

The three observation views share notable similarities in patterns of interspecific cranial shape variation (Fig. 3) readily linked with diversity in diet through particular influences on cranial performance and strength. First, the shape changes captured by the primary axis of shape variation (PC 1) reveals two cranial phenotypes: (1) relatively tall and broad crania with a short and steeply rising snout; and (2) relatively flat and gracile crania with an elongate, slender snout. Monitor lizard crania with greater widths and heights perform better at biting, pulling and shaking (as exemplified by V. komodoensis) (Moreno et al., 2008; D’Amore et al., 2011). The most extreme examples of this morphotype belong to durophagous species like V. exanthematicus, which are characteristically short and wide with a tall snout (McCurry et al., 2015). These traits are often coupled with deep mandibles and robust dentition (Rieppel & Lambhardt, 1979; D’Amore, 2015; McCurry et al., 2015). The gracile morphotype is most clearly observed in 2D in V. giganteus. The elongate cranial shape likely reflects a diet consisting of large prey and carrion (McCurry et al., 2015). Second, there are finer scale shape differences that are expected to be important in determining the strength of a space frame construction. For example, a greater ventral projection of the palatopterygoid articulation to form an obtuse angle, and a more vertical positioning of the epipterygoid increases structural support, and is observed in semi-frugivorous species feeding like V. olivaceous (Auffenberg, 1988). We also recover variation in angularity of the frontoparietal suture or mesokinetic joint. Cranial sutures are forms of articulation in which the bones are rigidly joined by fibrous tissue (synarthroses) (Di Ieva et al., 2014) and are expected to have important roles in cranial kinesis and reducing stresses during biting feeding (Moazen et al., 2009). The concordance among observation angles and links with diet suggests feeding performance imposes selective pressure across each view, resulting in high integration between the different views, and to certain degree among the cranial subunits described by each landmark configuration (Harmon et al., 2005; Perez, Bernal & Gonzalez, 2006; Klingenberg, 2008; Meloro et al., 2011).

Our analysis of dorsal, ventral and lateral cranium shape in monitors leads to subtle differences in interpretation of morphological diversification of the cranium as a whole. Given the dorsal landmark configuration describes fused and paired bones, we expect to recover a more conserved pattern of morphological diversification. Increased conservatism is evident through the strong phylogenetic signal, the greatest size–shape constraint among views, and a more clear separation of phylogenetic groups in phylomorphospace. The interplay of lateral and ventral views is apparent, further supporting a role for some developmental constraints in morphological diversification of the monitor cranium, but also highlighting the role of diet.

**IMPLICATIONS FOR 2D LANDMARK CONFIGURATION DESIGN**

Geometric morphometric methods have been widely applied in studies of head shape diversification for numerous vertebrate groups (Slice, 2007). External head morphology is primarily landmarked using the facial features in primates, and the scales of lizards, snakes and fish (Kalontzopoulou, 2011; Kerschbaumer & Sturmbauer, 2011; Baab, McNulty & Rohlf, 2012). The skull is frequently broken down into its constituent parts (including dentition), depending on the question of interest, and described by landmarks at the contacts between bones, tips of processes, origins and attachments of muscles, locations of joints and tips of lever systems (Slice, 2007). Although landmarks are frequently collected as surface marks on one tissue (i.e. scales or bone), they may relate to different tissues whose relationships vary with evolution, development, and growth (Slice, 2007; Oxnard & O’Higgins, 2009). For example, 2D landmark configurations on the surface of bone have been utilized in marmot skulls for all three cranial observation views, and the mandible. The ventral cranium seems to be the best morphological feature.
for recovering phylogenetic relationships in varanids, and it seems that the largest shape modifications occur in regions directly involved in the mechanics of mastication in several other groups (Cardini & O’Higgins, 2004; Caumul & Polly, 2005; Cardini & Thorington, 2006). The lateral cranium and mandible are least strongly correlated with phylogeny, and dorsal and lateral cranium morphology together reflects brain size and dermal bones (Cardini, Hoffmann & Thorington, 2005; Caumul & Polly, 2005).

The efficiency of geometric morphometrics methods to provide a detailed and accurate quantitative description of form ultimately increases with the amount of information contained within a landmark configuration (Cardini & Thorington, 2006). Outline-based geometric morphometric techniques are therefore a powerful addition to a 2D landmark configuration, as they can capture structural information on large areas of morphology only represented by surfaces, curves or outlines (Oxnard, 1978; Roth, 1993; Adams et al., 2004; Perez et al., 2006; Webster & Sheets, 2010). The outline-based approach is less widely applied than the landmark-based approach, having mainly been utilized in anthropological studies (Slice, 2007; Baab et al., 2012). However, three limitations of outline-based methods should be considered: (1) protrusions on one individual that do not appear on another can be poorly captured by the resulting semilandmark alignment; (2) it is often not clear how many semilandmarks should be used for any given outline; and (3) outline methods only define relative features (MacLeod, 1999; McCane, 2013; Finlay & Cooper, 2015). Regardless, their utility is highlighted in studies like ours, in which the clarity of results is considerably improved in the resulting semilandmark alignment; (2) it is often not clear how many semilandmarks should be used for any given outline; and (3) outline methods only define relative features (MacLeod, 1999; McCane, 2013; Finlay & Cooper, 2015). Regardless, their utility is highlighted in studies like ours, in which the clarity of results is considerably improved in the landmark configuration(s) that utilize both landmarks and outlines (Baab et al., 2012; Finlay & Cooper, 2015). By increasing the number of coordinates along a homologous contour or surface, it is ultimately possible to obtain more information on the shape of a structure. The optimal 2D observation view for analyzing cranial shape variation may thus be the view in which both landmarks and semilandmarks may be utilized.

In conclusion, our study adds to a growing body of evidence suggesting multiple carefully designed 2D landmark configurations can provide fairly accurate descriptions of interspecific variation in complex structures like the vertebrate head (Cardini & Thorington, 2006; Perez et al., 2009; Álvarez & Perez, 2013; Cardini, 2014; Finlay & Cooper, 2015; Klingenberg, 2015). The key benefits of 3D techniques may instead be in the extensions to assessing morphological patterns in internal structures (e.g. brain case), functional morphology (finite element modelling and mechanical strain) and reconstruction of ancestral shapes (evolutionary warping or morphing) that they offer (Parr et al., 2012).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1.** Cranial shape diversification among *Varanus*, after correcting for allometry, in (A) dorsal, (B) ventral, and (C) lateral views.

**Table S1.** A list of the cranial material used in morphometric analysis.

**Table S2.** Definitions of two-dimensional landmarks and semilandmarks used in the dorsal, ventral and lateral view landmark configurations.