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Geometric morphometric analysis of shell shape variation in *Conus* (Gastropoda: Conidae)

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Geometric morphometric techniques allow for the direct quantification and analysis of variation in biological shape and have been used in studies in systematic biology. However, these techniques have not been used for species discrimination in the gastropod genus *Conus*, a major taxon of significant tropical reef predators recognized for their peptide-based toxins. Here, we used landmark digitization and analysis to show that five species commonly studied for their conotoxins – *Conus consors*, *Conus miles*, *Conus stercusmuscarum*, *Conus striatus*, and *Conus textile* – can be effectively distinguished from each other by their shape, as manifested in the results of a principal components analysis (PCA) and the generated thin-plate splines. Two piscivorous species, *C. stercusmuscarum* and *C. striatus*, show clear overlaps in the PCA plot, although each taxon clusters within itself, as does each of the others. The loadings on the first two principal components show that the forms of the shells' aperture and spire are particularly important for discrimination. Phylogenetic analysis using neighbour-joining methods shows that group separations are comparable with published phylogenetic schemes based on molecular data and feeding mode (i.e. piscivory, vermivory, molluscivory). The results of this study establish the utility of geometric morphometric methods in capturing the interspecific differences in shell form in the genus *Conus*. This may lead to the utilization of these methods on other gastropod taxa and the creation of species-recognition programs based on shell shape.

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ADDITIONAL KEYWORDS: landmarks – neighbor-joining – principal components analysis – thin-plate splines.

INTRODUCTION

Morphometrics, the quantitative study of variation of biological shapes and their covariation with other variables (Rohlf, 1990; Adams, Rohlf & Slice, 2004), has been utilized in a wide range of studies across various disciplines. In biology, morphometrics has allowed for unique insights into evolution and development (Roth & Mercer, 2000). Systematics, when based on morphology, often utilizes morphometric techniques (Rohlf, 1990). Being a product of an organism's ontogeny, its form reveals important developmental and ecological patterns that could eventually lead to the understanding of biological processes and causal agents (Roth & Mercer, 2000).

Geometric morphometrics allows for the direct quantification and analysis of variation in biological shape. Many studies concerning geometric morphometrics techniques have utilized landmark analysis, involving data collected as two- or three-dimensional coordinates of biologically important landmarks on

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specimens (Roth & Mercer, 2000; Adams *et al*., 2004). The techniques are useful for revealing the patterns of multivariate variation in shape (Roth & Mercer, 2000). They also eliminate isometric size variation (David & Laurin, 1996) but size as a factor is not completely eliminated, as allometry is still usually present.

Geometric morphometrics has proven important in evolutionary studies of such diverse groups as the cichlids of Lake Tanganyika in Eastern Africa (Clabaut *et al*., 2007; Maderbacher *et al*., 2008); Maderbacher *et al*. (2008) were able to establish that geometric morphometrics is more useful in discrimination amongst populations than traditional morphometrics. One issue often raised regarding the use of morphometrics in phylogenetic studies is that morphometrics cannot supply homologous shape characters (Rohlf, 1998); morphometric data are continuous whereas cladistic procedures are based on discrete characters (Adams *et al*., 2004). One protocol that Adams *et al*. (2004) have proposed to overcome this hurdle is to use the original shape data, such as partial warp scores, as discrete characters. This is what Fink & Zelditch (1995) did to determine the monophyly of the piranha genus *Pygocentrus*. More recently, Catalano, Goloboff & Giannini (2010) described a method for the direct use of aligned landmark data in phylogenetic analysis. The approach is based on finding, for each of the ancestral nodes, the landmark positions that minimize the displacement along the branches of the phylogenetic tree.

Gastropod shell form has been a common subject of morphometric analyses. As the shell undergoes accretionary growth or stepwise addition of shell material at its margins, it maintains its shape (Ackerly, 1989). The coiling axis, around which the aperture grows in spirals, is often a reference point for models of shell growth (Ackerly, 1989; Johnston, Tabachnick & Bookstein, 1991; Van Osselaer & Grosjean, 2000), although its location in shells can be difficult to determine (Van Osselaer & Grosjean, 2000). Accretionary growth provides a detailed and continuous growth history throughout a snail's ontogeny (Johnson *et al*., 1991). In the development of the snail, shape changes can be traced, as by protoconch–teleoconch boundaries. Van Osselaer & Grosjean (2000) recommended that the older protoconch be analysed separately from the newer teleoconch regions, taking all dimensions into account simultaneously. In line with this, much of the variability in shell metric characters lies in the fact that size factors change as the shell grows, particularly in the teleoconch (Tursch & Greifeneder, 2001). This is because as the organisms grow, shell shape in the teleoconch region changes. This has long justified the more reliable use of protoconch measurements, which do not change with age, in systematic studies (Yasunori & Tomoki, 2000; Rolan *et al*., 2004). As gastropod shells and their shapes can remain unchanged over long periods of time, particularly in the protoconch region, they have provided insights into the history of certain gastropod taxa (Wagner, 1995; Samadi, David & Jarne, 2000).

Size and shape measures have been particularly useful for the study of the shells of certain gastropod genera, such as *Oliva* and *Conus*, because they lack meristic (i.e. countable) characters like spines (Tursch, 1998). Studies by Tissot (1984, 1988) on *Cypraea*, Merkt & Ellison (1998) on *Littoraria angulifera* Lamarck 1822, and Cruz & Vallejo (2009) on *Oliva* spp. utilized linear measurements and simple ratios of these size measures to represent shape. Such an approach has not been useful for many gastropod taxa; geometric morphometrics based on landmark data can be more useful for the discrimination of groups. Carvajal-Rodríguez, Conde-Padín & Rolán-Alvarez (2005) determined utilizing landmark data that there were differences in form amongst sympatric ecotypes of *Littorina saxatilis* Olivi 1792. By assigning 12 landmarks on digitized images of the shells and using relative warp analysis (RWA), they were able to differentiate amongst the ecotypes, which traditional morphometric approaches had failed to do.

Geometric morphometrics can be used to assess interspecific variations in form, particularly for taxa whose systematics has proven challenging (Tursch, 1998; Cruz & Vallejo, 2009). There have been difficulties in determining appropriate landmarks, and the unique accretionary growth of shells has been an issue (Johnson *et al*., 1991; Stone, 1998), but morphometrics is still a reliable tool for determining shell shape variations. However, geometric morphometrics has not seen much use with gastropod shells (Carvajal-Rodríguez *et al*., 2005).

One of the taxonomically problematic taxa is *Conus*, a large genus of gastropods that has been well preserved in fossil records since its first appearance about 55 Mya, in the Lower Eocene (Kohn, 2002). The nomenclature of the genus and its members has long been debated, with over 1000 species having been named but only slightly over 500 considered valid (Olivera *et al*., 1990). However, the genus is one of the most important amongst gastropods, both ecologically and medically. Cone snails are amongst the major predators in tropical reef communities (Leviten & Kohn, 1980; Olivera *et al*., 1990; Kohn, 2002). They inject venom to capture their prey through radular teeth modified into hypodermic needles unique to the genus (Kohn, 2002). This venom, which contains small peptides (conotoxins) targeted to neuromuscular receptors, has been studied extensively in the development of drugs (Carté, 1996; Livett, Gayler &

Khalil, 2004; Han *et al*., 2008); the first marine drug is Ziconotide, a potent calcium blocker that was developed based on knowledge of the peptides from the venom of *Conus magus* (Fenical, 2006). Conotoxins can be significant clade markers; they can be used to indicate common biological mechanisms of clades (Olivera, 2002). For instance, the κ A-conotoxins are found in all *Conus* species under Clade I (the piscivorous *Conus striatus* clade) but not in species belonging to any other clade, not even closely related Clade III (the piscivorous *Conus purpurascens* clade), with which Clade I otherwise shares predatory tactics and conserved conotoxins like the μ- and δ-conotoxins.

Cunha *et al*. (2005) mentioned that the issue of convergence hampers phylogenetic analysis of *Conus* based on morphology, but they cited only a 1992 study by Rolán that uses size but not shape factors. Shape of shell has been a major basis for the distinction between infrageneric groups of species within the taxon (Kohn & Riggs, 1975), and shell form is the likeliest candidate for critical evolutionary modifications in the genus (Kohn, 1990).

Biological shape is an important component of morphogenesis, a fundamental aspect of developmental biology. For an important yet problematic taxon like *Conus*, the elucidation of shape variation can lead to breakthroughs in the systematic studies on the genus with models and image recognition programs. The determination of evolutionary relationships within *Conus* based on morphology has not often met with success (Duda, Kohn & Palumbi, 2001).

This study therefore aimed to determine whether or not standard multivariate statistical methods applied to shape variables, as determined by landmark analysis, can be used to discriminate amongst several medically important species of the genus *Conus*; and to revisit and assess existing phylogenetic estimates for the genus and models on shell growth, and compare them based on the results of the analyses. It was also of interest to see if shape variations amongst the species reflect their differences in dietary habits.

MATERIAL AND METHODS

Samples

A total of 172 shells of adult specimens from five *Conus* species (Fig. 1A–E) was obtained from the laboratory collection of Professor Lourdes J. Cruz at the Marine Science Institute, Quezon City, Philippines. The species and corresponding sample sizes (N) are: the piscivorous *Conus consors* Sowerby II, 1833 ($N = 33$), *Conus stercusmuscarum* Linnaeus, 1758 ($N = 34$), and *Conus striatus* Linnaeus, 1758 ($N = 35$); the molluscivorous *Conus textile* Linnaeus, 1758 ($N = 35$); and the vermivorous *Conus miles* Linnaeus 1758 ($N = 35$). The species were selected from amongst those being studied in the laboratory of Professor Cruz based on the convenience of number of specimens available and their importance in conopeptide research. Although no conopeptides from any of these five species are being developed in clinical or preclinical trials (Livett *et al*., 2004; Fenical, 2006), the biochemical characteristics and activity of their conopeptides are being actively studied (West *et al*., 2002; Bulaj *et al*., 2005; Luo *et al*., 2007). Species identifications were confirmed by Professor Alan Kohn of the University of Washington. Prior to photographing and landmark digitization, the specimens were cleaned in a 50:50 bleach-water solution to remove the periostracum and other shell debris on the surface. Each shell specimen was subsequently

Figure 1. *Conus* species used in this study. A, *Conus consors* Sowerby ii, 1833; B, *Conus miles* Linnaeus, 1758; C, *Conus stercusmuscarum* Linnaeus, 1758; D, *Conus striatus* Linnaeus, 1758; E, *Conus textile* Linnaeus, 1758.

photographed with a mounted digital camera on both its aperture face (ventral) and dorsal face. The shells were orientated in such a way as to be able to show both the outer and inner edges of the apertural lip. Specimens with broken parts were not included in the analysis. Adams *et al*. (2004) have cited as a limitation of geometric morphometric methods the inability to account for missing areas on a biological form.

Shell landmarks

Landmarks were digitized on the specimens with tpsDig v. 1.40 (Rohlf, 2004). Figure 2 shows a specimen of *C. textile* with the 16 landmarks that were selected. By the definitions of Bookstein (1991), landmarks (LM) 1, 2, 3, 4, 5, and 6 are Type I landmarks, which are the most highly preferred, as they represent direct juxtapositions of tissues and may correspond to homologies. LM1 is the apex of the shell, whereas LM2–5 are upper and lower sutures of succeeding whorls. LM12, 13, 14, and 15 are duplications of LM5, 4, 3, and 2, respectively, on the left profile. The other landmarks are Type III and were designated based on overall form of aperture and the last major whorl. These points are not meant to capture homology but are used to capture the species' shell shape, particularly the aperture (LM7 and 8). Observation of the shapes of various *Conus* representatives suggests that it is mainly the shape of the last whorl and the aperture that distinguishes amongst species, with some having conical or ventricosely conical last whorls (*C. miles*), others being generally conoid-cylindrical (*C. consors*), and others still being ovate (*C. stercusmuscarum*, *C. striatus*, and *C. textile*). The coiling axis is not easily located here and in other gastropods because it is not discernible in real shells (Van Osselaer & Grosjean, 2000). The tpsDig program records the coordinates of the point selections made, which were subsequently used for data analysis. This program is freeware distributed at http://life.bio.sunysb.edu/morph.

Figure 2. Landmarks (LM) on a *Conus* specimen. LM1 – apex of the shell; LM2–5 – sutures between major whorls on right profile; LM6 – junction between end of suture and apertural lip; LM7 – outermost curve of aperture; LM8 – lowest point of aperture at base; LM9 – lowest point of last whorl at base; LM10 – most external point on left profile of last whorl; LM11 – shoulder on left profile, where last whorl curves; LM12 – point opposite to LM5 on left profile; LM13 – point opposite LM4 on left profile; LM14 – point opposite LM3 on left profile; LM15 – point opposite LM2 on left profile; LM16 – most external point on right profile of last whorl.

Analysis of the landmark data was performed using PAlaeontological STatistics (PAST) v. 1.90 (Hammer, Harper & Ryan, 2001), a free software available at http://folk.uio.no/ohammer/past. Procrustes transformation of the coordinates allows for the elimination of isometric size variation and orientation as factors and superimposes the specimens in a common coordinate system (Adams *et al*., 2004). A principal component analysis (PCA) of shape was performed to determine the linear combinations of variables that account for much of the variation in the data. Principal components 1 to 3 were run through a one-way multivariate analysis of variance (MANOVA) to determine if there are statistically significant differences amongst the species as grouped by diet (piscivorous, molluscivorous, vermivorous). Verification of the PCA results was performed by digitizing and analysing the landmarks of 42 specimens unidentified a priori and run through PCA with the previously identified specimens.

The scores on the first four principal components were then used in a canonical variate analysis (CVA) to determine how well these could be used to discriminate amongst the species. Thin-plate spline was used to help visualize shape variations as a deformation of configuration of landmarks from a reference configuration (Stone, 1998). This was accompanied by thin-plate spline grids and relative warp analysis, a PCA of principal warps.

A phylogenetic tree was generated from morphological distances (i.e. using landmark coordinates) to show the estimated phylogenetic relationships amongst the species. Using the PAST program, Euclidean distances were computed from the Procrustes-transformed landmark coordinates averaged for each species. These are distances in the space tangent to Kendall's shape space and are usually good approximations to the Procrustes distances in that space (Lockwood *et al.*, 2004). This was checked by computing a correlation between Procrustes distances and distances in the tangent space. A correlation of $r = 0.999999$ was obtained using the tpsSmall v. 1.20 software (Rohlf, 2003). The resulting distance matrix was subjected to the neighbour-joining method of Saitou & Nei (1987) through the PHYLogeny Inference Package (PHYLIP) v. 3.69 (Felsenstein, 2009). A distance-based method was used because shape variables represent continuous variation rather than discrete character states. The NEIGHBOR program of PHYLIP produces unrooted trees and assumes that shape differences amongst taxa provide phylogenetic information.

RESULTS

The PCA of the 32 coordinates of the 16 landmarks showed clear separations amongst the groups (*C. miles* is particularly well separated), although there are overlaps amongst others (most notably *C. stercusmuscarum* and *C. striatus*) (Fig. 3). Principal components 1 and 2 (PC1 and PC2, respectively) combine to account for 85.09% of the variation amongst the samples. Table 1 shows the loadings on the eigenvectors of the landmarks for the first two PCs, with the *x*- and *y*-coordinates corresponding to the particular orientation of the average shape used in this study. The first eigenvector has the largest positive loadings on the *x*-coordinates of LM7 and LM10 (width of main whorl and aperture) and the *y*-coordinate of LM12 (distance between shoulder and last suture); and the highest negative loadings on the *y*-coordinates of LM5 and LM6 (distance between last suture and apertural lip). The loadings for the second eigenvector are heaviest positively on the *x*-coordinates of LM7 and LM10, and negatively on the *x*-coordinate of LM8, which are all indicative of aperture width.

The MANOVA showed that there is a significant difference amongst centroids of PCs 1 to 3 across the species as grouped by diet (piscivorous, molluscivorous, vermivorous); the model is significant (Wilks' lambda = 0.048, $P < 0.0001$) for all three PCs taken as dependent variables. Contrasts matrices for the first two components show pairwise differences (Table 2). However, for PC1, the difference between piscivores and molluscivores is not statistically significant ($F = 1.08$, $P = 0.3000$). Figure 4 shows a plot of the PCA of the landmark coordinates but now separating the species in terms of their feeding groups. The groups are well separated, although *C. consors* does not group with its fellow piscivores when assessed by shape.

The phylogram in Figure 5 shows relationships amongst the taxa that are comparable with the PCA groupings and the cladistics in both Olivera (2002) and Duda & Kohn (2005); *C. stercusmuscarum* and *C. striatus* constitute a clade and *C. miles* is well separated from the others. The first separation is that of *C. consors* and *C. miles* from the three other species, amongst which the piscivorous *C. stercusmuscarum*

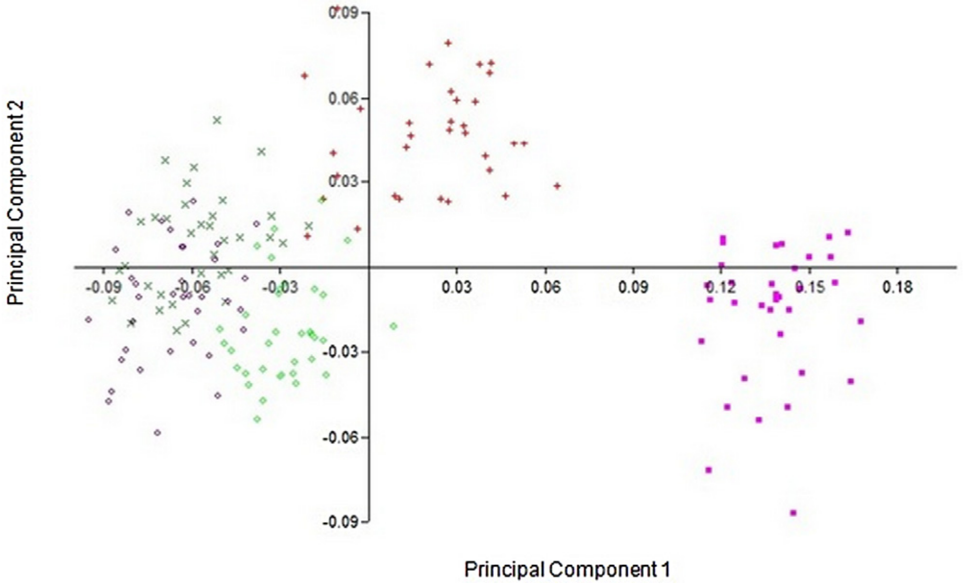

Figure 3. Plot of the results of the principal components analysis of the 32 coordinates of 16 landmarks on *Conus* specimens. +, *Conus consors*; ■, *Conus miles*; ×, *Conus stercusmuscarum*; ○, *Conus striatus*; ◇, *Conus textile*.

Table 1. Component loadings for principal component 1 (PC1) and PC2

Coordinates	PC1	PC2
X1	0.0094	–0.2391
Y1	–0.0148	0.0021
X2	–0.0685	–0.1670
Y2	–0.1538	0.0812
X3	–0.0910	–0.1438
Y3	–0.1780	0.0810
X4	–0.1156	–0.0941
Y4	–0.2202	0.0871
X5	–0.1339	–0.0139
Y5	–0.2551	0.0986
X6	–0.1766	0.1371
Y6	–0.2289	0.0380
X7	0.2881	0.6393
Y7	–0.1539	–0.0494
X8	0.1914	–0.2887
Y8	0.0995	0.0475
X9	0.1838	–0.2553
Y9	–0.0324	0.0942
X10	0.3202	0.2990
Y10	0.1352	–0.0300
X11	–0.1164	0.1980
Y11	0.2765	–0.1076
X12	–0.1293	0.0663
Y12	0.2981	–0.1038
X13	–0.0981	–0.0588
Y13	0.2502	–0.0892
X14	–0.0769	–0.1212
Y14	0.2082	–0.0878
X15	–0.0579	–0.1587
Y15	0.1693	–0.0863
X16	0.0712	0.2010
Y16	–0.1999	0.0243

X and Y refer to coordinates for each of the 16 landmarks. Eigenvalues are 0.0059 for PC1 and 0.0010 for PC2.

and *C. striatus* then form a cluster. There are thus marked morphometric differences amongst the species, particularly seen in *C. consors* and *C. miles*. As this is an unrooted tree, only relationships amongst the species can be described, without identifying common ancestry. The only inconsistency with the published schemes is that *C. textile* here is shown to be more closely related to *C. stercusmuscarum* and *C. striatus* than is *C. consors*.

For verification of the results of the PCA, 42 shells without a priori identification were subjected to the same methodology as the identified specimens. PC1, PC2, and PC3 cumulatively account for 88.37% of the variance. The resulting PCA plot of the landmark coordinates of both the original and the new specimens is shown in Figure 6. Later identification of the specimens determined that they come from at least seven *Conus* species, five of which are the original taxa of interest. As with the earlier results, the clustering of *C. miles* and *C. consors* is evident. Although there are overlaps amongst the remaining three species, the new specimens were found to cluster with their corresponding taxa. Six shells were identified as belonging to *Conus figulinus* Linnaeus, 1758, a relatively easy snail to recognize. The species is rather distant phylogenetically from *C. miles* as per Olivera (2002) and Duda & Kohn (2005), but it is, like *C. miles*, a vermivore. The best candidates for the identity of the unidentified vermivore species are *Conus capitaneus* Linnaeus, 1758 and *Conus mustelinus* Hwass in Bruguière, 1792, both of which belong to the same clade as *C. miles* (Olivera, 2002). *Conus capitaneus* is the sister taxon of *C. miles* in the phylogenetic scheme presented in Duda & Kohn (2005).

The loadings for this PCA are similar to those in the original PCA (see Table 1); PC1 is shown to have loadings being heaviest (negatively) for the x-coordinates of LM7 and LM10 and the y-coordinates of LM11 and LM12. Thus this component is a measure of main whorl and aperture width and location of the shoulder. In a manner also consistent with the original PCA, the loadings for the second eigenvector are heaviest positively on the x-coordinates of LM7 and LM10 and negatively on the x-coordinate of LM8, indicating a measure of the aperture. Aperture dimensions are thus appropriate for separating some of the important species, particularly vermivores from *Conus* with other diets.

CVA based on the first four PCs separated the groups in a similar way to the results of the PCA (Fig. 7). Canonical functions 1 and 2 (CF1 and CF2) together account for 96.1% of the variance. 86.6% of the original grouped cases are correctly classified, with *C. stercusmuscarum* and *C. striatus* being commonly misclassified. PC1 is most highly correlated with CF1 (Table 3).

Thin-plate splines show certain trends within and amongst groups (Fig. 8A–E). Within *C. consors* (Fig. 8A), certain shells show expansion of the spire whorls relative to the mean shape, whereas others show compression. The other groups exhibit higher intraspecific consistency. Distinct spire expansion characterizes *C. miles* (Fig. 8B), and in actual specimens this is represented by the characteristically lowered spire of the species. The last three taxa (Fig. 8C–E), which show degrees of overlap in the PCA, exhibit strong lateral expansion of the main whorl, most significantly in *C. stercusmuscarum* and *C. striatus*. Figure 9 shows the plot of relative warps 1 and 2 (RW1 and RW2), separating the groups in a similar fashion. RW1 and RW2 together explain 81.06% of the variation.

Table 2. Contrasts matrices of pair-wise comparisons in the multivariate analysis of variance of diet vs. principal components (PCs)

Contrast	df	Contrast SS	Mean square	*F* value	Pr > *F*
PC1					
1 vs. 2	1	0.77153993	0.77153993	647.23	< 0.0001
1 vs. 3	1	0.00128821	0.00128821	1.08	0.3000
2 vs. 3	1	0.47931710	0.47931710	402.09	< 0.0001
PC2					
1 vs. 2	1	0.02272340	0.02272340	29.72	< 0.0001
1 vs. 3	1	0.03313731	0.03313731	43.34	< 0.0001
2 vs. 3	1	0.00058032	0.00058032	0.76	0.3849

1, piscivore; 2, vermivore; 3, molluscivore; SS, sum of squares.

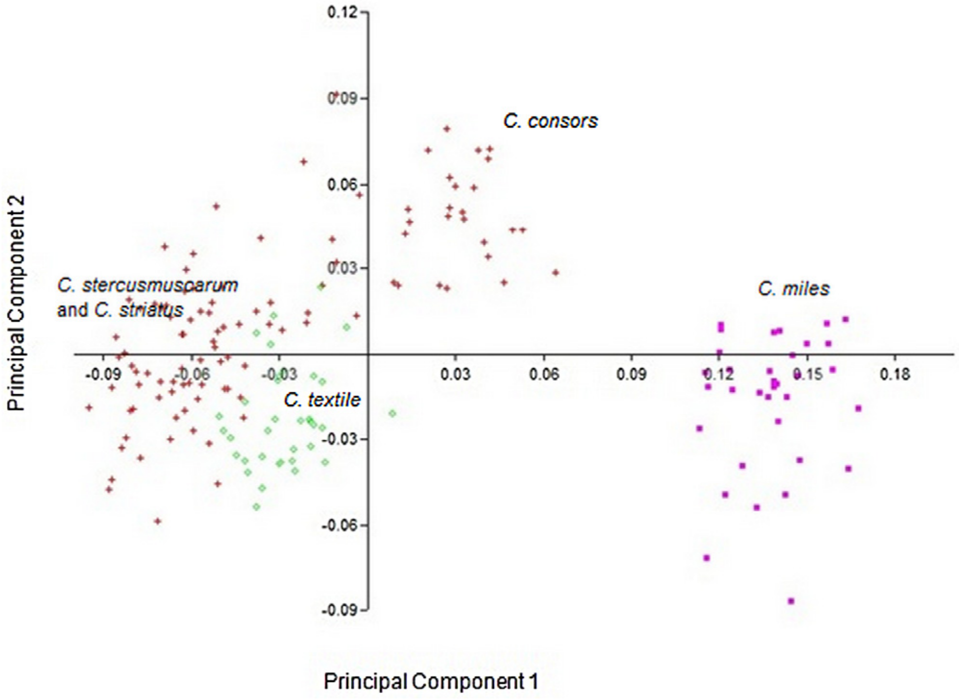

Figure 4. Plot of the results of the principal components analysis of the 32 coordinates of 16 landmarks on *Conus* specimens grouped by dietary requirements. +, piscivores; ■, vermivores; ◇, molluscivores.

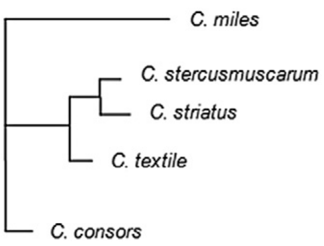

Figure 5. Phylogram depicting relationships amongst the five *Conus* species based on morphological distances (between landmark coordinates), generated by the NEIGHBOR program of PHYLIP 3.69.

DISCUSSION

The results of the PCA reflect and serve to quantify observable shape differences amongst some of the important *Conus* species. *Conus miles* is widely recognized as having a distinct shell; it has a conical or ventricosely conical last whorl. *Conus consors* has a generally conoid-cylindrical last whorl, whereas *C. stercusmuscarum*, *C. striatus*, and *C. textile* have last whorls that tend to be ovate in shape. The former two, which show the most overlap in the PCA, share a shoulder that is more sharply angulate and less rounded than *C. textile*. The loadings of the PCA

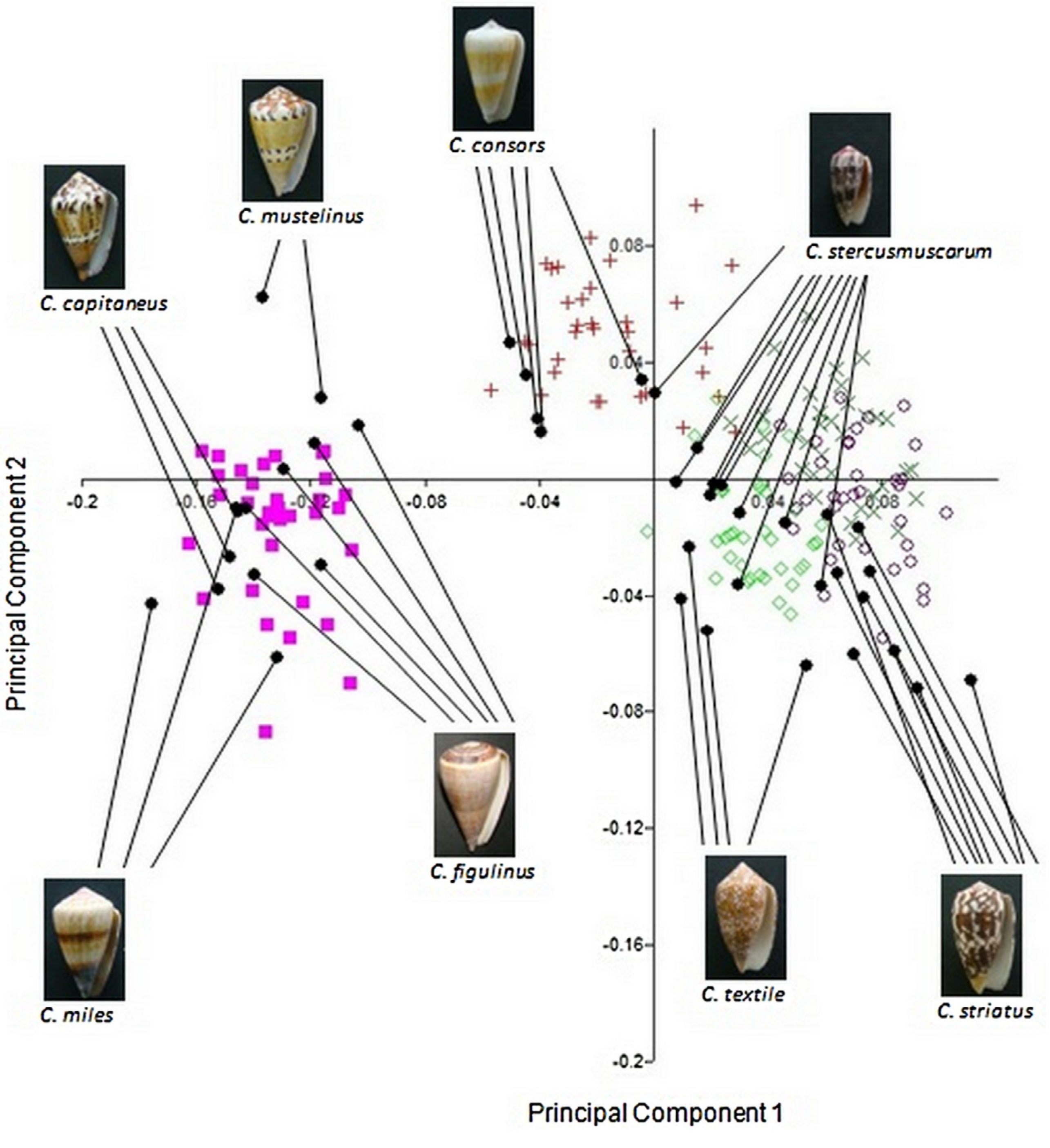

Figure 6. Plot of the results of the principal components analysis (PCA) with the identified *Conus* specimens and specimens unidentified a priori. Labels indicate identification of specimens after the PCA. +, *Conus consors*; ■, *Conus miles*; ×, *Conus stercusmuscarum*; ○, *Conus striatus*; ◇, *Conus textile*; ●, unidentified.

suggest that PC1 is primarily a measure of the width of the main whorl and the dimensions (both width and height) of the aperture and also suggestive of the artificial ratio circularity, which is aperture height divided by aperture width (Cruz & Vallejo, 2009). These patterns are evident from the spline plots of the relative warps (Fig. 8A–E). Kohn (1990) indicated that increase in the ratio of shell diameter to length has been one of the most important evolutionary trends in *Conus*. Traditional morphometric tech-

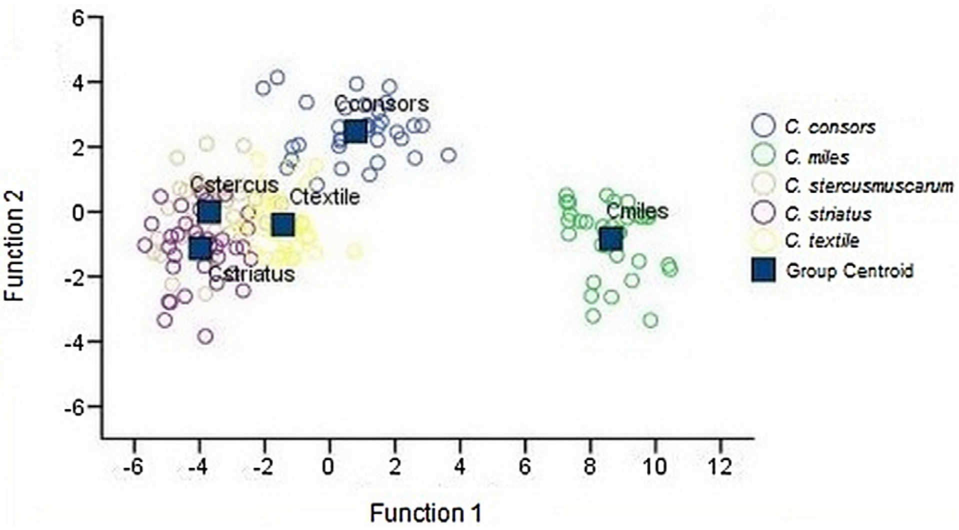

Figure 7. Plot of the results of the canonical variate analysis of scores on principal components 1–4 from landmark analysis.

Table 3. Pooled within-groups correlations between variables and canonical functions

	Function			
	1	2	3	4
PC1	0.974	0.178	–0.141	–0.017
PC2	–0.024	0.901	–0.430	0.057
PC3	0.005	0.325	0.940	0.102
PC4	0.001	–0.038	–0.039	0.999

PC, principal component.

niques determined that aperture height is the most discriminating character in the genus *Oliva* (Cruz & Vallejo, 2009). *Conus miles* and *C. consors* are thus distinct from the other species based on their apertural dimensions, particularly width and spire height. That the difference in PC1 between piscivores and molluscivores is statistically insignificant is consistent with the findings based on calmodulin sequences that the molluscivorous and piscivorous clades share ancestry and would suggest, based on the eigenvector loadings (see Table 1), that the apertural width and spire height are similar in form amongst these species. There are common foraging behaviours in gastropods with these diets, such as ambushing the prey from under the substrate (Kohn, 1956; Tursch & Greifeneder, 2001; Olivera, 2002; Stewart & Gilly, 2005).

The grouping of *Conus* species into clades similar to their dietary preferences indicates that feeding mode in the genus is highly conserved (Duda *et al*., 2001). It has already been established that *Conus* is highly specialized, and feeding activity plays an important role in the ecology of *Conus* populations (Leviten & Kohn, 1980). This may be linked to certain factors constraining diet within lineages, some of them structural, such as radula, venom, and shell gape (Duda *et al*., 2001). The general trend of low spire and conical form facilitates movement through the soft substrata in *Conus* habitats and allows expansion of the aperture, leading to a thickened last whorl that is highly defensive against predators whilst still allowing accommodation of large prey (Kohn, 1990). Shell structure can be said to reflect natural history features, such as feeding type (McClain, Johnson & Rex, 2004). As Table 1 shows, the loadings for PC1 and PC2 are both heavy on the x-coordinate of LM7, indicating that the width of the aperture is important in distinguishing amongst species. The radula, whose form (i.e. teeth type) is correlated with diet (Duda *et al*., 2001), emerges from the aperture via the animal's proboscis.

CVA in turn shows that PC1 and PC2 can effectively discriminate *C. consors*, *C. miles*, and to some degree *C. textile*. This statistical tool has been used in studies on gastropods, such as the one on *Oliva* by Cruz & Vallejo (2009).

There is some degree of consistency between the separation of the groups by the PCs and the assignment of clades based on molecular data (Table 4), which conveniently also separates the taxa by diet (Olivera, 2002). Species belonging to the same clade are likelier to have the same dietary requirements or preferences compared with species belonging to different clades (Kohn, 2002). *Conus stercusmuscarum* and *C. striatus* both belong to the *C. striatus* clade

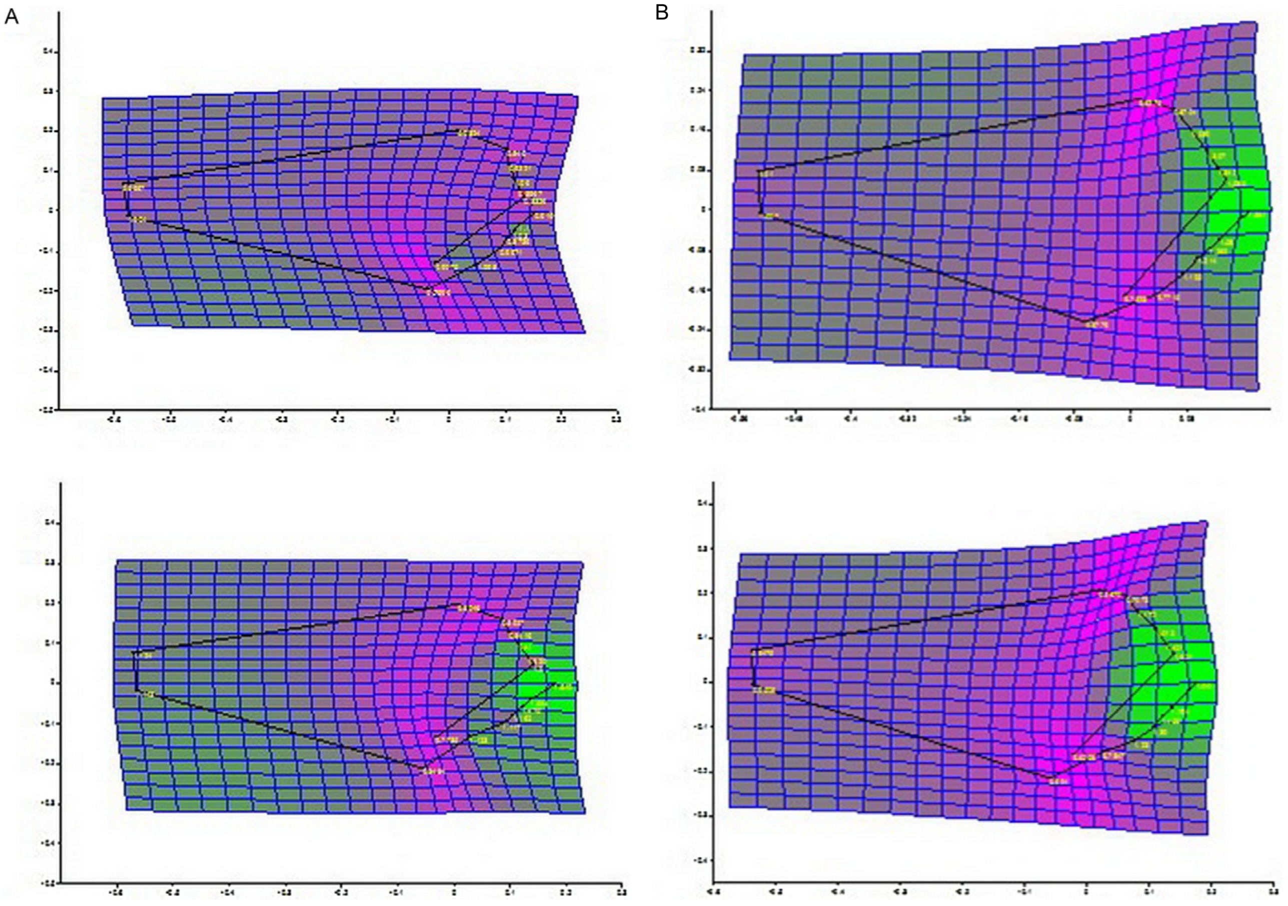

Figure 8. Thin-plate spline grids; warps in reference to mean shape. Numbers indicate area expansion or compression factors (i.e. degree of local growth). Green represents expansion, purple compression. A, *Conus consors*; B, *Conus miles*; C, *Conus stercusmuscarum*; D, *Conus striatus*; E, *Conus textile*.

(Clade I), as does *C. consors*. *Conus miles*, well separated, belongs to the *C. vexillum* clade (Clade XII), of which *C. capitaneus* and *C. mustelinus* are also members. That the distantly related vermivorous species *C. figulinus* also clusters with these illustrates a shared morphology consistent with shared diet. *Conus textile* is part of the mollusc-hunting Clade V named after the species. Phylogenetic analysis based on calmodulin sequences suggests that the molluscivorous clade that includes *C. textile* and the piscivorous clade that includes *C. consors*, *C. stercusmuscarum*, and *C. striatus* share a common ancestor, probably a vermivore (Duda *et al*., 2001).

Differences between the grouping in the phylogenetic tree and that in the schemes of Olivera (2002) and Duda *et al*. (2001) may be the result of morphometric changes being less costly evolutionarily than discrete changes that involve modification or disappearance of structures (Sánchez-Ruiz & Sanmartín, 2000). Although the existing cladograms may reflect evolution of the genus, the tree may reflect morphometric variance amongst closely related species, particularly the piscivores. Sánchez-Ruiz & Sanmartín (2000) propose that these less costly morphometric changes are more common amongst closely related, sympatric species. This may explain why *C. consors* is well separated from its fellow piscivores, but not why *C. stercusmuscarum* and *C. striatus* are clustered together. There is a significant size difference on average between adult forms of the latter two, however, and this probably indicates that they feed on very different fish species and thus do not overlap in diet.

Given the correlation between diet and conotoxins in the genus (Olivera, 2002; Duda & Palumbi, 2004), conotoxins are likely to have evolved adaptively, particular those of piscivores (Duda & Palumbi, 2004). Claude *et al*. (2004) were able to relate (through geometric morphometrics) morphology to diet and habitat in turtles of the superfamily Testudinoidea, revealing an adaptive radiation in the group that demonstrates a relationship between skull shape and lifestyle. It is uncertain how the shell shape correlates

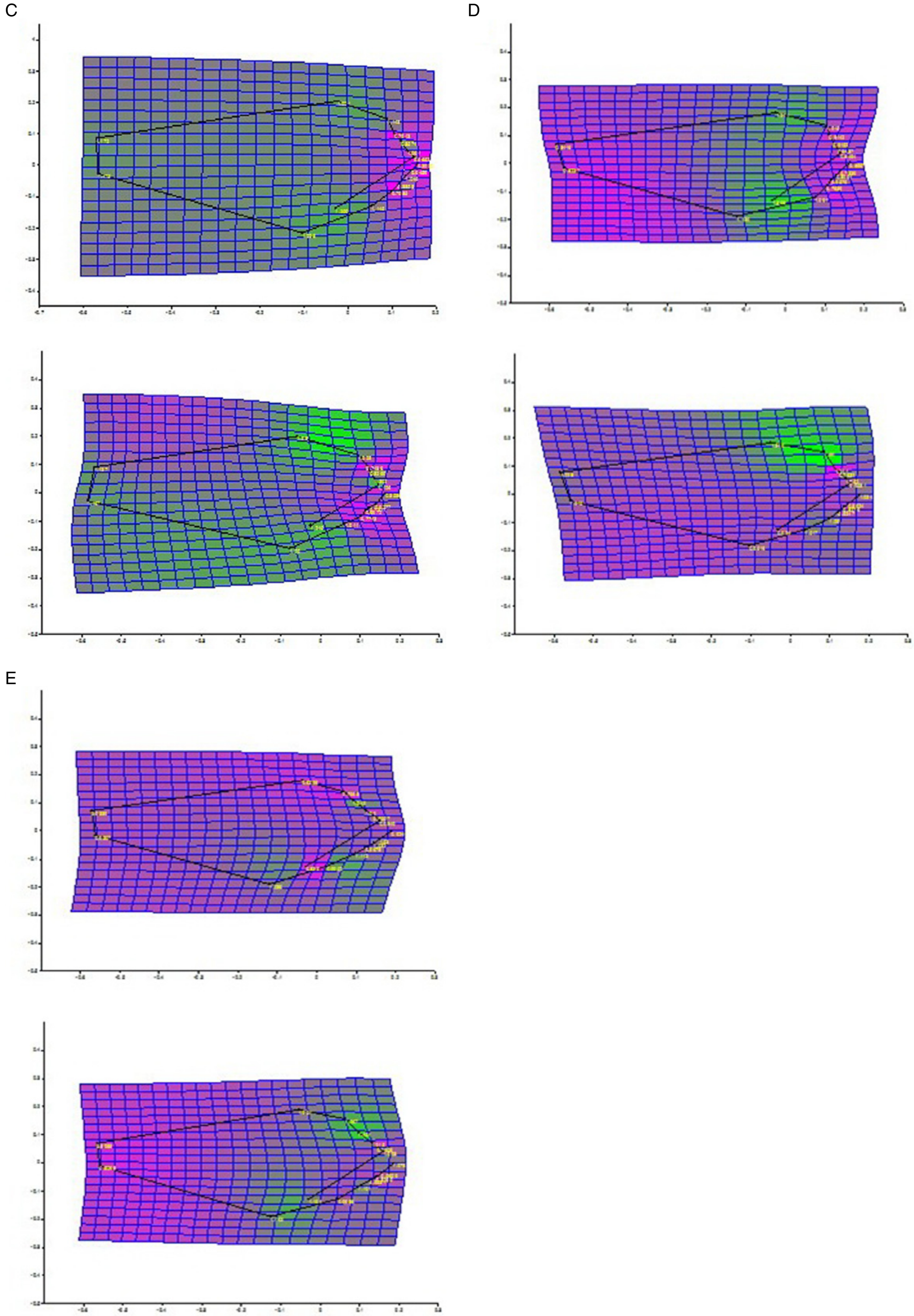

Figure 8. *Continued*

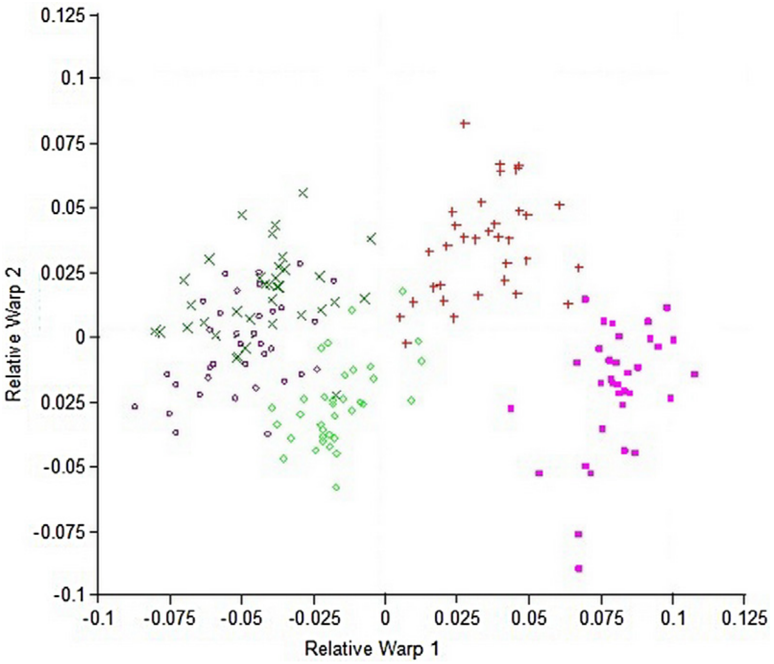

Figure 9. Plot of the results of the principal components analysis of relative warp 1 and relative warp 2. +, *Conus consors*; ■, *Conus miles*; ×, *Conus stercusmuscarum*; ○, *Conus striatus*; ◇, *Conus textile*.

Table 4. *Conus* classification, from Olivera (2002)

Clades	*Conus* species examples	Prey
Fish-hunting		
I	***striatus, stercusmuscarum***	**Fish**
II	*geographus*	Fish
III	*purpurascens*	Fish
IV	*radiatus*	Fish
Mollusc-hunting		
V	***textile***	**Gastropods**
VI	*marmoreus*	Gastropods
Worm-hunting		
VII	*lividus*	Hemichordates; polychaetes
VIII	*glans*	Errant polychaetes (Eunicidae)
IX	*planorbis*	Errant polychaetes (Eunicidae)
X	*betulinus*	Sedentary polychaetes (Capitellidae (?))
XI	*ebraeus*	Errant polychaetes (Eunicidae)
XII	***vexillum, miles***	**Errant polychaetes (Eunicidae)**
XIII	*virgo*	Sedentary polychaetes (Terebellidae)
XIV	*arenatus*	Sedentary polychaetes (Capitellidae)
XV	*sponsalis*	Errant polychaetes (Nereidae)
XVI	*tessulatus*	Errant polychaetes
XVII	*imperialis*	Errant polychaetes (Amphinomidae)

The clades of the species studied are in bold.

with diet in the natural setting, but it is likely that the particular shape of a species of *Conus* facilitates its burrowing activity and form of attack on its preferred prey. Kohn (1959) determined that feeding behaviour (i.e. burrowing, whether radula is left on the prey or undetached from proboscis, etc.) varies significantly across *Conus* species in relation to what they eat. It is interesting to note that snails of the genus *Oliva*, with shells of a generally ovate shape like those of *C. stercusmuscarum*, *C. striatus*, and *C. textile*, are active predators of other molluscs and invertebrates and are recognized as being amongst the quickest burrowers amongst gastropods (Tursch & Greifeneder, 2001). Given the tendency of piscivorous *Conus* species like *C. striatus* to ambush their fish prey from under the sand (Kohn, 1956; Olivera, 2002; Stewart & Gilly, 2005), they may benefit from the ovate shape in burrowing efficiently through the sand en route to their prey. The strong lateral expansion of the main whorl in *C. stercusmuscarum*, *C. striatus*, and *C. textile* in the thin-plate splines probably manifests itself as the adaptive ovate shape of these three species. That the expansion at the shoulder is shown here to be greater in *C. stercusmuscarum* and *C. striatus* than in *C. textile* may be indicative of their aforementioned differences in the texture of the shoulder.

Shell shape is essentially a record of the organism's ontogeny because shell structural change is accretionary, meaning that points on surfaces accumulate (Stone, 1998). Differences in shell shape amongst closely related species may therefore represent variations in shared developmental trajectories. Shape differences amongst *Conus* species may be easily seen and qualitatively described, and indeed common descriptions of species belonging to the genus often discriminate these groups detailing subtle variations of conical shape. However, there are advantages to quantifying these shape differences as has been carried out in this study. Quantification allows for the recognition of intermediate forms; judging degrees of proximity or similarity; and extrapolation or prediction of hypothetical and experimental extremes (Roth & Mercer, 2000).

Despite the ease with which geometric morphometric techniques can be applied to gastropod shells – and the wealth of traditional morphometrics studies on these specimens in the literature – application in this regard has not been commonly carried out (Carvajal-Rodríguez *et al*., 2005). Mammalian crania and facial features, particularly those of humans, have been more frequent subjects (Duarte *et al*., 2000; Delson *et al*., 2001; Zollikofer & Ponce de León, 2002; Mutsvangwa & Douglas, 2007). Regardless of subject specimen, however, techniques of geometric morphometrics have allowed for the separation of groups and a deeper understanding of ontogenetic effects than can be supplied by traditional morphometrics. Aside from traditional methods not being able to adequately capture shape even with ratios, geometric morphometrics is generally less time-consuming and allows the use of digital images, thus eliminating the need to kill and preserve specimens as is necessary in traditional morphometrics (Maderbacher *et al*., 2008). In the study by Maderbacher *et al*. (2008), data description by CVA by geometric morphometrics was more informative in discriminating populations of the *Tropheus moorii* Boulenger, 1898 species complex in the highly diverse Lake Tanganyika in eastern Africa. Like conspecifics of *Conus* sharing the same habitat, these species are models for allopatric speciation and differ more in colour than in morphology.

Geometric morphometrics, particularly landmark digitization and subsequent multivariate statistical analysis, can discriminate certain species of *Conus* through separation amongst taxa and clustering within a taxon in PCA plots, and through easily seen shape differences that can be visualized by thin-plate splines. The results of the study show the relatively unexplored potential of geometric morphometrics for distinguishing amongst taxa of gastropods, as it has successfully been employed in other organisms including mammals and fishes. The separation of groups by landmark analysis, through PCA and distance tree construction from morphological distances, is comparable with phylogenetic constructions based on conotoxins and diet, showing that phylogeny can be gleaned from morphological data. This contradicts the idea that morphometric data do not contain any phylogenetic signals, which in part is based on morphometrics not being able to supply homologous shape characters (Rohlf, 1998). The use of geometric morphometric data is especially helpful for such taxa as *Conus* that lack countable characters. Through analysis of the loadings of eigenvectors in the PCA, coordinates of landmarks corresponding to aperture dimensions (height or width) and height of spire from the shoulder were determined to be the most discriminating, emphasizing the relationship between shell shape and burrowing and feeding activities.

Shell growth is a significant ontogenetic record of gastropod shell development. There is the potential to produce recognition programs that can identify species through the analysis of shape from digital images. It is recommended that future studies using the same methodologies as here detailed be inclusive of more specimens across more species and dietary regimes to generate more accurate trends in shape. It would also be worthwhile to explore the application of these methods in determining shape variation amongst Conidae and related taxa such as Olividae and Turridae. In particular, the shapes of *Oliva* shells and recognized ovate forms of *Conus* may be compared. Shape of the proboscis and the radular teeth may also be correlated with shape of the shell.

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REFERENCES

Ackerly SC. 1989. Kinematics of accretionary shell growth, with examples from brachiopods and mollusks. *Paleobiology* **15:** 147–164.

Adams DC, Rohlf FJ, Slice DC. 2004. Geometric morphometrics: ten years of progress following the 'revolution'. *Italian Journal of Zoology* **71:** 5–16.

Bookstein FL. 1991. *Morphometric tools for landmark data*. Cambridge: Cambridge University Press.

Bulaj G, West PJ, Garrett JE, Watkins M, Zhang MM, Norton RS, Smith BJ, Yoshikami D, Olivera BM. 2005. Novel conotoxins from *Conus striatus* and *Conus kinoshitai* selectively block TTX-resistant sodium channels. *Biochemistry* **44:** 7259–7265.

Carté BK. 1996. Biomedical potential of marine natural products. *Bioscience* **46:** 271–286.

Carvajal-Rodríguez A, Conde-Padín P, Rolán-Alvarez E. 2005. Decomposing shell form into size and shape by geometric morphometric methods in two sympatric ecotypes of *Littorina saxatilis*. *Journal of Molluscan Studies* **71:** 313–318.

Catalano SA, Goloboff PA, Giannini NP. 2010. Phylogenetic morphometrics (I): the use of landmark data in a phylogenetic framework. *Cladistics* **26:** 539–549.

Clabaut C, Bunje PME, Salzburger W, Meyer A. 2007. Geometric morphometric analyses provide evidence for the adaptive character of the Tanganyikan cichlid fish radiations. *Evolution* **61:** 560–578.

Claude J, Pritchard PCH, Tong H, Paradis E, Auffray JC. 2004. Ecological correlates and evolutionary divergence in the skull of turtles: a geometric morphometric assessment. *Systematic Biology* **53:** 933–948.

Cruz RAL, Vallejo BM. 2009. Species discrimination in Philippine *Oliva* Linnaeus 1758 (Gastropoda: Olividae) using size and shape diversity measures. *Loyola Schools Review: Science and Engineering Edition* **8:** 85–104.

Cunha RL, Castilho R, Rüber L, Zardoya R. 2005. Patterns of cladogenesis in the venomous marine gastropod genus *Conus* from the Cape Verde Islands. *Systematic Biology* **54:** 634–650.

David B, Laurin B. 1996. Morphometrics and cladistics: measuring phylogeny in the sea urchin *Echinocardium*. *Evolution* **50:** 348–359.

Delson E, Harvati K, Reddy D, Marcus LF, Mowbray K, Sawyer GJ, Jacob T, Márquez S. 2001. The Sambungmacan 3 *Homo erectus* calvaria: a comparative morphometric and morphological analysis. *The Anatomical Record* **262:** 380–397.

Duarte LC, Monteiro LR, Von Zuben FJ, Dos Reis SF. 2000. Variation in mandible shape in *Thrichomys apereoides* (Mammalia: Rodentia): geometric analysis of a complex morphological structure. *Systematic Biology* **49:** 563–578.

Duda TF, Kohn AJ. 2005. Species-level phylogeography and evolutionary history of the hyperdiverse marine gastropod genus *Conus*. *Molecular Phylogenetics and Evolution* **34:** 257–272.

Duda TF, Kohn AJ, Palumbi SR. 2001. Origins of diverse feeding ecologies within *Conus*, a genus of venomous marine gastropods. *Biological Journal of the Linnaean Society* **73:** 391–409.

Duda TF, Palumbi SR. 2004. Gene expression and feeding ecology: evolution of piscivory in the venomous gastropod genus *Conus*. *Proceedings of the Royal Society Series B. Biological Sciences* **271:** 1165–1174.

Felsenstein J. 2009. *PHYLIP. PHYLogeny Inference Package, version 3.69*. Seattle, WA: University of Washington.

Fenical W. 2006. Marine pharmaceuticals: past, present, and future. *Oceanography* **19:** 110–119.

Fink WL, Zelditch ML. 1995. Phylogenetic analysis of ontogenetic shape transformations: a reassessment of the piranha genus *Pygocentrus* (Teleostei). *Systematic Biology* **44:** 343–360.

Hammer Ø, Harper DAT, Ryan PD. 2001. PAST. Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica* **4 (1):** 9pp. http://palaeo-electronica.org/2001_1/past/issue1_01.htm

Han TS, Teichert RW, Olivera BM, Bulaj G. 2008. *Conus* venoms: a rich source of peptide-based therapeutics. *Current Pharmaceutical Design* **14:** 2462–2479.

Johnston MR, Tabachnick RE, Bookstein FL. 1991. Landmark-based morphometrics of spiral accretionary growth. *Paleobiology* **71:** 19–36.

Kohn AJ. 1956. Piscivorous gastropods of the genus *Conus*. *Proceedings of the National Academy of Sciences, USA* **42:** 168–171.

Kohn AJ. 1959. The ecology of *Conus* in Hawaii. *Ecological Monographs* **29:** 47–90.

Kohn AJ. 1990. Tempo and mode of evolution in Conidae. *Malacologia* **32:** 55–67.

Kohn AJ. 2002. Comparative biology of *Conus* in the light of phylogeny: a preliminary report. *Bollettino Malacologico, Supplemento* **4:** 37–42.

Kohn AJ, Riggs AC. 1975. Morphometry of the *Conus* shell. *Systematic Zoology* **24:** 346–359.

Leviten PJ, Kohn AJ. 1980. Microhabitat resource use, activity patterns, and episodic catastrophe: *Conus* on tropical intertidal reef rock benches. *Ecological Monographs* **50:** 55–75.

Livett BG, Gayler KR, Khalil Z. 2004. Drugs from the sea: conopeptides as potential therapeutics. *Current Medicinal Chemistry* **11:** 1715–1723.

Lockwood CA, Kimbel WH, Lynch JM, Pilbeam D. 2004. Morphometrics and hominoid phylogeny: support for a chimpanzee-human clade and differentiation among great ape subspecies. *Proceedings of the National Academy of Sciences, USA* **101:** 4356–4360.

Luo S, Zhangsun D, Feng J, Wu Y, Zhu X, Hu Y. 2007. Diversity of the O-superfamily conotoxins from *Conus miles*. *Journal of Peptide Science* **13:** 44–53.

Maderbacher M, Bauer C, Herler J, Postl L, Makasa L, Sturmbauer C. 2008. Assessment of traditional versus geometric morphometrics for discriminating populations of the *Tropheus moorii* species complex (Teleostei: Cichlidae), a Lake Tanganyika model for allopatric speciation. *Journal of Zoological Systematics and Evolutionary Research* **46:** 153–161.

McClain CR, Johnson NA, Rex MA. 2004. Morphological disparity as a biodiversity metric in lower bathyal and abyssal gastropod assemblages. *Evolution* **58:** 338–348.

Merkt RE, Ellison AM. 1998. Geographic and habitat-specific morphological variation of *Littoraria* (*Littorinopsis*) *angulifera* (Lamarck, 1822). *Malacologia* **40:** 279–295.

Mutsvangwa T, Douglas TS. 2007. Morphometric analysis of facial landmark data to characterize the facial phenotype associated with fetal alcohol syndrome. *Journal of Anatomy* **210:** 209–220.

Olivera BM. 2002. *Conus* venom peptides: reflections from the biology of clades and species. *Annual Review of Ecology and Systematics* **33:** 25–47.

Olivera BM, Rivier J, Clark C, Ramilo CA, Corpuz GP, Abogadie FC, Mena EE, Woodward SR, Hillyard DR, Cruz LJ. 1990. Diversity of *Conus* neuropeptides. *Science* **249:** 257–263.

Rohlf FJ. 1990. Morphometrics. *Annual Review of Ecology and Systematics* **21:** 299–316.

Rohlf FJ. 1998. On applications of geometric morphometrics to studies of ontogeny and phylogeny. *Systematic Biology* **47:** 147–158.

Rohlf FJ. 2003. *tpsSmall*, version 1.20. Stony Brook, NY: Stony Brook University.

Rohlf FJ. 2004. *tpsDig, version 1.40*. Stony Brook, NY: Stony Brook University.

Rolan E, Guerra-Varela J, Colson I, Hughes RN, Rolan-Alvarez E. 2004. Morphological and genetic analysis of two sympatric morphs of the dogwhelk *Nucella lapillus* (Gastropoda: Muricidae) from Galicia (northwestern Spain). *Journal of Molluscan Studies* **70:** 179–185.

Roth VL, Mercer JM. 2000. Morphometrics in development and evolution. *American Zoologist* **40:** 801–810.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4:** 406–425.

Samadi S, David P, Jarne P. 2000. Variation of shell shape in the clonal snail *Melanoides tuberculata* and its consequences for the interpretation of fossil series. *Evolution* **54:** 492–502.

Sánchez-Ruiz M, Sanmartín I. 2000. Separation of *Aspidiotes* species using morphometric analysis (Coleoptera: Curculionidae). *European Journal of Entomology* **97:** 85–94.

Stewart J, Gilly WF. 2005. Piscivorous behavior of a temperate cone snail, *Conus californicus*. *Biological Bulletin* **209:** 146–153.

Stone JR. 1998. Landmark-based thin-plate spline relative warp analysis of gastropod shells. *Systematic Biology* **47:** 254–263.

Tissot BN. 1984. Multivariate analysis of geographic variation in *Cypraea caputserpentis* (Gastropoda: Cypraeidae). *Veliger* **27:** 106–119.

Tissot BN. 1988. Geographic variation and heterochrony in two species of cowries (genus *Cypraea*). *Evolution* **42:** 103–117.

Tursch B. 1998. Taxonomic problems in the genus *Oliva*. *Phuket Marine Biological Center Special Publication* **18:** 263–284.

Tursch B, Greifeneder D. 2001. *Oliva shells: the genus Oliva and the species problem*. Italy: L'Informatore Piceno.

Van Osselaer C, Grosjean P. 2000. Suture and location of the coiling axis in gastropod shells. *Paleobiology* **26:** 238–257.

Wagner PJ. 1995. Testing evolutionary constraint hypotheses with early Paleozoic gastropods. *Paleobiology* **21:** 248–272.

West PJ, Bulaj G, Garrett JE, Olivera BM, Yoshikami D. 2002. μ-Conotoxin SmIIIA, a potent inhibitor of tetrodotoxin-resistant sodium channels in amphibian sympathetic and sensory neurons. *Biochemistry* **41:** 15388–15393.

Yasunori K, Tomoki K. 2000. Taxonomic revision of *Pisulina* (Gastropoda: Neritopsina) from submarine caves in the tropical Indo-Pacific. *Paleontological Research* **4:** 107–131.

Zollikofer CPE, Ponce de León MS. 2002. Visualizing patterns of craniofacial shape variation in *Homo sapiens*. *Proceedings of the Royal Society of London Series B. Biological Sciences* **269:** 801–807.