Revista Mexicana de Biodiversidad 83: 1045-1052, 2012
DOI: 10.7550/rmb.26165

Threatened Neotropical mollusks: analysis of shape differences in three endemic snails from High Paraná River by geometric morphometrics

Moluscos neotropicales amenazados: análisis de diferencias de forma en tres caracoles endémicos del río Alto Paraná mediante morfometría geométrica

Roberto E. Vogler[1,2✉], Ariel A. Beltramino[1,3], Diego E. Gutiérrez-Gregoric[1,2], Juana G. Peso[4], Miguel Griffin[2,4] and Alejandra Rumi[1,2]

[1]*División Zoología Invertebrados, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata. Paseo del Bosque s/n (B1900FWA), La Plata, Argentina.*
[2]*Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.*
[3]*Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Argentina.*
[4]*Laboratorio de Plancton y Bentos, Facultad de Ciencias Exactas, Químicas y Naturales. Universidad Nacional de Misiones, Rivadavia 2370 (N3300LDX), Posadas, Argentina.*
[5]*División Paleozoología Invertebrados, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata. Paseo del Bosque s/n (B1900FWA), La Plata, Argentina.*
✉ *robertovogler@fcnym.unlp.edu.ar; robertovogler@yahoo.com.ar*

Abstract. Variation in shape among a living and 2 extinct aquatic snails of the genus *Aylacostoma*, using a geometric morphometric method of thin plate splines and multivariate analysis was investigated. The analysis was performed to evaluate the diagnostic capability of this method and to explore shell shape differences, due to the lack of other data, in an attempt to answer why only 1 of the species persisted in the wild. Sixteen landmarks in a bi-dimensional space for 32 shells of type, paratype and reference specimens deposited in museums of Argentina were defined. Analysis was successful in assigning individual specimens to particular species. Statistically significant differences in last whorl, aperture, and spire were found for the first 4 non-uniform components explaining an 85% of local variation observed. Differences could be related to a differential use of habitat and/or to the degree of exposure to water current. More globose shell found in the extinct species could be associated to habitats and substrata with the highest water currents, whereas the more stylized shell in the third species could be related to a preference for more protected habitats, like those where it presently occurs.

Key words: Argentina, *Aylacostoma,* freshwater, native species, Paraguay, TPS.

Resumen. La variación de forma entre una especie viviente y dos extintas de caracoles acuáticos del género *Aylacostoma*, fue investigada mediante el método de morfometría geométrica de "thin plate splines" y análisis multivariado. El análisis se realizó para evaluar la capacidad diagnóstica del método y explorar las diferencias de forma de conchilla, debido a la falta de otros datos, en un intento por responder por qué sólo una de las especies persistió en la naturaleza. Dieciséis "landmarks" fueron definidos en un espacio bi-dimensional para 32 conchillas de ejemplares tipo, paratipo y de referencia depositados en museos de Argentina. El análisis fue exitoso en la asignación de los individuos a especies particulares. Se encontraron diferencias significativas en el último anfracto, apertura y espira respecto de los 4 primeros componentes no uniformes, que explicaron el 85% de la variación observada. Estas diferencias podrían vincularse a un uso diferencial del hábitat y/o al grado de exposición a la corriente. La conchilla más globosa en las especies extintas pudo estar asociada a hábitats y sustratos con mayores corrientes, mientras que la conchilla más estilizada en la tercera especie podría estar relacionada con la preferencia por hábitats más protegidos, como los que actualmente habita.

Palabras clave: agua dulce, Argentina, *Aylacostoma*, especies nativas, Paraguay, TPS.

Introduction

Freshwater gastropods are found on every continent and in nearly all aquatic habitats (Strong et al., 2008). South America still lacks global estimates of species richness of mollusks (Lévêque et al., 2005), although local diversity estimates for large South American rivers (i.e. Uruguay, Paraná and Río de la Plata) and their tributaries, indicate that diversity of freshwater gastropods in this continent might be very high, including faunas that are sometimes extremely speciose and frequently do not occur in other continents (Lévêque et al., 2005; Gutiérrez-Gregoric et al.,

Recibido: 17 julio 2011; aceptado: 11 mayo 2012

2006; Rumi et al., 2006, 2008; Strong et al., 2008; Núñez et al., 2010). Nonetheless, many native snail populations are declining in numbers as a consequence of continuous degradation and destruction of their natural ecosystems because of unabated human activity (Rumi et al., 2006; Strong et al., 2008; Darrigran and Damborenea, 2011).

The genus *Aylacostoma* Spix, 1827 is included in the gastropod family Thiaridae Gill, 1871. The understanding of Thiaridae has notably increased in recent times (e.g. Glaubrecht, 1996, 1999, 2006; Michel, 2004; Gomez et al., 2011; Strong et al., 2011). However, although more than 30 species of *Aylacostoma* from Central and South America are reported in the literature (e.g. Simone, 2006), the genus has never been comprehensively revised. Excepting some anatomical data (i.e. Hylton-Scott, 1953; Morrison, 1954; Simone, 2001) and 2 phylogenetic studies based on comparative morphology (Simone, 2001, 2011), current knowledge of species of *Aylacostoma* is based solely on original descriptions and records of their occurrence as published in inventories of molluscan or benthic faunas (e.g. Souza et al., 2008; Jorcin et al., 2009).

The occurrence of species of this genus in Argentina and Paraguay was first reported by Hylton-Scott (1953, 1954). She described *A. guaraniticum* (Hylton-Scott, 1953), *A. chloroticum* Hylton-Scott, 1954 and *A. stigmaticum* Hylton-Scott, 1954 (Fig. 1). These species are considered viviparous, they reproduce by parthenogenesis and no males have been reported (Quintana and Mercado-Laczkó, 1997; Ostrowski de Núñez and Quintana, 2008). Of the 3 species, the only available anatomical data is for *A. guaraniticum* (Hylton-Scott, 1953), whereas for the other species only shells have been described (Hylton-Scott, 1954).

The 3 species were recorded in highly oxygenated freshwater habitats near the Yacyretá-Apipé rapids in the Paraná River (Argentina-Paraguay), between the Argentine cities of Ituzaingó (27°37' S, 56°40' W) and Posadas (27°20' S, 55°55' W) (Ostrowski de Núñez and Quintana, 2008). Fifty years later, these endemic species were threatened by extinction as the result of major alteration in the flow regime along the rapids. Such alteration was caused by the construction of the Yacyretá Binational Hydroelectric power plant (Seddon, 2000; Ostrowski de Nuñez and Quintana, 2008). Of the 3 species described until 1993 (before impoundment), only *A. chloroticum* still persists currently in 2 relictual populations at the upstream section of the reservoir (Ostrowski de Nuñez and Quintana, 2008) while *A. guaraniticum* and *A. stigmaticum* are categorized as extinct in the wild (Mansur, 2000a, b).

The 3 species from Argentina-Paraguay constitute the southernmost record of the entire South and Central American range of the genus and are supposed to represent closely related species (Castellanos, 1981). However, the lack of previous studies and the absence of soft parts

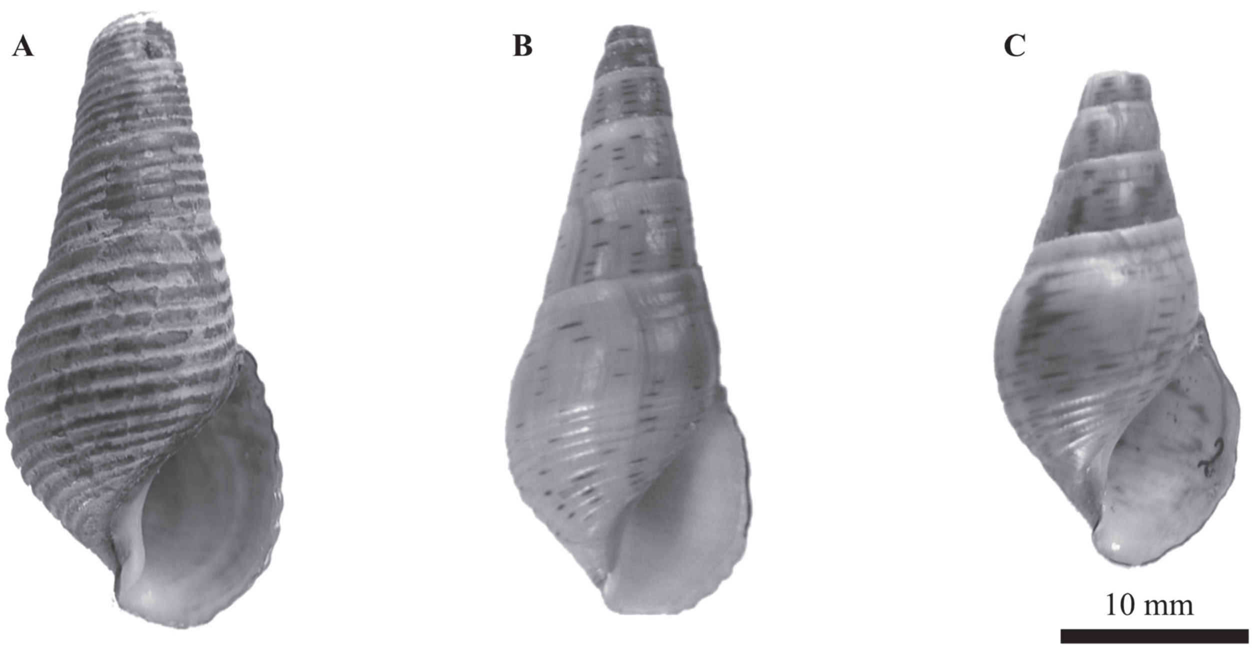

Figure 1. Specimens of the 3 endemic species of *Aylacostoma* from Argentina and Paraguay. Scale 10 mm. A, *A. guaraniticum* -MLP Nº 11213-; B, *A. chloroticum* -UNaM I CR1-; C, *A. stigmaticum* -MLP Nº 10964-.

deposited in museum collections, jointly with the extinction in the wild of *A. guaraniticum* and *A. stigmaticum*, have hampered further insight into their anatomy, biology, ecology and evolution.

Shape analysis is a fundamental part of much biological research and an indispensable technique in the identification of species (Adams et al., 2004), and may be a valuable tool for understanding species in which no other information is available. Information about the shape of an organism can be quickly and precisely captured by processing digital images with the "landmark methods" of geometric morphometrics that quantifies deformations of morphometric points in coordinate space and separate size and shape variation as a standard part of the analysis. Landmark methods have demonstrated to be very effective, particularly when combined with multivariate statistical techniques (Rohlf et al., 1996; Cadrin, 2000; Zelditch et al., 2004; Conde-Padín et al., 2007).

Considering that morphology could reflect special adaptations to some environmental features, we investigated if the geometric morphometric analysis will allow characterizing shell shape in these *Aylacostoma* species, in an attempt to answer why only 1 of these species persisted in the wild. Also we evaluate the diagnostic capability of this method for referring individual specimens to particular species; especially given that the anatomy is unknown.

Material and methods

Samples examined include 32 shells of *Aylacostoma* from Argentina and Paraguay hosted in the Argentine Museum of Natural Sciences "Bernardino Rivadavia" -MACN- (Buenos Aires, Argentina); La Plata Museum -MLP- (La Plata, Argentina) and the National University of Misiones -UNaM- (Posadas, Argentina). This study was based only on museum material, given the lack of living populations of *A. stigmaticum* and *A. guaraniticum* (Mansur, 2000a, b). Neither fixed specimens, soft parts, radulae nor previous studies of them (except for original descriptions) were found in museum collections and published reports.

All the material include type, paratype and reference adult specimens of *A. guaraniticum* (MACN-In Nº 29251; MLP Nº 11213; UNaM CR-1; total length range [last 3 whorls]: 24.70 – 34.76 mm); *A. stigmaticum* (MACN-In Nº 488-2; MLP Nº 10963/64/65; UNaM CR-1; total length range [last 3 whorls]: 21.89 – 27.88 mm) and *A. chloroticum* (MLP Nº 10958 and 11596; UNaM CR-1; total length range [last 3 whorls]: 23.17 – 33.76 mm).

All specimens were photographed with a Samsung SL-76 camera (8 mega pixels definition). The same shell orientation was used for all specimens, with coiling axis of shell on the y-axis and aperture on the same plane as the camera objective (Carvajal-Rodríguez et al., 2005, 2006). All images included a 5 cm graded scale. Sixteen landmarks were selected along the shell perimeter in order to capture differences in all regions of the shell (Fig. 2), following a criterion similar to that of Conde-Padín et al. (2007) (Table 1).

As stated by Carvajal-Rodríguez et al. (2005) in a study of *Littorina* snails, these points do not necessarily represent homologous landmarks -from a developmental point of view- in different specimens, although they allow to capture and analyze objectively and repeatably, shell shape in species of *Aylacostoma*. Six landmarks (LM4, LM5, LM8, LM9, LM11, LM12) were treated as sliding

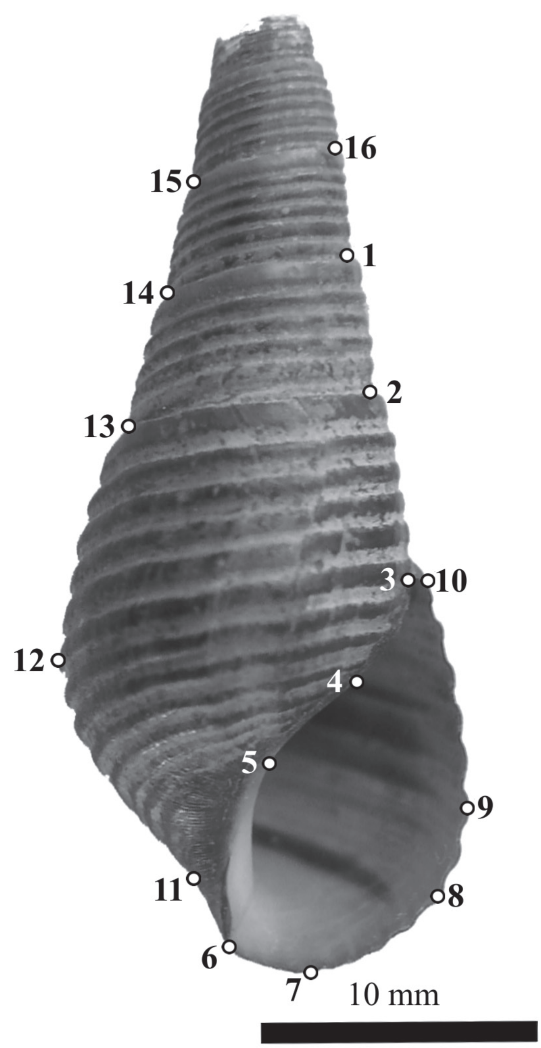

Figure 2. Location of the sixteen landmarks on shell photographs. Scale 10 mm.

Table 1. Position of the sixteen landmarks selected along the shell perimeter

Landmark	*Position*
LM1	On the right border of the profile of the shell at the end of the upper suture of the penultimate whorl.
LM2	On the right border of the profile of the shell at the end of the upper suture of the last whorl.
LM3	At the end of the suture of the last whorl.
LM4 and LM5	Respectively on the most external and internal points at the left profile of the shell aperture on their intersection with a perpendicular line to the axis from LM6.
LM6	At the end of the columella.
LM7	At the lowest point of the base.
LM8	At the most external point on the right profile of the last whorl at its intersection with a perpendicular line to the axis from LM6.
LM9	On the outermost point of apertural lip.
LM10	On the right outline of the shell at the end of the lower suture of the last whorl.
LM11	At the most internal point on the left outline of the last whorl.
LM12	At the most external point on the left outline of the last whorl.
LM13	On the left outline of the shell at the end of the upper suture of the last whorl.
LM14	On the left border of the outline of the shell at the end of the upper suture of the penultimate whorl.
LM15	On the left outline of the shell at the end of the upper suture of the antepenultimate whorl.
LM16	On the right outline of the shell at the end of the upper suture of the antepenultimate whorl.

semi-landmarks by using TPSUTIL version 1.44 (Rohlf, 2009), thus relaxing the homology criterion.

The raw coordinate configurations of all specimens were aligned (i.e. translated, rotated and scaled to match one another) using the Generalized Procrustes Analysis (GPA) procedure to eliminate variation due to differences in scale and orientation, which establishes an average configuration by minimizing the sum of squared distances between homologous landmarks from different specimens (Rohlf and Slice, 1990; Rohlf, 1999; Rufino et al., 2006). This average configuration of landmarks resulting from GPA or tangent configuration (Cavalcanti et al., 1999) served as the "reference configuration" in subsequent calculations. For each specimen, a variable for size (centroid size) and a set of variables for shape (uniform and non-uniform components) of shell shape were also obtained. Centroid size is obtained as a scaling factor during GPA (Bookstein, 1991; Conde-Padín et al., 2007). The 2 uniform components describe differences that affect equally all parts of the shell (global differences). In contrast, non-uniform components account for local shape deformations of the reference configuration at different spatial scales (Conde-Padín et al., 2007).

The coordinates of all aligned specimens were used for the thin plate splines -TPS- and relative warp analysis -RWA- (Bookstein, 1989, 1991; Rohlf, 1993), in order to analyze and display the direction of shape differences among species. In the TPS method, a hypothetical infinitely thin metal plate (grid) is fitted over the reference configuration, which is then deformed until it matches exactly the target shape (Bookstein, 1989, 1991; Rohlf et al., 1996). Relative warps (RWs) are the principal components of any kind of shape variables and reflect the major trends in shape variation (Rufino et al., 2006).

Landmark data were obtained by digitizing images of the shells as in Fig. 2, using software TPSDIG version 2.12 (Rohlf, 2008) to generate coordinates. Relative warp analysis and computation of partial warp scores were computed using TPSRELW version 1.49 (Rohlf, 2010) with the scaling option $\alpha = 0$ that weighs all landmarks equally and is considered to be more appropriate for systematic studies (Loy et al., 1993; Rohlf, 1993; Rohlf et al., 1996; Cavalcanti et al., 1999).

The first 4 derived morphometric variables studied (RWs1-4) were included in a non-parametric MANOVA (NP-MANOVA) (Anderson, 2001) based on Mahalanobis distance (9 999 permutations), in order to test the significance of the mean shell shape of the species. After NP-MANOVA, a canonical variates analysis (CVA) was conducted on the same first 4 RWs, in order to maximize the separation between groups (Zelditch et al., 2004), to estimate misclassification rates, and to evaluate the shape differences that best distinguish among the 3 species (Márquez et al., 2010). TPS deformation grids along the canonical axes were generated in TPSREGR version 1.38 (Rohlf, 2011).

Statistical analyses were performed with PAST, version 2.14 (Hammer et al., 2001). Most of the programs used in this study are available at http://life.bio.sunysb.edu/morph/

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Table 2. Classification matrix showing the number and percentage of individuals correctly classified for each species

	A. chloroticum	*A. guaraniticum*	*A. stigmaticum*	%
A. chloroticum	10	1	0	90.90
A. guaraniticum	1	13	0	92.85
A. stigmaticum	0	0	7	100
Total	11	14	7	94.58

Results

Relative warp analysis showed that approximately 85% of the local shell variation is explained by the first 4 RWs (i.e. RW1= 57.41%; RW2= 13.15%; RW3= 8.78%; RW4= 5.48%). The NP-MANOVA conducted in order to test the significance of the mean shell shape of the 3 species was higly significant (F= 7.164, *p*= 0.0001). Shape differences among species were maximized using CVA, and species were successfully discriminated (Fig. 3, Table 2). The CV1 (explaining 79.08% of the observed variation) can discriminate among the 3 species at the same time, while CV2 (explaining 20.92% of the observed variation) only discriminates between *A. guaraniticum* and the others (Fig. 3).

External landmarks and those that represent the aperture were connected by lines for an easier visualization of the meaning of deformations (Fig. 4). When analyzed from the lowest to the highest scores, first canonical axis can be mainly described as an expansion of the last whorl and aperture, involving almost all landmarks in the area. Also an antero-posterior contraction of spire was observed, showing an upward displacement of landmark pair defining the lower suture of penultimate whorl (LM13-LM2), as well as a downward and inwards displacement of landmark pair located on the upper suture of antepenultimate whorl (LM15-LM16) (Figs. 4A, B). In turn, when analyzed from the lowest to highest scores, the second canonical axis reveals no noticeable differences in spire shape, and variation was mostly associated to last whorl including an upward displacement of LM12 and a shrinking of LM6 and LM7 (Figs. 4C, D).

Discussion

Thin plate splines methods are increasingly used for morphometric research because they: *i)* allow complete separation of size and shape into distinct variables; *ii)* permit segregation of shape into uniform and non-uniform components; *iii)* have powerful means to visualize morphologic differences; *iv)* enable the incorporation of shape variables derived from geometric morphometric analyses into commonly used multivariate analyses; *v)* are cheap, and *vi)* employ user-friendly software (Anderson and Roopnarine, 2005; Carvajal-Rodríguez et al., 2005).

Application of geometric morphometric methods has demonstrated to be very useful in determining both intra (e.g. Palmer et al., 2004; Krapivka et al., 2007; Márquez et al., 2010; Valladares et al., 2010) and interspecific groups in mollusks (e.g. Ferson et al., 1985; Innes and Bates, 1999; Dommergues et al., 2003; Aguirre et al., 2006; Rufino et al., 2006). Like bivalves, gastropods have hard shells which make them excellent candidates for shape analysis by means of geometric morphometrics (e.g. Carvajal-Rodríguez et al., 2005, 2006), as no deformation occurs during manipulation.

In this study, significant differences in shell shape were found in the available museum material for *A. guaraniticum*, *A. chloroticum* and *A. stigmaticum* and almost 95% of the individual specimens were correctly assigned to species. We believe that the success of the method in revealing these differences seems to provide a robust basis to examine the morphological variation in these mollusks. This geometric sensivity has been noted by others in revealing shape differences in populations and specimens of sibling species of *Littorina* or British rough periwinkles (Carvajal-Rodríguez et al., 2005; Conde-Padín et al., 2007) and sibling species of *Nassarius* (Carvajal-Rodríguez et al., 2006). In the species studied here, the main shape differences evidenced along canonical axes can be summarized as changes in spire, last whorl and aperture. The most notorious discriminating differences

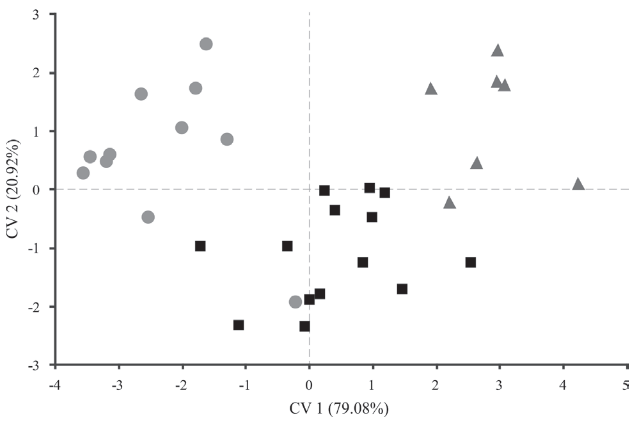

Figure 3. Scatterplot of individual scores from the canonical variates analysis (CVA) of all specimens. ●, *A. chloroticum*; ■, *A. guaraniticum*; ▲, *A. stigmaticum*.

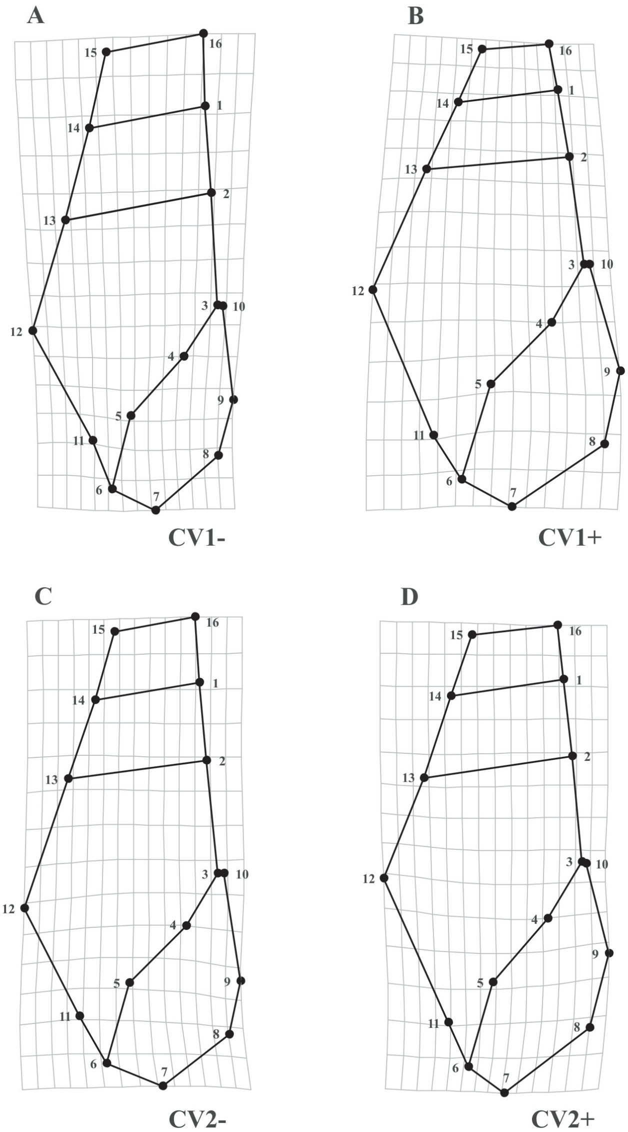

Figure 4. Deformations grids showing the most extreme negative and positive shape changes along the 2 canonical axes. A, B: CV1; C, D: CV2.

were concentrated on CV1, where the species shape varies in a range from more stylized shells in *A. chloroticum* to more globose shells in *A. stigmaticum*.

The detection of 2 statistically significant variables, which summarize the main differences in shape among the 3 species, is interesting because previous geometric morphometric studies in gastropods have suggested that an interpretation of shape in simple biological terms is possible (Carvajal-Rodríguez et al., 2005; Conde-Padín et al., 2007). As an example Conde-Padín et al. (2007) proposed that the more globose shell found in an ecotype of *Littorina saxatilis* (Olivi, 1792) is possibly related with the need for resisting crab attacks, which are common where this ecotype lives. Unfortunately, no interpretations of this nature -as could be resistance to molluscivorous fish- can be inferred for the *Aylacostoma* species from the Paraná River because of: *i)* the disappearance in 1993 of the rapids where the species lived (Quintana and Mercado-Laczkó, 1997); *ii)* the extinction in the wild of *A. guaraniticum* and *A. stigmaticum* (Quintana and Mercado-Laczkó, 1997; Mansur, 2000a, b), and *iii)* the lack of previous studies. Nevertheless, considering that the 3 species inhabited highly oxygenated shallow freshwater habitats in the rapids (Quintana and Mercado-Laczkó, 1997), it seems probable that the differences between the 3 species regarding last whorl, aperture and spire shapes could possibly be explained by a differential use of the habitat and/or perhaps by different degrees of exposure to water currents.

As previously demonstrated for other freshwater snails (Greenwood and Thorp, 2001), a larger foot could be related to the ability to avoid being dislodged by waves. Thus, we hypothesized that the more globose shell and more oval aperture found in *A. stigmaticum,* followed to a lesser degree by *A. guaraniticum*, may be associated to habitats and substrata with the highest water currents in the rapids. Contrarily, the stylized shell seen in *A. chloroticum* may be related to the preference for more protected habitats as those found at the upstream section of the Yacyretá Reservoir, where 2 relictual populations of *A. chloroticum* still persist (Ostrowski de Nuñez and Quintana, 2008). Further studies should be made on these populations to test our hypothesis.

Finally, we believe that geometric approaches should not be limited only to the species of *Aylacostoma* from the Argentina and Paraguay as we consider that they are valuable analytical tools for quantifying and exploring the shape, that together with other data (e.g. anatomical, morphological, ecological and genetical) could contribute to the revision of this particular group of mollusks.

Acknowledgements

We thank Dra. Verónica Núñez, Dr. Rolando González-José and two anonymous reviewers for their helpful comments and suggestions.

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