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Esteban Avigliano, María Eugenia Rolón, Juan José Rosso, Ezequiel Mabragaña & Alejandra Vanina Volpedo

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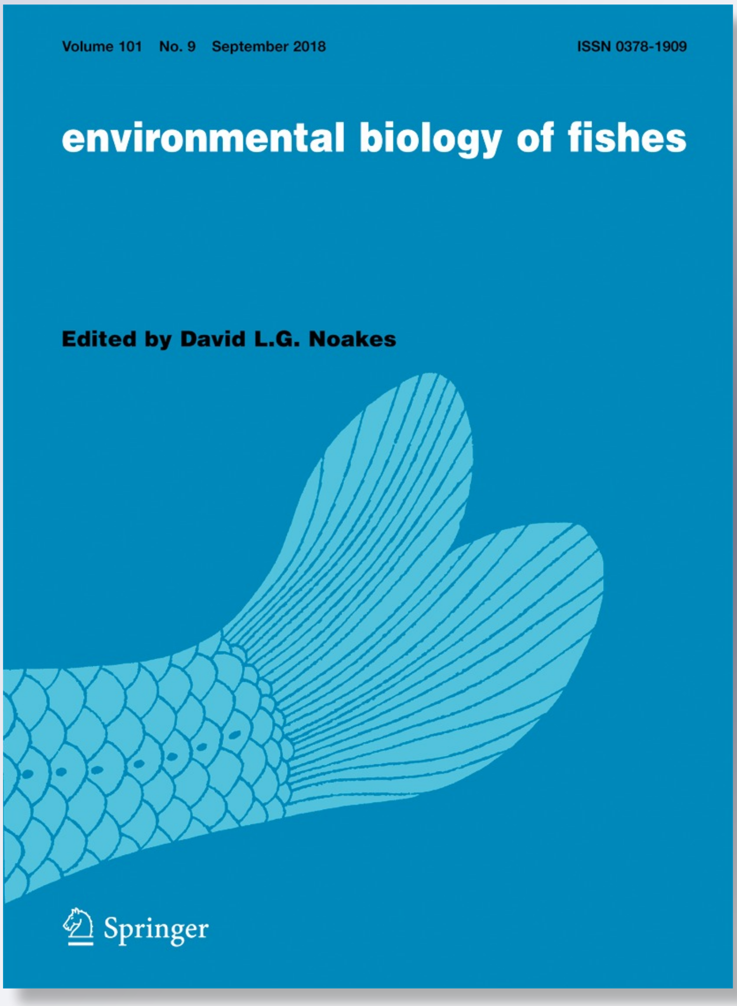

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Using otolith morphometry for the identification of three sympatric and morphologically similar species of *Astyanax* from the Atlantic Rain Forest (Argentina)

Esteban Avigliano · María Eugenia Rolón ·
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Alejandra Vanina Volpedo

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Abstract In several genera, the otolith shape is species-specific and the use of this structure provides a useful tool aiding in the species identification. In many studies regarding Neotropical fish fauna, species of the genus *Astyanax* are commonly identified at the genus level, mainly due to the phenotypic plasticity of the morphological characters traditionally used for species determination. In consequence, additional tools intended to better elucidate the taxonomic boundaries between species of *Astyanax* are certainly needed. In the last decade, the shape of otoliths has allowed to discriminate among closely related species. In this work, Fourier descriptors and shape indices of *lapill*us otolith were evaluated for the discrimination among three sympatric species of genus *Astyanax* inhabiting streams of the Atlantic Rain Forest (Argentina). Aspect ratio, roundness and ellipticity of otoliths were significantly different between the species ($p < 0.05$) while, no significant differences were found for circularity, rectangularity and form factor ($p > 0.05$). PERMANOVA analysis reveal significant differences between species using Fourier descriptors ($F = 96.7$, $0.0001 < p < 0.02$) and the reclassification rates of quadratic discriminant analysis were high, averaging 86.3% (82.7 - 88.6%). Multivariate analyses of shape indices were not effective to discriminate between species. Instead, high classification percentages suggest that the otolith outline is a potential tool for the identification of sympatric morphologically similar species of *Astyanax*. Our results could contribute to future taxonomic and phylogenetic studies and may be an interesting input for both paleontological and trophic studies in sympatric species.

Keywords *Astyanax* · Argentina · Fourier analysis · Morphology · Neotropical fish

Introduction

The genus *Astyanax*, with around 150 species (Eschmeyer and Fricke 2017), is the most species-rich genus in the order Characiformes and one of the most species diverse Neotropical fish genera. It is distributed in continental aquatic systems from South of USA (Ornelas-García et al. 2008) to Patagonia, Argentina (Almirón et al. 1997). This genus is of high ecological importance because it forms the food chain base for several predators, including other fish, mammals and birds (De La Ducommun et al. 2010; Rodrigues et al. 2014; Pereira et al. 2016). In low-nutrient Neotropical streams, genus *Astyanax* plays important roles in the recycling of nutrients, acting as keystone nutrient recyclers (Small et al. 2011). In addition, species of *Astyanax*

E. Avigliano (✉) · M. E. Rolón · A. V. Volpedo
Instituto de Investigaciones en Producción Animal (INPA), Facultad de Ciencias Veterinarias, Universidad de Buenos Aires (UBA)-CONICET, Av. Chorroarín 280, C1427CWO Buenos Aires, Argentina
e-mail: estebanavigliano@conicet.gov.ar

J. J. Rosso · E. Mabragaña
Grupo de Biotaxonomía Morfológica y Molecular de Peces, Instituto de Investigaciones Marinas y Costeras (IIMyC)-CONICET, Universidad Nacional de Mar del Plata (UNMdP), Buenos Aires, Argentina

are economically attractive as ornamental fish (Prang 2007) and show potential in both aquaculture and academic research (Bertolini et al. 2018).

Molecular (Javonillo et al. 2010) and morphological (Mirande 2010) evidence showed that species of the genus *Astyanax* do not conform a monophyletic genus and it is currently recognized as *incertae sedis* within the Family Characidae (Nelson et al. 2016). Roughly, the genus is defined by a set of morphological characters enlisted by Eigenmann (1921) almost one century ago. The genus contains many species with overlapping ranges in meristic and morphometric characters due to the phenotypic plasticity of the morphological characters traditionally used for species determination. Unfortunately, the use of some molecular tools, as DNA Barcoding, which has been widely used to aid in taxonomic questions, showed only partial satisfactory results (Rossini et al. 2016). Altogether, these taxonomic issues may explain why in many studies of the Neotropical fish fauna, the species of *Astyanax* are identified only at genus level. In consequence, additional tools intended to better elucidate the taxonomic boundaries between species of *Astyanax* are certainly needed. In this respect, the study of otoliths shape could be a promissory option.

In the last decade, the otoliths morphometry or shape has allowed to discriminate among closely related species (Reichenbacher et al. 2007; Bani et al. 2013; Tuset et al. 2013; Callicó Fortunato et al. 2014; Avigliano et al. 2015; Boudinar et al. 2016) contributing to solve these taxonomic issues. Otoliths are complex polycrystalline structures composed of calcium carbonate located in the inner ear of fish and have a role in hearing and equilibrium (Campana et al. 1997). An additional benefit of being able to discriminate species using otoliths is that these structures are often found in the stomach content of organisms (fish, mammals, birds) and as well as fossils in sediments being a very useful tool for food-chain, ecological and paleontological studies (Reichenbacher and Reichard 2014; Buckland et al. 2017; Giménez et al. 2017).

Recently, Avigliano et al. (2017a, b) described for the first time the otolith morphological characteristics of the species captured in the Atlantic Rain Forest (Argentina). In this sense, otolith morphometry could provide a useful tool aiding in the identification of the *Astyanax* species.

In this work, the potential use of otolith shape (elliptic Fourier analysis and shape indices) as a complementary tool to identify and discriminate sympatric and morphologically similar species of *Astyanax* was evaluated. Specifically, we tested whether otolith morphometry was able to discriminate among three commonly abundant and widespread (Rosso et al. 2013) species of *Astyanax* (*A. paris* Azpelicueta et al. 2002*, A. saguazu* Casciotta et al. 2003 and *A. xiru* de Lucena et al. 2013) coexisting (Flores et al. 2015) in streams of the southern most extreme of the Atlantic Rain Forest (Argentina).

Materials and methods

Study locations and collection

The study area is located among the highlands of the La Plata Basin (South America), surrounded by subtropical rainforests (Fig. 1). The major rivers of this region are the Uruguay River (geographical border between Argentina and Brazil), and the Paraná River (geographical border between Argentina and Paraguay).

Collection permits were granted by the Ministerio de Ecología y Recursos Naturales Renovables of the Misiones province. Fish were collected using trammel nets between May 2016 and March 2017 in six small tributaries of the Uruguay River (Ramos, Florida, Fortaleza, Garibaldi and Yabotí Miní streams) (Fig. 1). Upon capture, fish were sacrificed with an overdose of benzocaine, as recommended by the New South Wales Fisheries Animal Care and Ethics Committee (Barker et al. 2009). Sacrificed fish were kept refrigerated at 4 °C until reaching the laboratory, where they were identified, measured (standard length = SL in mm) and *lapilli* otoliths were extracted. *Lapilli* otoliths were used rather than *sagittae* otoliths because they were larger and allowed less measurement error.

The fish species were identified according to original descriptions (Azpelicueta et al. 2002; Casciotta et al. 2003; De Lucena et al. 2013) and the taxonomic key proposed by De Lucena et al. (2013). To avoid possible size fish effects on otolith shape, only individuals with a SL of 41–115 mm were selected. In total, 146 *A. saguazu* (SL mean ± SD, 87.7 ± 13.9 mm); 110 *A. paris* (61.4 ± 12.4 mm) and 35 *A. xiru* (77.2 ± 18.7 mm) were selected for the analysis. The otolith vouchers were deposited at

Fig. 1 Map of the study area. Numbers show the localities sampled for collecting the *Astyanax* spp. Each number represents more than one sampling site

the Universidad de Buenos Aires Fish Collection (COLV/Fish-UBA).

Otolith shape

The internal face of the right *lapilli* otoliths was photographed with a digital camera attached to a stereoscopic microscope (Leica EZ4-HD, Singapore) at 30× magnification (Fig. 2). The fields of the images were digitally cleaned and they were transformed to the BMP format.

Shape indices

The following morphometric variables were determined on the images using Image-Pro Plus 4.5

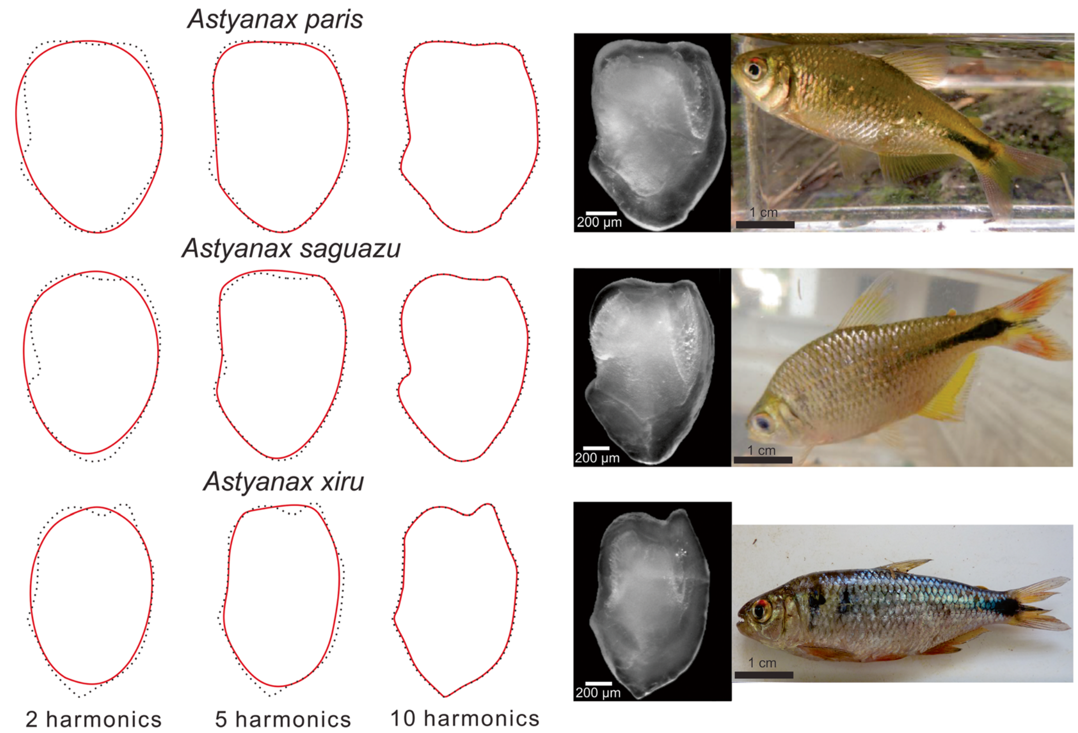

Fig. 2 *Astyanax* spp. otolith shape outlines reconstruction for successive cumulative contribution of the first 10 harmonics of the elliptical Fourier analysis (Fourier power spectrum = 99.9999%). Dotted line: original otolith outline; solid line: the cumulative contribution of harmonics

software according to the terminology used by Avigliano et al. (2014): otolith length (OL, mm), otolith width (OW, mm), otolith perimeter (OP, mm) and otolith surface (OS, mm^2). Then, six otolith shape indices were calculated (Tuset et al. 2003; Avigliano et al. 2015): aspect ratio, circularity, ellipticity, form factor, rectangularity and roundness (Table 1) (Eq. 1–6).

Table 1 Equations of the shape indices

Shape indices	Equation
Aspect ratio $= \frac{OW}{OL}$	1
Circularity $= \frac{OP^2}{OS}$	2
Ellipticity $= \frac{OL-OW}{OL+OW}$	3
Form factor $= \frac{4\pi OS}{OP^2}$	4
Rectangularity $= \frac{OS}{OLxOW}$	5
Roundness $= \frac{4OS}{\pi OL^2}$	6

OL, otolith length; OW, otolith width; OP, otolith perimeter and OS, otolith surface

Elliptic Fourier analysis

The Elliptic Fourier analysis allows delineating any object with a closed two dimensional outline. This method is based on the separate Fourier orthogonal decompositions of a curve into a sum of harmonically related ellipses (sine and cosine) that can be combined to reconstruct a closed outline. Each harmonic or descriptor is composed of four elliptic Fourier coefficients (FC) (a, b, c and d).

The FCs were obtained using Shape 1.3 software. Following Crampton (1995), the number of harmonics needed to obtain the best reconstruction of the otolith outline was estimated using the Fourier power spectrum. The first 10 harmonics achieved ~100% of the cumulated power (Fig. 2), then, 40 FCs were sufficient to describe the otoliths outline. FCs were normalized in order to be invariant with respect to size, rotation and beginning position of the trace (Ferson et al. 1985). These processes utilize parameters of the first harmonic, resulting in degeneration of the first three FCs (a, b and c ≈ 0) (Crampton 1995). Then, 37 FCs summarize the otoliths outline used in this study.

Data analysis

Shape indices and FCs were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene's tests respectively. Only ellipticity and 26 FCs displayed a normal distribution and homogeneity of variance. Circularity, form factor, rectangularity, roundness and the remaining 11 FCs did not meet such assumptions (Kolmogorov-Smirnov, $p < 0.05$; Levene, $p < 0.05$), even after transformation like square root, cubic, inverse or logarithm. Consequently, only ellipticity was retained for further parametric analyses whereas the remaining variables were subjected to non-parametric analyses.

To ensure that differences in fish length did not confound shape variation in otolith, the effect of standard length (SL) on all the shape indices and FCs was examined using Spearman correlation (Campana 2013). Shape indices and FCs were not significantly correlated with SL ($r < 0.3$, $p > 0.05$), hence it was not necessary to correct any variable.

All variables were corrected to avoid allometric effects of SL according to:

$$y_i = a_i \mathrm{x_i}^b \quad (7)$$

where y is an otolith shape variable (shape indices and FCs), x is the SL and a, and b are constants. Constant a_i depends on the particular individual and b is common for all the fish (Lleonart et al. 2000). The constant a an b are estimated for each shape variable as the y-intercept and slope of the regression between log($\mathrm{y_i}$) and log($\mathrm{x_i}$), respectively (Lleonart et al. 2000). For example, if the linear regression between the variables log(circularity) and log(SL) is:

$$\log(\text{circularity})_i = \log(a_i) + b^* \log(SL)_i \quad (8)$$

The constant a is log(a_i) and the slope is b. Each $\mathrm{y_i}$ value was transformed into y*, according to:

$$y*_i = y_i \left[\frac{x_0}{x_i}\right]^b \quad (9)$$

where y* is $\mathrm{y_i}$ value corrected for allometry, $\mathrm{x_0}$ is the mean standard length for all individuals (77.86 mm) and $\mathrm{x_i}$ is the standard length of the i-th specimen.

Ellipticity was compared between species with ANOVA and differences between level means were treated using Bonferroni test. Kruskal Wallis test were used to compare the aspect ratio, circularity, form factor, rectangularity and roundness between species. Multivariate statistics were also used to evaluate differences between species. In order to prevent the use of redundant variables and a false outcome in the multivariate analysis (Graham 2003), multicollinearity between variables was analyzed by means of Spearman coefficient of correlation. Aspect ratio was significantly correlated with ellipticity ($r = -0.97$, $p = 0.001$) and roundness ($r = -0.86$, $\mathrm{p} = 0.001$) while circularity significantly correlated with form factor ($r = 0.97$, $\mathrm{p} = 0.001$). On the other hand, FCs b2, a3, a4, b4, c5, b6, d6, a7, b7, b9, c9 and d10 significantly correlated with others ($r > 0.50$, $p < 0.05$). Then, aspect ratio and circularity were retained and ellipticity, roundness and form factor, as well as the named FCs were not included in the multivariate analysis.

Mardia's skewness and kurtosis tests showed multidimensional non-normality; therefore, permutational multivariate analysis of variance (PERMANOVA) was used instead of MANOVA (French et al. 2002) to detect differences in the otolith morphology between species. Two analysis were performed based on Mahalanobis distances (Anderson 2006) with 9999 permutations for shape indices and FCs, separately.

Because the assumption of homogeneity of variances-covariances matrices was not met (Box test, $p < 0.001$), quadratic discriminant function analysis (QDA) were used. In order to evaluate the efficiency of morphometric methods to discriminate the species, three QDA were performed; using shape indices and FCs separately, as well as together. Statistical tests were performed using the SPSS 19 (Stacks 1989), Infostat 2016 (Di Rienzo et al. 2011), Past 3.0 (Hammer 2011) and Ginkgo 1.7 (Bouxin 2005) programs.

Results

Descriptive statistics of shape indices are shown in Table 2. Aspect ratio, roundness ($28.5 < H < 30.4$, $p = 0.0001$) and ellipticity ($F = 15.6$, $p = 0.0001$) were significantly different among the species. Aspect ratio was found to be significantly lowest for *A. paris*, while no differences were found between *A. xiru* and *A. saguazu.* Ellipticity and roundness were highest for *A. paris* in relation to *A. xiru* and *A. saguazu* ($H = 42.4$; $p < 0.0001$). No significant differences ($3.9 < H < 4.6$, $p > 0.05$) between species for circularity, rectangularity and form factor were found.

PERMANOVA and QDA (Table 3, Fig. 3) analyses were not effective to discriminate between species using shape indices. PERMANOVA analysis did not show significant differences ($F = 1.4$, $p = 0.2$), and the percentages of well classified individuals obtained with the QDA were very low for *A. xiru* and *A. paris* (20.0%-24.4%). A moderate classification percentage was obtained for *A. saguazu* (71.2%) using only the shape indices (Table 3). Contrary to shape indices, multivariate analyses were found to be highly effective detecting differences in FCs between species. PERMANOVA analysis revealed multivariate significant differences for all comparisons of pairs between the three species using Mahalanobis distances ($F = 20.4$, $p = 0.0001$) (Fig. 3). Classification rates of QDA were high (mean = 86.3%), ranging from 82.7 to 88.6% (Table 3), when the FC were used. When the QDA was performed combining the shape indices with the FCs, the average percentage of well classified individuals obtained was 87.7%, showing only an increase of 1.4% with respect to the analysis using only the FCs (Table 3). Indeed, no differences in correct classification were obtained for *A. saguazu* and *A. paris* between the QDA of FCs and the combined approach including the shape indices. An improvement of 2.8% was registered with the combined approach for *A. xiru*.

Discussion

Because the otolith shape can be under genetic influence (Vignon and Morat 2010; Schwarzhans et al. 2012), the

Table 2 Descriptive statistic of the otolith shape indices

	N	Aspect ratio		Circularity		Ellipticity		Form factor		Rectangularity		Roundness	
		mean ± SD	range	mean ± SD	range	mean ± SD	range	mean ± SD	range	mean ± SD	range	mean ± SD	range
A. xiru	35	0.75 ± 0.10^{b}	0.66–1.28	5.39 ± 0.75^{a}	3.86–6.97	0.16 ± 0.03^{b}	0.10–0.21	18.43 ± 3.17^{a}	12.76–25.77	0.79 ± 0.02^{a}	0.74–0.83	1.37 ± 0.14^{b}	0.78–1.54
A. saguazu	146	0.72 ± 0.04^{b}	0.63–0.83	5.09 ± 1.06^{a}	0.47–8.58	0.17 ± 0.03^{b}	0.09–0.24	17.67 ± 3.54^{a}	3.77–30.18	0.78 ± 0.02^{a}	0.72–0.85	1.40 ± 0.10^{b}	1.14–1.66
A. paris	110	0.71 ± 0.14^{a}	0.60–1.49	5.21 ± 1.12^{a}	1.93–7.46	0.18 ± 0.03^{a}	0.07–0.24	18.07 ± 4.89^{a}	4.39–27.76	0.79 ± 0.02^{a}	0.70–1.06	1.46 ± 0.10^{a}	1.21–1.72

Different letters indicate statistical significant differences between species ($p < 0.05$). Ellipticity was compared among species with ANOVA while Kruskal Wallis test was used to compare the aspect ratio, circularity, form factor, rectangularity and roundness. N: sample size

Table 3 Cross-classification matrix of the quadratic discriminant analysis

	N	*A. xiru*	*A. saguazu*	*A. paris*
Shape indices				
A. xiru	35	20.0	68.6	11.4
A. saguazu	146	19.2	71.2	9.6
A. paris	110	8.2	65.5	26.4
Fourier descriptors				
A. xiru	35	88.6	2.9	8.6
A. saguazu	146	1.4	87.7	11.0
A. paris	110	5.5	11.8	82.7
Shape indices plus Fourier descriptors				
A. xiru	35	91.4	2.9	5.7
A. saguazu	146	1.4	87.7	11.0
A. paris	110	4.5	12.7	82.7

The numbers represent the classification percentage for each species of *Astyanax*. N: sample size

taxonomic value of otoliths is well recognized and several morphometric methods has been used to differentiate current and extinct species (Bani et al. 2013; Gierl et al. 2013; Reichenbacher and Reichard 2014; Zhuang et al. 2014; Avigliano et al. 2015). In particular, otolith contour analysis has been useful even to study species of extinct fish (Schulz-Mirbach and Reichenbacher 2008). However, environmental factors (e.g., deep and temperature) have been suggested to be responsible for inter- and intraspecific differences in shape otoliths (Lombarte and Lleonart 1993; Lombarte et al. 2010). Because of these characteristics, otolith shape has been widely used to identify nursery areas (Avigliano et al. 2017a, b), to discriminate fish stocks (Ferguson et al. 2011; Vasconcelos et al. 2017), and to describe ecomorphological patterns (Volpedo and Diana Echeverría 2003; Volpedo and Fuchs 2010; Jaramillo et al. 2014).

In this work, the fish were collected in very similar environments. Streams of the study area can be described as shallow oligotrophic environments (0.5–2 m) with similar environmental (temperature, pH, electrical conductivity, turbidity, dissolved oxygen, and total dissolved solids) and chemical (trace elements, nutrients and agrochemicals) characteristics (Avigliano and Schenone 2015, 2016). In this sense, it is possible that genetics would overcome environment and likely represent a major directive force influencing otoliths shape in the studied species of *Astyanax*. However, specific studies are necessary to understand what factors have real influence on the morphology of otolith in different species.

According to the univariate analyses, among the six shape indices used in this paper, only aspect ratio, ellipticity and roundness showed significant differences between some of the species pair comparisons. These three indices clearly separated *A. paris* from the remain species, but were not able to discriminate between *A. xiru* and *A. saguazu*. The values obtained for the indices suggest that the otoliths of *A. paris* tend to be rather elliptical and with a relatively low width/length ratio. On the other hand, the otoliths of *A. xiru* and *A. saguazu* do not differ from each other and present roundness and a relatively high width/length ratio.

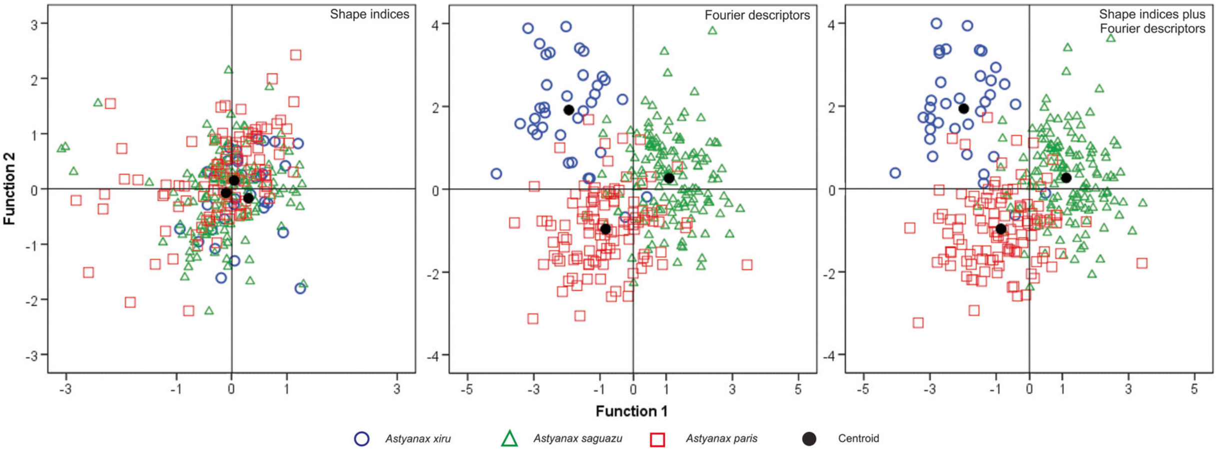

Fig. 3 Quadratic Discriminant Analysis of the otolith morphometry of three species of *Astyanax* from the Atlantic Rain Forest, in Argentina

In species where *sagittae* otoliths are conspicuous, in addition to the aspect ratio, circularity, ellipticity, form factor, rectangularity and roundness, it is common to also use relationships based on the *sulcus* and *rostrum* (Jaramillo et al. 2014; Zhuang et al. 2014; Avigliano et al. 2015). In the case of Characiformes such as the *Astyanax* genus, the *lapill*us otolith is the most conspicuous. This does not present *rostrum* and in the internal view, the *sulcus* is covered by the *Gibbus maculae* (Fig. 2), making it unfeasible to apply indices based on these structures. In this scenario, other indices such as compactness, convexity, eccentricity and triangularity or absolute measures such as the length and mass of the otolith (Tuset et al. 2013), could be further likely useful tools to separate species of Characiformes.

The multivariate analyses showed that shape indices alone were not efficient to separate the three species under study and with combination with FCs only slightly improved the correct classification percentages. Unlike the shape indices, the otolith outlines (as revealed by the elliptical Fourier analysis) showed significant multivariate differences between species and high classification percentages (Fig. 3) (Table 3) indicating that this aspect of the morphometry of the otolith is a potential tool for the identification of some *Astyanax* species. In general, when groups are well discriminated, the results are enhanced when methods separately effective are combined (Ferguson et al. 2011; Avigliano et al. 2017a, b). In this study, the combination of both techniques did not show substantial improvements in the classification of the species.

This work is probably the first to attempt to clarify the taxonomy of recent species using otolith contour analysis. Results shows that some sympatric and morphologically similar species of *Astyanax* may be discriminated using the shape of the otolith, especially the contour. This tool could contribute to future taxonomic studies and may be an interesting input for both paleontological and trophic works. Indeed, several studies have used morphology of the otoliths to perform diet studies of different vertebrate groups (Buckland et al. 2017; Giménez et al. 2017). Particularly, the otoliths of *Astyanax* have been used as a tool to study the diet of the Neotropical river otter *Lontra longicaudis* (Helder and De Andrade 1997).

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