



Taxonomy / Taxinomie

## Surfing among species, populations and morphotypes: Inferring boundaries between two species of new world silversides (Atherinopsidae)

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## ARTICLE INFO

## Article history:

Received 10 October 2015

Accepted after revision 18 November 2015

Available online 17 December 2015

## Keywords:

Atherinopsidae

Landmarks

Taxonomy

Meristic

Barcoding

Multivariate analysis

## ABSTRACT

Atherinopsidae are widespread freshwater and shallow marine fish with singular economic importance. Morphological, genetical and life cycles differences between marine and estuarine populations were already reported in this family, suggesting ongoing speciation. Also, coexistence and interbreeding between closely related species were documented. The aim of this study was to infer boundaries among: (A) *Odontesthes bonariensis* and *O. argentinensis* at species level, and intermediate morphs; (B) the population of *O. argentinensis* of Mar Chiquita Lagoon and its marine conspecifics. To achieve this, we integrated, meristic, Geometrics Morphometrics and DNA Barcode approaches. Four groups were discriminated and subsequently characterized according to their morphological traits, shape and meristic characters. No shared haplotypes between *O. bonariensis* and *O. argentinensis* were found. Significant-meristic and body shape differences between the Mar Chiquita and marine individuals of *O. argentinensis* were found, suggesting they behave as well differentiated populations, or even incipient ecological species. The fact that the *Odontesthes* morphotypes shared haplotypes with both, *O. argentinensis* and *O. bonariensis*, but also possess meristic and morphometric distinctive traits open new questions related to the origin of this morphogroup.

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### 1. Introduction

The order Atheriniformes is a monophyletic group diagnosed by ten characters, and composed by six families and 49 genera of generally small, silvery fish, which belong to the series Atherinomorpha, the most successful fish at

the surface layer of the ocean and of many freshwaters habitats [1,2]. The members of this Order commonly share (among others) the following characters: usually two separate dorsal fins, the first, if present, with flexible spines and the second preceded by a supplementary simple spine; anal fin usually preceded by a thorn; lateral line very weak or absent; pectoral fin usually located high in the flanks, abdominal pelvic fins in most species [3]. The New World silversides (Atherinopsidae) are widespread freshwater and marine fish commonly occurring in schools in shallow waters [2]. It is a family of singular economic importance, normally employed for farming and for game fishing, but

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also commercially exploited by the artisanal and commercial coastal fleets [4]. In Argentina, this family is represented by nine species commonly known as “pejerreyes”, including: *Odontesthes argentinensis* (Valenciennes, 1835); *O. bonariensis* (Valenciennes 1835); *O. incisa* (Jenyns 1841); *O. smitti* (Lahille, 1929); *O. hatcheri* (Eigenmann 1909); *O. humensis* de Buen 1953; *O. platensis* (Berg 1895); *O. nigricans* (Richardson 1848) and *O. perugiae* Evermann & Kendall 1906 [5,6].

Although most of *Odontesthes* species occurring in Argentina inhabit exclusively fresh or marine waters, *O. argentinensis* and *O. bonariensis*, are commonly found co-existing in brackish waters. These species are valuable resources for regional fisheries, being marketed fresh, but also very appreciated for game fishing in lakes, coastal lagoons and marine coastal areas [7]. Both have been reported in Mar Chiquita coastal lagoon (Buenos Aires, Argentina) and its freshwater tributaries, being in fact *O. argentinensis* much more abundant than *O. bonariensis* [8–10], which is a conspicuous silverside of the shallows lakes, small rivers and channels of the Pampa plain of Argentina [11]. However, distinguishing both species is not always straightforward for people with not expertise in fish identification.

*Odontesthes argentinensis* and *O. bonariensis* are genetically [12] and morphologically [13] closely related. Recent molecular evidence suggested that both species could be distinguished using several genetic markers [12,14]. However, interbreeding among both species was also reported [15]. Thus, it is likely to find intermediate forms in environments where both species coexist (i.e. coastal lagoons). Moreover, current identification keys are ambiguous for several characters [5,16,17]. For instance, Dyer [5] indicated that *O. argentinensis* has 26 to 28 gill rakers (GR) in the lower branch and the first dorsal fin is situated over or posterior to the anus, while *O. bonariensis* has 30–40 GR and first dorsal fin anterior to anus. However, Bemvenuti [16] and Cousseau et al. [17] stated that *O. argentinensis* has 19–25 GR in the lower branch. Unexpectedly, González-Castro et al. [9] found that most specimens of *O. argentinensis* collected in Mar Chiquita coastal lagoon shared characteristics of both species (20 to 25 GR and first dorsal fin anterior to anus).

Beheregaray and Sunnucks [18], based on molecular data, suggested that *O. argentinensis* is ongoing a recent speciation in brackish waters of South America. Moresco and Bemvenuti [19] stated that, in Río Grande do Sul (Brazil), *O. argentinensis* is represented by two populations: a resident population in the Patos Lagoon estuary and another one in the sea. Moreover, both populations showed evidence of spawning in its respective environment. Bemvenuti [20] found that the estuarine population of *O. argentinensis* has a spawning period during August–September. Moresco and Bemvenuti [19] characterized the reproductive biology of the marine population of this species and indicated that the spawning period extends from August to December. The ecological behaviour of *O. argentinensis* at higher latitudes than Brazil seems to be quite similar: there is a growing bulk of evidence about independent events of reproduction of marine and estuarine populations of this species. For example, the

spawning of *O. argentinensis* in the Mar Chiquita coastal lagoon (Argentina) has been documented [9]. The authors found ripe and spent females in the inner zone of the lagoon (where waters are mixo-oligohaline) between June and November, suggesting the spawning of the *O. argentinensis* inside Mar Chiquita coastal lagoon. Cousseau [6] indicates that *O. argentinensis* occur in coastal waters of the Province of Buenos Aires (Argentina) almost all over the year, but in late spring or early summer it “probably” migrates to estuarine waters with reproductive purposes. However, Llompert et al. [21] found that this species has a reproductive period in the coastal area of Bahía San Blas (Argentina, 40°S) between September and November, evidenced by an increase in the gonadosomatic index, with a peak in October corresponding to spawning. Therefore (as demonstrated for Brazil), the possibility of finding both, marine but also estuarine (or coastal lagoon) populations of *O. argentinensis* should not be ruled out.

All these previous results highlight a more than complex scenario where the accurate discrimination between *O. argentinensis* and *O. bonariensis* (and its plausible morphs), but also between the co-existing populations of *O. argentinensis* are desirable. In this respect, we noted that there is a lack of meristic, morphometric and molecular comparative data between *O. argentinensis* and *O. bonariensis*, but also between the presumptive coastal lagoon and marine populations of *O. argentinensis*. In this regard, a multidisciplinary approach is highly desirable for achieving valuable and robust results.

The aim of this study was to infer boundaries among: (A) *O. bonariensis* and *O. argentinensis* at the species level, and the eventual intermediate morphs that could inhabit Mar Chiquita Coastal Lagoon; (B) the putative populations of *O. argentinensis* from Mar Chiquita Coastal Lagoon and marine environments. To achieve this, we integrated meristic, landmarks-based morphometrics and molecular (DNA Barcode sequences) approaches.

## 2. Materials and methods

### 2.1. Study area

Mar Chiquita coastal lagoon is a temperate shallow estuary, separated from the sea by a littoral line of dunes with an inlet joining it to the ocean. It covers approximately 60 km<sup>2</sup>, with a maximum length of 25 km parallel to the sea. This lagoon, considered a World Reserve of Biosphere by UNESCO [22], is located in the south-west Atlantic (37°32′–37°45′S–57°19′–57°26′W). Salinity fluctuates over a wide range between 0 and 36 PSU, and it is extremely variable and influenced by the freshwater volume present in the lagoon, the tide, and the wind direction/intensity [23]. The fish composition of Mar Chiquita coastal lagoon has been studied during the last fifteen years and several fish species have been reported to make extensive use of the lagoon, in a permanent, seasonal or occasional way [8–10,24–28]. Moreover, González-Castro et al. [9] not only analysed the spatial and temporal patterns in fish assemblage composition and relative

abundance of fish species, but also evaluated the relative contribution of some environmental variables over these patterns.

## 2.2. Fish sampling

A total of 310 adult specimens belonging to genus *Odontesthes* were collected and analyzed, comprising three main continental, brackish and marine environments of the Province of Buenos Aires (Argentina): (a) fresh water environments, including Los Padres, La Brava, San Lorenzo, Chascomús, Pigué, Alsina and del Sol Lakes; (b) brackish water environments, i.e. Mar Chiquita coastal lagoon; (c) marine environments: Mar del Plata and Miramar coasts (Table 1).

Fish were transported to the laboratory, where they were measured, weighted and sexed macroscopically. A small portion of tissue muscle was excised from representative specimens inhabiting the three sampled environments.

### 2.2.1. Taxonomic (morphological) identification

Each specimen was, at first, taxonomically identified by means of morphological identification keys of Bemvenuti [13], Cousseau et al. [17] and Dyer [5].

### 2.2.2. Meristic data analysis

Meristic characters were considered for each specimen as follows: series of lateral scales (Lss), gill rakers count in the lower branch of the first arch (Gr), number of branched and unbranched rays of first dorsal fin (D1), second dorsal fin rays (D2) and anal fin rays (A). Also, the relative position of the first dorsal fin (D1) in relation to the anus was recorded and coded as 1 (D1 anterior to anus), 2 (D1 over to anus), and 3 (D1 posterior to anus).

As the second objective of the present paper is to infer limits between the putative population of *O. argentinensis* of Mar Chiquita Coastal Lagoon and its marine conspecifics, we further refer to *O. argentinensis* from oligohaline environment (Mar Chiquita) as Oarg\_Mch, and to those from marine localities (Mar del Plata, Miramar) as Oarg\_marine.

The uni/multivariate meristic analyses were separately performed for each objective: (A) groups considered for analyses: *O. argentinensis*, *O. bonariensis* and morphotypes and (B) groups considered for analyses: *O. argentinensis*

from Mar Chiquita coastal Lagoon and marine environments (coast of Mar del Plata and Miramar).

A non-parametric Kruskal–Wallis one-way ANOVA on ranks, followed by the Kruskal–Wallis Multiple-Comparison Z-value test [29], were performed in order to evaluate significant differences for the variables studied among *Odontesthes* groups included in objective (A). For the case of the objective (B), the two groups were tested for statistical significance by means of the Mann–Whitney test.

On the other hand, the compositional similarity of the meristic variables between *Odontesthes* groups was determined by a non-parametric multivariate analysis (ANOSIM: analysis of similarities) test [30,31]. The ANOSIM test was used to search for differences in the meristic variables between “groups”. This permutation test analyzes differences among replicates within “groups”, contrasted with differences between “groups”, computing an *R* statistics under the null hypothesis of “no difference between groups”. The *R* statistics falls between  $-1$  and  $1$ , so that *R* is approximately 0 if the null hypothesis is true and  $R = 1$  if all replicates within species are more similar to each other than any replicates from different species. A multidimensional scaling (MDS) analysis (group average sorting of the Bray–Curtis similarity measures based on non-transformed data) was also performed using the PRIMER software [31]. Similarity percentages (SIMPER) were used to identify which meristic variable characterized each “group” and which made the greatest contributions to any dissimilarity between “groups”.

Finally, the accuracy of classification of the specimens among the groups of *Odontesthes*, using meristic characters, was explored using a Discriminant Analysis (DA). The DA was followed by a Canonical Variate Analysis. This analysis is a special case of a Canonical Correlation Analysis for both, independent (meristic characters) and group (*Odontesthes* groups) variables that can be graphically displayed. All meristic variables were log transformed prior to this analysis.

### 2.2.3. Molecular genetics

Cytochrome oxidase subunit I (COI) was employed to test for species boundaries at the molecular level among groups tested in Objective A. Samples of white muscle tissue were excised from 51 individuals belonging to the *Odontesthes* groups (Table 1). In order to compare these

**Table 1**

Groups, group-code, collection sites and sample size of the specimens used for this study. *n*, total number of specimens; *n*<sub>COI</sub>, number of barcoded specimens; *n*<sub>M</sub>, number of specimens for meristic analyses; *n*<sub>IID</sub>, number of specimens for morphometric analyses based on interlandmark distances; *n*<sub>CM</sub>: number of specimens for geometric morphometric analyses.

Groups	Locality	ST length range (mm)	Group-code	<i>n</i>	<i>n</i> <sub>COI</sub>	<i>n</i> <sub>M</sub>	<i>n</i> <sub>IID</sub>	<i>n</i> <sub>CM</sub>
<i>O. argentinensis</i>	Mar Chiquita coastal lagoon	133–322	Oarg_Mch	190	19	190	55	40
<i>O. argentinensis</i>	Miramar coast; Mar del Plata coast	150–282	Oarg_marine	50	10	50	50	42
<i>O. morphotypes</i>	Mar Chiquita coastal lagoon	232–326	Omorph	19	5	19	10	10
<i>O. bonariensis</i>	Mar Chiquita coastal lagoon.	114–406	Obon	51	17	51	33	29
	Lake La Brava; Lake Chascomús; Lake Pigué; Lake Alsina; Lake del Sol; Lake San Lorenzo							
			Total <i>N</i>	310	51	310	121	87

groups with other *Odontesthes* species reported for the area, specimens of *O. platensis* ( $n = 2$ ), *O. smitti* ( $n = 3$ ) and *O. incisa* ( $n = 1$ ) were also included. Tissue muscles were preserved in 100% ethanol at  $-20^{\circ}\text{C}$  for genetic analysis. Specimens were labelled, photographed, formalin fixed (with further alcohol long-term preservation) and deposited as vouchers in the fish collection of the Universidad Nacional de Mar del Plata, Argentina.

DNA extraction, polymerase chain reaction (PCR), and sequencing of the COI gene were performed according to standard DNA barcoding protocols [32] and primer cocktails developed for fish [33,34]. Extraction and amplification were performed at the International Barcode of Life Argentinean Reference Barcode Laboratory of CONICET at the Museo Argentino de Ciencias Naturales, Buenos Aires, Argentina. Amplification of the 5' region of COI, corresponding to base positions 6474 to 7126 of the *Danio rerio* mitochondrial genome [35], was first attempted using FF2d\_t1/FR1d\_t1 primer combination and C\_FishF1t1/C\_FishR1t1 primer cocktails [32]. The primer combinations C\_FishF1t1 and C\_FishR1t1 both contained two primers (FishF2\_t1/VF2\_t1 and FishR2\_t1/FR1d\_t1, respectively). PCR reactions were performed in 96-well plates. The reaction master mix consisted of 825  $\mu\text{l}$  water, 125  $\mu\text{l}$  106 buffer, 62.5  $\mu\text{l}$   $\text{MgCl}_2$  (25 mM), 6.25  $\mu\text{l}$  dNTP (10 mM), 6.25  $\mu\text{l}$  each primer (0.01 mM) and 6.25  $\mu\text{l}$  Taq DNA polymerase (5 U/ $\mu\text{l}$ ). This mixture was prepared for each plate, and each well contained 10.5  $\mu\text{l}$  of solution and 2  $\mu\text{l}$  of genomic DNA. The PCR reaction profile was comprised of an initial step of 2 min at  $95^{\circ}\text{C}$ , and 35 cycles of 30 s at  $94^{\circ}\text{C}$ , 40 s at  $52^{\circ}\text{C}$ , and 1 min at  $72^{\circ}\text{C}$ , with a final extension at  $72^{\circ}\text{C}$  for 10 min. For specimens that failed to be amplified using the primer combinations above, the primer combinations C\_VF1LFt1/C\_VR1LRt1 (34) consisting of VF1\_t1/VF1d\_t1/LepF1\_t1/VFli\_t1 and VR1\_t1/VR1d\_t1/LepR1\_t1/VRli\_t1 primer sets respectively were tried. All primers were appended with M13 tails to facilitate sequencing.

Amplicons were visualized on a 2% agarose E-Gel H 96-well system (Invitrogen). Sequencing reactions applied M13 forward and reverse primers using the BigDyeH Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems Inc.), and the reaction profile was comprised of an initial step of 2 min at  $96^{\circ}\text{C}$  and 35 cycles of 30 s at  $96^{\circ}\text{C}$ , 15 s at  $55^{\circ}\text{C}$ , and 4 min at  $60^{\circ}\text{C}$ . Products were directly sequenced using an ABI 3730 capillary sequencer according to the manufacturer's instructions. Sequencing was performed at the Canadian Centre for DNA Barcoding (CCDB) in Ontario, Canada.

**2.2.3.1. Molecular data analysis.** DNA sequences were aligned using the Muscle [36] tool and further double-checked visually. Each distinct sequence was considered a different haplotype. The K2P+G model was chosen for comparison purposes, as it was determined as the best-fit model under Akaike information criterion for NJ, ML and MP analyses. A neighbour-joining (NJ) analysis was performed to provide a graphic representation of divergences between species [37].

Nevertheless, as distance-based models erase all character-based information [38], the best nucleotide

substitution model was also employed to perform a maximum-likelihood (ML) and Maxima-Parsimonia analyses. All these analyses were performed using MEGA version 5.0 [37]. Robustness of trees was tested using bootstrap analysis [39] with 1000 replicates. The Barcode Index Number System (BINs) was used to reinforce species identification. BINs is "an online framework that clusters barcode sequences algorithmically, generating a web page for each cluster. Since clusters show high concordance with species, BINs can be used to verify species identifications as well as document diversity when taxonomic information is lacking." [40].

Data analysis of COI sequences for the *Odontesthes* groups was complemented with the nucleotide diagnostic (ND) approach proposed by Wong et al. [41]. Nucleotide diagnostics, either simple (single nucleotide position) or compound (multiple single nucleotide positions; see Sarkar et al. [42]) are molecular characters that are unique for a particular species relative to the pool of species in which the ND was identified. Nucleotide diagnostics have already been shown to be useful in aid for species identification [43,44] and may further enhance DNA Barcode applications by overcoming the ambiguity inherent to the distance-based identification processes. Moreover, it has been recently demonstrated that using the ND approach as a complementary methodology of analysis was useful to reinforce the utility of the DNA barcoding technique to identify species for a large set of Neotropical fish species with low K2P divergence values ( $< 2\%$ ) [45].

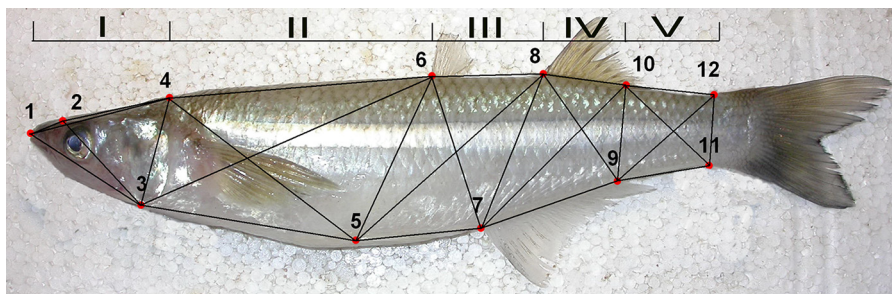
All sequence assemblies, electropherogram (trace) files, primer sequences and specimen provenance data were deposited in the "Odontesthes of Argentina" Project (Project-code: OdArg) on BOLD (Barcode of life Data System). This includes digital images of the morphological voucher specimens, sex and ontogenetic stage (juvenile or adult), total and standard body length as well as GPS coordinates for all collection localities. The sequences were deposited in GenBank (accession numbers: pending).

#### 2.2.4. Morphometry

Two types of variables were employed in order to achieve both objectives: (1) interlandmark distances (IID); (2) coordinates data (landmarks). Accordingly, two different morphometric approaches were performed.

**2.2.4.1. Morphometric analysis based on IID.** Twenty-five morphometric variables were taken as interlandmark distances over the left side of all specimens, using a digital calliper (0.05-mm precision). These variables were based on 12 landmarks obtained by truss network [46], defined on the basis of external anatomy and are homologous among the species (Fig. 1). Statistics and mathematics procedures for IID analysis followed González-Castro et al. [47,48]. The morphometric characters were organized according to the groups defined for each objective: (A) groups considered for analyses, *O. argentinensis*, *O. bonariensis* and morphotypes, and (B) groups considered for analyses, *O. argentinensis* from oligohaline environments and *O. argentinensis* from marine environments.

A normalization technique to scale the data that exhibit an allometric growth was used according to Leonart et al.



**Fig. 1.** Box truss showing the interlandmark distances collected in the silversides analysed, based on 12 anatomical landmarks. (Number of Box truss in Roman). Landmarks: 1, tip of premaxilla; 2, second nostril; 3, caudal extreme of the subopercular; 4, projection of the external border of the opercular towards the mid-dorsal line; 5, anterior insertion of the pelvic fin; 6, anterior insertion of the first dorsal fin; 7, anterior insertion of the anal fin; 8, anterior insertion of the second dorsal fin; 9, posterior insertion of the anal fin; 10, posterior insertion of the second dorsal fin; 11, ventral insertion of the caudal fin; 12, dorsal insertion of the caudal fin.

[49]. The standard length (SL) was used as the independent variable, whilst the remaining eight morphometric characters were considered as dependent ones.  $SL_0$  represents a reference value of size (250 mm in this paper) to which all individuals are reduced (or amplified) [50]. This transformation scales the data that exhibit allometric growth [49]. After transformation, a new matrix was constructed containing the corrected matrices for each species, and Principal Component Analysis (PCA) was performed using MULTIVARIADO<sup>®</sup> software [51]. Finally, principal components scores (PCs) were submitted to cross-validated discriminant analysis (DA) using SPSS<sup>®</sup> v.13.0, in order to build a predictive model of group membership based on observed characteristics of each case. This procedure generates a set of discriminant functions based on linear combinations of the predictor variables that provide the best discrimination between the groups. The functions are generated from a sample of cases for which group membership is known; the functions can then be applied to new cases with measurements for the predictor variables but unknown group membership.

**2.2.4.2. Geometric morphometric analysis based on coordinate data (landmarks).** The analysis was performed on the Cartesian coordinates of the 12 anatomical landmarks, reconstructed from distance measurements among the landmarks (after its normalization procedure, as previously explained for IID analysis), based on the proposed box truss scheme (Fig. 1) and using MORPPHEUS<sup>®</sup> Software [52]. Reconstruction of the form (shape plus size) from truss measures provides Cartesian coordinates for landmarks and allows estimation of, and compensation for, the measurement error [53].

The landmark coordinates for each specimen were scaled, translated and rotated using the generalized Procrustes superimposition (GLS, also called GPA). Scaling, translation and rotation was employed to minimize the Procrustes distance, the sum of squared distances between the corresponding landmarks. In summary, the objects were centred at the origin by subtracting the coordinates of its centroid from the coordinates of each landmark. Then each object was scaled (to unit centroid size) by dividing each coordinate of each landmark by its centroid size [54]. One landmark configuration was used as a reference and all

others were rotated to minimize the partial Procrustes distance. The average shape was then calculated and became the new reference to which all objects were rotated again. This step was repeated until rotation ceased to occur.

The thin-plate spline (TPS) procedure was employed to compare shape differences among the groups, using both the uniform and non-uniform shape components and an upward/downward arching effect of the fish's body was observed. This effect was not related to biological factors (size or species) or to the preservation technique (freezing), but was rather due to slight posture differences between fish during interlandmarks distances capture. This distortion associated with the specimen's posture was already addressed in fish by Valentin et al. [55]. These authors proposed a method that effectively removes this artefact from the data, coupling a PCA-based model of the arching with Burnaby's orthogonal projection. This method also has the property of making the correction directly on landmark coordinates. Then, the methodology of Valentin et al. [55] was applied and the new unbiased coordinates were re-subjected to GLS and TPS (using TPSRELW Software (ver 1.46) [56]). The thin-plate spline is a method that projects data from shape space into a tangent space that is Euclidian and generates deformation grids, which depict shape changes over the entire object by interpolating between landmarks [54,57]. This method is not only an effective visualization tool, but its coefficients (partial warp scores) represent the non-uniform shape variation between specimens-consensus and can be used in descriptive and inferential statistical tests as well [58]. A principal components analysis (PCA) of the partial warps matrix was performed (usually named as relative warp analysis, RWA), in order to describe the major trends in shape variation. To examine the potential for differences in shape in classifying unknown specimens, the relative warps scores were submitted to discriminant analysis (SPSS ver. 13.0). This was carried out using cross-validation.

### 3. Results

#### 3.1. Taxonomic (morphological) identification

The silversides analyzed were morphologically identified as:

- *Odontesthes bonariensis* (Obon): all the specimens collected from Pampa Plain Lakes ( $n = 48$ ) and a some ( $n = 3$ ) from the inner (oligohaline) zone of Mar Chiquita Coastal Lagoon;
- *Odontesthes argentinensis* (Oarg): all the specimens collected in the marine localities ( $n = 50$ ) and specimens collected from the oligohaline zone of Mar Chiquita Coastal Lagoon ( $n = 190$ );
- morphotypes: in this morphological identification process, we detected several specimens ( $n = 19$ ) that failed to be assigned to either *Odontesthes bonariensis* (Obon) or *Odontesthes argentinensis* (Oarg). They presented intermediate values for the gill rakers counts in the lower branch of the first gill arch. Moreover, these specimens did not match any diagnosis of the remaining species reported for the area. Consequently, this third group was regarded as morphotypes (Omorph) for further statistical analyses.

### 3.2. Meristic

The basic statistics on meristic data for the four groups identified in the present work are summarized in Table 2.

#### 3.2.1. Objective A

The Kruskal–Wallis one-way ANOVA on ranks showed that both species of *Odontesthes* and the morphotypes significantly differed in all but D1 meristic characters. The largest difference was found in the number of soft rays in the anal fin which were significantly ( $\chi^2$ : 54.58;  $P < 0.001$ ) different in all pairwise comparisons. *Odontesthes bonariensis* and morphotypes also displayed a higher number of scales in the lateral line ( $\chi^2$ : 110.95;  $P < 0.001$ ), a higher number of rays in D2 ( $\chi^2$ : 54.34;  $P < 0.001$ ) and a higher gill raker count ( $\chi^2$ : 165.77;  $P < 0.001$ ) than *O. argentinensis*. The analysis of contingency showed that the D1 fin is more ( $\chi^2$ : 10.77;  $P < 0.029$ ) frequently inserted anterior to the anus in all three groups.

Analyses of similarities showed that there was significant differences in overall among groups in terms of meristic variables (Global test of the ANOSIM:  $R = 0.92$ ;  $P = 0.1\%$ ). Pairwise comparisons within ANOSIM showed significant differences between all compared pair groups (Tables 3A and 3B). According to the ANOSIM test, the highest differences occurred between Oarg and Obon (Tables 3A and 3B). These results were also evident in the ordination analysis performed (MDS), in which specimens

of the same group were clustered together as well as groups distinguished each other (Fig. 2A). SIMPER identified Gr and Lss as the meristic variables responsible for these differences (Tables 3A and 3B).

In the DA, the linear discrimination function that was used to elucidate the *a priori* classification significantly included all but D1 meristic characters. The DA further showed that individuals from the three groups were correctly classified in 99%. The Canonical Variate Analysis significantly extracted all the information (100%) in the first two variates. The spatial ordination of samples along the first and second variates is depicted in Fig. 3.

#### 3.2.2. Objective B

The Mann–Whitney test showed that lateral line scales (Z-value: 6.25), number of soft rays in the anal fin (Z-value: 7.2) and gill raker counts (Z-value: 8.94) significantly differed between the two putative populations of silversides. Particularly, marine wanderers of *O. argentinensis* displayed a higher number of lateral line scales, soft anal fin rays and gill rakers than those of *O. argentinensis* from Mar Chiquita. In addition, marine specimens displayed a more ( $\chi^2$ : 116.99;  $P < 0.0001$ ) posteriorly inserted first dorsal fin, and only 8 out of 50 specimens presented this fin inserted anterior to anus. Conversely, 122 of 190 specimens from Mar Chiquita displayed the insertion of first dorsal fin anterior to the anus.

The number of rays in both dorsal fins did not significantly contribute to the linear discrimination function of populations of *O. argentinensis*. DA further discriminated both populations by means of the remaining meristic characters. A percentage of 79.2% of correct classification for group membership was achieved. Overall, group classification reached 90% and 89.5% for marine and Mar Chiquita populations, respectively.

Analyses of similarities showed significant difference among both Oarg (Mar Chiquita vss marine) groups in terms of meristic variables (Global test of the ANOSIM:  $R = 0.373$ ;  $P = 0.1\%$ ). These results were also observed in the performed ordination analysis (MDS), in which most specimens of the same group were clustered together as well as groups distinguished each other (Fig. 2B). SIMPER identified Gr and Lss as the meristic variables responsible for these differences (Tables 3A and 3B).

### 3.3. Molecular analysis

As COI is not intended to be used at population level, the results are mainly focused on objective A.

**Table 2**  
Basic statistics on meristic data for the four groups of silversides analysed.

Groups	GR		Lss		D1		D2		A		D1-anus (%)	
	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Ant	Over/post
Oarg_Mch	21.7 (1.1)	17–24	53.2 (2.0)	50–58	4.9 (0.6)	4–6	10.0 (0.6)	9–12	18.3 (0.8)	16–20	64.2	35.8
Oarg_marine	23.8 (1.0)	21–25	55.4 (1.7)	52–59	4.8 (0.6)	4–6	10.0 (0.7)	8–11	19.5 (0.9)	18–21	16.0	84.0
Omorph	27.3 (1.0)	25–29	58.6 (2.6)	53–64	4.8 (0.5)	4–6	11.1 (0.7)	10–12	19.2 (0.5)	19–20	61.1	38.9
Obon	32.9 (1.6)	30–36	58.0 (2.6)	52–66	5.0 (0.6)	4–6	10.6 (0.7)	9–12	17.7 (0.7)	15–19	68.6	31.4

GR: gill rakers counts; Lss: lateral series scales; D1: first dorsal fin counts; D2: second dorsal fin counts; A: anal fin counts; D1-anus: origin of first dorsal fin in relation to the anus (ant: anterior to anus; over/post: over or posterior to anus). **Group-code as in Table 1.**

**Table 3A**

The contribution of meristic variables to observed differences among groups determined by SIMPER analyses.

Groups pairwise	A							
	ANOSIM		SIMPER					
	RS	SL	AA <sub>1</sub>	AA <sub>2</sub>	AD	D/Sd	C%	Cu%
<i>Oarg</i> - <i>Obon</i>	0.984	0.1						
GR			22.18	32.94	4.55	5.11	60.09	60.09
Lss			53.69	58.08	1.97	1.58	25.98	86.07
A			18.61	17.74	0.46	1.10	6.02	92.08
<i>Oarg</i> - <i>Omorph</i>	0.811	0.1						
GR			22.18	27.58	2.31	2.71	41.36	41.36
Lss			53.69	58.58	2.18	1.72	38.88	80.24
D2			10.09	19.16	0.47	1.41	8.42	88.66
A			18.61	11.11	0.43	1.22	7.63	96.30
<i>Obon</i> - <i>Omorph</i>	0.692	0.1						
GR			32.94	27.58	2.17	2.63	47.15	49.35
Lss			58.08	58.58	1.11	1.23	25.20	74.55
A			17.74	19.16	0.61	1.80	13.81	88.36
D2			10.60	11.11	0.31	1.02	7.05	95.41

Meristic variables are listed in descending order of percentage contribution and only categories contributing > 2% to observed differences are shown. One-way ANOSIM results for meristic variables of the groups analysed. AA<sub>1</sub>: Average abundance of the *i*-group; AD: average dissimilarity; D/Sd: dissimilarity standard deviation; C%: percentage contribution; Cu%: cumulative percentage contribution. RS: R significance. SL: Statistical level percentage.

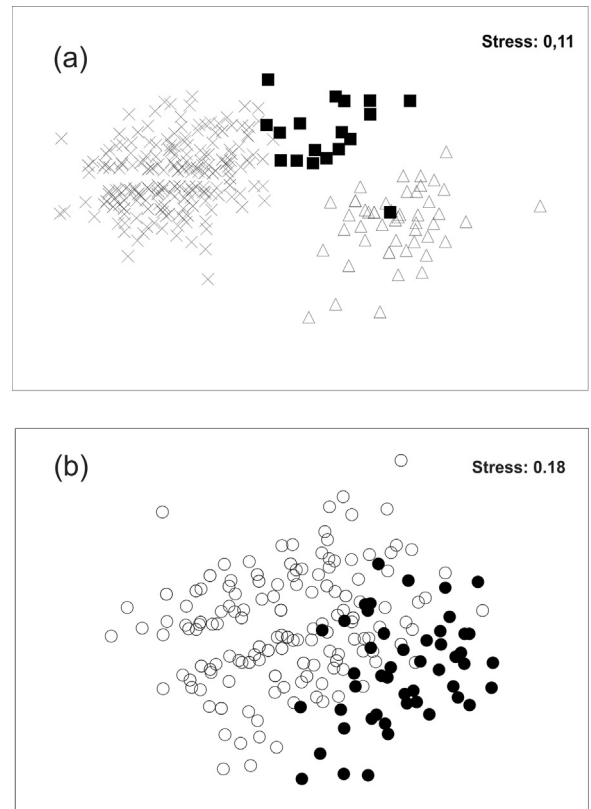
**Table 3B**

The contribution of meristic variables to observed differences among groups determined by SIMPER analyses.

Groups pairwise	B					
	SIMPER					
	AA <sub>1</sub>	AA <sub>2</sub>	AD	D/Sd	C%	Cu%
Lss	55.42	53.23	1.26	1.42	37.55	37.55
GR	23.80	21.75	0.96	1.62	28.81	66.35
A	19.52	18.37	0.58	1.31	17.42	83.77
D2	10.06	10.09	0.28	0.97	8.45	92.22

Meristic variables are listed in descending order of percentage contribution and only categories contributing > 2% to observed differences are shown. AA<sub>1</sub>: Average abundance of marine *Odontesthes argentinensis* (*Oarg\_marine*) AA<sub>2</sub>: only categories contributing > 2% to the observed differences are shown. AA<sub>2</sub>: Average abundance of *Odontesthes argentinensis* from Mar Chiquita lagoon (*Oarg\_Mch*); AD: average dissimilarity; D/Sd: dissimilarity standard deviation; C%: percentage contribution; Cu%: cumulative percentage contribution. RS: R significance. SL: statistical level percentage. Group-code as in Table 1.

The NJ, ML and MP analyses (based on K2P + G model) generated trees with nearly identical topologies (data not shown). *Odontesthes platensis*, *O. smitti* and *O. incisa* clustered separately and were assigned to three different BINs. Conversely, *O. argentinensis* and *O. bonariensis* clustered together and were assigned to the same BIN (data not shown). Interestingly, no shared haplotypes were found between *O. bonariensis* and *O. argentinensis* specimens. Moreover, a compound nucleotide diagnostic



**Fig. 2.** a: multidimensional scaling (MDS) performed on meristic data in order to infer boundaries among *O. bonariensis* (*O\_bon*) (white triangles), *O. argentinensis* (*Oarg*) (black cruxes), and the intermediate morphs (*O\_morph*) (black squares); b: multidimensional scaling (MDS) performed on meristic data in order to infer boundaries among the putative populations of *O. argentinensis* from Mar Chiquita coastal lagoon (*Oarg\_Mch*) (white circles) and *O. argentinensis* from marine coastal areas (*Oarg\_marine*) (black circles).

allowed us to discriminate between *O. argentinensis* and *O. bonariensis* by the exclusive occurrence of characters in two informative sites (124 T + 421 A).

Sequence analysis revealed 13 variable sites corresponding to 10 haplotypes, distributed within *Obon*, *Oarg\_marine*, *Oarg\_Mch* and *Omorph* groups (Table 4). Indeed, *Obon* displayed only two different haplotypes, being both shared with *Omorph* specimens. The remaining eight haplotypes corresponded to *O. argentinensis*: one of them was shared with a single *Omorph* specimen (Hp 3), two were unique haplotypes of *Oarg\_marine* (Hp 6 and Hp 9), and three were unique haplotypes for *Oarg\_Mch* (Hp's 7, 8 and 10) (Table 4).

### 3.4. Morphometry

#### 3.4.1. Objective A

**3.4.1.1. Morphometric analysis based on ILD.** The 25 normalized interlandmark distances, which were analyzed by PCA of the correlation matrix, produced eight eigenvalues greater than one (data not shown). The first four PCs explained more than 63% of the variance in the data. Only

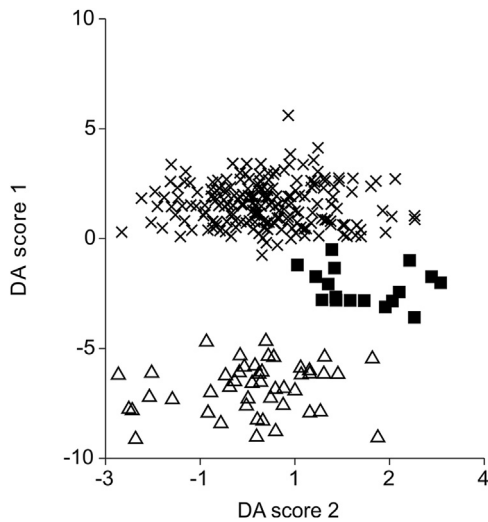


Fig. 3. Discriminant analysis performed on meristic data to infer boundaries among *O. bonariensis*, *O. argentinensis*, and the intermediate morphs. Symbols: *Odontesthes bonariensis* (O\_bon) (white triangles); *Odontesthes* morphotypes (O\_morph) (black squares); *O. argentinensis* (black cruxes).

correlations (between variables and components) higher than 0.59 were taken as significant (data not shown). PCA based on IID allowed graphic segregation of the three groups analyzed, with a slightly degree of overlap between them (data not shown). In this respect, Obon was located in the fourth quadrant (data not shown), with higher loadings for the 1–4, 1–3, 2–4 interlandmark distances (which represents the head length) but also 5–7 (the distance

between the origins of the ventral and anal fins). Moreover, Obon specimens displayed higher values for the 1ld that constitutes the third Box Truss (PC1 vs. PC3, data not shown) and lower values for the 10–11, 8–9, 9–10 variables (interlandmark distances of fourth and third Box- Truss). The specimens of Omorph were located basically in the first quadrant (data not shown) and characterized by higher loadings for the 3–4 (head width) and 6–7/8–9 (variables related to the origin of first and second dorsal fins, with respect to the Anal fin); lower values were obtained for the 4–6 variable, which represent the distance between the end of the head and the origin of the first dorsal fin, thus indicating that this fin originates very near to the head, when compared with the other *Odontesthes* groups analysed (data not shown). In this respect, Oarg was the most spread group and showed higher loadings for the 4–6 8–9, 9–10 and 10–11 variables and lower ones for 2–4, 3–4, 1–4 (head shape) and 6–7.

The data corresponding to the 25 PCs of the PCA where employed to perform the Discriminant Analysis. DA for the 148 individuals of *Odontesthes* produced two significant canonical discrimination functions. The first one explained 90.0% of the total variance in the data, (Wilks' lambda = 0.092,  $P < 0.000$ ). The DA correctly classified 96.6% of the *Odontesthes* individuals according to the three groups defined *a priori*, whereas the cross-validated analysis correctly classified 90.2% of the fish according to their body shape (Table 5). Accordingly, group misclassifications were scarce, with a highest rate of 10.0% of Omorph misclassified as Oarg (Table 5). Three groups were defined, accordingly to those defined *a priori*, and their centroids and individuals were separated both on the first and second discriminant functions (Fig. 4).

Table 4  
Distribution of the haplotypes (Hp) of the four groups analysed. Group-code as in Table 1.

Groups	n	Hp 1	Hp 2	Hp 3	Hp 4	Hp 5	Hp 6	Hp 7	Hp 8	Hp 9	Hp 10
Obon	17	13	4								
Omorph	5	2	2	1							
Oarg_marine	10			1	6	1	1			1	
Oarg_Mch	19			12	2	2		1	1		1
Total	51	15	6	14	8	3	1	1	1	1	1

Table 5  
Cross-validated Discriminant Analysis for the Objective A, based on the PC scores of: (1) interlandmark distances and (2) landmark coordinates. Group-code as in Table 1.

Morphometric approach	Objective	Predicted Group Membership (%)				
		Groups	Obon	Omorph	Oarg	Total
1	A	Percent				
		Obon	93.94	6.06	0	100
		Omorph	10	80	10	100
		Oarg	2.86	6.67	90.48	100
Note: 90.5% of cross-validated grouped cases correctly classified						
2	A	Obon	82.8	13.8	3.4	100
		Omorph	20.0	50.5	30.0	100
		Oarg	2.4	9.8	87.8	100
		Note: 83.5% of cross-validated grouped cases correctly classified				



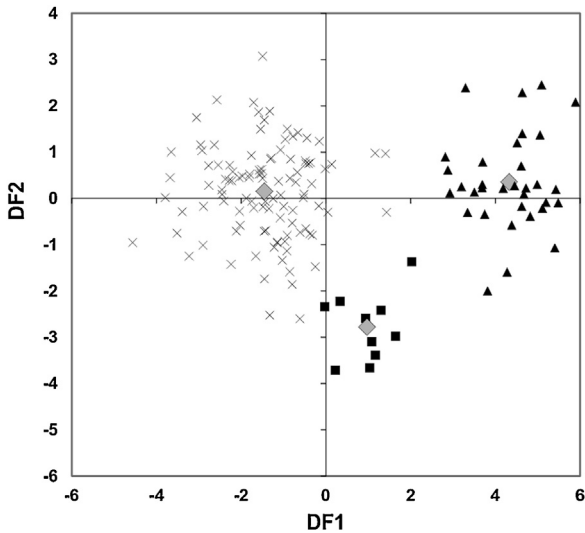


Fig. 4. Discriminant Analysis based on interlandmarks distances, in order to infer boundaries among *O. bonariensis*, *O. argentinensis*, and the eventual intermediate morphs. Symbols: centroid of each species group (big grey diamonds); *Odontesthes bonariensis* (O\_bon) (white triangles); *Odontesthes* morphotypes (O\_morph) (black squares); *O. argentinensis* (black cruxes).

Remarkably, there was no overlap for the centroids/specimens of Obon, Omorph and Oarg (Fig. 4).

3.4.1.2. Geometric morphometric analysis based on coordinates (landmark data). The first four RW explained 68.13% (31.76, 17.72, 12.30 and 6.36% respectively) of the total

variance for the GLS/RWA analysis of the body shape of the three silversides groups analysed in this Objective A. The pattern of morphological variation described by the first two relative warps is shown in Fig. 5.

The shape variation along the first RW was basically expressed by the depression (negative RW1 scores) or expansion (positive RW1 scores) of the body and head along the dorso-ventral axis (i.e. the body height along by the second, third and fourth box trusses), but also the placement and length of the dorsal and anal fins (Fig. 5). Accordingly, the head shape vary from shorter and depressed (RW1–), which is typical of those *O. argentinensis* specimens located in the third quadrant, to deeper (in height) and longer (RW1+), as can be observed in O\_morph and those samples of *O. argentinensis* located in the fourth quadrant (Fig. 5). The relative position of the dorsal fins (D1 and D2) was in accordance with the morphological traits observed in the previous analyses: the *O. argentinensis* specimens (those related to RW1) exhibited D1 posterior to anus and, a shorter D2; in the opposite, the individuals of O\_morph and *O. argentinensis* related to (RW1+) showed the D1 located anterior to anus (Fig. 5). Moreover, the third and fourth box trusses of the O\_morph (and *O. argentinensis* related to RW1+) individuals changed dramatically: D2 is forward displaced and the ventral and anal fins (particularly its origin) are downwardly displaced.

The shape of the RW2 basically allowed differentiating O\_bon specimens (RW2+) from the remaining groups. The former was characterized by having the shortest caudal peduncle, the longest snout, and a longer head; a forward displacement of the origin of ventral fin, backward

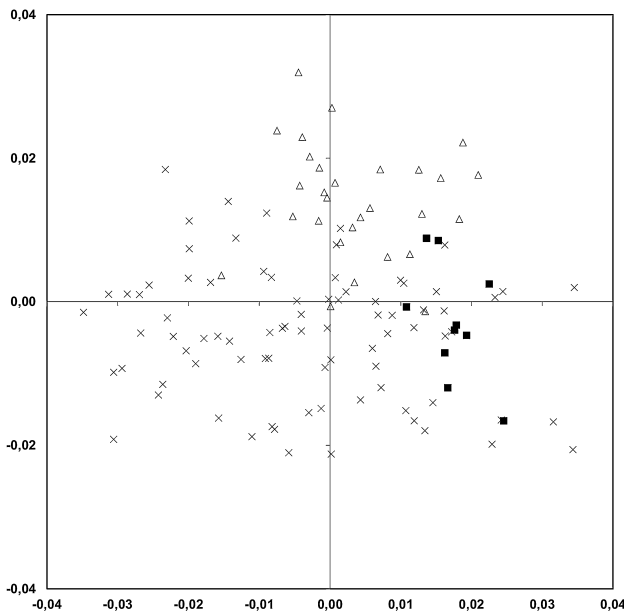


Fig. 5. Relative Warp analysis (RW1 vs. RW2) based on landmarks coordinates to infer boundaries among *O. bonariensis*, *O. argentinensis*, and the intermediate morphs. Thin-plate spline transformation grids for the extreme points of RW1/RW2 are shown; they were superimposed on the shapes predicted when the average landmark configuration of all specimens was deformed into that of a hypothetical specimen positioned at the extreme of the RW of interest. Symbols: *Odontesthes bonariensis* (O\_bon) (white triangles); *Odontesthes* morphotypes (O\_morph) (black squares); *O. argentinensis* (black cruxes).

displacement of D1 fin and a smaller anal fin base (forward displacement of its insertion) (Fig. 5).

The data corresponding to the 20 RWs of the RWA were employed to perform the Discriminant Analysis. DA for the 121 original grouped cases of silversides classified by groups (Obon, Omorph and Oarg) produced two significant canonical discrimination functions, were the first one explained 87.3% of the total variance in the data (Wilks' lambda = 0.164,  $P < 0.000$ ). The DA correctly classified 90.1% of the original grouped cases, whereas the cross-validated analysis correctly classified 83.5% of the fish according to their body shape (Table 5). Three groups were defined, and its centroids and individuals were separated both on the first and second discriminant functions (data not shown). There was no overlap for the centroids of the three groups. Misclassifications (cross-validated analysis) ranged between 9.8–30.0% according the analysed groups (Table 5).

### 3.4.2. Objective B

**3.4.2.1. Morphometric analysis based on IID.** The 25 normalized interlandmark distances, which were analyzed by PCA of the correlation matrix, produced eight eigenvalues greater than one (data not shown). The first four PCs explained more than 64% of the variance in the data. Only correlations (between variables and components) higher than 0.59 were taken as significant (data not shown). PCA based on IID allowed almost a total graphic segregation of the two groups analyzed (data not shown). In this respect, Oarg\_marine was located in the second and third quadrants (data not shown), with higher loadings for the 4–6, 3–6 interlandmark distances (which represents the distance between the posterior part of the head and the first dorsal fin), but also 7–9 (the anal fin base length), and lower values for the 3–4, 1–4, 2–4 and 2–3 variables (interlandmarks distances of first box truss, which

represents the head) and 8–9, 8–10 variables (interlandmark distances of fourth box truss). The specimens of Oarg\_Mch were located basically in the first and fourth quadrants (data not shown) and characterized by higher loadings of the variables related to the head shape (1–4, 2–3, 2–4, 3–4), and 7–8, 8–9, 8–10 and 9–10 (variables related to the fourth box truss, which represents the body at the level of the second dorsal and anal fins), and lower values for the 4–6 and 3–6 variables, thus indicating that the first dorsal fin is closer to the head, when compared with the marine *O. argentinensis* specimens.

The data corresponding to the 25 PCs of the PCA were employed to perform the Discriminant Analysis. DA for the 148 individuals of *Odontesthes* produced one significant canonical discrimination function, which explained 100% of the total variance in the data, (Wilks' lambda = 0.059,  $P < 0.000$ ). It is noticeable that both, the original and the cross-validated DA, correctly classified 100% of the *Odontesthes* individuals of the two groups included in this Objective B, according to their body shape.

**3.4.2.2. Geometric morphometric analysis based on coordinates (landmarks data).** The first four RWs explained 75.38% (45.24, 11.65, 10.53 and 7.96% respectively) of the total variance for the GLS/RWA analysis of the body shape of the two *O. argentinensis* groups (Mar Chiquita and marine) analyzed in this Objective B. The pattern of morphological variation described by the first two relative warps is shown in Fig. 6. A complete segregation of the groups was observed, which explain the dispersion along the first RW axis observed in the RW analysis of Objective A (Fig. 5).

The shape variation between the two groups analyzed in this objective B was exclusively related to the first RW. It was basically expressed by the depression (negative RW1 scores) or expansion (positive RW1 scores) of the head (as a consequence of the displacement of the landmarks 4 and

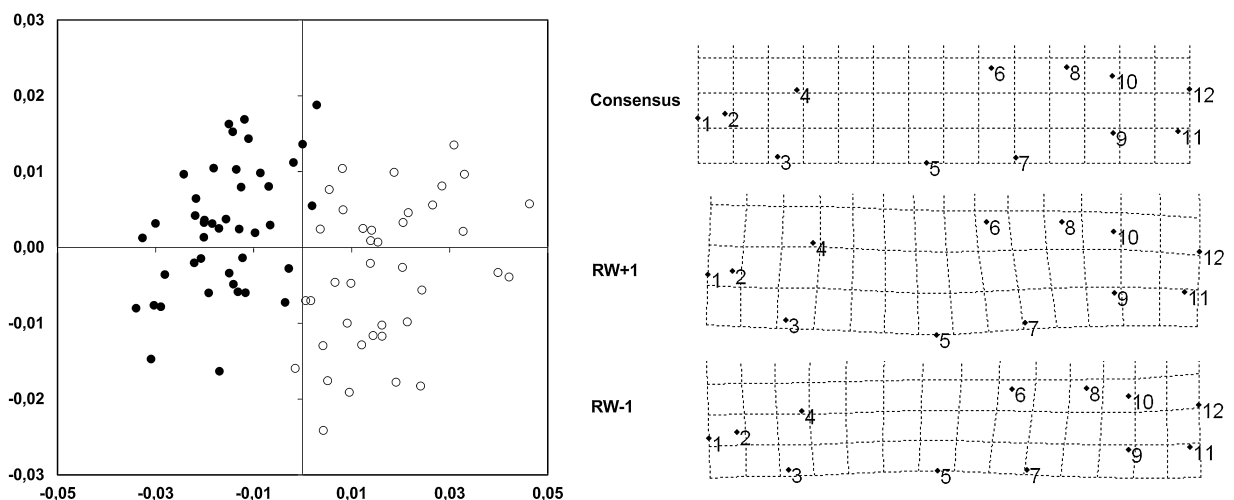


Fig. 6. Relative Warp analysis (RW1 vs. RW2) based on landmark coordinates performed on the populations of *O. argentinensis* from Mar Chiquita Coastal Lagoon and marine environment. Thin-plate spline transformation grids for the extreme points of RW1 are shown; they were superimposed on the shapes predicted when the average landmark configuration of all specimens was deformed into that of a hypothetical specimen positioned at the extreme of the RW of interest. Symbols: *O. argentinensis* from Mar Chiquita coastal lagoon (Oarg\_Mch) (white circles); *O. argentinensis* from marine environments (Oarg\_marine) (black circles).

3) and the body (related to the displacement of the dorsal, ventral and anal fins) (Fig. 6). Accordingly, the head shape vary from shorter and depressed (RW1–) typical of Oarg\_marine specimens, to deeper (in height) and longer (RW1+), as can be observed in those Oarg\_Mch individuals (Fig. 6). The relative position of the dorsal fins (D1 and D2) was in accordance with the morphological traits observed in the previous analyses: the Oarg\_marine specimens (RW1) exhibited a backward displacement of the D1 fin and a shorter D2; to the opposite, the Oarg\_Mch individuals (RW1+) showed a forward displacement of the D1 fin (Fig. 6). Moreover, the third and fourth box trusses of the Oarg\_Mch (RW1+) individuals have dramatically changed: D2 is forward displaced and the ventral and anal fins (its origins) are downwardly displaced; also, the insertion of the anal fin (landmark 9) is forwardly displaced, resulting in a narrower anal fin. At last, Oarg\_marine individuals were also characterized by a wider anal and a smaller caudal peduncle (Fig. 6).

The data corresponding to the 20 RWs of the RWA were employed to perform the Discriminant Analysis. DA for the 83 original grouped cases of silversides classified by groups (Oarg\_Mch and Oarg\_marine) produced one significant canonical discrimination function (Wilks' lambda = 0.087,  $P < 0.000$ ). As in the morphometric analysis based on ILLD, both the original and the cross-validated DA correctly classified 100% of the individuals of the two groups analyzed in this Objective, according to their body shape.

#### 4. Discussion

The present study represents the first attempt to analyze together the meristic, genetic and landmarks-based morphometric characters of *O. argentinensis* and *O. bonariensis*. This multidisciplinary approach allowed us to detect and characterize four groups by means of both their body shape and meristic features: *O. bonariensis*, *O. argentinensis* from marine environments, *O. argentinensis* from oligohaline environments (Mar Chiquita Lagoon) and *Odontesthes* morphotypes. *Odontesthes bonariensis* was characterized by having 30–36 GR, 52–67 Lss, a long head, a deep body at the level of the second and third box trusses, the anal fin located posteriorly in the body and a narrow anal fin base; *O. argentinensis* from marine environments, have 21–25 Gr, 52–59 Lss, a small head (short and depressed) and dorsal fins (D1–D2) inserted posteriorly in the body; *O. argentinensis* from Mar Chiquita lagoon, showed 17–24 Gr, 48–58 Lss, the widest head, dorsal fins inserted anteriorly and a robust body at the third and fourth box trusses (Fig. 1). Finally, the group of *Odontesthes* morphotypes showed intermediate values of Gr/Lss between *O. bonariensis* and *O. argentinensis* (25–29 and 53–64, respectively), a higher and rather long head too (higher and mid loadings of 3–4/1–4 variables, respectively) and a D1 inserted closer to the head (anterior to the anus), D2 forwardly displaced and anal fin downwardly displaced.

One of the morphological traits employed in the identification keys to distinguish *O. argentinensis* from *O. bonariensis* is the position of first dorsal fin in relation to the anus [5,13,17]. These authors agreed that

*O. argentinensis* (adult specimens) has the first dorsal fin (D1) situated over or posterior to the anus, while in *O. bonariensis* the D1 is located anterior to the anus. Surprisingly, we found that 64% of *O. arg\_Mch* ( $n = 190$ ) presented the D1 anterior to the anus, while only 16% of the marine wanderers (*O. arg\_marine*;  $n = 50$ ) showed this state. Furthermore, 31.4% of the *O. bon* ( $n = 51$ ) analyzed had the D1 over (or even posterior) to the anus. Finally, *Odontesthes* morphotypes ( $n = 19$ ) presented the latter state in 39% of the specimens, while the remaining 61% displayed D1 anterior to the anus. These results evidenced that using the current taxonomic identification keys could lead to misidentifications. Therefore, we suggest that this character alone should not be included in the identification keys.

Sufficient evidence has been presented to accept the assumption that landmark-based morphometry can discriminate among species, populations and even morphotypes [48,59–62]. With respect to Atheriniformes, ÓReilly and Horn [63] and, more recently, Flucker et al. [64] and Crichigno et al. [65] presented landmarks-based studies, employing the body or head shape to elucidate the phenotypic variation, at the population or specific taxonomical level. In the present work, a multidisciplinary methodological approach was employed, which includes genetic, meristic and morphometric analyses. Moreover, the fact that two complementary morphometric approaches were used contributed to a better understanding of the taxonomic differences related to the body shape of these fish.

Depending on the kind of fish under study, achieving a standard or neutral posture for each individual is not straightforward. The fish body usually is not a rigid structure, and the specimen's shape could be influenced by its posture during landmark capture. This issue was recently stated by Valentin et al. [55], who detected an upward or downward arching effect in the morphometric data set of a multidisciplinary study on redfish (genus *Sebastes*) in the north-west Atlantic Ocean [66]. These authors proposed an approach, coupling a PCA-based model of the arching with Burnaby's orthogonal projection for removing the artefact from the data. This approach was successfully employed by González-Castro et al. [48] for a morphometric data set of seven species of Mugilidae (Actinopterygii). In the present work, the same kind of arching effect was encountered for the approaches based on landmark coordinate data. Valentine et al.'s [55] methodology was applied, and the arching effect was removed, yielding satisfactory results as evidenced by the RWA and the correct classification rates in the DA (Figs. 5 and 6).

COI sequences are probed to be highly effective to discriminate at the species level [33,67–69]. However, despite so far *O. argentinensis* and *O. bonariensis* are regarded as two valid species, their COI sequences were assigned to the same BIN. A lack of discrimination among closely related fish species using COI was already reported [68,70,71]. Moreover, Heras and Roldan [12], using several mitochondrial DNA markers, discriminated between *O. bonariensis* and *O. argentinensis*, but noted that “genetic distances between them for all molecular markers were

within the range of *Odontesthes* intraspecific levels. Moreover, *O. bonariensis* and *O. argentinensis* comprised a common lineage in all phylogenetical analyses consistent with their shared morphological characters." In fact, the three specimens of *O. argentinensis* employed by Heras and Roldán [12] corresponded to the same sampling sites (Mar Chiquita lagoon and Mar del Plata coast) as those of the present work. Although no delimitation between species was obtained using DNA barcoding, no shared haplotypes between *O. bonariensis* and *O. argentinensis* were found, and the compound nucleotide diagnostic character analysis allowed us to discriminate between *O. argentinensis* and *O. bonariensis*. The fact that Omorph specimens shared haplotypes with both *O. argentinensis* and *O. bonariensis* coupled with the meristic and morphometric diagnostic features of this species opens new questions about the origin of this group: could this morphogroup be the result of natural hybridization between *O. argentinensis* and *O. bonariensis* (as was reported by Tejedor [15])? Should it just be considered a bunch of rare specimens resulted from the intraspecific morphological variability of *O. argentinensis*, which can be linked to their novel colonization to the estuarine habitat?

*Odontesthes argentinensis* is considered a widely distributed western Atlantic coastal species, occurring in marine and estuarine environments from the Sao Paulo State, in Brazil, southwards from the province of Chubut, Argentina [5]. Atherinids show a high degree of plasticity, which is inherent within their genetic make-up. This plasticity pre-adapts the species to radiate into habitats showing more stable environmental characteristics, representative of any part of their endemic range [72]. Moreover, the extent and pattern of divergence between estuarine and marine populations of *O. argentinensis* of southern Brazil indicated that speciation is occurring in the Patos Lagoon estuary [73]. This is an example of speciation associated with significant behavioural and ecological divergence. Indeed, while inshore sheltered waters and estuaries remain the principal habitat of the Atheriniformes, these fish show a striking ability to invade and speciate within vacant freshwater niches [72]. Bloom et al. [74] shown that transitions from marine to freshwater environments result in accelerated speciation and extinction rates, and that these rate differences may help explain the remarkable disparity in species richness between continents and oceans. García et al. [75] pointed out that promiscuous and recent contact between incipient species of silversides blurs species boundaries, yielding complicated taxonomy and species delimitation among silverside genus *Odontesthes*. Interestingly, in the present work, we found significative-meristic and body shape differences between the Mar Chiquita coastal lagoon and the marine populations of *O. argentinensis*, as it was already pointed out by Bemvenuti [76] for *O. argentinensis* of marine and estuarine areas of Rio Grande do Sul State (Brazil). Beheregaray and Sunnucks [18] suggested that ecological shifts due to colonization of estuarine habitats seem to have promoted rapid adaptive divergence and reproductive isolation in estuarine populations of *O. argentinensis*, which were considered as incipient ecological species. This conclusion is supported by the existence of a set of

environmental factors required for successful reproduction of estuarine ecotypes. The estuarine (Oarg\_Mch) and marine (Oarg\_marine) populations of *O. argentinensis* studied here are meristically and morphometrically (this study), as well as genetically (Heras and Roldán [12] and this study), distinguishable and appear to behave as well differentiated populations, or even incipient ecological species according to Beheregaray and Sunnucks [18]. Even though COI haplotypes of estuarine and marine populations are shared (and thus no discrimination between both groups were obtained using NDC), a dominant haplotype in each group was observed. Indeed, Heras and Roldán [12], using several markers, found some minor genetic difference between both groups, although the sample used was relatively low ( $n = 3$ ) to strongly support this. Moreover, González-Castro et al. [9] stated that there is a reproductive isolation of *O. argentinensis* in Mar Chiquita coastal lagoon. They found ripe and spent females in the inner zone of the lagoon (Zone III in González-Castro et al. [9]) between June and November, confirming reproductive events of *Odontesthes argentinensis* inside the coastal lagoon. All these results strongly suggest that each population should be treated separately with regard to management and conservation plans, specially taking into account that Mar Chiquita coastal lagoon is a World Reserve of Biosphere. It would be desirable to employ additional molecular markers (i.e., microsatellites or nuclear genes) in order to delimit and characterize this population/incipient species of *O. argentinensis* that inhabit Mar Chiquita coastal lagoon.

#### Disclosure of interest

The authors declare that they have no competing interest.

#### Acknowledgments

The authors would like to thank: Julio Mangiarotti (forest guard of Mar Chiquita Biosphere Reserve), Marcelo Pons (fishermen of Mar Chiquita), Mónica Iza, Florencia Celesia and Gladys Eiras (Mar Chiquita Town Hall) and Town Hall Mar Chiquita authorities (Luis Facca, Jorge Paredi); Matías Delpiani (BIMOPE Laboratory of UNMdP, Argentina) for collaboration in the field sampling; Darío Lijtmaer for assistance in barcode protocols. This work was funded by CIC (CIC 0823/05), UNMdP (15/E525, EXA 577/2) and PIP-CONICET No. 112201301003390 grants.

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